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The Trail Pheromone of a Stingless Bee, *Trigona corvina* (Hymenoptera, Apidae, Meliponini), Varies between Populations

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Abstract

Stingless bees, like honeybees, live in highly organized, perennial colonies. Their eusocial way of life, which includes division of labor, implies that only a fraction of the workers leave the nest to forage for food. To ensure a sufficient food supply for all colony members, stingless bees have evolved different mechanisms to recruit workers to foraging or even to communicate the location of particular food sites. In some species, foragers deposit pheromone marks between food sources and their nest, which are used by recruited workers to locate the food. To date, pheromone compounds have only been described for 3 species. We have identified the trail pheromone of a further species by means of chemical and electrophysiological analyses and with bioassays testing natural gland extracts and synthetic compounds. The pheromone is a blend of wax type and terpene esters. The relative proportions of the single components showed significant differences in the pheromones of foragers form 3 different colonies. This is the first report on a trail pheromone comprised of esters of 2 different biogenetic origins proving variability of the system. Pheromone specificity may serve to avoid confusions between the trails deposited by foragers of different nests and, thus, to decrease competition at food sources.

Key words: chemical communication, esters, GC-EAD, labial gland secretions, nestmate recruitment, social bees

Introduction

Stingless bees are eusocial insects that live in the tropical and subtropical regions of the world in colonies ranging from a few dozens to 100 000 or more adult workers (Michener 2000). Like other eusocial insects, they developed a variety of communication mechanisms to effectively allocate the workers of a colony to different tasks (Wilson 1971). Thus, in order to collect sufficient amounts of food to nourish the entire nest population, foraging workers inform their nestmates about the presence and, in several species, about the location of food sources with thorax vibrations and body contacts exhibited within the nest, with footprint secretions and pheromone marks deposited in the field, or with a combination of these signals and cues (Lindauer and Kerr 1958, 1960; Kerr et al. 1963; Kerr 1969; Nieh 2004; Barth et al. 2008; Hrncir 2009; Jarau 2009). Among the recruitment mechanisms found in stingless bees, the communication of food locations by means of pheromone marks deposited between a food site and the nest is particularly precise and effective (Lindauer and Kerr 1958, 1960; Jarau et al. 2003). Foragers of species that use this mode of communication can recruit large numbers of workers to a precise location at a certain direction, distance, and height from their nest in a short time (Lindauer and Kerr 1958, 1960; Kerr and Cruz 1961; Kerr et al. 1963; Jarau et al. 2003; Schmidt et al. 2003; Nieh et al. 2004; Sánchez et al. 2004, 2007).

The pheromones used for marking were long thought to be secreted by the bees' mandibular glands (Lindauer and Kerr 1958, 1960; Kerr et al. 1963; Nieh et al. 2003; Nieh 2004). However, proper experiments that corroborate this assumption were never provided (Jarau 2009), and recent experiments have unequivocally shown that the trail

pheromones of *Trigona recursa* (Jarau et al. 2004, 2006), *T. spinipes* (Schorkopf et al. 2007), *Geotrigona mombuca* (Stangler et al. 2009), and *Scaptotrigona pectoralis* (Hemmeter 2008, cited in Jarau 2009) are secreted from the foragers' labial glands. Mandibular gland secretions, by contrast, have a clear deterrent effect at food sources and play an important role in alarm communication and defense (Jarau et al. 2004; Schorkopf et al. 2009). The chemical structures of trail pheromone compounds have only been elucidated for a few species to date. Hexyl decanoate is the main component from labial gland secretions of *T. recursa* foragers and acts as a key compound for triggering trail-following behavior in newly recruited workers of this species (Jarau et al. 2006). However, the attractiveness of this ester is reduced as compared with natural labial gland extracts, which indicates that the entire trail pheromone of *T. recursa* is composed of a blend of compounds (Jarau et al. 2006). In *T. spinipes*, the single dominant component of labial gland secretions, octyl octanoate, was as efficient in triggering trail-following behavior as the complete labial gland extract (Schorkopf et al. 2007). Stangler et al. (2009) identified a series of terpene- and wax-type esters from labial gland secretions of *G. mombuca*, with farnesyl butanoate as major component. Thus, the trail pheromone of *G. mombuca* is composed of esters, but the specific role of single compounds needs to be clarified by further investigations testing synthetic substances (Stangler et al. 2009).

An interesting question arising from the chemical analyses of stingless bee trail pheromones is whether their composition is fixed for a certain species or shows colony-specific variations, for example, as adaptation to different environmental conditions or due to the use of different food sources. In the present study, we identified the composition of the trail pheromone of *Trigona corvina* (a species exhibiting scent marking behavior; Aguilar et al. 2005) by means of chemical and electrophysiological analyses as well as with bioassays testing a synthetic pheromone blend. Because the glandular origin of pheromone marks deposited by *T. corvina* foragers has not been investigated yet, we first tested whether labial or mandibular gland secretions elicit trail-following behavior in newly recruited bees of this species. Finally, we compared the pheromone compositions of foragers collected from different populations in order to clarify whether the composition of the trail pheromone of *T. corvina* shows intraspecific variability.

