Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2019; 7(2): 257-268

Received: 04-01-2019
Accepted: 08-02-2019

Bridget O Bobadoye
Federal College of Forestry, Department of Horticulture and Landscape Technology, P.M.B 5054, Jericho Hills, Ibadan Oyo, Nigeria

Correspondence
Bridget O Bobadoye
Federal College of Forestry, Department of Horticulture and Landscape Technology, P.M.B 5054, Jericho Hills, Ibadan Oyo, Nigeria

Potential cues signalling nest mate recognition behaviour in African meliponine bee species (Hymenoptera: Meliponini)

Bridget O Bobadoye

Abstract
The cognitive ability to identify and respond differently to the presence of either a nest mate or non-nest mate exists in many organisms and is vital for members of most social insect colonies. Bioassay experiments showed that all four bee species could successfully discriminate nest mates from non-nest mates, as they all exhibited more aggression when exposed to hetero-specific nest mate extracts than when exposed to con-specific nest mate extracts (within or between nest), although aggression between same species colonies was not significantly different, with the species *Meliponula ferruginea (black)* exhibiting the most aggression, followed by *Hypotrigona ruspoli*, and *Plebeina hildebrandti*, while the least aggressive was *Hypotrigona gribodoi*. A high number of guard bees opened their mandibles and even proceeded to attack at their nest entrances when presented with an extract from (between nest) con-specific non nest mates and (between species) hetero-specific non-nest mates compared to when presented with a solvent control. Gas chromatography revealed similar patterns of recognition cue compounds present in cuticular profiles and nest materials (nest entrance and involucrum sheaths) from the four African meliponine bee species. This comprised of alkanes, alkenes and methyl-branched alkanes ranging from C8-C35 with trace amounts of acids, esters, aldehydes and ketones. The electro-antennography response to 9-Hexadecenoic acid and β-Farnesene (*E*) is consistent with that in *Apis mellifera* which showed positive responses to tricosene and the 16-C and 18-C fatty acids in particular, which suggests a generality of signal function in nest mate recognition between these closely related bees of the same family.

Keywords: nest mate recognition, cues, meliponine bees, electro-antennography

Introduction
Social insects are known to possess a highly developed recognition system that facilitates either passive behaviors towards their nest mates or aggressive behaviour towards non-nest mates. This cognitive ability is particularly crucial for colony survival by offering protection from parasites during territorial interactions when defending their colonies and also during essential daily activities such as foraging [1, 2]. The use of certain mechanisms to transfer information between individuals to initiate certain behaviors has long been confirmed in honeybees [3–6]. Such discriminatory behaviour is majorly based on recognition cues, as members of a colony rely on the existence of a signature odor to fully carry out this function when in contact with each other either at an individual or colony level. CHCs amongst other channels may play a crucial role to function as contact pheromones, as surface hydrocarbons are essential cues for recognition in both solitary and social insects [7–10]. The cuticle of most insects is coated with a lipid layer, with hydrocarbons forming a dominant group of chemical components of this layer [7, 11].
Cuticular hydrocarbons (CHs) have been categorized to typically range from C8- C40 [12] with 3 major structural classes: n-alkanes, n-alkenes and mono-, di-, tri- methyl- branched alkanes [10] with additional components found in trace amounts such as fatty acids, glycerides, sterols, ketones, long chain alcohols and aldehydes [9, 10, 13]. Two of these major classes of chemical components have been speculated to play different physiological functions respectively: n-alkanes form impermeable layers on the insects cuticle which help to form resistance against desiccation, while n-alkenes form permeable layers, that play a vital role in chemical communication [14, 15]. These hydrocarbons can be exchanged between individuals by means of trophallaxis, self and allo-grooming [16]. These hydrocarbons serves as unique chemical signatures, as they further help to maintain the social structure of colonies by differentiating

individuals according to caste and functions [10, 17]. They also function as an attractant or repellant during courtship [18-20] as they enhance the assessment of colony membership, and subsequent recognition allows individuals to act non-aggressively towards nest mates and aggressively towards non-nest mates [21–23].

The chemical identity of recognition cues in the honey bee *Apis mellifera* has been intensively studied [2, 8, 24–27] and their role at either individual and population levels confirmed. In *Apis mellifera*, adults emerge without any "signature odors" which could serve as recognition cues for specific purposes [28–31]. Hence, individual worker bees earn such "signature odors" comprising majorly alkenes and fatty acids, only after coming in contact with chemical stimuli such as comb wax to acquire a "distinctive signature template" [28, 30]. Wax based nesting materials have been known to be viable acquisition channels for nest mate recognition in *Apis mellifera*. However, the major acquisition channels of recognition cues at both individual and nest-specific levels remain largely unknown in African meliponine bees. Therefore we carried out experiments to determine if wax based nesting materials (Involucrum sheaths and the nest entrance tubes) form additional acquisition channels to acquire recognition cues, apart from cuticular based hydrocarbons in these African meliponine bees species. We further investigated the components of these meliponine bee nesting materials for dominant compounds which have been implicated in nest mate recognition systems in the honey bee, *Apis mellifera.* We also bio-assayed synthetic forms of these putative dominant compounds including a representative alkane, an alkene, an aldehyde and a wax ester to predict recognition behaviors' within-nest, between-nest con-specifics and between-nest hetero-specifics. By establishing which compounds, if any, affect nest mate recognition.

Not until recently, has recognition behaviour been documented in some meliponine bee species [10, 32, 33] given that meliponine bees like honey bees are highly eusocial and should be able to recognize nest mates from non-nest mates. However, little is known about their recognition cue chemistry, what acquisition channels is utilized and how such cues shape recognition behaviour in these African bee species.

Unlike honeybees, meliponine bees construct distinctive nests from endogenously produced wax, and they are likely to include more exogenously produced materials from the environment such as mixtures of resin and floral oils into their nests principally for construction [34, 35]. Studies have confirmed the use of a range of externally derived compounds as recognition cues in most social insects such as honey bees' waxes, where the dominant hydrocarbons are odd-chained alkanes (C-21 to C-35) [36] and are primarily used as recognition cues. Lactones (fatty acid derivatives) had been shown to also function as recognition cues in the asocial sweat bee, *Lasioglossum zephyrum* [37, 38] and environmentally derived odors playing an important role in nest mate recognition in some ants [39-41], some species of social wasps largely depend on methyl-branched alkanes [2, 26], however, floral odors seem to be relatively unimportant in honey bee nest mate recognition [4, 13]. Honeybees are also known to exhibit recognition behaviors primarily at nest entrances, which should similarly occur in meliponine bee species, as they are believed to also be territorial at food sources like the honey bee; however nest mate recognition in these bees may also be expressed away from the nest as well as at the nest entrance [28, 41, 42, 28] revealed that the Neo-tropical meliponine bee *Tetragonisca angustula* could recognize con-specifics from hetero specifics even at nest entrances. Breed & Page (1991) also investigated nest mate recognition in some species of *Meliponula* and discovered that *M. quadrifasciata* and *M. rufiventris* were more tolerant of nest mates than of non-nest mates. In other studies focusing on meliponine recognition mechanisms, *Trigona minangkabau* [43] and *Hypotrigona gribodoi* [44] rejected con-specific non-nest mate at experimental feeding sites.

Therefore this study sought to test the hypothesis that a) Similar recognition cues used by the honey bee, *Apis mellifera* could also be employed by African meliponine bees b) Additional acquisition channels (nest materials) can be used to acquire cues for discrimination in these bee species. These underlying olfactory cues responsible for recognition behaviour can be employed during foraging and territorial nest defense, which are the two most crucial behaviors for the survival of any colony. Using the well- researched honeybee as a reference point, we investigated whether these African meliponine bee species uniquely utilize either endogenous derived cues (cuticular compounds) more than exogenous derived cues (components from both nest entrance and the involucrum) in nest mate recognition or even a combination of both exogenous and endogenous derived cues.

Materials and Methods

Experimental colonies

Studies were conducted between October, 2015 and February, 2016 at the laboratory of the behavioral and chemical ecology unit of the International Centre of Insect Physiology and Ecology (*Icipe*), Duduville campus (1º 17′S, 36º 49′E) in Nairobi, Kenya. Colonies were surveyed in February, 2014 from Taita taveta county (03° 20' S, 38º 15' E) and then transported to the meliponary section of the International center for insect physiology and ecology (*icipe*) where they were maintained throughout the experimental period. Four colonies in replicates of *Meliponula ferruginea (black), Hypotrigona gribodoi, Hypotrigona ruspoli and Plebeina hildebrandti* were used in the experiments. The colonies were queen right and estimated to be approximately similar in size and fitness, having similar number of workers (approx 500-600 individuals. They were hived inside wooden boxes (45 x 20 x 10 cm) and left to forage freely on nearby vegetation.

