Animal Behaviour 85 (2013) 225–232

Contents lists available at SciVerse ScienceDirect

Animal Behaviour

journal homepage: www.elsevier.com/locate/anbehav

Learnt information in species-specific 'trail pheromone' communication in stingless bees

Christian Reichle [a], Ingrid Aguilar [b], Manfred Ayasse [a], Robert Twele [c], Wittko Francke [c], Stefan Jarau [a,*]

[a] Institute for Experimental Ecology, University of Ulm, Ulm, Germany
[b] Centre for Tropical Bee Research (CINAT), National University of Costa Rica, Heredia, Costa Rica
[c] Institute for Organic Chemistry, University of Hamburg, Hamburg, Germany

ARTICLE INFO

Article history:
Received 19 July 2012
Initial acceptance 20 September 2012
Final acceptance 15 October 2012
Available online 28 November 2012
MS. number: 12-00557

Keywords:
odour learning
pheromone
recruitment communication
Scaptotrigona spp.
scent trail
signature mixture
stingless bee

Specificity in biological signalling systems is often important to keep information private. Foragers of several species of stingless bees deposit chemical marking signals to guide nestmates to food sources. The markings show species- and colony-specific compositions and primarily attract a bee's nestmates. An interesting question is whether the bees innately recognize specific trail markings or learn their particular composition from nestmates. To investigate this question, we tested whether *Scaptotrigona pectoralis* and *Scaptotrigona subobscuripennis* workers taken from their mother colonies and workers that emerged from combs transferred to foster colonies of the congeneric species are attracted to the marking compounds of workers from their natal colony or from the foster colony. A significant majority of workers were attracted to extracts prepared from foragers of the nest they inhabited, regardless of whether this was the original mother or the congeneric foster colony. Thus, the preference of stingless bee workers for specific food-marking scent mixtures is not innate, but is influenced by the odour they experience within their colony. Despite marked differences in the chemical composition of the scent marks in labial gland secretions of the two investigated species they also shared some main components. We hypothesize that recruitment trail information in stingless bees is composed of one or a few key pheromone compounds acting in conjunction with an additional signature mixture that is species and colony specific and must be learnt by recruited workers.

The transmission of species-specific information in biological signalling systems is important in some situations, for example for finding a conspecific mate by means of sex pheromones, but not in others, such as in the responses elicited by alarm pheromones (Wyatt 2003). Privacy is also advantageous for scent marks deposited by social insect foragers at or around a food source in order to communicate its location to their nestmates but not to foragers of other colonies. Not surprisingly, social insects have thus evolved species- and colony-specific chemical recruitment signals allowing them to avoid interactions with competitors at resources (Traniello 1980; Akino & Yamaoka 2005; Jarau 2009; Jarau et al. 2010, 2011; John et al. 2012).

The chemical compounds used for marking by social stingless bees are mixtures of carboxylic acid alkyl- and terpenyl esters secreted from the foragers' labial glands (Jarau et al. 2004, 2006, 2010, 2011; Schorkopf et al. 2007; Barth et al. 2008; Jarau 2009; Stangler et al. 2009; Lichtenberg et al. 2011). At a resource, bees deposit the scent marks that attract additional foragers they had recruited within the nest, thereby enhancing their domination at and quick exploitation of the resource (Lindauer & Kerr 1958, 1960; Jarau et al. 2003; Lichtenberg et al. 2010). Complete scent trails laid between a food source and the nest are not needed for successful recruitment (Nieh et al. 2003, 2004a). Rather, just a few chemical markings at the food sources provide sufficient information for recruits to find them (Schorkopf et al. 2011). None the less, the deposited compounds may potentially be detected and exploited by workers from neighbouring conspecific colonies, or even from other species with similar food requirements, that eavesdrop on the information provided (Wyatt 2003; Nieh et al. 2004b; Slaa & Hughes 2009). Competition for food is generally high among social insect colonies. Thus, eavesdropping may be a beneficial strategy to find new food sources with reduced search effort and increased foraging efficiency (Slaa & Hughes 2009). However, eavesdropping may impose costs not only for the individuals that originally deposited the food-marking signals but also for the eavesdroppers themselves. This is particularly true for social insect species that aggressively defend resources and for which the resulting fights lead to the death of many workers, such as in the stingless bees *Trigona corvina* or *Trigona hyalinata* (Jarau et al. 2010;

* Correspondence: S. Jarau, Institute for Experimental Ecology, University of Ulm, Albert-Einstein-Allee 11, 89081 Ulm, Germany.
E-mail address: stefan.jarau@uni-ulm.de (S. Jarau).

0003-3472/$38.00
http://dx.doi.org/10.1016/j.anbehav.2012.10.029

Lichtenberg et al. 2011). Thus, 'private' communication channels by means of species- and colony-specific compositions of chemical food markings that attract only a depositor's own nestmates are advantageous. Jarau et al. (2010, 2011) indeed found that scent trails of *T. corvina* and *Scaptotrigona pectoralis* are colony specific in terms of their effectiveness in triggering trail-following behaviour in recruited bees. The chemical specificity of the scent marks from different colonies arises from differences in the quantitative proportions of its single components (Jarau 2009; Jarau et al. 2010; John et al. 2012). Recently, we found that foragers of *S. pectoralis* that were exposed to a specific bouquet of scent mark compounds during recruitment in the nest were subsequently attracted to it when searching for food in the field (Reichle et al. 2011). This was even true when the scent-marking secretion from foragers of a foreign conspecific colony were presented, indicating that recruited bees learn the composition of the chemical components specific to a colony within the nest (Reichle et al. 2011).

Whether learning also plays a role in species-specific scent mark recognition and when and how the bees learn their colony's specific composition of the food site marking secretion remain interesting questions that need to be answered. The bees might learn odours from labial gland secretions of adult bees in the larval stage from the food they encounter within the brood cells, provided that it contains the crucial compounds, and then show a preference for the respective odour blend later in life as adults. The influence of preimaginal learning of olfactory cues on an individual's host-searching behaviour as adult has already been demonstrated for another hymenopteran insect, the parasitoid *Hyssopus pallidus* (Gandolfi et al. 2003). Alternatively, bees may learn the chemical components as adults only after emergence from the brood cells; for example, guards of the eusocial sweat bee *Lasioglossum zephyrum* learn the odour of their nestmates and use it as a recognition template to discriminate kin from nonkin (Buckle & Greenberg 1981; Greenberg 1988). Stingless bees mass provision their brood cells, close them after an egg has been laid by the queen, and larvae and pupae develop without any direct contact with the adult individuals of a colony. Therefore, it should be possible to distinguish between the two alternatives for odour learning, that is, during either the immature or the adult stage, as well as learning by innate recognition.