Materials and methods

Study site and bee nests

Field experiments (artificial scent trail bioassays; see below) were carried out between February and May 2006 at the Centre for Tropical Bee Research (CINAT) of the National University of Costa Rica in Heredia, Costa Rica (lat 9°58′22′′N, long 84°07′45′′W). We used 2 nests of *T. corvina* Cockerell, 1913. Nest A was brought from Atenas, Alajuela province (lat 9°59′03′′N, long 84°22′45′′W), to the CINAT, where it was placed on a table under a large tree. Nest B naturally occurred on a small tree approximately 2 m above the ground on the University campus. The 2 nests were separated from each other by a distance of 180 m.

Test substances for bioassays

We tested whether one or more of the following substances release trail-following behavior in *T. corvina* recruits: 1) mandibular gland extract, 2) labial gland extract from foragers of the recruits' parental nest, 3) labial gland extract from foragers of a foreign nest, 4) a synthetic blend of compounds that were found to release a physiological response in gas chromatography with electroantennographic detection analyses (GC-EAD; see below), and 5) pure hexane (solvent control experiments).

Gland extracts were prepared from *T. corvina* individuals that had been collected during foraging at sugar solution feeders. The bees were sacrificed by freezing at –8 °C, and their glands dissected in saline solution under a stereo microscope by carefully separating them from any tissue other than the respective glandular epithelia and reservoirs. For all extracts, the amount of hexane was adjusted to 100 μL per pair of glands (e.g., 10 labial glands in 500 μL hexane). Thus, 100 μL of the pooled extracts used for bioassays corresponded to the gland content of one individual bee (one bee equivalent). The glands were left in the solvent for 24 h at room temperature (about 24 °C on average). The extracts were then stored in a freezer (–8 °C), unless they were in use for the bioassays. The synthetic blend of electrophysiologically active compounds (see Results) was diluted to 1 μg compounds per 100 μL hexane.

Artificial scent trail bioassays

The general experimental setup and procedure followed the method for scent trail bioassays described in Jarau et al. (2006). Foragers from the nest under study were trained to collect 0.5 mol/L unscented aqueous sugar solution from a "training feeder," which was moved stepwise to the final experimental position 30 m away from the nest. The trained foragers were marked with water-based acrylic color on their thoraces for their identification. Recruitment never occurred during the training phase. Thus, recruited bees had not already been in the field searching for food when the artificial scent trails were installed. Once all trained bees had arrived at the training feeder at its final position, it was replaced by a "recruitment feeder" (RF) mounted on wooden stakes (50 cm high). The RF contained a 3 mol/L sugar solution in order to cause the foragers to deposit pheromone marks and to recruit additional workers inside their nest. As soon as the first forager began to mark a scent trail from the RF toward the nest, we installed an artificial scent trail between the nest and the RF. The experimental trail

branched off from the bees' natural trail 20 m away from the nest and led to a "test feeder" (TF) that was identical to the RF in appearance and food supply. It consisted of 10 small wooden stakes (2.3 × 2.3 × 50 cm) positioned between the branching point and the TF in intervals of 1 m (for an illustration of the experimental setup, see Figure 1 in Jarau et al. 2006). To each stake, a thin cardboard rectangle impregnated with 10 μL of test substance (corresponding to 0.1 bee equivalent of gland extract or 0.1 μg synthetic ester blend) was attached on a headless nail. The amount of applied test substances proved to be effective in an earlier study (Jarau et al. 2006). Unbaited stakes and cardboards were placed at 1-m intervals from the branching point toward the RF in order to provide equal structural conditions for the last 10 m toward the TF and RF, respectively. The artificial scent trail was renewed 15 min after the beginning of an experiment, which in total lasted for 30 min. The artificial scent trail was installed either to the left or to the right from the natural scent trail in different experiments in order to avoid side bias.

The first naïve bees arriving at the RF after the start of an experiment were marked as foragers until their number reached 15. These foragers were allowed to recruit at the RF during the whole experiment. Every other bee that arrived at the RF and all bees arriving at the TF were immediately captured and stored away until the end of an experiment. Thus, the bees were not allowed to communicate the location of the TF to their nestmates. All captured bees were color marked before they were released to allow their identification as feeder experienced bees in subsequent tests. For the statistical analyses, we only counted the unmarked, feeder naïve recruits captured during an experiment in order to avoid pseudoreplications and possible learning effects. Their distribution was determined by calculating the relative proportions at RF and TF from the total number of captured unmarked recruits.