Extraction of CHCs for bioassays

Cuticular hydrocarbons from five nurse bees of each species were sourced from colonies and extracted (45) in replicates. Nurse bees were collected and freeze-killed by placing on ice for approximately 20 minutes. Cuticular hydrocarbons were extracted by washing them in 500µl of pentane for 10 minutes followed by a re-concentration of the extract (to rid it of excess solvent) under a stream of nitrogen gas and stored in -20 ºC until ready to use for bioassays. Ten extracts were prepared from each of the four species along with a control (pentane) in the same manner. These extracts were used as sources of chemical stimuli in mandibular opening response (MOR) [45] and nest entrance defense bioassays [46].

Behavioral experiment 1: Mandibular opening response (MOR) bioassay

On the day when each bioassay was to be conducted, worker bees were captured at their nest entrance while returning from foraging bouts and then immobilized by placing them on ice

for five minutes. A harnessing method described by (45) was employed for the selected worker bees from each colony (N = 25) and then isolated with minimal disturbance for a period of one hour in order to accustom each individual bee to the harness (Fig 1). Aggressive behavior was thereafter quantified by presenting five different types of stimuli to the bees from four different species respectively, 1) *Hypotrigona gribodoi* extract 2) *Hypotrigona ruspoli* extract 3) *Plebeina hildebrandti* extract 4) *Meliponula ferruginea* extract and 5) control solvent extract (pentane, 99% purity). An approximate volume of 10 µl of pure pentane or pentane based hydrocarbon extract was applied to the tip of a glass Pasteur pipette and then held upright to evaporate the solvent from the tip before usage for the mandibular opening response (MOR) bioassay.

For each test bee, extract from its own colony and species (con-specifics) served as nest mate stimuli, while extract from different species (hetero-specifics) served as non-nest mate stimuli. Only one stimulus was presented to each test bee by touching the antennae with the tip of the Pasteur pipette bearing the stimuli for an average period of 10 seconds. Aggressive behavior was scored as (1) when the test bee continuously opened its mandible (Fig 2) while non-aggressive behavior was scored as (0) when the test bee repeatedly shook its antennae (Fig 3). A total of 25 bees from each species were subjected to this test assay, with one singular stimulus presented randomly to only one harnessed bee. Observations were considered to be null if the bee showed neither any of these behaviors.

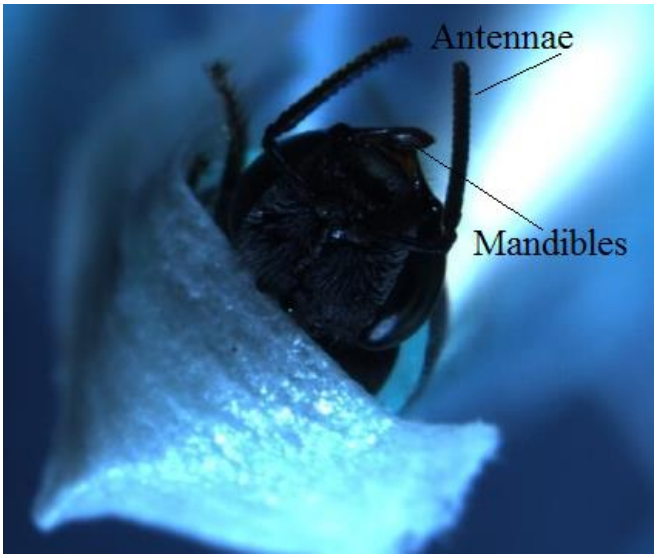

Fig 1: Harnessing set-up showing an individual bee, *Hypotrigona ruspoli* harnessed and conditioned prior to the mandible opening response bioassay (MOR).

Behavioral experiment 2: Nest entrance defense (NED) bioassay

Ten guard bees were used for this experiment to quantify aggression to both endogenous cues (nest mate and non-nest-mate stimuli) and exogenous cues (nest entrance extract and nest material extract). To induce bees into initiating either an aggressive or non-aggressive behaviour, guard bees were exposed to 12 different types of stimuli from the four different species in this bioassay: 1) *Hypotrigona gribodoi* CHC and nest entrance extract 2) *Hypotrigona ruspoli* CHC, nest entrance and cerumen extract 3) *Plebeina hildebrandti* CHC, nest entrance and cerumen extract 4) *Meliponula ferruginea* CHC, nest entrance and cerumen extract and 5) control solvent extract (Pentane, 99% Purity). *Hypotrigona gribodoi* is known not to produce involucrum sheaths.

For each test colony, pentane based extracts from both its own colony and species (con-specifics) served as nest mate stimuli, while extracts from another species (hetero – specifics) served as non-nest mate stimuli. The behaviour of the guard bees toward each presented treatment was observed for five minutes starting from the first interaction. Aggressive behaviour was confirmed and recorded when one or more guard bees left the nest entrance, and proceeded to bite with open mandibles, while a non-aggressive behaviour was recorded when one or more guard bees retreated from the entrance into the hive or simply touched the stimuli bearing pipette tip only with its antennae.

Experiments were considered null if all guard bees present at the entrance exhibited neither of these behaviors within five minutes. Moreover, since we wanted to be sure that both aggressive and non-aggressive behaviors occurred after close monitoring of the guard bee (s) (and that the respective treatments have been perceived by the guards) and were not based on visual stimuli, the assay was paused for a period of one hour before commencing another replicate with a different treatment.

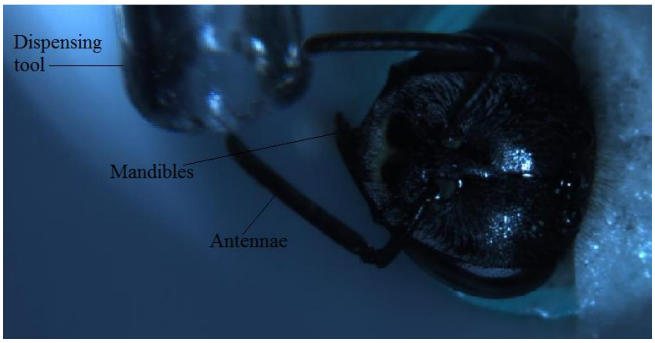

Fig 2: A harnessed bee showing aggressive response (continuous opening of mandibles) when presented with a hetero-specific non-nest mate extract from another bee species.

Fig 3: A harnessed bee exhibiting non- aggressive response (Continous antennation) when presented with a con-specific nest mate extract from another colony.

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

To determine if foragers can detect and positively respond to dominant compounds found in natural extracts of con-specific or hetero-specific foragers, we conducted coupled gas chromatography-electroantennogram detection (GC-EAD) analyses. Excised antennae of foragers of the four meliponine bee species: 1) *Hypotrigona ruspoli* 2) *Hypotrigona gribodoi* 3) *Meliponula ferruginea* (black) 4) *Plebeina hildebrandti* were exposed to natural extracts from their species and other

hetero-specific species. We used an HP-5 column (30 x 0.25 mm ID X 0.25 µm, Agilent, US) with nitrogen (2 ml/min) as the carrier gas. The oven temperature was 50 °C for 2 min and then increased at 10 °C/min to 230 °C. The Flame Ionization Detector (FID) was heated to 300 °C to detect all compounds. The electro-antennogram (EAG) system was connected to this GC system with a custom, 40 cm heated (250 °C) transfer line. The EAD signals and FID signals were separately recorded. We replicated EADs with three individual foragers from each of the four species.

Extraction of headspace volatiles (CHCs) for chemical analyses

Nurse bees, forager bees, nest entrance tubes and involucrum sheaths of all four species had their headspace volatiles extracted. Cuticular hydrocarbons of both nurse bees and foraging bees were routinely extracted using the protocol described by (45), by washing five bees in one ml of pentane for ten minutes, thereafter evaporating the solvent under a gentle stream of nitrogen gas. Extracts were stored in -20 ºC until ready to use for chemical analyses. A pure pentane control was subjected to similar evaporation process. Volatile extraction for both nest entrance tubes and involucrum sheaths followed the same procedure [45].

