



Recruitment Behaviour in an African Meliponine Bee Species (hymenoptera, Apidae: Meliponini): Understanding Glandular Origin and Pheromonal Components

Bridget O. Bobadoye^{1*}, Olaniyi T. Alamu¹, Jennifer A. George-Onaho¹, John A. Ete¹, Ayangbade E. Ayandokun¹, Ifeoluwa S. Agboola¹, Olubusola T. Adeoye¹ and Popoola J. Adekola²

¹Forestry Research Institute of Nigeria, P.M.B. 5054, Jericho Hills, Ibadan Oyo State, Nigeria.

²Federal College of Forestry, P.M.B. 5057, Jericho Hills, Ibadan Oyo State, Nigeria.

*Corresponding author, email: bridgetbobadoye@gmail.com

ORCID ID: 0000-0001-6042-4065

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Abstract

Meliponine bees are speculated to use a variety of communication mechanisms to effectively recruit workers of a colony to collect sufficient amounts of food to nourish the entire nest population. Mechanisms used to convey such information include thoracic vibrations and trophallaxis within the nest; footprint secretions and pheromone marks deposited in the field, or a combination of these signals and cues. There have been numerous discrepancies about the origin of trail pheromone production from the head, thorax, abdomen and leg regions of meliponine bees. Because the glandular origin of pheromone marks deposited by African meliponine bee's species has not yet been investigated, we first confirmed if these species actually carry out scent marking and recruitment behaviour at visited food sources. Secondly, we tested if either nasonov or tarsal gland secretions elicited trail-following behaviour in newly recruited bees by means of chemical and electro-physiological analyses as well as with bioassays testing both natural extracts and synthetic pheromone compounds from both glands. Significant differences were observed in the foraging patterns of the four bee species on collected resources (nectar, pollen and water) as the synthetic compound, (*E*)- β -farnesene was significantly as attractive to foragers of the four species when compared to the natural nasonov gland extract. Our results showed a significantly higher proportion of foragers from the four species been attracted to food resources baited with natural extracts from their own glands and recruited additional foragers to such baited food sites.

Keywords: Meliponine bee species, recruitment behaviour, nasonov glands, tarsal glands.

Introduction

Chemical compounds play vital roles in the communication systems of many living organisms (Wyatt 2003). These compounds are commonly used for scent marking at food sources and generally termed as "footprint pheromone" or "trail pheromone" (El-Sayed 2012, Reichle et al. 2013) which are perceived through olfactory cues and also possibly chemo-tactically. Apart from honey

bees (Hymenoptera, Apidae, Apini), meliponine bees (Hymenoptera, Apidae, Meliponini) are another group of eusocial bees that have developed an advanced level of organization. The first evidence that meliponine bee foragers exchange food odours to fellow nest mates inside the nest via footprint pheromones came from an experiment conducted by Lindauer and Kerr (1958). These "footprints" were long thought

to be secreted by the bees' mandibular glands (Lindauer and Kerr 1960, Kerr 1969, Nieh et al. 2003, Nieh et al. 2004). However, proper experiments that confirmed this assumption were never documented (Hrncir et al. 2016).

Mandibular gland secretions by contrast, have clear deterrent effects at food sources and play vital roles in alarm communication and defense (Jarau et al. 2003, 2006, Stangler et al. 2009). More recent studies moved to negate this theory and revealed that the trail pheromones of some meliponine bee species such as *Trigona recursa* (Jarau et al. 2003), *Trigona spinipes* (Schorkopf 2007), *Geotrigona mombuca* (Stangler et al. 2009) and *Scaptotrigona pectoralis* (Sawaya et al. 2009) are secreted from the foragers' labial glands. The chemical structures of trail pheromone compounds have only been elucidated for a small number of meliponine species to date. Hexyl decanoate, an ester, is the main component from labial gland secretions of *Trigona recursa* foragers and acts as a key compound for triggering trail-following behaviour in newly recruited workers of this species (Jarau et al. 2003). However, the attractiveness of this ester is reduced when compared with natural labial gland extracts, which indicates that the entire trail pheromone of *Trigona recursa* is composed of a diverse blend of compounds (Hrncir et al. 2016). In *Trigona spinipes*, the single dominant component of labial gland secretions, octyl octanoate, was as efficient in triggering trail-following behaviour 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 *Geotrigona mombuca*, with farnesyl butanoate as major component. Thus, conclusively stating that the trail pheromone of *Geotrigona mombuca* is composed of esters, but the specific roles of single compounds needed to be clarified by further investigations testing synthetic compounds (Stangler et al. 2009). In addition, Jarau et al. (2006, 2010), Schorkopf et al. (2007) and Stangler et al. (2009) demonstrated that trail pheromones are exclusively secreted from the foragers' labial glands in *Geotrigona mombuca*. Therefore, it