In the present study, we investigated whether workers of *S. pectoralis* and *Scaptotrigona subobscuripennis*, which emerged from brood cells that we transferred to a colony of the respective congeneric species, showed a preference for their own species' scent marks or for the foreign odour bouquet of their nestmates from the foster colony. We predicted that workers living in a foreign colony would show preferences for the foreign pheromone if the bees had learnt the respective odour blend after they emerged from the brood cells. If the bees still preferred their own pheromone, we would expect an underlying innate preference, learning of the odour bouquet from the food store in the cells or learning of their own gland secretion's composition. In addition to the bioassays we analysed the composition of the labial gland secretions from foragers of the two studied species. In particular, we asked whether scent marks of bees that spent their adult life in the nest of a foreign species resembled the secretion of their congeneric nestmates or retained its species-specific composition.

METHODS

Bee Nests and Study Site

For this study we used two nests of *S. pectoralis* and two nests of *S. subobscuripennis* (Hymenoptera, Apidae, Meliponini). The two species occur sympatrically in Costa Rica and can be easily distinguished from each other by their body coloration: orange in *S. pectoralis* (Fig. 1a) and black in *S. subobscuripennis* (Fig. 1b). The nests were collected in the surroundings of Atenas, Alajuela Province, Costa Rica, and transferred to the Centre for Tropical Bee Research (CINAT) of the National University in Heredia, Costa Rica (9°58.377′N, 84°07.754′W), where the experiments were carried out between January and June 2010. Each colony was kept in a wooden nestbox and the bees had free access to the outside. The experiments comply with the current laws of Costa Rica and Germany.

Nest Manipulation and Behavioural Bioassays

Transfer of brood combs

Prior to the bioassays we transferred brood combs containing larvae and pupae of *S. pectoralis* into a colony of *S. subobscuripennis* and vice versa. Thus, for both species we obtained bees that emerged from brood cells and lived in their mother colony, as well as bees that grew up in a foster nest of the respective congeneric species after emerging from their cells. Newly emerged bees of both species were accepted by the workers of their foster colonies. A few weeks after the introduction of the combs they were observed to work as foragers at the nest entrances. Owing to the distinct colour differences between the two species (Fig. 1) the introduced bees could easily be distinguished from the nests' native workers.

Test substances for bioassays

We prepared extracts of the cephalic parts of the labial glands that produce the food-marking signals (Jarau 2009) from foraging bees that were collected at the entrance of their nests and killed by

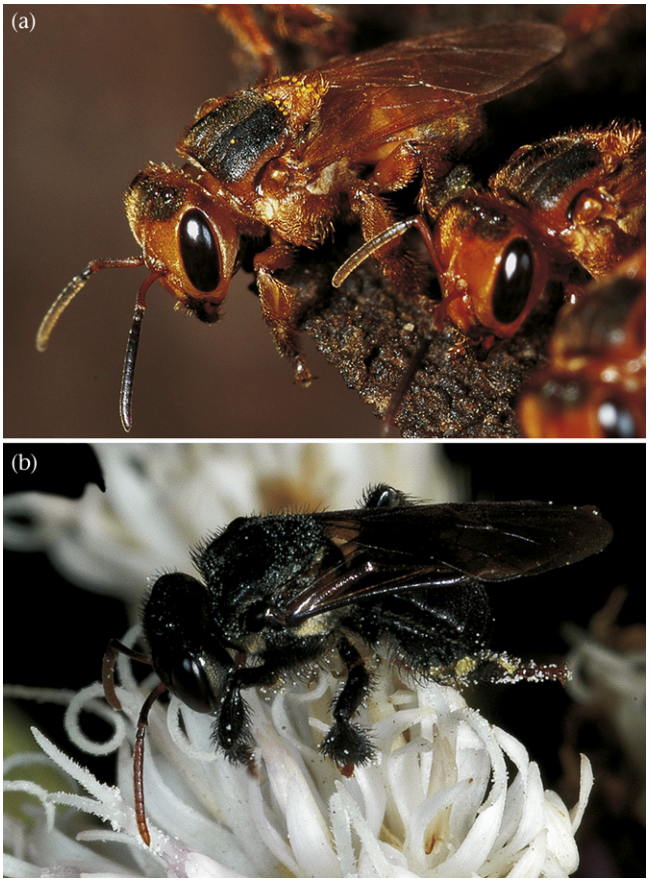

Figure 1. The two stingless bee species used for this study: (a) *Scaptotrigona pectoralis* and (b) *Scaptotrigona subobscuripennis*.

freezing at −15 °C. The glands were dissected in saline solution under a stereo microscope by carefully removing all tissues other than the gland acini and placed in hexane for 24 h at room temperature. The resulting extracts were stored in a freezer (−15 °C). For the bioassays we extracted five pairs of labial glands in 500 μl hexane. Thus, 100 μl of extract corresponded to the gland content of one individual bee.

Bioassay set-up

We tested whether bees taken from their mother colony and bees that emerged in a foster colony of a congeneric species were attracted by the scent mark volatiles of sister workers from their original (mother) colonies or by the volatiles of the unrelated workers from the foster colonies. In control experiments, bees taken from the mother colony of each species had to choose between pure solvent and the blend of labial gland compounds from their sister nestmates. Thus, for each species we registered the choice of foragers collected from their mother nest (1) between the labial gland secretion of their sister nestmates and that of the foreign species or (2) between the labial gland secretion of their sisters and the solvent hexane, as well as (3) the choice of foragers collected from their foster nests between the labial gland secretion of their sisters and that of nestmate foragers of the foreign species.