Chemical Analyses

Coupled gas chromatography/mass spectrometric (GC/MS) analysis was carried out on an Agilent Technologies 7890A gas chromatograph equipped with a capillary column HP-5 MS (30 m × 0.25mm ID ×0.25µm film thickness) and coupled to a 5795C mass spectrometer. An aliquot (1 µl) of the extracts from the different species was injected in the split less mode (Inlet temperature = 250 °C, Pressure = 6.8 psi), and helium was used as the carrier gas at 1.0 ml/min. The injector port was maintained at 280 °C. The oven temperature was then held at 35 °C for 5 min, increased to 280 °C at 10 °C/min, and then held at 280 °C for 5.5 min. Mass spectra were recorded at 70 ev. Dominant n-alkanes, n-alkenes and methyl-branched alkanes 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 (47). For compound quantification, peak areas were compared to an external standard corresponding to 5ng/µl of Eicosane (C20).

Chemicals

The following chemicals were to be used as synthetic standards: *n*-Octane, *n*-Hexadecane, *n*-Octadecane, *n*-Docosane, *n*-Tricosane, n-Hexacosane, *n*-Triacontane, *n*-Pentacosane, *n*-Heptacosane, *n*-Octacosane, *n*-Tetracosane, *n*-Heneicosane, *n*-Pentatriacontene, 1-Docosene, Octadecanol acetate, Methylhentriacontane, Tridecanol, n-Octadecanol, 2-Methyl-*E*-7-octadecene, Cyperotudone, Octamethyl, Cyclododecanemethanol, Cyclocolorenone (*Epi*), Cyclohexane, Cyclopentane, Zierone, β-amyrin, Farnesyl acetate (*2E*,6*E*) and α-amyrin. However, they were narrowed down to: *n*-Eicosane, Oleic acid, 9-Hexadecenoic acid (Z), β-Farnesene (*E*) with the purity of >99%, obtained from Aldrich Chemical Company (UK).

Behavioral experiment 3: Synthetic compounds tested in bioassay.

Bioassays were conducted in January 2016, N=25 bees each (con-specifics and hetero-specific foragers) originating from four different colonies were collected from their respective nest entrances while returning from foraging and treated with pure synthetic compounds to estimate aggressive responses. The following compounds selected were based on the following criteria: a) GC-MS analyses showing compounds to have a relative abundance of > 5%. (b) demonstrated to affect nest mate recognition in *Apis mellifera*; (c) dominant in *Apis* and/ or *meliponine* wax/cerumen; and (d) represent the diversity of compound classes found in wax/ cerumen of meliponine bees. Dominant compounds selected were from the following: Nurse bees: Eicosane (C20); worker bees (foragers): 9-Hexadecanoic acid; nest entrance tube: Oleic acid and involucrum sheaths (cerumen): (*E*)-β-Farnesene. This was applied systematically by dispensing 10µl of the compound from a Pasteur pipette tube directly to the thorax of each individual bee. These treatment concentrations are similar to those used by [30].

Separate bioassays (N=25) were performed by placing con-specifics in pairs (treated and untreated) from within-nest (nest mates), between-nest (con-specifics) and between species foragers (hetero specifics) in a large Perspex Petri dish (9 cm in diameter) mounted on a bioassay platform measuring (19.5cm length x 9.5 cm width). Aggressive behavior was quantified and had a specified range (biting or grappling of body parts: legs, wings or thorax) which was recorded as a bite, or one that typically escalated from a bite to grappling of body parts. These behaviors were observed for a time period of 10 minutes.

Statistical Analyses

The aggressive responses of all four meliponine bees species was subjected to one sample chi-square test by testing the differences of aggressive responses when exposed to natural extracts on both individual level (MOR), colony level (NED) and the tested synthetic compounds: Eicosane (C20), 9-Hexadecanoic acid, Oleic acid and β-Farnesene (*E*). Further analysis was carried out to quantify the levels of aggression between the paired bees from the four species by subjecting the log-transformed data to Kruskal-Wallis ANOVA test. A canonical discriminant analysis was carried out to determine which of the tested compounds significantly caused aggressive behavior between con-specific nest mates, con-specific non nest mates or hetero-specifics. All statistical analyses were carried out using Sigmaplot V 11.0 statistical software (Systat Software, San Jose, CA 2011).

Results

Mandibular opening response (MOR) Bioassay

All four bee species successfully discriminated nest mates from non-nest mates. The number of bees that opened their mandibles when presented with a natural cuticular extract was significantly higher compared to when presented with a solvent control (Wald's χ^2=106.5, df = 2, P<0.005) (Fig 4). All species exhibited more aggression when exposed to hetero-specific nest mate cuticular extracts than when exposed to con-specific nest mate cuticular extracts (within or between nest), although aggression between same species colonies was not significantly different (P=0.066), with the species *Meliponula ferruginea (black)* (76.01%, N= 25) exhibiting the most aggression, followed by *Hypotrigona ruspoli* (69.3%, N= 22), and *Plebeina hildebrandti* (66.7%, N= 25), while the least aggressive was *Hypotrigona gribodoi*(62.7%, N= 21). There was less aggressive behaviour exhibited when closely related bees were presented with

treatments from within-nest foragers (con-specific nest mates) than between-nest foragers (con-specific non-nest mates) or between species foragers (hetero specific non-nest mates) (Wald's χ^2=70.5, df = 1, P < 0.005). In general, the levels of aggression (biting of body parts) increased significantly when a bee was exposed to a non-nest mate stimulus (between nest) (Wald's χ^2=17.9, df = 1, P = 0.001) or (between species) (Wald's χ^2=46.0, df = 1, P < 0.005) compared to a solvent control (Wald's χ^2=6.6, df = 1, P < 0.005).

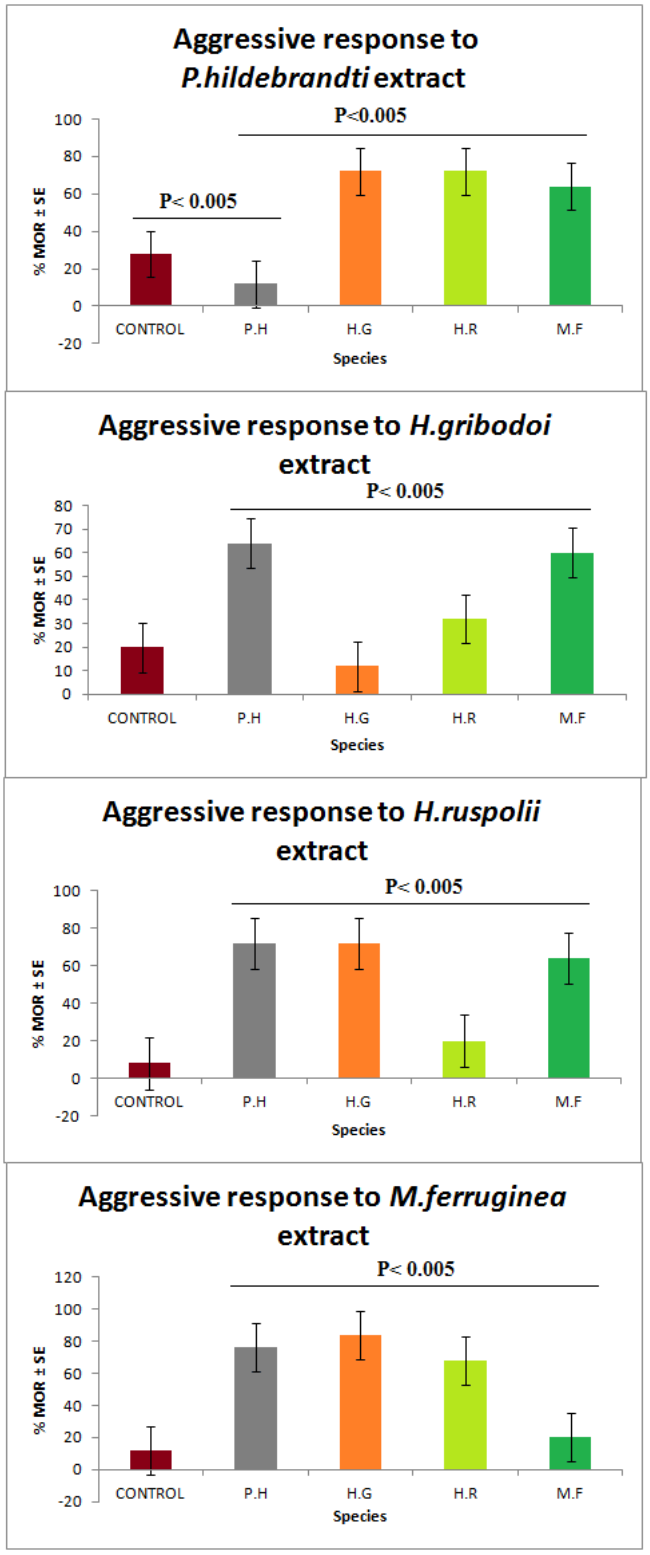

Fig 4: Aggressive responses exhibited by four meliponine bee species during the mandible opening response bioassay when presented with both con/hetero-specific stimuli (cuticular hydrocarbons).