was reasonable to come to a conclusion that labial gland secretions in foragers of these species are involved in trail pheromone communications.

Kerr and Rocha (1988) raised another parallel hypothesis that compounds used for scent marking food sources by foragers of *Melipona ruiventris* and *Melipona compressipes* came from abdominal liquids (nasonov secretions which are a blend of six mono-terpenes including (*E,E*)-farnesol) which are excreted at sugar baited feeders after food uptake. However, this conclusion was only made on behavioural observations without demonstrating if these bees are actually attracted by the same anal droplets or confirming the chemical identities of these anal droplets which seriously undermine the hypothesis that these anal droplets function as attractive food-marking substances. Interestingly, another gland that was investigated for trail pheromone production and supported with strong evidence is the tarsal (Arnhart) gland (Arnhart 1923) which was inferred from studies carried out with *Melipona seminigra* by Hrncir et al. (2004).

This has spurred greater interests to determine the origin of production of these trail pheromones. Recently, some studies revealed that meliponine bee foragers efficiently utilize scent trails laid out with secretions produced solely from their labial glands in order to guide their nest-mates to a food site (Schorkopf et al. 2007, Stangler et al. 2009). Other studies also demonstrated that secretions from the labial glands of *Scaptotrigona pectoralis* foragers elicited trail following behaviour in recruited workers (Reichle et al. 2011). This has raised another unanswered question if meliponine bee species solely utilize secretions from either of these glands (nasonov glands, tarsal glands) to lay pheromone trails and recruit other nest mates to a food source (Barth et al. 2008, Hrncir 2009). The other most obvious glands that could be implicated with strong evidence in the secretion of footprint pheromones as against other potential locations of origin are the tarsal (Arnhart) glands (Arnhart 1923) this was inferred from studies carried out with *M. seminigra* by Hrncir et al. (2004).

These glands are situated in the fifth tarsomeres of hymenopterans' hind-legs of adult queen bees, workers and drones. The tarsal (arnhart) gland appear to be a flattened sac within each of the last tarsal segments of each leg (Hölldobler and Palmer 1989, Jarau et al. 2012) and consists of a unicellular layer which surrounds and secretes into a sac-like cavity forming the reservoir of the glandular secretions. The unicellular layer of epithelial cells contains a vast abundance of cellular organelles consistent with secretory activity (Jarau et al. 2012). These pheromones are then deposited by the terminal arolium between the tarsal claws as the bee walks on a surface. In addition to the feet, it is deposited by the tip of the abdomen, which often trails over any surface as the bee walks (Barth et al. 2008, Jarau et al. 2012). This trail laying secretions was shown to affect the behaviour of other nest mates of *M. seminigra* as demonstrated by Hrncir et al. (2004).

Both contradictions between the apparent use of attractive footprint secretions from the labial glands by *Scaptotrigona pectoralis* and *M. seminigra* foragers at food sources on the one hand and the lack of openings of the tarsal (arnhart) glands on the other hand were resolved by the discovery of a different system of glands within the bees' legs (Jarau et al. 2004) which are composed of a distinct claw retractor tendon running from the leg's femur through its tibia and tarsus and connecting to the base of the pre-tarsus which possesses a specialized glandular epithelia within the femur and tibia where they are secreted to the external environment as footprint pheromones. Sugar feeders baited with extracts of these tarsal glands, dissected from *M. seminigra* foragers, attracted foragers in the same pattern as feeders naturally marked by foragers themselves (Jarau et al. 2004). The chemical structures of compounds deposited by meliponine bees at food sources have so far been elucidated for only this species (*Melipona seminigra*) to-date consisting majorly of 12 alkanes, eight alkenes, one methyl alkane, and one aldehyde (Jarau et al. 2003). The dominant compounds, each constituting $\geq 10\%$ of the total amount