GC-EAD

To identify compounds from the labial gland extracts of *T. corvina* foragers to which the chemoreceptors of worker antennae are sensitive, we carried out GC-EAD analyses using a HP 6890 gas chromatograph fitted with a DB-5MS column (30 m × 0.25 mm inner diameter [i.d.], 0.25 μm film thickness, J & W Scientific) and a flame ionization detector (FID). Hydrogen was used as carrier gas (constant linear flow rate 2 mL/min). The effluent was split with a variable outlet splitter (SGE) at a split ratio of FID:EAD = 1:3, and the outlet for the EAD was lead into a cleaned, humidified airflow that was directed over a worker's antenna. Antennae were mounted between 2 capillary glass electrodes filled with saline solution and attached to Ag-AgCl wires. The electrodes were connected to a high-impedance DC amplifier (Syntech), and the flame ionization (FID) and electroantennographic (EAD) signals were simultaneously recorded on a PC using the program GC-EAD 2000 (Syntech). For each run, 1 μL gland extract was injected splitless at 50 °C onto the column. After 1 min, the split valve was opened and the temperature increased by 10 °C/min until it reached 310 °C. A peak was classified as electrophysiologically active when it coincided with an EAD baseline deflection in at least 50% of the 28 analyzed runs.

Chemical analyses

To compare the chemical composition of trail pheromones from bees of different nests, we collected foragers from nests A and B in Heredia and from a third nest (henceforth nest C) from the Tropenstation La Gamba, Puntarenas Province, in the southwest of Costa Rica (lat 8°42′02′′N, long 83°12′07′′W). For quantitative chemical analyses, the labial glands of 12 foragers from each nest were dissected as described above and extracted individually in 200 μL hexane for 24 h at room temperature. Prior to gas chromatographic analysis, each extract was concentrated to 50 μL, and 1 μg *n*-pentadecane was added as an internal standard. One microliter per sample was injected into a Thermo Finnigan Trace GC (Rodano) that was equipped with a DB-5MS column (30 m × 0.25 mm i.d., 0.25 μm film thickness, J & W Scientific). Hydrogen was used as carrier gas (constant linear flow rate 2 mL/min). The GC was operated splitless at 50 °C for 1 min, followed by a programmed increase to 310 °C at a rate of 10 °C/min and held at the final temperature for another 17 min.

For structure elucidation of compounds from the labial glands, pooled extracts from 5 foragers were used. The samples were analyzed with a Fisons Instruments gas chromatograph series 8008 linked to a Fisons MD800 mass spectrometer (Fisons Instruments). Separations were performed with 30 m × 0.25 mm i.d. unpolar fused silica columns coated with CP8912 VF-1MS and CP8944 VF-5MS, respectively (Varian), and 70 eV mass spectra were taken in electron impact mode. The temperature was initially held at 60 °C for 5 min, then increased by 10 °C/min to 300 °C and held at this temperature for 33 min. Helium served as carrier gas. Identification of compounds was based on comparisons of mass spectra with literature data (McLafferty and Stauffer 1989) and with mass spectra and retention times of authentic reference substances. Saturated hydrocarbons as well as squalene and ethyl (9Z)-octadecenoate were purchased from Aldrich. All other esters were synthesized in our laboratory by reacting commercial (Aldrich) acid chlorides with alcohols in the presence of pyridine according to standard textbook procedures. Crude products were purified by chromatography on silica gel. Mass spectra and nuclear magnetic resonance (NMR) data were in accordance with expected data (Francke et al. 2000).

Statistical analyses

Statistical tests on the bioassay data were carried out with SimgaStat (Version 3.5). Kruskal–Wallis and Dunn's tests

or Mann–Whitney tests, respectively, were used to check for significant effects of artificial trail treatment on the proportion of recruits that followed the trails and landed on the TF during the experiments. The distribution of newcomers at the RFs and TFs did not differ between the experiments conducted with nest A and B when parental and foreign labial gland extracts, mandibular gland extracts, or hexane were tested (Mann–Whitney tests; parental labial gland extract: $T = 43.0$, $N_1 = 6$, $N_2 = 7$, $P = 0.945$; foreign labial gland extract: $T = 37.0$, $N_1 = N_2 = 6$, $P = 0.247$; mandibular gland extract: $T = 13.0$, $N_1 = 3$, $N_2 = 4$, $P = 0.857$; hexane: $T = 18.0$, $N_1 = N_2 = 4$, $P = 1.0$). Therefore, the data obtained in the bioassays testing natural gland extracts were pooled for the analyses. The proportion of newcomers reaching the TFs in experiments with synthetic ester blend significantly differed between the experiments conducted with the 2 nests (Mann–Whitney test: $T = 50.0$, $N_1 = 5$, $N_2 = 7$, $P = 0.003$) and the respective data were treated separately.

For the comparison of the composition of trail pheromones from nests of different populations, we calculated the relative proportions of the physiologically (GC-EAD) active gland constituents for all foragers collected from nest A, B, and C ($N = 12$ each). A canonical discriminant function analysis (DFA) with these data was then carried out with SPSS 11 for Mac. The standardized discriminant function coefficients were used to assess the importance of individual compounds for the separation of foragers from different nests. Classification by the discriminant functions was done using the leave-one-out method.