Nest entrance defense (NED) Bioassay

Bioassays conducted at the nest entrance of all four bee species revealed that guard bees were able to successfully predict and discriminate nest mates extract from non-nest mate extract (cuticular hydrocarbons, nest entrance tubes and involucrum sheaths). A higher number of guard bees opened their mandibles and even proceeded to attack when presented with an extract from (between nest) con-specific non-nest mates and (between species) hetero-specific non-nest mates compared to when presented with a solvent control (Wald's χ^2=128.3, df = 2, P<0.001). *Plebeina hildebrandti* guard bees exhibited the highest level of aggression to non- nest mate stimuli (con-specifics and hetero-specifics) (Wald's χ^2=51.9, df = 2, P<0.001) than *Meliponula ferruginea (black)* (Wald's χ^2=36.7, df = 2, P<0.001), *Hypotrigona ruspoli* (Wald's χ^2=22.4, df = 2, P<0.001) and *Hypotrigona gribodoi* (Wald's χ^2=17.3, df = 2, P<0.001). Levels of aggression by guard bees increased significantly when a non-nest mate stimulus was presented to its nest entrance, but this varied significantly between treatments (ANOVA: $F_{1,25}$ =0.74, N=130, P=0.002).

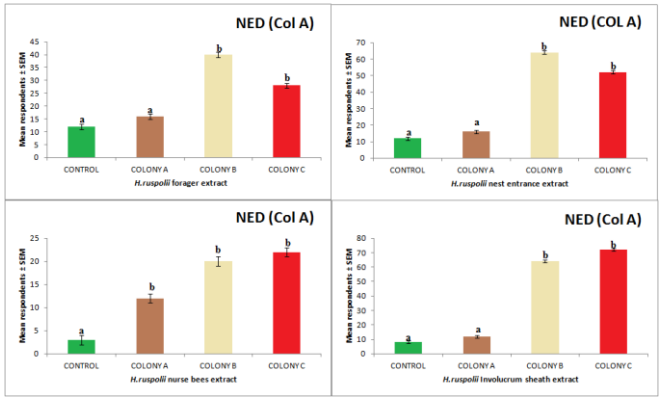

Fig 5: Aggressive responses exhibited by the meliponine bee species, *Hypotrigona ruspoli* during the nest entrance defense bioassay when presented with respective con-specific stimulus (between nests).

5.2.4.3 Cuticular profiles of four African meliponine bee species.

Cuticular profiles from the four African meliponine bee species revealed similar composition as they are composed of a dominant complex mixture of alkanes, alkenes and methyl-branched alkanes ranging from C8-C35 (Figure 6a) with trace amounts of acids, esters, aldehydes and ketones. The n-alkanes had retention times and mass spectra that matched with those of authentic standards (El-Sayed, 2009). 12 major components 9- Hexadecanoic acid, Hexadecane (C16), Octadecane (C18), Eicosane (C20), 3-methylheneicosane (C21), Tricosane (C23), Tetracosane (C24), Hexacosane (C26), Heptacosane (C27), Triacontane (C30), Dotriacontane (C32), Pentatriacontene (C35), dominated both cuticular profiles of both nurse bees and worker bees (foragers) of these species as the proportions of short-chained alkanes in the cuticular extracts remained constant, with no significant difference (P=0.689) in the relative abundance of both alkenes and methyl-branched alkanes (Figure 6a-g). However, both the nest entrance and the involucrum sheaths of all four species largely comprised of terpenoids and aldehydes such as (*E*)-β Farnesene and a combination of the straight chained alkanes.

These different four species could be distinguished by using the transformed peak areas of these 12 compounds that

dominantly occurred among the species. Using the stepwise DA, six variables grouped the bees according to their species with function 1 explaining 81.24% of the variation separating species 1 and 2 from both species 3 and 4, and function 2 explaining 18.76% of the variation further separating species 3 and 4 from species 2 and 1. The discriminating compounds selected by the stepwise DA were: *3*-methylheneicosane, *n*-Pentatriacontene, 9-Hexadecenoic acid (Z), β-Farnesene (*E*) and Heptacosane.

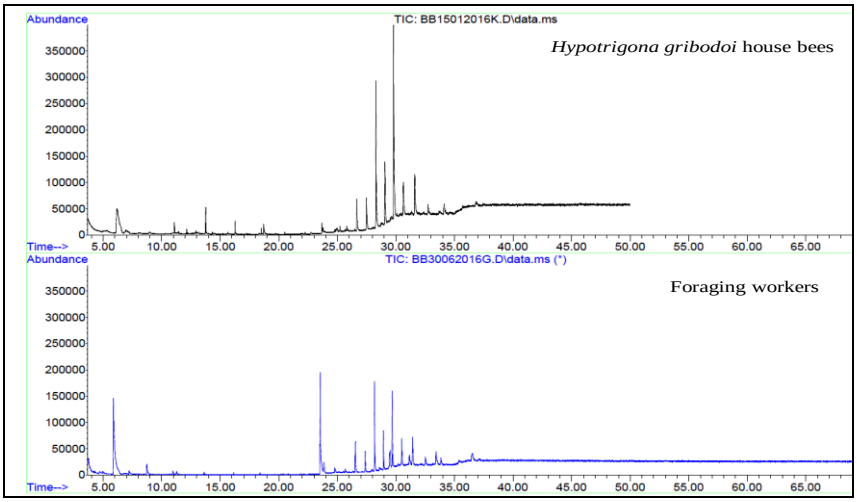

Fig 6a: Cuticular hydrocarbon profile of *H. gribodoi* house bees and foragers.

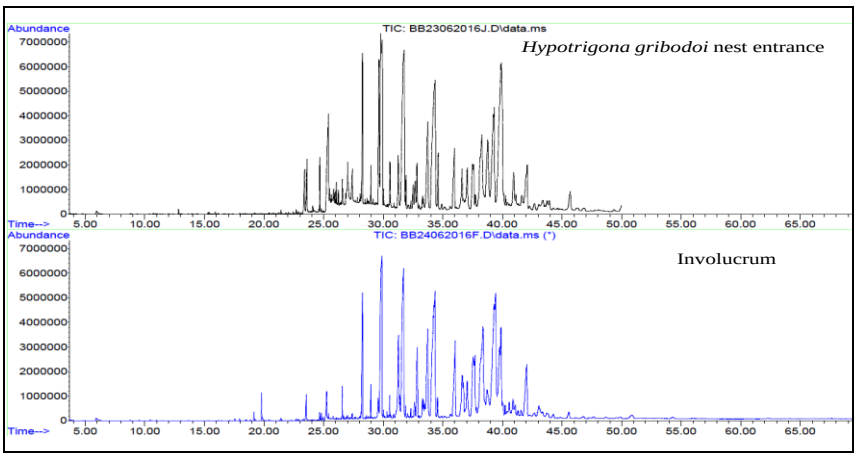

Fig 6b: Cuticular hydrocarbon profile of *H. gribodoi* nest entrance and involucrum.

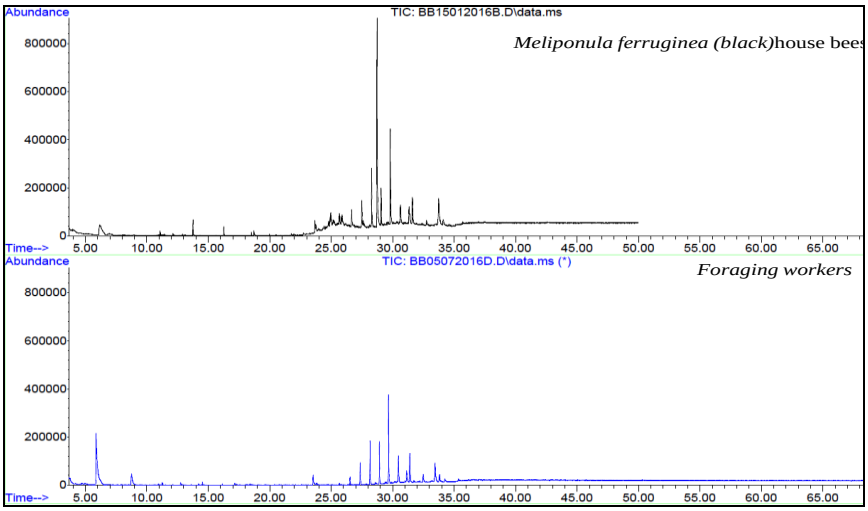

Fig 6c: Cuticular hydrocarbon profile of *M. ferruginea* (black) house bees and foragers.

