ECOLOGICAL, BEHAVIOURAL AND BIOCHEMICAL TRAITS OF AFRICAN MELIPONINE BEE SPECIES (Apidae:Meliponini) IN A BIODIVERSE HOTSPOT OF KENYA.

BY

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DECLARATION

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This thesis is my original work and has not been presented for a degree in any other university or any other award.

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DEDICATION

This work is dedicated to Almighty God, my ever present help, my constantly supportive parents (Mr. Sunday and Mrs. Cecilia Aitokhuehi) for their continous prayers, help and huge encouragement, my husband (Ayodotun Bobadoye), and finally my blessed child (Oluwasindara Ofure Osemobor Benedicta Bobadoye).

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ABBREVIATIONS AND ACRONYMS

CHCs   Cuticular Hydrocarbons
FID   Flame Ionisation Detector
GC   Gas Chromatograph
GC-EAD   Gas Chromatography-Electroantennographic Detection
GC-MS   Gas Chromatography-Mass Spectrometry
MS   Mass Spectrometry
PCR   Polymerase Chain Reaction

ABSTRACT

This study was carried out with the aim of assessing habitat ecology and behavioral mechanisms critical for the survival of African meliponine bee species. This will help to fully decipher how they effectively communicate information amongst colony members during two vital ecological processes (colony defense and foraging). Surveys of the floral phenology of potential food plants of African meliponine bees (Apidae: meliponini) in six diverse habitat gradients, observed that most flowering plants overlapped across seasons, which could potentially provide both floral resources (nectar and pollen) to foraging; meliponine bee species. Approximately 80 different plant species belonging to 34 families were recorded, with high proportions from Fabaceae and Asteraceae families dominating flowering plants in both lowland and highland habitats. This indicates that such diverse vegetation found in these habitats could invariably sustain nutritional requirements essential for the survival of insect pollinators such as native meliponine bee species.

Further surveys conducted in these habitats confirmed the natural occurrence of four different meliponine bee species; *Hypotrigona gribodoi*, *Hypotrigona ruspolii*, *Meliponula ferruginea* (black) and *Plebeina hildebrandti*. The abundance of feral colonies were observed to be dissimilar across the habitats, with *Hypotrigona gribodoi* exhibiting the highest level of plasticity (abundance and diversity) in nesting preferences across the six habitats sampled, while *Plebeina hildbrandti* demonstrated the lowest level of plasticity, which may be attributed to their flexibility in nesting in varying habitat types. Diversity profiles indicates that MDW (mixed deciduous wood lands) presented itself as a much preferred habitat for nesting and trees as a preferred nesting substrate, as profile curves indicated that more species from all four species could be identified with increased sampling transects in this habitat and on more tree nesting substrates compared to other sampled habitats, it unmistakably signifies the negative effects that disturbed habitats play in predicting the diversity of bee species within an ecosystem. Discrimination of the meliponine bee species was further demonstrated by the analysis of their mitochondrial cytochrome oxidase 1(mt*COI*) gene and wing venation patterns. Sequence analysis demonstrated high divergence enough to characterize bee specimens according to their species, while the wing venation pattern provided sufficient proof to support the level of phylogenetic segregation.

Discrimination between nest-mates from non-nest mates from the four African meliponine bee species is facilitated through olfactory cues. Behavioral and electrophysiological assays together with coupled GC-MS analyses revealed that these species correctly detect members of their own colony based on the existence of a signature odor (alkanes, alkenes and methyl- branched alkenes), but were significantly defensive when exposed to the extract of a non-nest mate. This may imply that surface CHCs amongst other exogenous acquisition channels (nest entrance and nest construction materials) could play additional roles as recognition cues for individuals to locate con-specifics and discriminate hetero-specifics.

Behavioral assays on foraging patterns of the four meliponine bee species showed insignificant differences between them. In trail laying bioassays, components of the volatiles from nasonov glands were twice more attractive to foragers compared to tarsal glands. Coupled GC-MS analyses identified the biological active peaks to be dominantly terpenes and esters. Additional trail laying bioassays with the dominant volatile compound (*E*)-*β* Farnesene identified from both the tarsal and nasonov glands showed that these bees may potentially produce trail pheromones from the nasonov glands, but deposit and disperse them via the openings located in the tarsal glands.