Results

Scent trail bioassays with natural gland extracts

The total number of recruits reaching the feeding tables in the single experiments proved to be similar for the different test substances applied to the artificial scent trails (mean ± standard deviation, N = number of experiments; hexane: 33.1 ± 20.7, range 16–63, $N = 8$; mandibular gland extract: 44.6 ± 25.8, range 17–81, $N = 7$; foreign labial gland extract: 54.3 ± 24.3, range 22–113, $N = 12$; parental labial gland extract: 102.9 ± 69.1, range 27–275, $N = 13$; synthetic ester blend: 63.0 ± 44.8, range 15–161, $N = 12$). The behavior of recruited workers, however, significantly differed in response to artificial scent trail following, depending on the test substance applied to the trails (Kruskal–Wallis test, $H_3 = 26.947$, $P < 0.001$; Figure 1). In the control experiments, the proportion of recruits that reached the TF at the end of the hexane baited trail equaled 0.0 (0.0/0.0) % (median and first quartile and third quartile, respectively). Likewise, almost no bees were distracted by the trails scented with mandibular gland extract (0.0 [0.0/1.2] %) or with labial gland extract prepared from foragers of a foreign colony (1.9 [0.0/12.7] %). The small differences in the proportions of recruits captured at the TFs in the control experiments

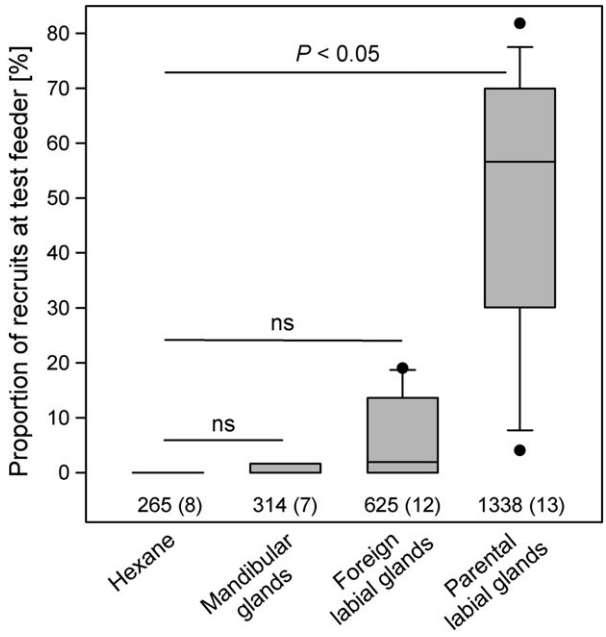


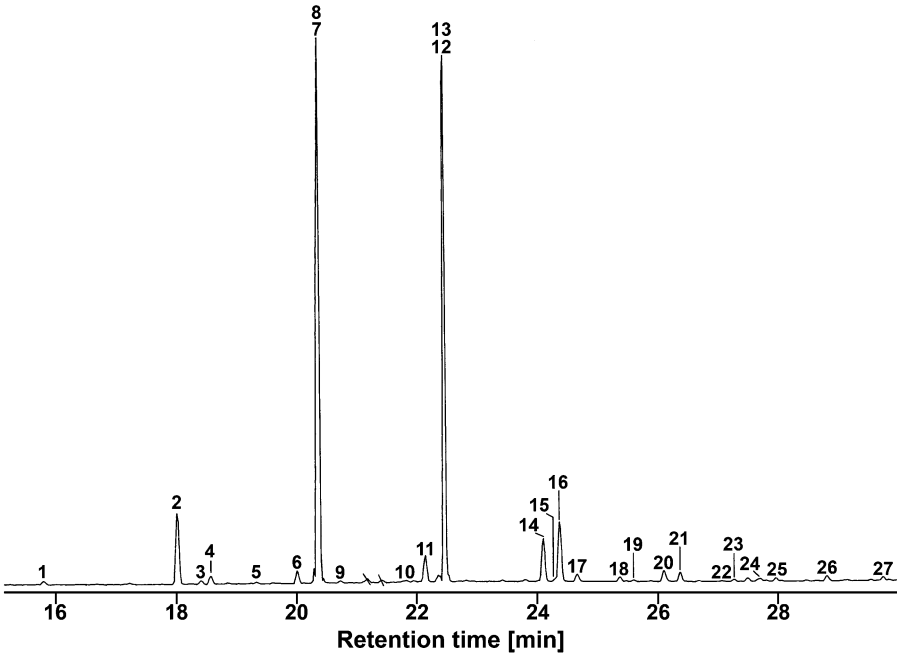
Figure 1 Percentage of newly recruited *Trigona corvina* foragers that followed the artificial scent trails baited with the pure solvent hexane or with different gland extracts and reached the TFs (100% = sum of recruits at TF and RF). Data for experiments done with nest A and B were pooled; boxes include the median and the 25th and 75th percentiles, whiskers give the 5th and 95th percentiles, and dots represent outlying values. Significance levels were calculated with a Kruskal–Wallis test followed by Dunn's tests; ns, not significant. Total numbers of individual bees tested and number of conducted experiments (in brackets) are given below the boxes.

and in the bioassays testing mandibular and foreign labial gland extract are not significantly different (Dunn's tests; mandibular glands: $Q = 0.300$, $P > 0.05$; foreign labial glands: $Q = 1.336$, $P > 0.05$). By contrast, significantly more bees were distracted from their natural scent trails by the experimental trails when they were baited with parental labial gland extract prepared from nestmate foragers (56.6 [34.1/69.7] %) as compared with the hexane trails (Dunn's test, $Q = 4.281$, $P < 0.05$; Figure 1).