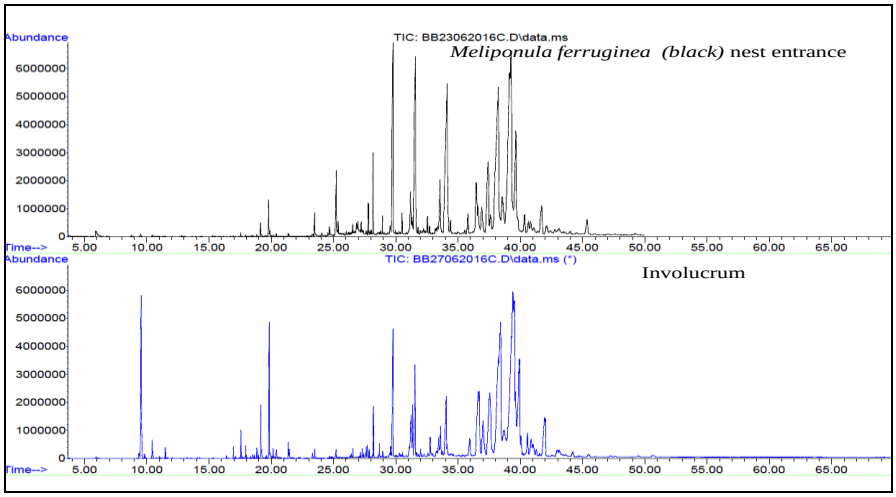

Fig 6d: Cuticular hydrocarbon profile of *M. ferruginea* (black) nest entrance and involucrum.

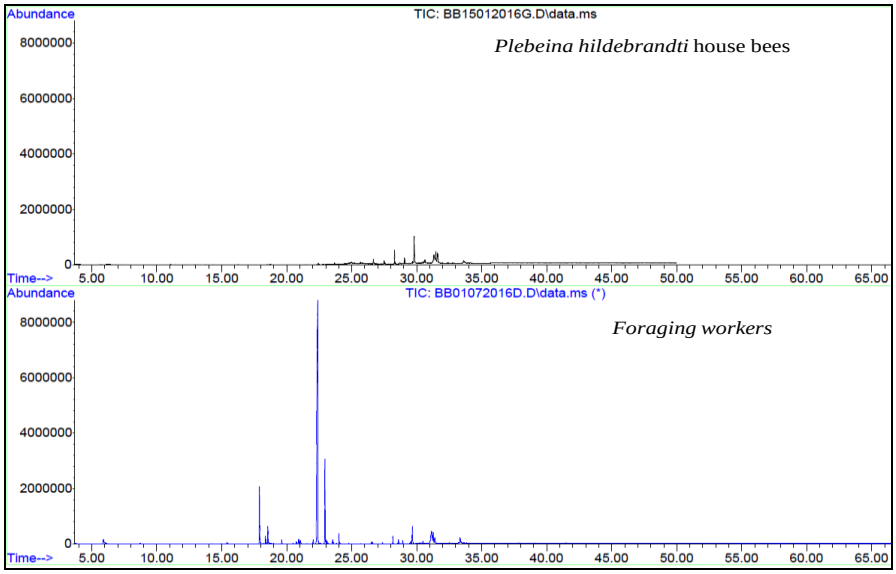

Fig 6e: Cuticular hydrocarbon profile of *Plebeina hildebrandti* house bees and foragers.

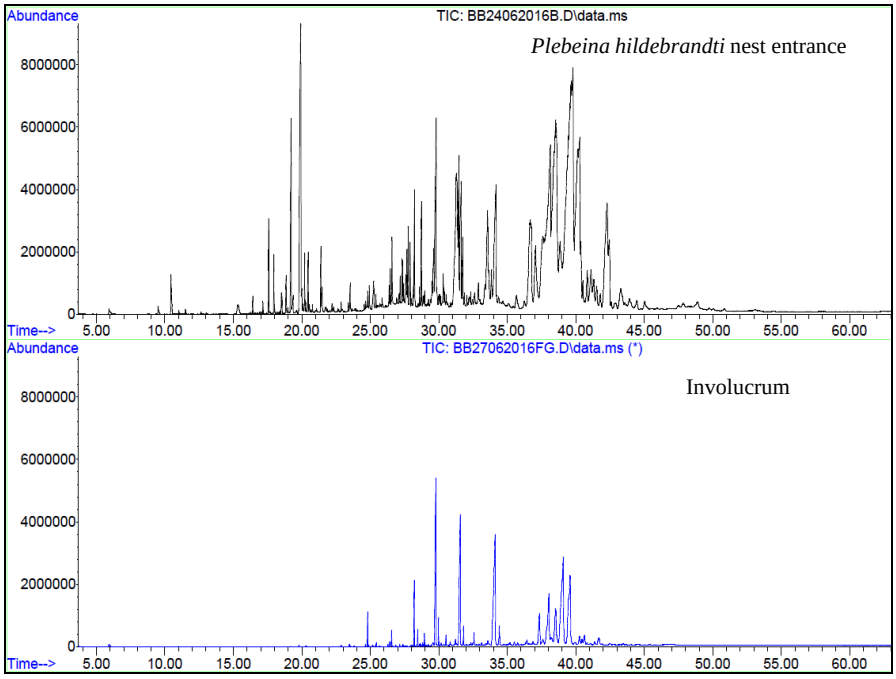

Fig 6f: Cuticular hydrocarbon profile of *Plebeina hildebrandti* nest entrance and involucrum.

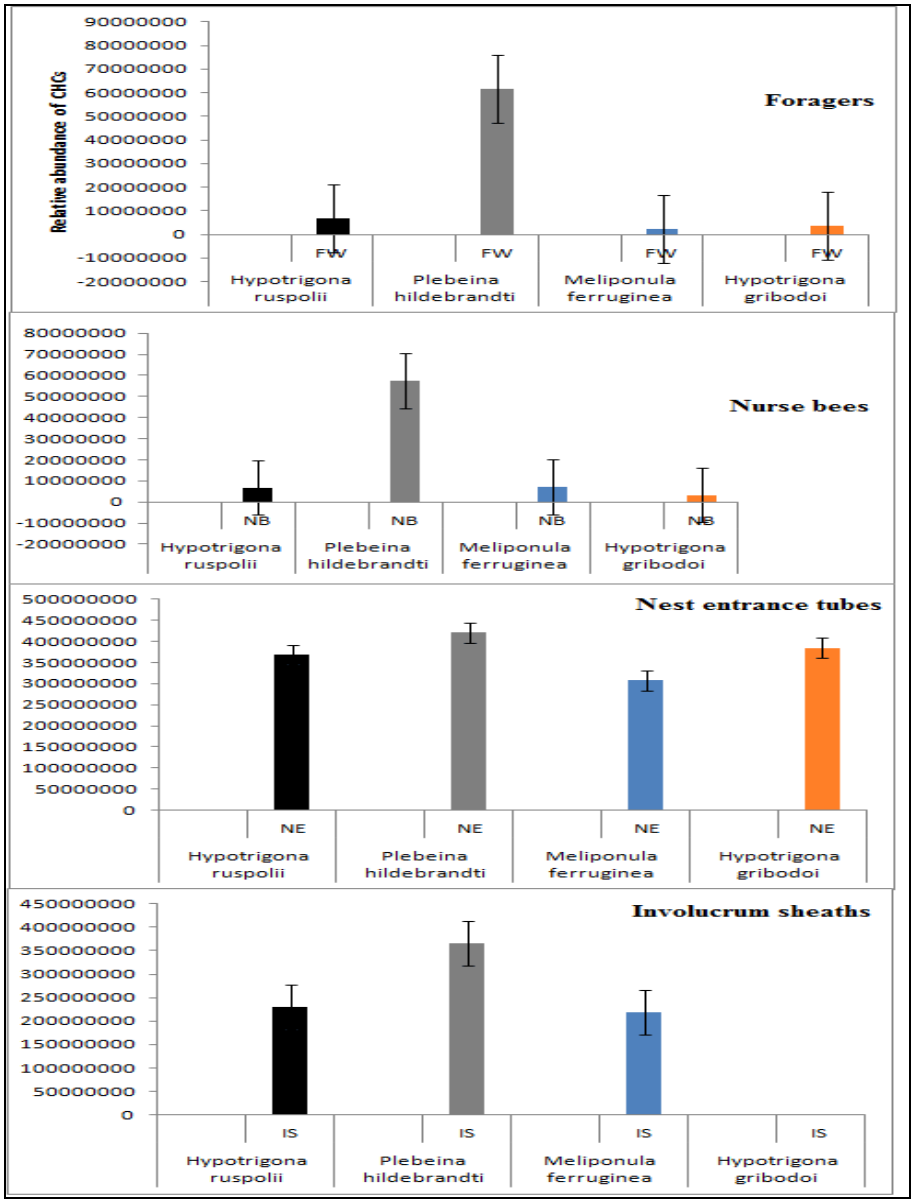

Fig 6g: Relative abundance of cuticular hydrocarbons (alkenes and methyl-branched alkanes) from the different stimulus (foragers, nurse bees, nest entrance tubes and involucrum sheaths) of the four meliponine bee species.