of the identified volatiles, were pentacosane, heptacosane, corresponding alkenes, 7-(Z)-pentacosene and 7-(Z)-heptacosene. The same compounds were also detected in extracts collected from the tarsal glands of *Melipona seminigra* as well as from its last tarsomeres. These extracts also contained additional forty-one compounds, comprising mainly esters, acids, and methyl branched alkanes (Jarau et al. 2003, Stangler et al. 2009). These identified compounds from *M. seminigra* scent marks are somewhat similar to the compounds reported from bumble bee scent marks (Eltz et al. 2001, Leonhardt et al. 2010) resulting in similar effects on the behaviour of foragers. This study was intended to confirm if African meliponine bees species scent mark at food sources by identifying the components of both nasonov and tarsal gland secretions and elucidate its effects on the recruitment behaviour of four species of African meliponine bee species. This study was carried out to test the hypotheses that a) African meliponine bee species carry out scent marking behaviour at food sources and effectively recruit other foragers b) pheromones responsible for scent marking behaviour may possibly originate from the nasonov gland but maybe deposited by the tarsal glands.

Materials and Methods

Experimental colonies

Behavioural experiments were conducted between April and September, 2016 at the Behavioural and Chemical Ecology Laboratory of the International Centre of Insect Physiology and Ecology (ICIPE), Dugesi campus (1°17'S, 36°49'E) in Nairobi, Kenya. In February, 2014 colonies which had been sourced from Taita Taveta County (03°20' S, 38°15' E) were transported to the Meliponary Section of ICIPE where they were further stabilized and maintained throughout the experimental period. Three colonies each of *Plebeina hildebrandti*, *Meliponula ferruginea* (black), *Hypotrigona gribodoi* and *Hypotrigona ruspolii* used in the experiments were queen right colonies and estimated to be approximately similar in size and fitness, having similar numbers of

workers (> 500) individuals. They were placed at a distance of 1 m from each other and left to forage freely on nearby vegetation throughout the experimental period.

Glandular extraction for bio-assays

Both nasonov and tarsal glands from five foragers of each species returning from foraging bouts were collected from each colony. Bees were collected and immobilized by placing on ice for ~20 minutes. Prior to gland extraction, hind legs bearing any substance (pollen, nectar or resin) which could be possible sources of contamination were excised. Gland extraction procedure and concentration of gland extracts were routinely carried out as described by Jarau et al. (2006). Glands were dissected in saline solution under a stereo microscope by carefully separating them from any tissue other than the targeted glandular epithelia, thereafter soaked in pentane for 24 hours at room temperature (24 °C) (Figure 1). For all extracts, the amount of pentane was adjusted to 100 µl per pair of glands (e.g., 10 nasonov/tarsal glands in 500 µl pentane). Twelve extracts were prepared from each of the four species along with a control (pentane) in the same manner. These extracts were stored in -20 °C until ready to use for bio-assays.

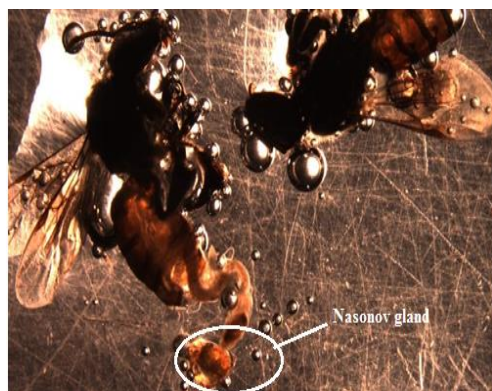


Figure 1: Excised abdominal region containing the nasonov gland (glandular epithelia) from *H. ruspollii* prior to solvent extraction.

Behavioural experiment 1: Scent marking behaviour on food resources

A total of 12 marked artificial feeders were randomly baited with different artificially made food sources (nectar, pollen and water). The experimental setup and procedure followed the method for scent trail bio-assays described by Jarau et al. (2006). Foragers from each colony were gradually trained over a period of two months (February-March) to collect these unscented resources from the individually marked artificial feeder prior to conducting these observational bio-assays. Observations were made between 09:30 and 15:00, for twenty-five minutes per hour on each feeder. Throughout all observations, the species identity, number of bees landing on each baited feeder and time of collection was recorded. Most importantly, the observations of scent marking behaviour were observed and confirmed when bees raised their abdomens at an angular length in the air while simultaneously fanning their wings or rubbed their abdomen against their tarsal region (metatarsus/tarsus) (Figure 2) after landing on the feeders.