Chemical and electrophysiological analyses

We focused our chemical analyses on labial gland extracts because the bioassays have demonstrated that the trail pheromone of *T. corvina* is produced by these glands. The mean total amount of volatiles found in labial glands was 2.40 ± 1.08 µg ($N = 36$). We identified 25 compounds from the labial gland extracts, which are dominated by carboxylic acid alkyl and terpene esters (Figure 2).

GC-EAD analyses done with 28 worker antennae revealed 7 peaks that elicited responses of the chemoreceptors in more than 50% of the trials. These peaks correspond to 9 compounds: octyl hexanoate, octyl octanoate, octyl decanoate, decyl hexanoate, decyl octanoate, decyl decanoate, geranyl octanoate, geranyl decanoate, and one unidentified terpene ester, which was present only in minor quantities (Figure 3,



1	*n*-Tetradecane	15	Ethyl (9*Z*)-octadecenoate
2	Octyl hexanoate	16	Decyl decanoate
3	*n*-Hexadecane	17	(2*E*,6*E*)-Farnesyl hexanoate
4	Isopropyl dodecanoate	18	Tricosene *
5	*n*-Heptadecane	19	*n*-Tricosane
6	Geranyl hexanoate	20	*unidentified* (terpene ester)
7	Octyl octanoate	21	(2*E*,6*E*)-Farnesyl octanoate
8	Decyl hexanoate	22	Pentacosene *
9	*n*-Octadecane	23	*n*-Pentacosane
10	*unidentified* (terpene ester)	24	(2*E*,6*E*)-Farnesyl decanoate
11	Geranyl octanoate	25	*n*-Hexacosane
12	Octyl decanoate	26	*n*-Heptacosane
13	Decyl octanoate	27	Squalene
14	Geranyl decanoate		

Figure 2 Gas chromatographic separation of the compounds extracted from labial glands of a *Trigona corvina* forager. Asterisks indicate components with an unknown double-bond position.

Table 1). The physiological activity of the identified esters was verified in GC-EAD runs with synthetic compounds.

The relative proportions of the GC-EAD active compounds differed considerably between extracts prepared from foragers of nests A, B, and C, respectively (Table 1). A canonical DFA based on the physiologically active labial gland ester bouquet resulted in 2 discriminant functions (f1: $\chi^2 = 137.127$, degrees of freedom [df] = 16, $P < 0.001$; f2: $\chi^2 = 23.722$, df = 7, $P < 0.001$) that clearly grouped the foragers belonging to a specific nest and separated the nests originating from different populations (Figure 4). The compounds that mainly contributed to the separation of nests in both functions were octyl octanoate (discriminant function coefficients; f1: 8.724, f2: 2.875), decyl hexanoate (f1: 6.530, f2: 1.922), and decyl octanoate/octyl decanoate (f1: 4.908, f2: 2.078). In addition, geranyl octanoate (f1: 4.598), octyl hexanoate (f1: 2.179), decyl decanoate (f1: 1.559), and geranyl decanoate (f1: 1.408) had discriminant function coefficients >1 for the first function. In the cross-validated classification, 100% of the bees from nest B were correctly classified, whereas for nest A only 58% of the foragers were classified to their own nest (the remaining 42% to nest C) and 83% for nest C (the remaining 17% to nest A). Overall, 81% of the foragers were classified to their correct nest.

Scent trail bioassays with synthetic esters

To test whether the physiologically active compounds from the labial glands constitute the behaviorally active trail pheromone of *T. corvina*, we conducted a further set of scent trail bioassays. We baited the experimental trails with a blend of esters that was mixed according to their relative abundance

in labial gland secretions of foragers collected from nest A. The blend consisted of the following 5 (out of 9) electrophysiologically active compounds: octyl hexanoate, octyl octanoate, geranyl octanoate, decyl octanoate, octyl decanoate (the remaining 4 compounds were not available as synthetic substance at the time of bioassay conduction).