Bioassays with synthetic compounds

At least one compound from each representative group tested yielded a significant increase in aggression over control levels. 9-Hexadecanoic acid and β-Farnesene (*E*) significantly increased levels of aggression between hetero-specifics in all four species, while Eicosane (C20) and Oleic acid had no significant effect on aggression or recognition process. Figure 7 showed the response observed by the pairs of bees from the same colony and different species in which one bee was treated by exposure to the respective synthetic compounds: Eicosane (C20), 9-Hexadecanoic acid, Oleic acid (C18) and β-Farnesene (*E*).

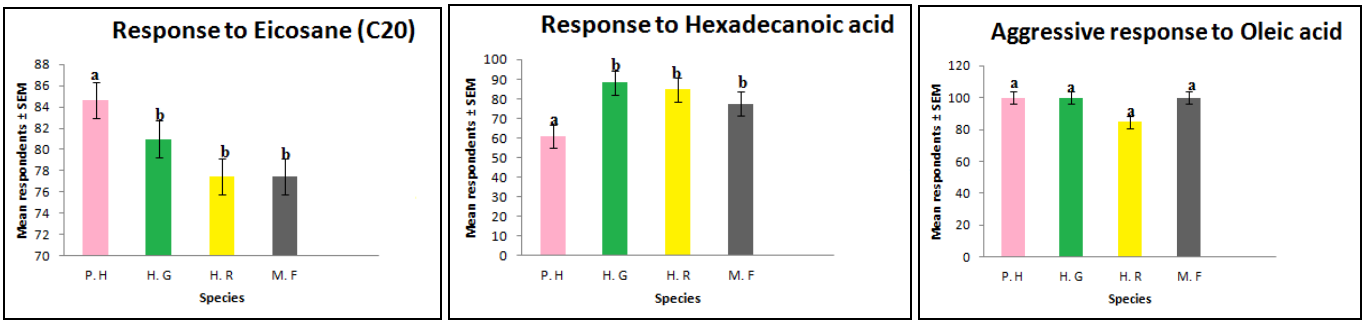

Fig 7: Aggressive responses exhibited by four meliponine bee species during the (MOR) mandible opening response bioassay when presented with selected synthetic compound stimuli found to dominate their cuticular profiles. *M.F: *Meliponula ferruginea*, H.R: *Hypotrigona ruspoli*, H.G: *Hypotrigona gribodoi*, P.H: *Plebeina hildebrandti*.

Discussion

Our findings have shown that similar recognition cue compounds utilized in Afro-tropical meliponine bee species is similar to the honeybee *Apis mellifera*, which is corroborated by our findings. Bioassays revealed *Meliponula ferruginea* (black), *Plebeina hildebrandti*, *Hypotrigona gribodoi* and *Hypotrigona ruspoli* all positively responded to a group of compounds similar to responses elicited from *Apis mellifera* [31]. The positive antennal response to a trans fatty acid (Z) 9-Hexadecenoic acid and a sesquiterpene, β-Farnesene (*E*) is consistent with that in *Apis mellifera* [31, 48] which similarly revealed positive responses to (Z) 9-tricosene and 16-C and 18-C fatty acids, suggesting a generality of signal function in nest mate recognition between these closely related bees of the same family. Nest mate recognition system of these four meliponine bee species operates remarkably in a similar to that of the honey bee, *Apis mellifera*, and this further confirms that individuals can predict the difference between the odor of a nest mate, and that of another bee they encounter coming from either a different nest or species [30]. Aggression levels observed in these four bee species could have resulted from differing olfactory perception for these compounds or an insignificant effect of some other compounds found in trace amounts being less important than others in making nest mate/non-nest mate recognition decisions. Such minute differences between an expected and an actual odor may definitely take longer to detect and process compared to compounds which make up a large proportion resulting in a rapid detection and hence a shorter time to exhibit aggression.

Due to the probability that wax could be an additional acquisition channel of nest mate recognition cues in bees, it is noteworthy to observe that the composition of the nest material (nest entrance tube and involucrum sheaths) from these African meliponine bee species all contained slightly similar hydrocarbon and lipid content, especially the composition of the involucrum sheaths which is a mixture of wax and plant resin. The relative amounts from *Meliponula ferruginea* (black) contained 67% hydrocarbons, 21% fatty acids and 10% esters; *Hypotrigona gribodoi* contained 54% hydrocarbons,12% fatty acids and 10% esters; *Hypotrigona ruspoli* contained 71% hydrocarbons, 31% fatty acids and 11% esters and *Plebeina hildebrandti* containing 43% hydrocarbons, 37% fatty acids and 18% esters revealed the same degree of similarity when compared with *Apis mellifera* wax which contained 16% hydrocarbons, 35% esters and 14% fatty acids (50)(Hart and Ratnieks, 2002; Patricio *et al*., 2002). Similarly, the waxes of *Trigona buyssoni* and *Trigona atomaria* consist of 59% hydrocarbons, 27% monoesters and 5% free acids, and 71% hydrocarbons, 26% monoesters and 2% free acids, respectively [4, 52, 53, 54] found that the mixture of compounds in the involucrum sheaths of *Melipona bicolor* was more identical to those obtained from *Trigona* species than to that of *Apis mellifera*, although the involucrum sheaths of *Melipona bicolor* had significantly higher proportions of monoesters (23%) compared to these four African meliponine bees, *Meliponula ferruginea* (black) (10%), *Hypotrigona gribodoi* (10%), *Hypotrigona ruspoli* (11%), *Plebeina hildebrandti* (18%) [55]. Suggested that (16-C) palmitoleic and (18-C) oleic acids are the metabolic source for alkenes in *Melipona bicolor* wax, where (18-C) has been reported to function as dominant recognition cues [56] (Breed, 1998b). In this study, our results with the even chained alkane: Eicosane (20-C) on the four meliponine bee species is quite consistent with that of *Apis mellifera* as octadecane (18-C) significantly affected nest mate recognition. This indicates that these compounds, if present in substantial amounts in both their nest entrances and building structures (cerumen), could serve as additional channels to acquire recognition cues.

The most important nest mate recognition cues in *Apis mellifera* are the free fatty acids [57] (Breed, 1998b), which also showed substantial behavioral activity in three out of the four meliponine bee species. Oleic acid (Z)-9-Octadecenoic acid yielded negative results in *Meliponula ferruginea* (black), *Hypotrigona gribodoi* and *Hypotrigona ruspoli* species except *Plebeina hildebrandti*, with 9-Hexadecenoic acid which is an unsaturated 18-C fatty acid yielding positive results in these three species. 16-C and 18-C fatty acids are prominent components in *Apis mellifera* wax (Tulloch, 1980) and are present in most meliponine bee waxes that have been studied [54, 58], although no information is available for the wax composition of these four African meliponine bee species, these fatty acids have higher melting points than alkanes and alkenes and may add important structural characteristics to bees' waxes [51].

The dominant compounds found in the involucrum sheaths and nest entrance tubes of these bee species especially *Plebeina hildebrandti* may further point to the use of exogenous cues to discriminate nest mates from non-nest mates. Environmental odors are known to affect nest mate recognition in many eusocial insect species [59] and the use of these exogenous odors derived from the environment gives a complexity to nest mate templates, making room for more precise recognitions compared to the limited range of compounds found only in their cuticular profiles. Unlike honeybees, meliponine bees utilize more plant materials during nest construction, such as resins in addition to wax [60] which may contribute to a more complex blend. The use of a wider range of acquisition channels for recognition by all four bee species reveals that signals originating from endogenously produced cuticular hydrocarbons need not be the only acquisition channel of recognition cues in these species. Exogenous volatiles, such as those found in resins, when brought into the nest during construction and maintenance may also serve as readily available cue sources.