Behavioural experiment 2: Scent marking behaviour on food resources baited with natural gland extract

Experiments were carried out on food sources (nectar, pollen and water) baited with both nasonov and tarsal gland natural extracts from the four species. Approximately 10 µl of gland extract were applied on the landing base of each feeder. Observations were made between 09:30 and 15:00, for 25 minutes per hour on each feeder, for 30 days.

Most importantly, the observations of scent marking behaviour were observed and confirmed to be initiated when bees raised their abdomens at an angular length in the air while simultaneously fanning their wings or rubbed their abdomen against their tarsal region (metatarsus/tarsus) (Figure 2) after landing on the feeders.



Figure 2: A *Hypotrigona ruspolii* forager exhibiting scent marking behaviour on a baited feeder provisioned with nectar.

Electrophysiological (GC-EAD) responses to natural extracts of forager bees

To identify compounds from both nasonov and tarsal gland extracts of bee foragers to which the chemo-receptors of their antennae are sensitive, coupled gas chromatography-electro-antennogram detection (GC-EAD) analyses were conducted. This was to establish if meliponine foragers can detect and positively respond to compounds responsible for scent marking behaviour dominant or either common to both nasonov and tarsal gland extracts. Excised antennae of foragers from the four meliponine bee species; *Hypotrigona ruspolii*, *Hypotrigona gribodoi*, *Meliponula ferruginea* (black) and *Plebeina hildebrandti* were mounted between two capillary glass electrodes filled with saline solution. The electrodes were connected to a high-impedance DC amplifier (Syntech), and the flame ionization (FID) and electro-antennographic (EAD) signals were simultaneously recorded on a PC using the program GC-EAD 2000 (Syntech). For each run, 3 μl gland extract was injected in splitless mode at 50 $^{\circ}\text{C}$ into the column. The Flame Ionization Detector (FID) was heated to 300 $^{\circ}\text{C}$ to detect all eluted compounds. A HP-5 column (30 \times 0.25 mm ID \times 0.25 μm , Agilent, US) with nitrogen (2 ml/min) was used as the carrier gas. The oven temperature was 50 $^{\circ}\text{C}$ for 2 min and then increased at 10 $^{\circ}\text{C}/\text{min}$ to 230 $^{\circ}\text{C}$. The electro-antennogram (EAG) system was connected to the GC system with a custom, 40 cm heated (250 $^{\circ}\text{C}$) transfer line. Separate recordings of both EAD and FID signals

were done. EADs were replicated with three individual foragers from each of the four species. A peak was classified as electrophysiologically active when it coincided with an EAD baseline deflection (Syntech 2004)

Glandular extraction for chemical analyses

Head space volatiles from both nasonov and tarsal glands from ten foraging bees were routinely extracted using the protocol described by Jarau et al. (2006). Glands were dissected by excising the 6th and 7th abdominal tergite region (nasonov gland) between the tarsus and metatarsus region (tarsal gland) in sterile saline solution and soaking in 1 ml of pentane for 24 hours at room temperature (24 $^{\circ}\text{C}$), thereafter evaporating the solvent under a gentle stream of nitrogen gas to adjust 100 μl per pair of glands (e.g., 10 nasonov/tarsal glands in 500 μl pentane), thus 100 μl of the pooled extracts corresponded to the gland content of one individual bee (one bee equivalent). Extracts were stored at -20 $^{\circ}\text{C}$ until ready to use for chemical analyses. A pure pentane control was subjected to similar evaporation process.