To test the attractiveness of the different labial gland extracts to foragers, we recorded their choice behaviour in Y-tube olfactometers (glass tubes with 0.8 cm inner diameter; stem length 12 cm; length of side arms 10 cm; angle between side arms 55°). To avoid any influence of visual cues on the bees' behaviour we conducted the experiments in a dark room under red light. The two arms of the Y-tubes were connected to a motor pump (Laboratory Power Supply, PS-302A, Volcraft) with silicone tubes. A cylindrical borosilicate glass cartridge packed with activated charcoal (ORBO-32, Supelco) filtered and cleaned the air from atmospheric pollutants before it was directed into the arms of the Y with a constant flow of 100 ml/min. For each test, small filter papers (4 × 15 mm) baited with 10 μl of a labial gland extract, or treated with pure solvent, were placed at each end of the shorter Y-tube arms. A single worker bee, used only once for a test, was then released into the long arm of the Y-tube, and its choice at the branching arms was recorded. A side was registered as 'chosen' when the bee touched the filter paper at the end of the tube. For each test, a new, ethanol-cleaned and dried Y-tube and new filter papers were used. Each experimental series testing the choice between two gland extracts was repeated with at least 40 individual workers. Control experiments testing a gland extract against the pure solvent were carried out with at least 20 workers. To account for potential side preferences by the bees the positions of the arms bearing the extract or the solvent control papers were changed between trials.

Chemical Analyses

For quantitative chemical analyses of the labial gland secretions, we collected foraging bees from the *S. pectoralis* and the *S. subobscuripennis* nests that served as foster colonies for the bees of the respective congeneric species. From each nest we collected original foragers as well as bees of the foreign species (8–10 individuals each). Labial glands of workers were dissected as described above, extracted individually in 200 μl hexane for 24 h at room temperature, and subsequently stored in a freezer (−15 °C). Prior to gas chromatographic analyses, each extract was concentrated to 45 μl. We injected 1 μl per sample into a gas chromatograph (GC; HP 5890 Series II, Palo Alto, CA, U.S.A.) equipped with a DB-5MS column (30 m × 0.25 mm inner diameter, 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 the extracted compounds, the samples were analysed by a Fisons Instruments gas chromatograph series 8008 linked to a Fisons MD800 mass spectrometer (Fisons Instruments). Separations were performed on 30 m × 0.25 mm inner diameter nonpolar fused silica columns coated with CP8912 VF-1MS or CP8944 VF-5MS (Varian), respectively, 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 the carrier gas. Structure assignments of compounds were based on comparison of mass spectra with literature data (McLafferty & Stauffer 1989) and with mass spectra and retention times of authentic reference substances. Determination of double bond position in unsaturated compounds was carried out by GC/MS-investigations of bis(methylthio)-derivatives (Francis & Veland 1981; Buser et al. 1983), which were prepared by the addition of dimethyldisulphide to crude extracts. Assignment of branching positions in methylalkanes was based on their mass spectra (Pomonis et al. 1978; Doolittle et al. 1995).

Noncommercial compounds were synthesized for reference: racemates of long chain secondary alcohols were obtained by reduction of methylketones with lithium aluminium tetrahydride. Carboxylic acid esters were prepared by reaction of corresponding alcohols with appropriate acid chlorides according to laboratory standards. Alkenes were prepared by Wittig-reaction of corresponding aldehydes with appropriate alkyltriphenylphosphonium bromides. Methylalkanes were synthesized by Wittig-reaction of corresponding methylketones with appropriate alkyltriphenylphosphonium bromides according to laboratory standards. The obtained branched alkenes were hydrogenated over Pd/C-catalyst.

Statistics

The behaviour of workers in the Y-tube experiments was analysed with chi-square tests using absolute choice numbers to detect significant deviations of the bees' choice from a random choice. Prior to the chi-square tests, 2 × 2 chi-square analyses of contingency tables were conducted for each species to check whether data obtained for the different nests could be pooled for further analyses. To compare the chemical composition of the labial gland secretions of *S. pectoralis* and *S. subobscuripennis* foragers taken from their mother colonies or from the respective congeneric host nests in which they emerged, we conducted nonparametric multivariate analyses based on 30 compounds shared by foragers of both species using the program PAST version 2.15 (Hammer et al. 2001). We performed a nonmetric multidimensional scaling (NMDS) analysis based on the calculation of Bray–Curtis distances to visualize differences between species and bees from different nests, respectively. Bray–Curtis distances were also used for a one-way analysis of similarities (ANOSIM) with 10 000 permutations to test for significance in dissimilarities between the chemical profiles of workers of different species and nest origins and between single pairs of species and nest origins (sequential Bonferroni-adjusted *P* values). Compounds that predominantly contributed to the Bray–Curtis dissimilarities between workers collected from different species and nests were identified by calculating similarity percentages (SIMPER).

RESULTS

Scent Mark Preference

The bioassay data obtained in the Y-tube experiments did not differ between the experiments conducted with the two nests of

each species (2×2 chi-square analyses of contingency tables: *S. pectoralis* from mother nest: $\chi^2_1 = 4.1 \times 10^{-15}$, $P = 1$; *S. pectoralis* from host nest: $\chi^2_1 = 0.154$, $P = 0.693$; *S. subobscuripennis* from mother nest: $\chi^2_1 = 3.3 \times 10^{-15}$, $P = 1$; *S. subobscuripennis* from host nest: $\chi^2_1 = 1.0 \times 10^{-14}$, $P = 1$). Therefore, data for each species were pooled for further analyses. Workers of both species showed a significant preference for the labial gland extract of their genetic sisters over the extract of foragers from the congeneric species when they were taken from their mother colony. In *S. pectoralis* 68% of the tested bees chose their own pheromone (chi-square test: $\chi^2_1 = 4.9$, $N = 40$, $P < 0.05$; Fig. 2) and in *S. subobscuripennis* 77% (chi-square test: $\chi^2_1 = 12.1$, $N = 40$, $P < 0.001$; Fig. 3). In contrast, the bees that emerged from combs transferred to a nest of the respective congeneric species significantly preferred the labial gland volatiles of nestmate foragers from the foster colony over the volatiles of their genetic sisters. In *S. pectoralis* 80% (chi-square test: $\chi^2_1 = 14.4$, $N = 40$, $P < 0.001$; Fig. 2) and in *S. subobscuripennis* 73% (chi-square test: $\chi^2_1 = 8.1$, $N = 40$, $P < 0.01$; Fig. 3) of the bees chose the foreign scent mark compounds. In the control experiments, the bees were significantly attracted by the labial gland extract of their sister nestmates rather than by pure solvent (chi-square tests: *S. pectoralis*: $\chi^2_1 = 5.0$, $N = 20$, $P < 0.05$; Fig. 2; *S. subobscuripennis*: $\chi^2_1 = 9.8$, $N = 20$, $P < 0.01$; Fig. 3).