Conclusion

The results of both mandibular opening response (MOR) and nest entrance defense (NED) bioassays suggest that these species do make use of CHCs but in varying proportions, but the chemical profiles of both nest entrances and involucrum sheaths do suggest that these bee species do employ a mechanism to distribute chemical components, with very minute differential substances as unique compounds in their colonies, which could be responsible for the ability for these species to precisely recognize their nest mates from non-nest mates. This further confirms that Afro- tropical meliponine bee species can distinctly recognize nest mate using CHCs. However, other exogenously derived cues can potentially play a role in successful discrimination of nest mates from non-nest mates.

References

1. Evans T, Inta R, Lai J, Lenz M. Foraging vibration signals attract foragers and identify food size in the drywood termite, Cryptotermes secundus. Insectes Soc [Internet]. 2007 [cited 2016 Jan 11]; Available from: http://link.springer.com/article/10.1007/s00040-007-0958-1

2. Goulson D, Stout JC, Langley J, Hughes WOH. Identity And Function Of Scent Marks Deposited By Foraging Bumblebees. J Chem Ecol. 2000; 26(12).
3. Biesmeijer J, Slaa E. Information flow and organization of stingless bee foraging. Apidologie [Internet]. 2004 [cited 2016 Jan 14]; Available from: http://www.apidologie.org/index.php?option=com_article&access=standard&Itemid=129&url=/articles/apido/pdf/2004/02/M4203.pdf
4. Nieh J, Contrera FL, Yoon R, Barreto L, Imperatriz-Fonseca V. Polarized short odor-trail recruitment communication by a stingless bee, Trigona spinipes. Behav Ecol Sociobiol [Internet]. 2004 Sep 20 [cited 2016 Jun 21]; 56(5). Available from: http://link.springer.com/10.1007/s00265-004-0804-7
5. Schorkopf D, Jarau S. Spitting out information: Trigona bees deposit saliva to signal resource locations. R … [Internet]. 2007 [cited 2017 Apr 21]; Available from: http://rspb.royalsocietypublishing.org/content/274/1611/895.short
6. Slaa E, Hughes W. Local enhancement, local inhibition, eavesdropping, and the parasitism of social insect communication. Food Exploit by Soc insects … [Internet]. 2009 [cited 2016 Jan 14]; Available from: https://books.google.com/books?hl=en&lr=&id=kto_CQAAQBAJ&oi=fnd&pg=PA147&dq=Nest+specificity+of+the+trail+pheromone+of+Trigona+corvina+workers+(Apidae,+Meliponini)+and+its+genetic+context&ots=4Zn4QdlF_r&sig=HFOvXZLhswmSgM7g5XCZPGTAABQ
7. Leonhardt SD, Blüthgen N. A Sticky Affair: Resin Collection by Bornean Stingless Bees. Biotropica. 2009; 41(6):730-6.
8. Nunes T, Nascimento F, Turatti I. Nestmate recognition in a stingless bee: does the similarity of chemical cues determine guard acceptance? Anim Behav [Internet]. 2008 [cited 2016 Jan 19]; Available from: http://www.sciencedirect.com/science/article/pii/S0003347207006185
9. Yusuf A, Pirk C, Crewe R. Nestmate recognition and the role of cuticular hydrocarbons in the African termite raiding ant Pachycondyla analis. J Chem [Internet]. 2010 [cited 2016 Jan 7]; Available from: http://link.springer.com/article/10.1007/s10886-010-9774-6
10. Zweden J van, d'Ettorre P. Nestmate recognition in social insects and the role of hydrocarbons. Insect Hydrocarb Biol [Internet]. 2010 [cited 2017 Apr 13]; Available from: https://books.google.com/books?hl=en&lr=&id=brc3SLVzo-oC&oi=fnd&pg=PA222&dq=recognition+cues+in+honey+bees&ots=3AuUBvnOWJ&sig=ylqYAfsdEl47AuN3m5nYN3v24mg
11. Gastauer M, Campos LAO, Wittmann D. Handling sticky Resin by Stingless Bees : Adhesive Properties of Surface Structures. 2013; 85:1189-96.
12. Akino T, Yamaoka R. Trail discrimination signal of Lasius japonicus (Hymenoptera: Formicidae). Chemoecology [Internet]. 2005 [cited 2016 Jan 14]; Available from: http://link.springer.com/article/10.1007/s00049-005-0288-6
13. Katzerke A, Neumann P, Pirk C. Seasonal nestmate recognition in the ant Formica exsecta. Behav Ecol… [Internet]. 2006 [cited 2016 Jan 6]; Available from: http://link.springer.com/article/10.1007/s00265-006-0245-6
14. Gibbs JP, Stanton EJ. Habitat fragmentation and arthropod community change: Carrion beetles, phoretic mites, and flies. Ecol Appl. 2001; 11(1):79-85.
15. Foley JA, DeFries R, Asner GP, Barford C, Bonan G, Carpenter SR, *et al*. Global Consequences of Land Use. Science (80-). 2005; 309(5734).
16. Huang Q, Wang W. Studies on feeding and trophallaxis in the subterranean termite Odontotermes formosanus using rubidium chloride. Entomol… [Internet]. 2008 [cited 2016 Jan 11]; Available from: http://onlinelibrary.wiley.com/doi/10.1111/j.1570-7458.2008.00764.x/full
17. Nunes T, Turatti I, Mateus S. Cuticular hydrocarbons in the stingless bee Schwarziana quadripunctata (Hymenoptera, Apidae, Meliponini): differences between colonies, castes and age. Genet Mol… [Internet]. 2009 [cited 2016 Jan 19]; Available from: http://www.funpecrp.com.br/gmr/year2009/vol8-2/pdf/kerr012.pdf
18. Torto B, Boucias DG, Arbogast RT, Tumlinson JH, Teal PEA. Multitrophic interaction facilitates parasite-host relationship between an invasive beetle and the honey bee. Proc Natl Acad Sci U S A. 2007; 104(20):8374-8.
19. Barth F, Hrncir M, Jarau S. Signals and cues in the recruitment behavior of stingless bees (Meliponini). J Comp Physiol A [Internet]. 2008 [cited 2017 Apr 21]; Available from: http://link.springer.com/article/10.1007/s00359-008-0321-7
20. Chen L, Sharma KR, Fadamiro HY. Fire ant venom alkaloids act as key attractants for the parasitic phorid fly, Pseudacteon tricuspis (Diptera: Phoridae). Naturwissenschaften. 2009; 96:1421-9.
21. Jungnickel H, Costa A Da. Chemical basis for inter-colonial aggression in the stingless bee Scaptotrigona bipunctata (Hymenoptera: Apidae). J insect… [Internet]. 2004 [cited 2016 Jan 19]; Available from: http://www.sciencedirect.com/science/article/pii/S0022191004000988
22. Schorkopf D, Hrncir M. Mandibular gland secretions of meliponine worker bees: further evidence for their role in interspecific and intraspecific defence and aggression and against their role. J. [Internet]. 2009 [cited 2016 Jan 14]; Available from: http://jeb.biologists.org/content/212/8/1153.short
23. Simone-Finstrom M, Spivak M. Propolis and bee health: the natural history and significance of resin use by honey bees. Apidologie [Internet]. 2010; 41(3):295-311. Available from: http://link.springer.com/10.1051/apido/2010016
24. Aguilar I, Fonseca A, Biesmeijer J. Recruitment and communication of food source location in three species of stingless bees (Hymenoptera, Apidae, Meliponini). Apidologie [Internet]. 2005 [cited 2016 Jan 14]; Available from: http://www.apidologie.org/index.php?option=com_article&access=standard&Itemid=129&url=/articles/apido/pdf/2005/03/M4064.pdf
25. Akino T, Yamamura K. Direct behavioral evidence for hydrocarbons as nestmate recognition cues in Formica japonica (Hymenoptera: Formicidae). Appl Entomol…