Chemical analyses

Coupled gas chromatography/mass spectrometric (GC/MS) analyses were carried out on an Agilent Technologies 7890A gas chromatograph equipped with a capillary column HP-5 MS (30 m \times 0.25 mm ID \times 0.25 μm film thickness) and coupled to a 5795C mass spectrometer. An aliquot (1 μl) of the gland extracts from different species was injected in splitless mode (Inlet temperature = 250 $^{\circ}\text{C}$, Pressure = 12.1 psi), and helium was used as the carrier gas at 1.0 ml/min. The injector port was maintained at 280 $^{\circ}\text{C}$. The oven temperature was then held at 35 $^{\circ}\text{C}$ for 5 min, increased to 280 $^{\circ}\text{C}$ at 10 $^{\circ}\text{C}/\text{min}$, and then held at 280 $^{\circ}\text{C}$ for 5 min. Mass spectra were recorded at 70 eV. All the alkanes, alkenes, ethers, alcohols, organic acids, esters and aldehydes were identified by comparing their retention times and mass spectral data with those recorded from the NIST 08 spectral library and by co-injection with

authentic standards, while the alkenes and aldehydes were identified by using EI diagnostic ions (El-Sayed 2012). For compound quantification, peak areas were compared to an external standard corresponding to 5 ng/ μ l of 2-heptanol.

Behavioural experiment 3: Scent marking behaviour on food resources baited with synthetic compound, (*E*)- β -farnesene

Bio-assays were conducted in December 2016, where pairs of forager bees (N = 25) originating from four different colonies and species were collected from their respective nest entrances while returning from foraging and then immobilized on ice for approximately five minutes to minimize the possibility of the bees producing any alarm pheromones. Food sources baited with a synthetic form of the dominant compound from both the nasonov and the tarsal glands: (*E*)- β -farnesene were used to carry out scent marking bio-assays. Initiation of scent marking behaviour in response to the synthetic compound were conducted in a dual choice test bio-assay Perspex platform measuring 13 \times 5.7 cm and sealed with a glass lid. An aliquot of this synthetic pheromone (25 μ l) was dispensed round a food source placed onto a filter paper (Whatman No.1) which was placed on one side of the bio-assay chamber while the other chamber was provisioned with an untreated food resource (negative control).

Statistical analyses

The foraging pattern of individual foragers on each food resource was analysed using descriptive statistics. Student Newman Keuls (SNK) tests were used to check for significant effects on foraging behaviour to a preference of either treatment (un-baited and baited food sources). Data from scent marking behaviour by all four meliponine bees' species were subjected to one sample chi-square test by testing for significant differences when exposed to natural extracts of both nasonov and tarsal glands and the tested synthetic compound: (*E*)- β -farnesene. In order to compare the gland composition of trail pheromones of the four different species,

the relative peak areas of both nasonov and tarsal gland compound constituents of *H. gribodoi*, *H. ruspolii*, *M. ferruginea* (Black) and *P. hildebrandti* were calculated, then log-transformed and data subjected to Kruskal-Wallis test. All statistical analyses were carried out using Sigmaplot V 11.0 statistical software (Systat Software, 2011).

Results

Behavioural experiments 1, 2 and 3: Foraging and scent marking behaviour on food resources

Significant differences were observed in the foraging patterns of each of the four bee species on collected resources (nectar, pollen and water) between 11:00 hours and 14:00 hours; *M. ferruginea* (Black) ($F_{3,116} = 5.61$, $p < 0.001$), *H. gribodoi* ($F_{3,116} = 6.46$, $p < 0.001$), *H. ruspolii* ($F_{3,116} = 2.81$, $p = 0.042$) and *P. hildebrandti* ($F_{3,116} = 4.19$, $p = 0.007$). In all the four species, the total number of bees landing and initiating scent marking progressed with increasing foraging hours. Foraging activity peaked between 11:00 and 14:00 hours as 70% of all foraging bouts gradually declined before and after this observation period. *Plebeina hildebrandti* species showed the highest foraging activity on both baited and un-baited nectar sources, as workers began landing on the feeders as from 11:05 and peaked at 13:00 hours, while *H. gribodoi*, *H. ruspolii* and *M. ferruginea* (Black) all foraged till much later, signifying similar commencement of foraging but having longer peak periods which lasted until 15:00 hours. In general, the collection of nectar started to decrease after this peak period until cessation. Nectar was always the most collected resource by *P. hildebrandti* (N = 220), *M. ferruginea* (Black) (N = 117), *H. ruspolii* (N = 124), *H. gribodoi* (N = 109), throughout the whole observational period, while water was the second most collected resource by *P. hildebrandti* (N = 101), *H. gribodoi* (N = 97), *H. ruspolii* (N = 94), *M. ferruginea* (Black) (N = 71), followed lastly by pollen: *M. ferruginea* (black) (N = 84), *H. gribodoi* (N = 60), *H. ruspolii* (N = 61) and *P. hildebrandti* (N = 73). The foraging activity for pollen followed the same