In conclusion, this study has revealed how natural habitats converted to agro-ecosystems shape the diversity of African meliponine bee species in this biodiverse hotspot in Kenya. It also shows the use of olfactory cues by individual foragers to discriminate nest mates from non nest mates, which similarly occurs in honey bees during colony defense. It also implicates the nasonov gland as a likely source of trail pheromone production, while the tarsal glands facilitate the deposition and distribution of these essential compounds during foraging.

CHAPTER ONE
INTRODUCTION

1.1 General Introduction

Bees are believed to originate from a single spheci-form species (Michener, 2000) comprising of seven families with over 400 genera, which include the familiar honey bee (*Apis mellifera*), bumblebees (*Bombus terrestris*) and meliponine bees (*Melipona* species). There are over 16,000 described bee species distributed worldwide (Michener, 2001), with a vast majority categorized as solitary species. Bees are highly adaptive which makes them most successful pollinators due to their high dependence on nectar and pollen from flower resources for feeding; they exhibit among the highest floral visitation rates in the world, making them the single most important group of pollinators (Potts *et al*., 2006; Klein *et al*., 2007; Abrol, 2012).

Bees form keystone mutualisms with their host plants thereby maintaining the biodiversity of most terrestrial eco-systems (Potts *et al*., 2006; Morris 2010; Frund *et al*., 2013). They also play a vital role in the reproduction of most angiosperms by pollinating flowers (Steffan-Dewenter and Klein, 2006; Bradbear, 2009; Gill *et al*., 2016). Through this ecosystem service, they contribute to the preservation and maintenance of genetic diversity of flowering plants (Eltz *et al*., 2001; Sharma *et al*., 2011; Frund *et al*., 2013). This is particularly notable in agro-ecosystems, where bees are known to contribute to an increase of crop yields both qualitatively and quantitatively (Klein *et al*., 2007; Garibaldi *et al*., 2015). The value of crop pollination by the most important managed pollinator, the honey bee *A. mellifera*, is estimated to be 5-14 billion dollars per year in the United States alone (Kremen and M'Gonigle, 2015) with a global estimate of US$ 65-70 billion (Kremen *et al*., 2007; Giannini *et al*., 2015; Munyuli, 2010). Recent reviews indicate that 35% of total crop production volume and 70% of major global crops rely solely on animal pollination (Klein *et al*., 2003; Klein *et al*., 2007). In agricultural landscapes, bees have long been reputed to be vital for successful fruit production (Sheffield *et al*., 2008). According to Potts *et al*., (2003) an estimated 60-70% of flowering plant species are dependent upon insects for pollination.

While bees contribute to preservation of ecosystems, they too benefit from on the resilience and stability of these ecosystems for survival. Indeed, their diversity, distribution and abundance are related to environmental conditions as well as anthropogenic impacts in the ecosystems they live in (Brown and De Oliveira, 2014; Brown and Albrecht, 2001; Goulson *et al.*, 2015). However, pollination success of insect-pollinated crops is not solely dependent on a single, highly specialized pollinator species, but on a diverse community of pollinators (Steffan-Dewenter and Westphal, 2008).

In the face of global declines in pollinator populations, a combination of factors such as climate change and anthropogenic activities (e.g. deforestation for timber use, charcoal production, increased agricultural production, habitat fragmentation and isolation) have been shown to jeopardize the survival and stability of bees in habitats (Goulson *et al.*, 2015). Due to this negative trend, there has been renewed interest targeting alternative pollinators, such as meliponine bees, that can function in the same capacity as the honey bee, *Apis mellifera*. Meliponine bees of African origin belong to one of three subfamilies of the family *Apidae* (Roubik, 2006b; Michener, 2000a). They are a group of small-to-average sized bees with atrophied stings which share certain important traits, such as production of similar hive products, colony organization and behavioral patterns with the honey bee (Heard, 2000; 2001). Meliponine bees are known to be important pollinators in agricultural ecosystems (Heard, 1999; Slaa *et al.*, 2006; Brown and Oliveira, 2014) and in non-crop plant species in natural landscapes (Harrison *et al.*, 1999; Slaa *et al.*, 2006), It is therefore necessary to gain more insight about the ecological requirements of African meliponine bee species essential for their survival, understand their behavioral patterns and determine the underlying mechanisms that explain such behaviors, especially during foraging, which would facilitate easier domestication for pollination purposes. It is expected that this study shall generate useful information on the ecology of African meliponine bee species, their territorial defensive behaviors and the mechanisms that facilitate their communication to ensure successful foraging bouts. This information is imperative to enhanced and sustainable utilization of these pollinators and in future conservation efforts.