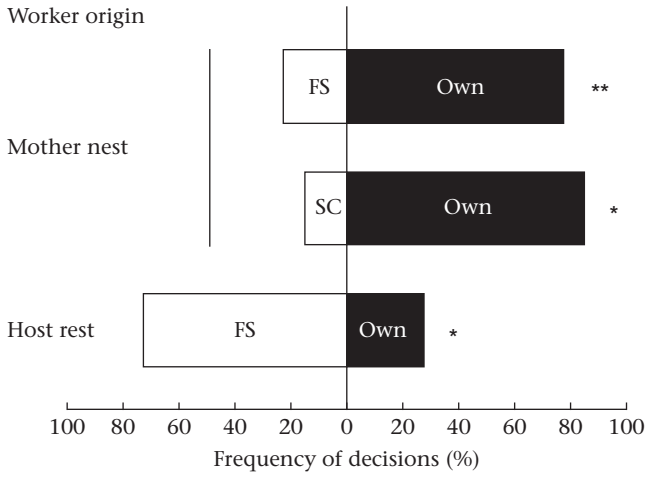

Figure 3. Attraction of *Scaptotrigona subobscuripennis* workers that grew up in their mother nest or in a *Scaptotrigona pectoralis* host nest to labial gland extracts of their own species (Own), to labial gland extracts of the foreign species (FS) or to the solvent control (SC). Data for experiments testing two gland extracts are based on 40 bees that chose one side of the Y-tube; data for the solvent control are for 20 reacting bees. $^*P \leq 0.05$; $^{**}P \leq 0.001$ (chi-square tests). Throughout the experiments nine additional bees did not react in the olfactometer and were not counted.

Labial Gland Chemistry

From the labial gland extracts of *S. pectoralis* 54 compounds were identified (Fig. 4, Table 1). Esters, comprising 28 compounds, represented the main class of volatiles in the labial glands of this species. The group was dominated by decyl hexanoate, decyl octanoate and its isomer, dodecyl hexanoate, each comprising more than 10% of the glands' content, followed by (5*Z*)-tetradecenyl butyrate and tetradecyl butyrate. Esters of methyl carbinols such as 2-pentadecyl butyrate represent an interesting subgroup of chiral volatiles; however, the enantiomeric compositions remain unknown. As indicated above, isomeric wax type esters such as decyl decanoate, dodecyl octanoate, tetradecyl hexanoate and hexadecyl butyrate formed small clusters. In addition to esters we detected five *n*-alkanes, two alkenes, eight alcohols (two primary alcohols and six methyl carbinols), three aldehydes, seven methyl ketones and one isoprenoid in rather small amounts (less than 1% of the entire blend; Table 1).

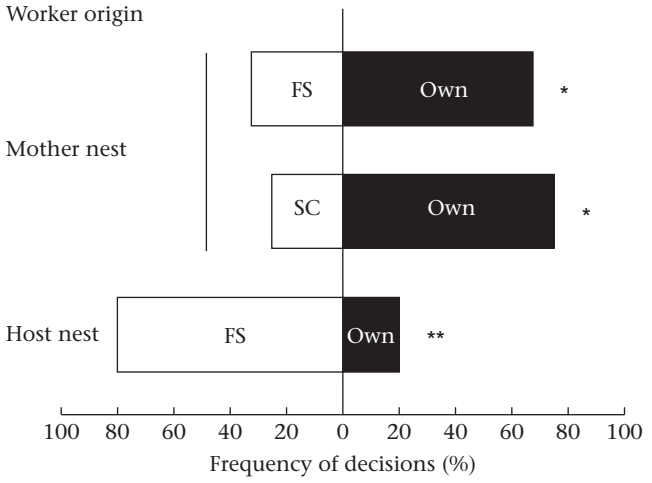

Figure 2. Attraction of *Scaptotrigona pectoralis* workers that grew up in their mother nest or in a *Scaptotrigona subobscuripennis* host nest to labial gland extracts of their own species (Own), to labial gland extracts of the foreign species (FS) or to the solvent control (SC). Data for experiments testing two gland extracts are based on 40 bees that chose one side of the Y-tube; data for the solvent control are for 20 reacting bees. $^*P \leq 0.05$; $^{**}P \leq 0.001$ (chi-square tests). Throughout the experiments seven additional bees did not react in the olfactometer and were not counted.

From the labial gland extracts of *S. subobscuripennis* 51 compounds were identified (Fig. 4, Table 1). Similar to *S. pectoralis*, carboxylic acid alkyl esters (16 compounds) were among the most abundant components but straight chain and branched, saturated and unsaturated hydrocarbons (together 23 compounds) dominated the labial gland secretions of *S. subobscuripennis* (Table 1). The main compounds were the esters (5*Z*)-tetradecenyl butyrate and decyl octanoate, as well as the alkenes (9*Z*)-pentacosene and (9*Z*)-heptacosene. In addition to esters and hydrocarbons we found three alcohols (all methyl carbinols), one aldehyde, six methyl ketones and two isoprenoids in minor quantities in the *S. subobscuripennis* extracts (Table 1). The occurrence of branched hydrocarbons in *S. subobscuripennis* is interesting because they were completely lacking in the labial gland secretions of *S. pectoralis*.

Comparison of the labial gland secretions of the two species reveals similar qualitative compositions; however, distinct differences in relative proportions both in some esters and some hydrocarbons are obvious (see following section).