sequence across all the four species, but with no significant difference in activity. Two species showed similar foraging peaks for this resource from 12:00 hours for 50% of the observational period; *H. gribodoi* and *H. ruspolii* foragers, which was characterized by constant number of bees landing on the feeders with pollen and eventually decreased as the day progressed compared to *M. ferruginea* (Black) and *P. hildebrandti*. Notable, however, was the foraging pattern for water which was observed to be more regular after 13:00 hours.

Chemical and electro-physiological analyses

Chemical analyses of both nasonov and tarsal gland extracts demonstrated that the trail pheromone of *P. hildebrandti*, *H.*

gribodoi, *H. ruspolii* and *M. ferruginea* could be potentially produced by nasonov glands but mechanically deposited on any surface through the tendon retractor claws located on the hind legs, based on scent marking observations. Four dominant compounds were identified from the nasonov gland extracts (Figure 3a) and two dominant compounds from the tarsal gland extract (Figure 3b) which are sesquiterpenes. GC-EAD analyses done with thirty worker bee antennae revealed one peak that elicited consistent responses of the chemoreceptor's in more than 30% of the trials. These peaks correspond to the compound (*E*)- β -farnesene. The physiological activity of (*E*)- β -farnesene was verified in subsequent GC-EAD runs with its synthetic derivative.

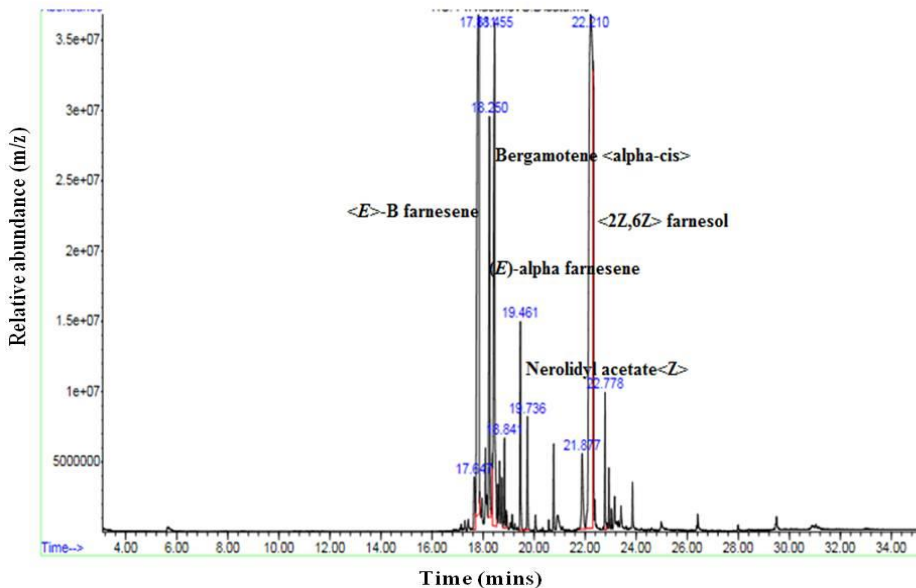


Figure 3a: GC-MS chromatogram showing dominant compounds identified from the nasonov epithelial gland extract of a representative meliponine bee species, *Hypotrigona ruspolii*.