[Internet]. 2004 [cited 2016 Jan 6]; Available from: http://jlc.jst.go.jp/DN/JALC/00242606480?from=Google
26. Akino T, Yamamura K, Wakamura S, Yamaoka R. Direct behavioral evidence for hydrocarbons as nestmate recognition cues in Formica japonica (Hymenoptera: Formicidae). Appl Entomol Zool [Internet]. 2004; 39(3):381-7. Available from: https://www.jstage.jst.go.jp/article/aez/39/3/39_3_381/_pdf
27. Zweden J van, d'Ettorre P. Nestmate recognition in social insects and the role of hydrocarbons. Insect Hydrocarb Biol … [Internet]. 2010 [cited 2016 Jan 19]; Available from: https://books.google.com/books?hl=en&lr=&id=brc3SLVzo-oC&oi=fnd&pg=PA222&dq=cuticular+hydrocarbons+in+the+stingless+bee&ots=3AqVCxpHWK&sig=VB52TF6TVOFrJMkGV_mQ8QPftMo
28. Bowden R, Williamson S, Breed M. Floral oils: their effect on nestmate recognition in the honey bee, Apis mellifera. Insectes Soc [Internet]. 1998 [cited 2017 Apr 13]; Available from: http://www.springerlink.com/index/FPR5W8J10VU66CHD.pdf
29. Breed M, Bennett B. Kin recognition in highly eusocial insects. Kin Recognit Anim by David JC… [Internet]. 1987 [cited 2016 Jan 6]; Available from: http://agris.fao.org/agris-search/search.do?recordID=US201302067801
30. Breed M, Perry S, Bjostad L. Testing the blank slate hypothesis: why honey bee colonies accept young bees. Insectes Soc [Internet]. 2004 [cited 2017 Apr 13]; Available from: http://link.springer.com/article/10.1007/s00040-003-0698-9
31. Breed M, Leger E, Pearce A, Wang Y. Comb wax effects on the ontogeny of honey bee nestmate recognition. Anim Behav [Internet]. 1998 [cited 2017 Apr 13]; Available from: http://www.sciencedirect.com/science/article/pii/S0003347297905819
32. Burger H, Dötterl S, Ayasse M. Host-plant finding and recognition by visual and olfactory floral cues in an oligolectic bee. Funct Ecol. 2010; 24(6):1234-40.
33. Nunez C, Oliveira M De, Lima R. Chemical analyses confirm a rare case of seed dispersal by bees. Apidologie [Internet]. 2008; 39:618-26. Available from: http://link.springer.com/article/10.1051/apido:2008047
34. Potts SG, Vulliamy B, Roberts S, apos O, Toole C, Dafni A *et al*. Role of nesting resources in organising diverse bee communities in a Mediterranean landscape. Ecol Entomol. 2005; 30(1):78-85.
35. Roubik DW. Review article stingless bee nesting biology. Apidologie. 2006; 37:124-43.
36. Simone-Finstrom M, Spivak M. Propolis and bee health: the natural history and significance of resin use by honey bees. Apidologie [Internet]. 2010 [cited 2017 Jun 4]; Available from: http://www.apidologie.org/articles/apido/full_html/2010/03/m09142/m09142.html?mb=0
37. Human H, Nicolson SW. Nutritional content of fresh, bee-collected and stored pollen of Aloe greatheadii var. davyana (Asphodelaceae). Phytochemistry. 2006; 67:1486-92.
38. Leonhardt SD, Kaltenpoth M. Microbial communities of three sympatric Australian stingless bee species. PLoS One [Internet]. 2014; 9(8):e105718. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25148082
39. Hölldobler B, Wilson E. The ants [Internet]. 1990 [cited 2016 Jan 6]. Available from: https://books.google.com/books?hl=en&lr=&id=R-7TaridBX0C&oi=fnd&pg=PA1&dq=The+Ants&ots=sK0PebGN5c&sig=grVrRDFWeCtjBppHIP4KppoNNOY
40. Jackson BD, Morgan ED. Insect chemical communication: Pheromones and exocrine glands of ants. Chemoecology [Internet]. 1993 Sep [cited 2016 Jun 21]; 4(3, 4):125-44. Available from: http://link.springer.com/10.1007/BF01256548
41. Stuart R, Herbers J. Nest mate recognition in ants with complex colonies: within-and between-population variation. Behav Ecol [Internet]. 2000 [cited 2016 Jan 7]; Available from: http://beheco.oxfordjournals.org/content/11/6/676.short
42. Dos Santos GM, Antonini Y. The traditional knowledge on stingless bees (Apidae: Meliponina) used by the Enawene-Nawe tribe in western Brazil. J Ethnobiol Ethnomed. 2008; 4:19.
43. Suka T, Inoue T. Nestmate recognition of the stingless beeTrigona (Tetragonula) minangkabau (Apidae: Meliponinae). J Ethol [Internet]. 1993 [cited 2017 Apr 13]; Available from: http://www.springerlink.com/index/t47k207xt120j7t3.pdf
44. Kirchner W, Friebe R. Nestmate discrimination in the African stingless bee Hypotrigona gribodoi Magretti (Hymenoptera: Apidae). Apidologie (France) [Internet]. 1999 [cited 2017 Apr 13]; Available from: http://agris.fao.org/agris-search/search.do?recordID=FR1999003440
45. Guerrieri F, d'Ettorre P. The mandible opening response: quantifying aggression elicited by chemical cues in ants. J Exp Biol [Internet]. 2008 [cited 2016 Jan 6]; Available from: http://jeb.biologists.org/content/211/7/1109.short
46. Shackleton K, Al Toufailia H, Balfour NJ, Nascimento FS, Alves DA, W Ratnieks FL. Appetite for self-destruction: suicidal biting as a nest defense strategy in Trigona stingless bees, 2014.
47. El-Sayed A. The pherobase. 2009 [cited 2016 Jan 6]; Available from: https://scholar.google.com/scholar?q=The++pherobase&btnG=&hl=en&as_sdt=0%2C5#4
48. Page R, Breed M. Kin recognition in social bees. Trends Ecol Evol [Internet]. 1987 [cited 2017 Apr 13]; Available from: http://www.sciencedirect.com/science/article/pii/0169534787900346
49. Frake AM, De Guzman LI, Rinderer TE. Comparative resistance of Russian and Italian honey bees (Hymenoptera: Apidae) to small hive beetles (Coleoptera: Nitidulidae). J Econ Entomol. 2009; 102(1):13-9.
50. Hart AG, Ratnieks FLW. Task-partitioned nectar transfer in stingless bees: Work organisation in a phylogenetic context. Ecol Entomol. 2002; 27(2):163-8.
51. Patricio EFLRA, Cruz-López L, Maile R, Tentschert J, Jones GR, Morgan ED. The propolis of stingless bees: Terpenes from the tibia of three Frieseomelitta species. J Insect Physiol. 2002; 48(2):249-54.
52. Johnson LK, Haynes LW, Carlson MA, Fortnum HA,

Gorgas DL. Alarm substances of the stingless bee, Trigona silvestriana. J Chem Ecol [Internet]. Kluwer Academic Publishers-Plenum Publishers; 1985 Apr [cited 2016; 11(4):409-16. Available from: http://link.springer.com/10.1007/BF00989552
53. Slaa EJ, Cevaal A, Sommeijer MJ. Floral constancy in Trigona stingless bees foraging on artificial flower patches: a comparative study. J Apic Res [Internet]. 1998; 37(3):191-8. Available from: isi:000078695300007
54. Biesmeijer JC, Giurfa M, Koedam D, Potts SG, Joel DM, Dafni A. Convergent evolution: Floral guides, stingless bee nest entrances, and insectivorous pitchers. Naturwissenschaften. 2005; 92(9):444-50.
55. Velthuis HHW, Koedamb D, Imperatriz-Fonseca VL, Elthuisa HHW V, Oedamb DK, Onsecab VLIM. The males of Melipona and other stingless bees, and their mothers. Apidologie. 2005; 36:169-85.
56. Breed M. Chemical cues in kin recognition: criteria for identification, experimental approaches, and the honey bee as an example. 1998 [cited 2017 Apr 13]; Available from: http://agris.fao.org/agris-search/search.do?recordID=US1997062412
57. Wilson AL, Ward D, Brown M, Johnson SD. Seed production in a threatened Aloe is not affected by bird exclusion or population size. Plant Ecol. 2009; 203(2).
58. Blomquist G. Chemistry, biochemistry, and physiology of insect cuticular lipids. Arch Insect… [Internet]. 1987 [cited 2016 Jan 6]; Available from: http://onlinelibrary.wiley.com/doi/10.1002/arch.940060404/pdf
59. Downs S, Ratnieks F. Adaptive shifts in honey bee (*Apis mellifera* L.) guarding behavior support predictions of the acceptance threshold model. Behav Ecol [Internet]. 2000 [Cited 2017 Apr 13]; Available from: http://beheco.oxfordjournals.org/content/11/3/326.short
60. Eltz T, Brühl CA, Imiyabir Z, Linsenmair KE. Nesting and nest trees of stingless bees (Apidae: Meliponini) in lowland dipterocarp forests in Sabah, Malaysia, with implications for forest management. For Ecol Manage. 2003; 172(2, 3):301-13.