Influence of Nest Environment on Labial Gland Chemistry

NMDS revealed that *S. pectoralis* and *S. subobscuripennis* workers were fully differentiable on the basis of their labial gland chemical profiles, whereas the profiles of workers of a particular species taken from their mother nest and from a host nest showed some degree of overlap (Fig. 5). ANOSIM results, however, demonstrated large dissimilarities between all compared groups of worker species and nest origins (global $R = 0.741$, $P < 0.0001$), as well as between all pairwise comparisons: *S. pectoralis* versus *S. subobscuripennis* species comparison: $R = 0.589$, $P < 0.0004$; *S. pectoralis* workers from host colony versus their *S. subobscuripennis* nestmates: $R = 0.963$, $P < 0.0001$; *S. subobscuripennis* workers from host colony versus their *S. pectoralis* nestmates: $R = 0.570$, $P < 0.0001$; *S. pectoralis* workers from mother versus host colony: $R = 0.823$, $P < 0.0002$; *S. subobscuripennis* workers from mother versus host

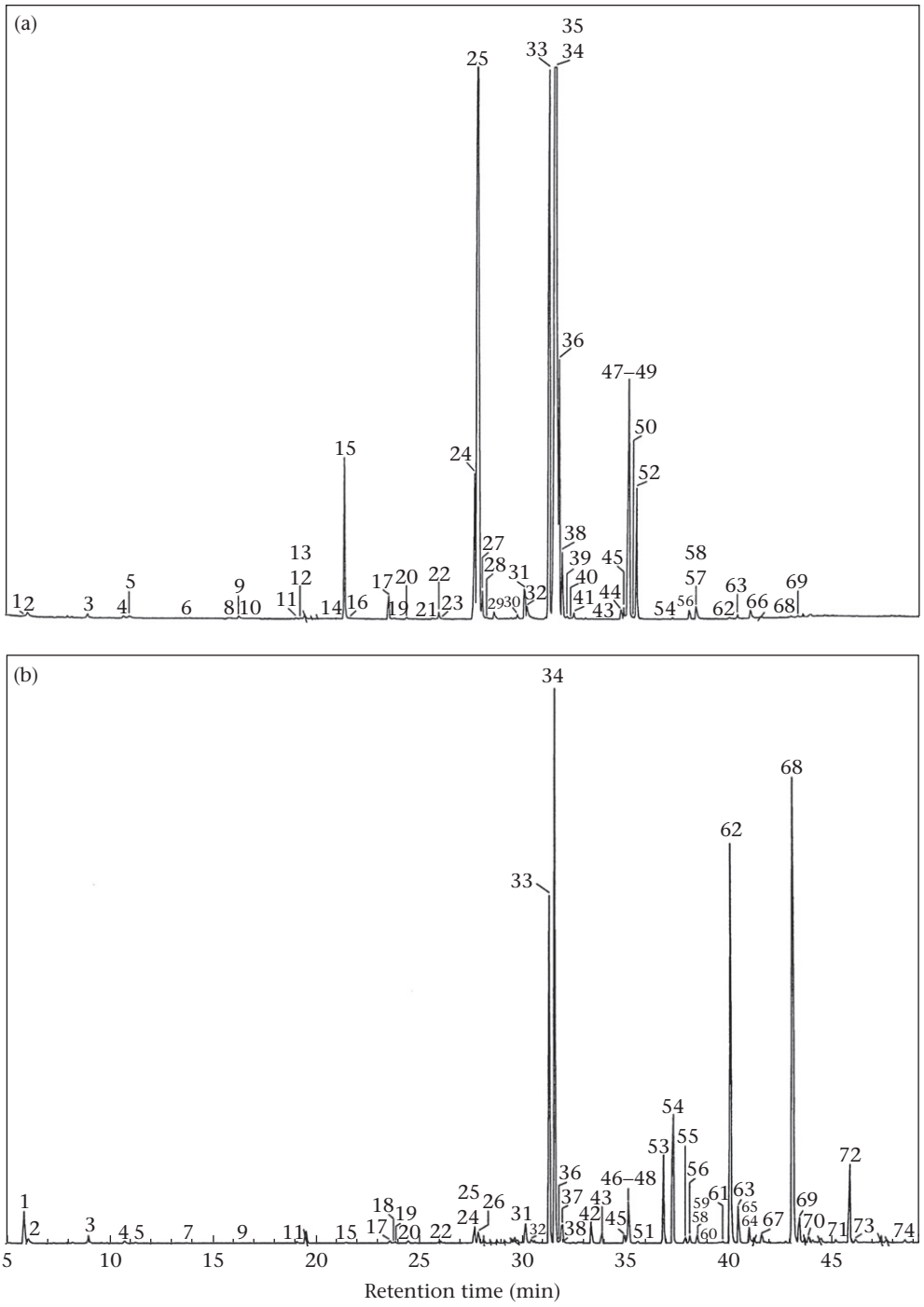

Figure 4. Gas chromatographic separation of the compounds extracted from labial glands of (a) *Scaptotrigona pectoralis* and (b) *Scaptotrigona subobscuripennis* foragers. Names of identified compounds are given in Table 1 according to the peak numbers in this figure.

colony: $R = 0.603$, $P < 0.001$. Results of SIMPER analyses showed that the compounds that predominantly (over 10% each) contributed to the dissimilarities between the composition of labial gland secretions of the two species were (5*Z*)-tetradecenyl butyrate, octyl octanoate and decyl octanoate. The same compounds also accounted for the differences between *S. subobscuripennis* workers from a host colony and their *S. pectoralis* nestmates. Decyl hexanoate, (5*Z*)-tetradecenyl butyrate and decyl octanoate caused the dissimilarities between *S. pectoralis* workers from a host colony and their *S. subobscuripennis* nestmates. Dissimilarities between *S. pectoralis* workers from their mother nest and the host colony were mainly due to decyl hexanoate, octyl octanoate and decyl octanoate and dissimilarities between *S. subobscuripennis* workers from their mother nest and the host colony were caused by (5*Z*)-tetradecenyl butyrate and decyl octanoate.

DISCUSSION

Stingless bees use species- and colony-specific chemical markings at food sources that allow a forager to communicate the location of food primarily to her nestmates (Jarau 2009; Jarau et al. 2010, 2011). However, it was not known whether the recognition of

Table 1
Relative abundance of compounds identified from labial gland secretions of *Scaptotrigona pectoralis* and *Scaptotrigona subobscuripennis* foragers