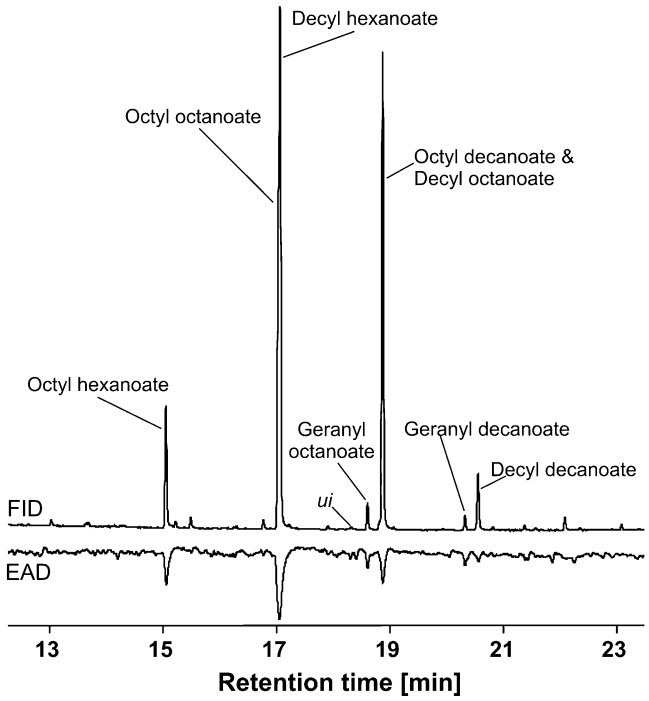


Figure 3 Gas chromatogram (FID) of a labial gland extract from a *Trigona corvina* forager and simultaneous recording of the chemoreceptors' responses (EAD) of an antenna from a worker taken from the same nest. Names of physiologically active compounds are given in the chromatogram. ui, unidentified compound (=peak 10 in Figure 2).

The synthetic ester blend had clearly different effects on newly recruited bees from nest A and nest B, respectively. A significantly higher proportion of recruits was distracted from their natural scent trails by the ester trails (18.8 [13.0/22.1] %) as compared with hexane trails (0.0 [0.0/1.6] %) in the experiments done with nest A (Mann–Whitney test, $T = 10.0$, $N_1 = 4$, $N_2 = 5$, $P = 0.016$), whereas the synthetic blend was as unattractive as the hexane trails to recruits of nest B (blend: 0.0 [0.0/0.0] %; hexane 0.0 [0.0/0.0] %; Mann–Whitney test, $T = 22.0$, $N_1 = 4$, $N_2 = 7$, $P = 0.788$) (Figure 5).

Discussion

Trail pheromone origin and chemistry

The results of our bioassays show that the trail pheromone of *T. corvina* is exclusively produced in the foragers' labial glands. This is in accordance with recent studies conducted with *T. recursa*, *T. spinipes*, *G. mombuca*, and *S. pectoralis* (Jarau et al. 2004, 2006; Schorkopf et al. 2007; Jarau 2009; Stangler et al. 2009) and again refutes the long assumed role of mandibular gland secretions for scent trail marking in stingless bees (Lindauer and Kerr 1958, 1960; Kerr et al. 1963; Nieh et al. 2003; Nieh 2004).

The compounds from labial gland extracts detected by the chemoreceptors on the foragers' antennae belong to the major chemical class of esters (6 carboxylic acid alkyl esters and 2 terpenyl esters). The bioassays with synthetic ester blends had shown that the tested compounds are part of the trail pheromone of *T. corvina*. However, the natural labial gland extract was more attractive to newly recruited workers as compared with the synthetic blend. We assume that this was the case because the synthetic pheromone, which consisted of 5 of the 9 physiologically active compounds, was incomplete. In particular, the lack of decyl hexanoate, which proved to be an important compound for the nest specificity

Table 1 Relative proportions (%) of the GC-EAD active compounds from labial glands of foragers collected from *Trigona corvina* nests from 3 different populations (mean ± standard deviation, $N = 12$ for each nest)

Compound	Nest A	Nest B	Nest C	χ^2	P
Octyl hexanoate	2.67 ± 0.98[a]	6.13 ± 2.16[b]	1.62 ± 0.92[a]	19.964	<0.001
Octyl octanoate	54.33 ± 20.99[a]	19.79 ± 5.56[b]	57.42 ± 15.85[a]	19.718	<0.001
Decyl hexanoate	12.93 ± 18.63[a]	28.4 ± 4.69[b]	12.14 ± 3.62[a]	17.754	<0.001
Unidentified (peak 10)	1.09 ± 0.95	0.42 ± 0.38	1.38 ± 2.41	1.911	=0.385
Geranyl octanoate	16.46 ± 13.27[a]	2.44 ± 1.60[a]	0.68 ± 0.94[b]	16.785	<0.001
Octyl decanoate and decyl octanoate*	9.41 ± 3.23[a]	33.09 ± 4.02[b]	24.48 ± 12.34[b]	19.115	<0.001
Geranyl decanoate	0.25 ± 0.23[a]	2.74 ± 3.51[b]	0.14 ± 0.06[a]	23.502	<0.001
Decyl decanoate	0.36 ± 0.12[a]	5.13 ± 1.07[b]	0.49 ± 0.18[a]	25.297	<0.001

All but the unidentified compound significantly contribute to nest-specific distinctions of the labial gland volatiles (Kruskal–Wallis tests with df = 2; Dunn's tests, different letters (a,b) indicate significantly different proportions between the nests with $P < 0.05$).
*The exact ratio between octyl decanoate and decyl octanoate could not be determined.