1.2 Problem statement

Bee communities, both wild and managed, have been declining over the last half century as various causes such as pesticide use in agricultural and urban areas have increased tremendously, amidst other anthropogenic factors. Changes in land use have equally resulted in patchy distributions of vegetation which has gradually shaped food and nesting resources for pollinators. In such unpredictable environments, it's therefore expedient to make intelligent decisions regarding everyday foraging and defense strategies. Since foragers communicate information about the environment to the colony, a combination of different communication channels between the colony and environment is indispensable. Odors and pheromones are omnipresent as signals and cues and are fundamental carriers of information in most arthropods. In social insects in particular, communication critically depends on chemical signaling, because when individual foragers pass information at a local level, the colony's decisions are thus also made locally. In other words, intelligent defense and foraging choices is not entirely a collective decision but an individualistic one, as the ability of a forager to find a profitable food source and recruit its nest mates to exploit it, is dependent on certain mechanisms employed by it. Even more impressive is the fact that decisions made to recognize a mate from a non-mate and to transmit the location of potential food sources could vary both widely and rapidly, making it hard to confirm if they make use of similar mechanisms like the honey bee to guide their behaviour. This information has rarely been documented for African meliponine bees in order to validate how these species search large regions for food sources, and what mechanisms they use to identify a potential threat to them as an individual to the whole colony. It is also uncertain if these bee species have developed other ways of communicating information between individuals and if such exchange of information between the individuals could lead to the selection of food sources in unpredictable environments. The purpose of this study is therefore to investigate the ecology, behavior and biochemical traits critical for the survival of these species. Linking foraged resources of these bee species to their behavior, will help to better understand how they effectively communicate information amongst colony members during the initiation, location and collection of food resources and also during defense.

1.3 Objectives

The overall objective of this study was to assess the biodiversity of meliponine bees and the mechanisms driving their foraging and nest-mate recognition behaviors.

1.3.1 Specific objectives

This study had three specific objectives:

1. To determine diversity of meliponine bees species within the Plateau-Mountainous ecosystems of Taita hills.
2. To determine the olfactory cues that influence nest-mate recognition behavior in meliponine bees.
3. To elucidate the role of trail pheromones in foraging in African meliponine bee species.

1.4 Justification

As majority of landscapes in Kenya drastically change due to habitat destruction and land conversion, the composition of pollinator communities is altered unknowingly and deficits are unintentionally created (Samejima *et al*., 2004; Winfree *et al*., 2009). This in turn threatens the normal functioning of pollination, which is one of the most critical reproductive processes essential for plant survival (Winfree and Kremen., 2009; Kennedy *et al*., 2013; Giannini *et al*., 2015). Pollinators are important species because plants and ecosystems largely depend on pollinators for stability due to the multiple roles they play in maintaining the viability of pollinator-dependent plants which in turn supports herbivore and carnivore survival within a food chain(García and Martínez., 2012; Martins *et al*., 2015). Meliponine bees are a group of eusocial insects that form part of this niche and they play an important role in the pollination process of plant life, particularly plants in natural and semi-natural habitats (Heard, 1999; Wille, 1983). They are also considered to be crucial pollinators in tropical forests (Roubik, 1989; Roubik *et al*., 1999; Corlett, 2004) and visit more than 100 plant species in a given habitat (Wilms *et al*., 1996).   In Africa, recent studies have been conducted on the taxonomy, biology and domestication of meliponine bee species (Kajobe, 2009; Kwapong *et al*., 2010; Nkoba *et al*., 2012) while overlooking more intricate studies on how these pollinators

actually survive within its habitat. Such detailed studies on the ecology and the influence of different landscape characteristics on their nesting and foraging behaviour are lacking.