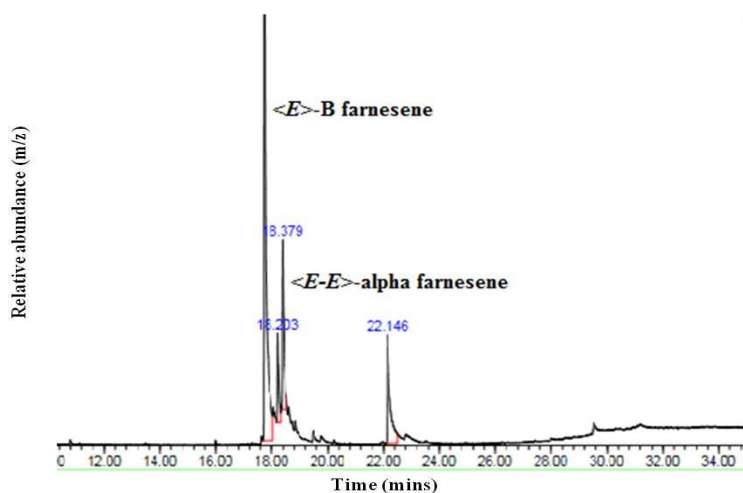


Figure 3b: GC-MS chromatogram showing dominant compounds identified from the tarsal gland extract of a representative meliponine bee species, *Hypotrigena rufipolii*.

Bio-assays with synthetic compounds

Scent trail bio-assays with synthetics

To test whether the physiologically active compounds from both nasonov and tarsal glands constitute the behaviourally active trail pheromone of these species, further sets of trail bio-assays were conducted. Significantly higher proportions of foragers from the four species were attracted and recruited additional foragers to food resources baited with natural extracts from their own nasonov glands: *M. ferruginea* (Black) ($t = 4.097$, $df = 58$, $p < 0.001$), *H. rufipolii* ($t = 0.633$, $df = 58$, $p = 0.005$), *H. gribodoi* ($t = 2.64$, $df = 58$, $p = 0.004$) and *P. hildebrandti* ($t = 12.92$, $df = 58$, $p < 0.001$) over the control (un-baited food resource), ($F = 95.77$, $df = 4$, 145 , $p < 0.001$). This similarly occurred when compared to food sources baited with natural extracts from their own tarsal glands or from other species; with no significant preference for any species: *M. ferruginea* (Black) ($t = 2.41$, $df = 58$, $p = 0.011$), *H. rufipolii* ($t = 2.49$, $df = 58$, $p = 0.015$), *H. gribodoi* ($t = 2.52$, $df = 58$, $p = 0.014$) and *P. hildebrandti* ($t = 2.85$, $df = 58$, $p = 0.006$) over the control (un-baited food resource) ($F = 1.22$, $df = 4$, 145 , $p = 0.304$), as no significant differences were observed between respective treatments. The synthetic compound (*E*)- β -farnesene (Sigma-Aldrich, USA) was significantly as attractive to

foragers of the four species as when compared to the natural nasonov gland extract but not natural tarsal gland extracts ((*E*)- β -farnesene: ($F = 19.01$, $df = 4$, 145 , $p < 0.001$), nasonov gland extract: ($F = 95.77$, $df = 4$, 145 , $p < 0.001$), tarsal gland extract: ($F = 1.13$, $df = 4$, 145 , $p = 0.304$).

Discussion

The results of our bio-assays show that these bee species carry out scent marking at food sources and trail pheromones of these four species are exclusively produced in the foragers' nasonov glands. This is in accordance with recent studies conducted with *Scaptotrigona pectoralis*, *Geotrigona mombuca*, *Trigona recursa* and *Trigona spinipes* (Jarau et al. 2000, 2003, 2006, 2010, Stangler et al. 2009, Reichle et al. 2013) and further disclaims the long assumed role of mandibular gland secretions for scent trail marking in meliponine bees species (Lindauer and Kerr 1958, 1960, Kerr et al. 1963, Nieh et al. 2003, 2004, Kuhn-Neto et al. 2009, Lichtenberg et al. 2011).

The major compound (*E*)- β -farnesene (Sigma-Aldrich, USA) from nasonov gland extracts detected by the chemo-receptors on the foragers' antennae from these four species belongs to the chemical class of terpenoids. Gas chromatographic analyses had shown that this compound, (*E*)- β -

farnesene constitutes a dominant part of the trail pheromone in these species. However, the natural nasonov gland extract was more attractive to recruited foragers, compared to the singular compound, (*E*)- β -farnesene. The reason is that this physiologically active compound may be incomplete as a synthetic pheromone trail bouquet, which has been shown to contain varied amounts of geraniol and citral in some studies (Jarau et al. 2003, Stangler et al. 2009, Hrnecir et al. 2016).