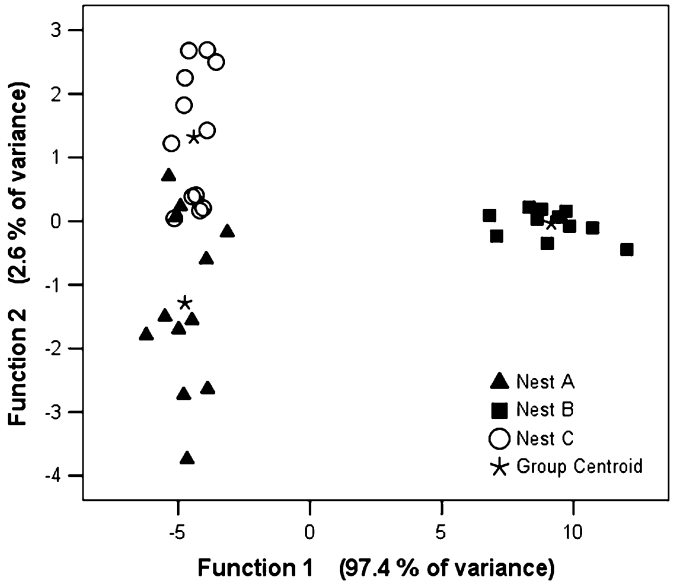


Figure 4 Comparison of the trail pheromone compositions from labial glands of *Trigona corvina* foragers collected from 3 nests at different locations. The relative proportions of the physiologically (GC-EAD) active compounds were used for a DFA. Foragers from the different nests can be well distinguished by means of their pheromone bouquet (discriminant function 1: $\chi^2 = 137.127$, df = 16, $P < 0.001$; discriminant function 2: $\chi^2 = 23.722$, df = 7, $P < 0.001$). The percentage of explained variance is given for each function.

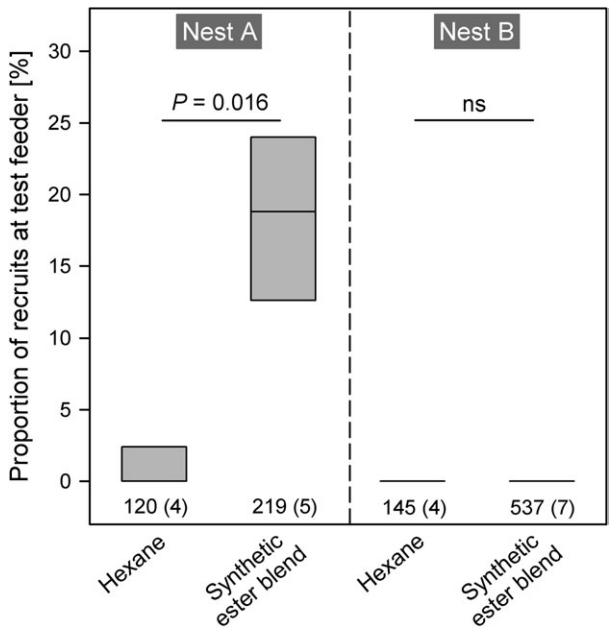


Figure 5 Percentage of newly recruited *Trigona corvina* foragers that reacted to artificial scent trails baited with a synthetic blend of esters resembling the trail pheromone of foragers from nest A. Boxes include the median and the 25th and 75th percentiles. Significance levels were calculated with Mann–Whitney tests; ns, not significant. Total numbers of individual bees tested and number of conducted experiments (in brackets) are given below the boxes.

of the pheromone bouquet in the DFA, may have caused this result.

Some of the compounds we have found in the labial glands of *T. corvina* are also known from cephalic secretions (head or gland extracts) of several other stingless bee species belonging to the genera *Trigona*, *Geotrigona*, and *Tetragona* (Kerr et al. 1981; Johnson et al. 1985; Francke et al. 2000; Stangler et al. 2009) but so far only hexyl decanoate (*T. recursa*: Jarau et al. 2006) and octyl octanoate (*T. spinipes*: Schorkopf et al. 2007) had been demonstrated to elicit trail-following behavior in recruits. Our study, therefore, extends the list of known trail pheromone compounds of stingless bees, and it can be assumed that the esters identified from head or gland extracts of other trail laying species may constitute their respective trail pheromones. Indeed, the physiochemical similarities between these compounds on the one hand and with the most frequently occurring compound in moth sex pheromones, (*Z*)-9-tetradecenyl acetate (Schultz 2001), on the other, may indicate their ideal properties as volatile signals to attract flying recruited workers over longer distances (Jarau 2009). Terpenyl esters are also used as marking compounds by solitary bees, and bumblebee males deposit carboxylic acid alkyl esters (among other substances) on twigs or leaves, which attract females, as well as other males, for the purpose of mating (Bergström 2008).