	Peak no. in Fig. 4	Relative abundance	
		S. pectoralis	*S. subobscuripennis*
n-Alkanes			
Dodecane	7		+
Tetradecane	11	+	+
Hexadecane	19	+	+
Heneicosane	43		+
Docosane	51		+
Tricosane	54	+	+
Tetracosane	60		+
Pentacosane	63	+	++
Heptacosane	69	+	+
Nonacosane	73		+
Methyl branched alkanes			
11-Methyl tricosane	55		+
3-Methyl tricosane	59		+
13-Methyl pentacosane	64		+
11-Methyl pentacosane	65		+
11-Methyl heptacosane	70		+
Alkenes			
Heneicosene	42		+
(9*Z*)-Tricosene*	53		++
(9*Z*)-Pentacosene*	62	+	++++
Hexacosene	67		+
(9*Z*)-Heptacosene*	68	+	++++
(9*Z*)-Nonacosene*	72		++
Hentriacontene	74		+
Alkadiene			
Pentacosadiene	61		+
Alcohols			
Heptan-2-ol	2	+	+
Nonan-2-ol	5	+	+
Decan-1-ol	7	+	
Undecan-2-ol	10	+	
Dodecan-1-ol	14	+	
Tridecan-2-ol	16	+	
Pentadecan-2-ol	23	+	
Heptadecan-2-ol	32	+	+
Aldehydes			
Decanal	6	+	
Dodecanal	13	+	
Octadecenal	37		+
Octadecanal	40	+	
Ketones			
Heptan-2-one	1	+	++
Nonan-2-one	4	+	+
Undecan-2-one	9	+	+
Tridecan-2-one	15	++	+
Pentadecan-2-one	22	+	+
Heptadecan-2-one	31	+	+
Nonadecan-2-one	43	+	
Esters			
2-Heptyl hexanoate	12	+	
Decyl butyrate	17	+	+
2-Heptyl octanoate	18		+
Isopropyl dodecanoate	20	+	+
Decyl pentanoate	21	+	
Octyl octanoate	24	++	+
Decyl hexanoate	25	++++	+
Dodecyl butyrate	26		+
2-Tridecyl butyrate	27	+	
Tetradecyl acetate	28	+	
2-Pentadecyl acetate	29	+	
Dodecyl pentanoate	30	+	
(5*Z*)-Tetradecenyl butyrate	33	+++	++++
Decyl octanoate	34	++++	++++
Dodecyl hexanoate	35	++++	
Tetradecyl butyrate	36	+++	+
2-Pentadecyl butyrate	38	++	+
Hexadecyl acetate	39	+	
2-Heptadecyl acetate	41	+	
(5*Z*)-Tetradecenyl hexanoate	44	+	
Hexadecenyl butyrate	45	+	+
Ethyl (9*Z*)-octadecenoate	46		+
Decyl decanoate	47	++	+
Dodecyl octanoate	48	++	+
Tetradecyl hexanoate	49	++	
Hexadecyl butyrate	50	+	
2-Heptadecyl butyrate	52	++	
(5*Z*)-Tetradecenyl octanoate	56	+	+
Dodecyl decanoate	57	+	
Tetradecyl octanoate	58	+	+
Decyl tetradecenoate	66	+	
Isoprenoids			
Limonene	3	+	+
Squalene	71		+

+: <1%; ++: >1%; +++: >5%; ++++: >10% of the entire composition of the blend.
* Double bond position and geometry verified only for *S. subobscuripennis*.

species-specific blends of volatiles by recruited workers is innate or the outcome of a learning process, a distinction necessary to understand the mechanisms underlying recruitment communication. Recently, Wyatt (2010) distinguished between pheromones and signature mixtures. Pheromones are defined as chemical compounds that act as evolved signals between two individuals of the same species and elicit a specific, innate reaction, whereas signature mixtures are variable subsets of molecules in an animal's chemical profile that are learnt by other conspecifics and used to recognize individuals or members of a particular social group, such as a social insect colony (Wyatt 2010). The important difference is that pheromones are chemical signals that are fixed within a species, whereas in signature mixtures the differences between the odour composition of different individuals or social groups are the message.

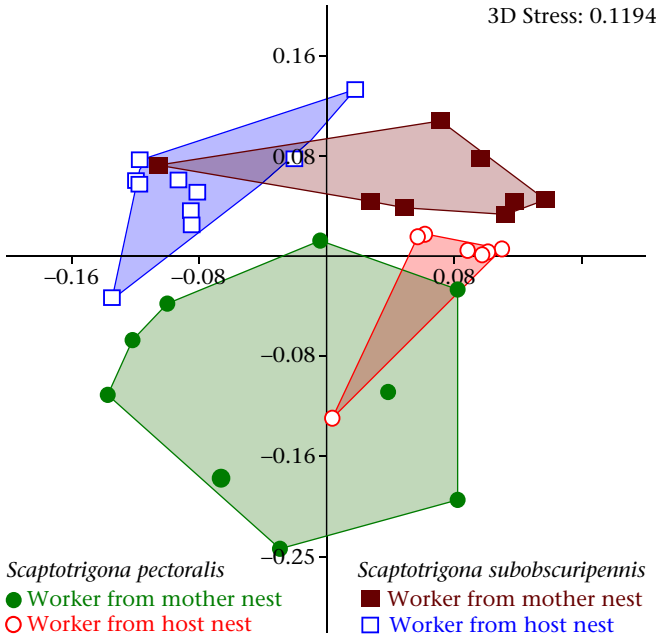

Figure 5. Nonmetric multidimensional scaling visualization of differences in the chemical composition of labial gland secretions of *Scaptotrigona pectoralis* and *Scaptotrigona subobscuripennis* foragers (35 individuals in total) collected from their mother nests or from host nests of the respective congeneric species in which they emerged from experimentally introduced brood combs.

Our bioassays have shown that workers of *S. pectoralis* and *S. subobscuripennis* learn the composition of specific recruitment scent marks within their nest and are subsequently attracted to the respective blend of chemical compounds. This is true even when the specific blend of volatiles is that of a foreign species. Most importantly, our results show that the preference for a specific 'trail pheromone', as the respective scent marks are usually called (Jarau 2009), in workers of stingless bees is not innate. Rather, the bees learn the chemicals specific to their nest within the colony as they do with floral odours (stingless bees: Reichle et al. 2010; honeybees: Farina et al. 2007; Farina & Grüter 2009; Reinhard & Srinivasan 2009; bumblebees: Molet et al. 2009). We cannot exclude, however, the possibility that the bees identify the gland secretion of the other species by certain key compounds. Both species share (5*Z*)-tetradecenyl butyrate and decyl octanoate as major constituents of their labial glands. These compounds alone could indicate that foragers marked a food source. However, our experiments show that recognition of such key compounds, which could be innate, is not sufficient to attract the workers. A more complex blend of compounds from the labial glands is apparently needed to specify or enhance the activity of the signal communicated by the key compounds, as was suggested for another scent trail-laying stingless bee species, *Trigona recursa* (Jarau et al. 2006). The labial gland secretions of *S. pectoralis* and *S. subobscuripennis* showed distinct differences, despite the shared abundant esters (see Table 1). These species-specific differences were also found in workers that lived in a congeneric host nest, as revealed by ANOSIM and SIMPER analyses, and were primarily caused by the glands' main components. In accord with the distinction made by Wyatt (2010) we hypothesize that stingless bee recruitment trail information is composed of one or a few key pheromone components that act in conjunction with an additional signature mixture, which is specific for a colony and has to be learnt by recruited workers.