The ability to exploit available food sources is vital for the survival of any existing meliponine bee's colony. Nevertheless, competition for these resources during foraging cannot be avoided for most insect pollinators either at intra- or inter-specific level. Effective communication through chemical and/or behavioral cues is known to be of paramount importance to meliponine bees during foraging (Nagamitsu and Inoue, 1997; Jha and Kremen, 2013; Aleixo *et al*., 2016) . However, the mechanisms that influence these bees' foraging behavior are poorly understood, particularly with respect to how they predict the location of such resources (Eltz *et al*., 2002; Slaa, 2003, 2006). It is also unclear what foraging habits and defensive strategies these bees employ to deter other hetero-specifics from a visited foraged site. By answering these questions will help to fully understand how they effectively communicate information amongst colony members during the initiation, location and collection of food resources and also during defense interactions. This study will provide useful information about ecological requirements of meliponine bees of African origin, their behaviours during foraging, territorial defence and the biochemical traits essential for survival and colony fitness in altered/ disturbed landscapes.

CHAPTER TWO

LITERATURE REVIEW

2.1 Classification of meliponine bees

Meliponine bees are members of the family Apidae and they are closely related to honey bees, bumble bees and orchid bees (Roubik, 1989, 1992, 2006b; Roubik *et al*., 2005; Vit *et al*., 2012). Over 300 species of meliponine bees have been described worldwide (Kennedy *et al*., 2013; Kleijn *et al*., 2015; Kremen *et al*., 2007; Williams and Tarpy, 2010) out of which 300 are categorized as social and live in organized colonies with elaborate caste systems (Hartfelder and Makert, 2006; Nogueira *et al*., 2014; Ribeiro *et al*., 2006). They are the largest group of eusocial bees with a fossil history dating back to about 65 million years ago (Camargo and Pedro, 2002; Pedro, 2014; Posey and Camargo, 1985). They are widely distributed throughout most tropical and Neo-tropical regions of the world (Engel and Michener, 2013; Michener, 2000b). Meliponine bees (Sub-family: Meliponinae) are divided into two tribes: *Meliponini* and *Trigonini*, with the latter having a higher number of genera and sub-genera (Eardley *et al*., 2015). *Meliponini* has 23 genera with 18 sub-genera, while *Trigonini* has 50 genera with 32 sub-genera (Engel and Michener, 2013; Michener, 2000b; Velthuis *et al*., 2005). Generally, meliponine bees are an understudied group of social bees with highly organized colonies (Pedro, 2014).

2.1.1 Geographical distribution and diversity of African meliponine bee species

Meliponine bees (Apidae, Meliponinae) occur in all tropical regions of the world where they are abundant in species and numbers. These diverse species are widely distributed ranging from tropical through subtropical regions of the world such as Africa, Australia, Southeast Asia, and parts of South America (Michener, 2000), where they thrive under ecologically diverse habitats such as forests and dryland savannahs (Eltz *et al*., 2003; Omoro *et al.*, 2010; Pfeifer *et al*., 2012). The diversity of meliponine bee species is high in Neotropical regions with as high as sixty species found in a single habitat > 20 ha (Roubik, 2006b; Roubik *et al*., 2005). Cortopassi-Laurino *et al.,* (2006) reported about 45 species of meliponine bees within an Asian community.