Nest specificity of trail pheromones

In the present study, we found clear nest specificity in the trail pheromone of *T. corvina*, both in terms of chemical composition and effectiveness in triggering trail-following behavior. The finding, that recruits are significantly attracted to scent trails baited with labial gland extracts prepared from their nestmates but not from foragers of a foreign colony, can be explained by the demonstrated differences in the relative proportions of trail pheromone components in foragers from different nests. This conclusion is corroborated by the bioassays with trails baited with synthetic esters, which were blended according to their relative abundance in labial gland secretions of foragers from nest A and attracted recruits from the same nest but not from nest B.

Our study is the first to demonstrate nest-specific composition and effectiveness of trail pheromones in a stingless bee. Even for ant recruitment trail pheromones, object of a disparate higher number of investigations, only few cases of colony specificity have been reported (Hölldobler and Wilson 2009). Regnier et al. (1973) mention colony-specific chemical cues in homing trails of *Pogonomyrmex badius* harvester ants that enable workers to find the right trail back to their nest (however, without presenting underlying data). Likewise, *Pogonomyrmex barbatus* and *P. rugosus* trunk trails, which arise from recruitment trails and are used by workers during foraging and homing, help to partition the foraging grounds of neighboring conspecific colonies and thus to avoid aggressive encounters of their foragers (Hölldobler 1976). The recruitment trail pheromones of

Pogonomyrmex species, which induce general trail-following behavior in recruited workers, are secreted from the poison glands of foragers (Hölldobler and Wilson 1990). Recently, Hölldobler et al. (2004) reported that the preference of *P. rugosus* foragers for trails marked by their nestmates to trails marked by foreign conspecific workers likely is due to Dufour's gland secretions, which contain nest-specific patterns of volatiles (mainly hydrocarbons and esters) and may be deposited in addition to the poison gland content. A colony-specific effect of hindgut extracts in releasing trail-following behavior was demonstrated in *Lasius neoniger* (Traniello 1980). By contrast, in both *L. japonicus* and *L. nipponensis*, the actual trail pheromone extracted from worker gasters is not specific in releasing following behavior, but colony specificity is added to the trails by footprint hydrocarbons deposited by workers along the trails (Akino and Yamaoka 2005; Akino et al. 2005). Thus, within a species, ant recruitment trail pheromones appear to release trail-following behavior regardless of whether depositor and follower are from the same colony, but colony specificity can be achieved by volatiles originating from other glands than the pheromone itself (with the apparent exception of *L. neoniger*). This differs from *T. corvina*, where colony-specific trail pheromones are secreted from a single source, the foragers' labial glands, and vary in the relative proportions of their actual constituents.

Trail pheromone specificity and its implications

The proximate mechanisms that lead to different pheromone compositions in the labial glands of foragers from different nests remain to be revealed. The biosynthesis of a specific pattern of the single pheromone components may be controlled by genetic mechanisms being inherent to all workers of a colony. Maybe the pheromone blend is more flexible and depends on the food sources used by the bees at a given time and location. Regardless of the mechanism that leads to nest-specific differences in trail pheromones, however, it is likely that foragers are able to detect the pheromones deposited by workers of foreign colonies. Nest specificity is achieved by altering the relative amounts of the same set of compounds, and foragers from all nests of a species should be able to perceive these compounds. Therefore, the recruits' discrimination between trails laid by nestmates and trails deposited by foragers from a foreign colony likely is based on the recognition of their specific pheromone blends. It remains to be answered whether recruits simply do not recognize a foreign pheromone due to the different blend of its components or whether they actively decide not to follow the pheromone of foreign conspecific foragers.

Ignoring foreign scent trails appears disadvantageous because they reliably indicate the location of a food source. So why do the bees (scouts and recruits alike) not eavesdrop on foreign trails? The selective advantage of nest-specific trail pheromones may be that they allow avoiding competition and conflicts at food sources between foragers of neighboring colonies. This is of particular importance for aggressive, group foraging bees, such as *T. corvina* (Johnson and Hubbell 1974; Roubik 1981; Johnson 1983; see also Biesmeijer and Slaa 2004). When workers from different nests of this species meet at food sources, they become entangled in fierce fights, which usually lead to the death of many individuals (Johnson and Hubbell 1974). Hence, by limiting aggressive encounters between foragers of neighboring nests and, thus, the loss of large numbers of workers, the colonies' fitness could be increased. The nest-specific composition and use of trail pheromones we have demonstrated for *T. corvina* may well be a mechanism for competition avoidance and for resource partitioning between conspecific foragers. The nests from the 3 populations we used in our study were too far away from each other for an encounter of their workers. Importantly, however, John (2008) analyzed the composition of trail pheromones from *T. corvina* foragers collected from neighboring nests within populations by means of chemical analyses and DFAs (similar to the study at hand) and found that pheromone differences based on the relative proportion of their constituents are even more pronounced between neighboring nests as compared with nests taken from different populations. This finding corroborates the hypothesis that the selective pressure to distinguish between the trail pheromones of nestmates and of foreign foragers is particularly strong when workers from different colonies have overlapping foraging areas.

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