The composition of the labial gland secretions of foragers of both species that lived in the colony of the respective congeneric species differed from the labial gland secretions of their genetic sisters that were left in the mother colony. Importantly, however, the pheromone of foragers living in a foreign nest did not match the composition of the pheromone of the congeneric workers of their host nest. The demonstrated differences may be explained by different diets of the bees (C. Reichle, personal observation). Unfortunately, little is known about the influence of the diet of bees on their gland constituents. Nevertheless, our findings indicate that the biosynthesis of labial gland secretions is not fixed for workers of a particular colony. As differences in colony nutrition caused by changes in available resources at different times of the year may influence the biosynthesis of the foragers' labial gland secretions, learning a particular signature mixture that adds specificity to the trail pheromone is indeed essential for colony recruitment to work.

The present study provides a new view of the mechanisms of 'trail pheromone' communication in social insects by emphasizing the importance of learning in this context. Unfortunately, the intranidal recruitment behaviour of trail-laying species of stingless bees remains largely unknown, and details about how the chemical blends comprising trail pheromones and nest-specific signature mixtures are released by foraging bees and perceived by recruits remain elusive. Future studies should focus on the recruitment process within the nest to illuminate how, when and where learning of signature mixtures takes place in scent trail-laying social insects.

Acknowledgments

We thank Javier Guevara from the Ministerio del Ambiente y Energia (MINAE) in Costa Rica for his help in obtaining a research permit (190-2009-SINAC), as well as the German Academic Exchange Service (DAAD, scholarship to C. R.) and the German Research Foundation (DFG, project JA 1715/3-1 granted to S. J.) for financial support.

References

Akino, T. & Yamaoka, R. 2005. Trail discrimination signal of *Lasius japonicus* (Hymenoptera: Formicidae). *Chemoecology*, **15**, 21–30, http://dx.doi.org/10.1007/s00049-005-0288-6.

Barth, F. G., Hrncir, M. & Jarau, S. 2008. Signals and cues in the recruitment behavior of stingless bees (Meliponini). *Journal of Comparative Physiology A*, **194**, 313–327, http://dx.doi.org/10.1007/s00359-008-0321-7.

Buckle, G. R. & Greenberg, L. 1981. Nestmate recognition in sweat bees (*Lasioglossum zephyrum*): does an individual recognize its own odour or only odours of its nestmates? *Animal Behaviour*, **29**, 802–809.

Buser, H.-R., Arn, H., Guerin, P. & Rauscher, S. 1983. Determination of double bond position in mono-unsaturated acetates by mass spectrometry of dimethyl disulfide adducts. *Analytical Chemistry*, **55**, 818–822.

Doolittle, R. E., Proveaux, A. T., Alborn, H. T. & Heath, R. R. 1995. Quadrupole storage mass spectrometry of mono- and dimethylalkanes. *Journal of Chemical Ecology*, **21**, 1677–1695.

Farina, W. M. & Grüter, C. 2009. Trophallaxis: a mechanism of information transfer. In: *Food Exploitation by Social Insects. Ecological, Behavioral, and Theoretical Approaches* (Ed. by S. Jarau & M. Hrncir), pp. 183–197. Boca Raton: CRC Press.

Farina, W. M., Grüter, C., Acosta, L. & Mc Cabe, S. 2007. Honeybees learn floral odors while receiving nectar from foragers within the hive. *Naturwissenschaften*, **94**, 55–60, http://dx.doi.org/10.1007/s00114-006-0157-3.

Francis, G. W. & Veland, K. 1981. Alkylthiolation for the determination of double-bond positions in linear alkenes. *Journal of Chromatography*, **219**, 379–384.

Gandolfi, M., Mattiacci, L. & Dorn, S. 2003. Preimaginal learning determines adult responses to chemical stimuli in parasitic wasps. *Proceedings of the Royal Society B*, **270**, 2623–2629, http://dx.doi.org/10.1098/rspb.2003.2541.

Greenberg, L. 1988. Kin recognition in the sweat bee, *Lasioglossum zephyrum*. *Behavior Genetics*, **18**, 425–438.

Hammer, Ø., Harper, D. A. T. & Ryan, P. D. 2001. PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica*, **4**, 9, http://palaeo-electronica.org/2001_1/past/issue1_01.htm.

Jarau, S. 2009. Chemical communication during food exploitation in stingless bees. In: *Food Exploitation by Social Insects. Ecological, Behavioral, and Theoretical Approaches* (Ed. by S. Jarau & M. Hrncir), pp. 223–249. Boca Raton: CRC Press.

Jarau, S., Hrncir, M., Schmidt, V. M., Zucchi, R. & Barth, F. G. 2003. Effectiveness of recruitment behavior in stingless bees (Apidae, Meliponini). *Insectes Sociaux*, **50**, 365–374, http://dx.doi.org/10.1007/s00040-003-0684-2.

Jarau, S., Hrncir, M., Zucchi, R. & Barth, F. G. 2004. A stingless bee uses labial gland secretions for scent trail communication (*Trigona recursa* Smith 1863). *Journal of Comparative Physiology A*, **190**, 233–239, http://dx.doi.org/10.1007/s00359-003-0489-9.

Jarau, S., Schulz, C. M., Hrncir, M., Francke, W., Zucchi, R., Barth, F. G. & Ayasse, M. 2006. Hexyl decanoate, the first trail pheromone compound identified in a stingless bee, *Trigona recursa*. *Journal of Chemical Ecology*, **32**, 1555–1564, http://dx.doi.org/10.1007/s10886-006-9069-0.

Jarau, S., Dambacher, J., Twele, R., Aguilar, I., Francke, W. & Ayasse, M. 2010. The trail pheromone of a stingless bee, *Trigona corvina* (Hymenoptera, Apidae, Meliponini), varies between populations. *Chemical Senses*, **35**, 593–601, http://dx.doi.org/10.1093/chemse/bjq057.