This study therefore adds to the existing list of known trail pheromone compounds used by meliponine bee species, and it can be assumed that the terpenyl esters identified from nasonov or tarsal gland extracts of other trail laying species may constitute their respective unique trail pheromones. Indeed, the chemical similarities between these compounds such as the terpenyl esters in these meliponine bees are also used as marking compounds by some solitary bees and bumblebees by depositing carboxylic acid alkyl esters on twigs or leaves for mating purposes (Bergström 2008).

In this present study, a generality of compounds from the terpenyl esters group in the trail pheromones of *Plebeina hildebrandti*, *Meliponula ferruginea* (Black), *Hypotrigona gribodoi* and *Hypotrigona ruspolii* in terms of composition, which was sufficient in triggering trail-following behaviour. This confirmatory finding, that foragers are significantly attracted to food sources baited with nasonov gland extracts prepared from their nest-mates over foragers of a foreign colony, may be explained by the differences in the relative proportions of trail pheromone components of foragers from these different species.

Though there seemed to be some minute disparity in the scent marking components of these bee species, which is either linked to their morphology such as body and gland size capable of influencing the relative abundance of these scent marking compounds. It was observed that the gland components of larger sized bees, *Plebeina hildebrandti* was dominated by larger amounts of terpenoids compared to much smaller sized species, *Hypotrigona gribodoi*, *Hypotrigona ruspolii*

and *Meliponula ferruginea* (black). It may be that these smaller sized bees significantly make use of other compounds such as cuticular hydrocarbons to lay trails. This has been observed from certain studies suggesting that cuticular hydrocarbons could also provide and function as footprint cues in social wasps and some bee species to recognize their nest entrance at close range (Soroker et al. 1998). Similarly, these same footprint hydrocarbons are informative to foraging bees, and are readily used to discriminate against either visited or already depleted food sites (Goulson et al. 2000, 2002, Barth et al. 2008, Jarau et al. 2012). Although this discrimination behaviour originally was believed to be based on active deposition of lipid "scent-marks" by bees, studies (Jarau et al. 2012) suggested that these chemicals are deposited wherever the bees walk, and were used as footprint cues rather than pheromonal signals (Eltz et al. 2001). This sheds more light to the dual functionality that cuticular hydrocarbons may play in communication mechanisms. *Bombus terrestris* workers were reported to deposit a similar range of compounds, mostly long chain alkanes and alkenes, in essentially similar concentrations at food, nest, and neutral sites (Goulson et al. 2000). These findings suggest that these hydrocarbon marks are deposited involuntarily, regardless of the behavioural context and source (Nieh and Roubik 1995, Schmidt et al. 2003, Hrnecir et al. 2004). Recently, Hölldobler and Palmer (1989) reported that the preference of *P. rugosus* foragers for food-sites marked by their nest mates over food-sites marked by foreign con-specific workers is likely due to similar gland secretions, which contain nest-specific patterns of volatiles (mainly hydrocarbons and esters) deposited in addition to the abdominal gland content (Hölldobler and Palmer 1989). A colony-specific effect of abdominal extracts in releasing trail following behaviour was demonstrated in the primitive ant *Lasius neoniger* (Traniello 1989) but by contrast, the actual trail pheromone extracted from workers abdominal region was not specific in initiating scent marking behaviour in other

closely related species, *Lasius japonicus* and *Lasius nipponensis* (Saran et al. 2006). However, colony specificity was added to these trails by footprint hydrocarbons deposited by these workers along their own trails (Akino and Yamaoka 2005, Akino et al. 2005).

Conclusion

Regardless of the dominant presence of (*E*)- β -farnesene in *Plebeina hildebrandti*, *Meliponula ferruginea* (black), *Hypotrigona gribodoi* and *Hypotrigona ruspolii* and its use as a trail marking cue, it is likely that foragers are able to detect and distinguish scent trails deposited by workers of both same and foreign species by recognizing other compounds secreted in minute quantities. Avoiding foreign scent trails appears advantageous to these bees because they reliably indicate the location of an already visited food source which could help in avoiding both competition and conflicts at food sources between foragers of different species. This is of particular importance for the survival of less aggressive meliponine bee species. A forager's ability to discriminate between trails laid by a different forager of another species is most likely based on the recognition of additional but minute compounds in their specific pheromone bouquets.

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