Jarau, S., Hemmeter, K., Aguilar, I. & Ayasse, M. 2011. A scientific note on trail pheromone communication in a stingless bee, *Scaptotrigona pectoralis* (Hymenoptera, Apidae, Meliponini). *Apidologie*, **42**, 708–710, http://dx.doi.org/10.1007/s13592-011-0070-4.

John, L., Aguilar, I., Ayasse, M. & Jarau, S. 2012. Nest-specific composition of the trail pheromone of the stingless bee *Trigona corvina* within populations. *Insectes Sociaux*, **59**, 527–532, http://dx.doi.org/10.1007/s00040-012-0247-5.

Lichtenberg, E. M., Imperatriz-Fonseca, V. L. & Nieh, J. C. 2010. Behavioral suites mediate group-level foraging dynamics in communities of tropical stingless bees. *Insectes Sociaux*, **57**, 105–113, http://dx.doi.org/10.1007/s00040-009-0055-8.

Lichtenberg, E. M., Hrncir, M., Turatti, I. C. & Nieh, J. C. 2011. Olfactory eavesdropping between two competing stingless bee species. *Behavioral Ecology and Sociobiology*, **65**, 763–774, http://dx.doi.org/10.1007/s00265-010-1080-3.

Lindauer, M. & Kerr, W. E. 1958. Die gegenseitige Verständigung bei den stachellosen Bienen. *Zeitschrift für vergleichende Physiologie*, **41**, 405–434.

Lindauer, M. & Kerr, W. E. 1960. Communication between the workers of stingless bees. *Bee World*, **41**, 29–41 &; 65–71.

McLafferty, F. W. & Stauffer, D. R. (Eds). 1989. *Wiley/NBS Registry of Mass Spectral Data*. New York: Wiley Interscience.

Molet, M., Chittka, L. & Raine, N. E. 2009. How floral odors are learned inside the bumblebee (*Bombus terrestris*) nest. *Naturwissenschaften*, **96**, 213–219, http://dx.doi.org/10.1007/ s00114-008-0465-x.

Nieh, J. C., Contrera, F. A. L. & Nogueira-Neto, P. 2003. Pulsed mass recruitment by a stingless bee, *Trigona hyalinata*. *Proceedings of the Royal Society B*, **270**, 2191–2196, http://dx.doi.org/10.1098/rspb.2003.2468.

Nieh, J. C., Contrera, F. A. L., Yoon, R. R., Barreto, L. S. & Imperatriz-Fonseca, V. L. 2004a. Polarized short odor-trail recruitment communication by a stingless bee, *Trigona spinipes*. *Behavioral Ecology and Sociobiology*, **56**, 435–448, http://dx.doi.org/10.1007/s00265-004-0804-7.

Nieh, J. C., Barreto, L. S., Contrera, F. A. L. & Imperatriz-Fonseca, V. L. 2004b. Olfactory eavesdropping by a competitively foraging stingless bee, *Trigona spinipes*. *Proceedings of the Royal Society B*, **271**, 1633–1640, http://dx.doi.org/10.1098/rspb, 2004.2717.

Pomonis, J. G., Fatland, C. F., Nelson, D. R. & Zaylskie, R. G. 1978. Insect hydrocarbons: corroboration of structure by synthesis and mass spectrometry of mono- and dimethylalkanes. *Journal of Chemical Ecology*, **4**, 27–39.

Reichle, C., Jarau, S., Aguilar, I. & Ayasse, M. 2010. Recruits of the stingless bee *Scaptotrigona pectoralis* learn food odors from the nest atmosphere. *Naturwissenschaften*, **97**, 519–524, http://dx.doi.org/10.1007/s00114-010-0662-2.

Reichle, C., Aguilar, I., Ayasse, M. & Jarau, S. 2011. Stingless bees (*Scaptotrigona pectoralis*) learn foreign trail pheromones and use them to find food. *Journal of Comparative Physiology A*, **197**, 243–249, http://dx.doi.org/10.1007/s00359-010-0605-6.

Reinhard, J. & Srinivasan, M. V. 2009. The role of scents in honey bee foraging and recruitment. In: *Food Exploitation by Social Insects. Ecological, Behavioral, and Theoretical Approaches* (Ed. by S. Jarau & M. Hrncir), pp. 165–182. Boca Raton: CRC Press.

Schorkopf, D. L. P., Jarau, S., Francke, W., Twele, R., Zucchi, R., Hrncir, M., Schmidt, V. M., Ayasse, M. & Barth, F. G. 2007. Spitting out information: *Trigona* bees deposit saliva to signal resource locations. *Proceedings of the Royal Society B*, **274**, 895–898, http://dx.doi.org/10.1098/rspb.2006.3766.

Schorkopf, D. L. P., Morawetz, L., Bento, J. M. S., Zucchi, R. & Barth, F. G. 2011. Pheromone path attached to the substrate in meliponine bees: helpful but not obligatory for recruitment success. *Journal of Comparative Physiology A*, **197**, 755–764, http://dx.doi.org/10.1007/s00359-011-0638-5.

Slaa, E. J. & Hughes, W. O. H. 2009. Local enhancement, local inhibition, eavesdropping, and the parasitism of social insect communication. In: *Food Exploitation by Social Insects. Ecological, Behavioral, and Theoretical Approaches* (Ed. by S. Jarau & M. Hrncir), pp. 147–164. Boca Raton: CRC Press.

Stangler, E. S., Jarau, S., Hrncir, M., Zucchi, R. & Ayasse, M. 2009. Identification of trail pheromone compounds from the labial glands of the stingless bee *Geotrigona mombuca*. *Chemoecology*, **19**, 13–19, http://dx.doi.org/10.1007/s00049-009-0003-0.

Traniello, J. F. A. 1980. Colony specificity in the trail pheromone of an ant. *Naturwissenschaften*, **67**, 361–362.

Wyatt, T. D. 2003. *Pheromones and Animal Behaviour. Communication by Smell and Taste*. Cambridge: Cambridge University Press.

Wyatt, T. D. 2010. Pheromones and signature mixtures: defining species wide signals and variable cues for identity in both invertebrates and vertebrates. *Journal of Comparative Physiology A*, **196**, 685–700, http://dx.doi.org/10.1007/s00359-010-0564-y.