Klumpp (2007) also reported approximately 12 species that are widely distributed in Australia, while Eardley (2004) reported about 20 species comprising of six genera: *Meliponula*, *Plebeina*, *Hypotrigona*, *Cleptotrigona*, *Liotrigona* and *Dactylurina* that are widely distributed in Africa. Although knowledge of the exact number of meliponine bees' species in Kenya is hampered by inadequate nationwide bee surveys and wrongly identified species (Gikungu, 2006), though a number of species have recently been confirmed in recent studies by Nkoba (2012). These include *Hypotrigona gribodoi* and *Meliponula ferruginea* (black and brown), which are localized in Arabuko sokoke forest (Macharia and Raina, 2010), *Hypotrigona araujo* which has been reported in Mwingi, *Meliponula bocandei*, *Meliponula ferruginea* (reddish brown), *Meliponula lendiliana*, *Hypotrigona ruspolii* and *Plebeina hildebranti* has also been reported to occur in Kakamega forest (Macharia and Raina, 2010; Mwangi *et al*., 2012). Because of their high biodiversity and their great abundance in tropical forests, these bees are important pollinators in tropical ecosystems.

2.1.2 Meliponine bees and their nesting biology

Meliponine bees belong to the family Apidae as they are closely related to honey bees, bumble bees and orchid bees (Roubik, 2002). Generally, meliponine bees are small in size ranging from 1.5 – 15 mm. The smallest *Trigonisco duckel* measures only about 1.7mm with the largest *Melipono fuliginoso*, measuring <15 mm in length (Araújo *et al*., 2004; Martins *et al*., 2015; Nogueira *et al*., 2014). Till date, taxonomic descriptions have revealed a substantially small number of meliponine bees in Africa (Eardley and Kwapong, 2013; Kwapong *et al*., 2010), probably due to sketchy field surveys as unpublished information sources reveal a much greater diversity (currently under taxonomic identification).

African meliponine bees are less diverse than species found in Neo-tropical regions (South Americas) (Araújo *et al*., 2004; Martins *et al*., 2015; Nogueira *et al*., 2014). The nest is the central place from which meliponine bees mate, forage and pass through different life stages, and its architecture has often been used as a key feature in according species status to morphometrically and genetically similar taxa, because they are more

elaborate and complex than those of *Apis mellifera* (Hurtado-Burillo *et al*., 2013; Lucas and Fresneau, 2002). Most meliponine bees use cerumen, a mixture of wax and plant resin, as the main building material in the construction of brood combs, storage pots and involucrum (Engel and Michener, 2013). Species such as *Trigona spinipes* use additional materials such as leaves and other plant vegetation parts mixed with resin, whereas *Partamona* spp use mud and sometimes feces in their nest construction (Potts *et al*., 2005; Roubik, 2006b; Taki *et al*., 2008). These nests are immobile fixtures and potentially long-lived, reflecting a highly visible aspect of meliponine bee behavior (Halcroft, 2007; Kajobe, 2008). They build these nests in a wide range of places such as underground, crevices in tree trunks, abandoned birds' nests, termite nests and human-made buildings (Souza *et al*., 2006). These species-specific nests may be totally or partially exposed (Antonini and Martins, 2003; Eltz *et al*., 2003). Most species are recognizable from their unique nest entrance which is usually built of pure wax or a mixture of wax and mud. The structure of the nest entrance varies from one species to another, which is used in nest orientation while offering a more effective defense against predators (Roubik, 2006b). Visible differences may occur geographically, as documented for nest entrance tubes of the Amazonian meliponine bee, *Ptilotrigona lurida* (Pedro, 2014) which has a peculiar nest entrance. Such variation in architecture, such as the elaboration of the nest entrance (Biesmeijer *et al*., 2005; Couvillon *et al*., 2008; Kelly *et al*., 2014) could likely be linked to either the nest age or the micro-environment.

After the entrance, there is a tubular passage way built mainly of propolis that leads to the storage pot area. Deposits of resins have been reported next to the entrance tube and various other locations within the hive, which is frequently used by the bees. Inside the nest, there are varying shapes and arrangements of brood cells and food storage containers, the oval-shaped storage pots are built with cerumen and range from small to large spheres, conical or even cylindrical shapes, honey and pollen are stored separately in these 'pots' surrounding the brood area, while stored nectar or ripened honey are sealed up in far end extremities of the nest cavity. The brood cells are spherical to ovoid in shape as some larger sized bees of the *Plebeia* family are observed to build a regular pancake-like stack of brood cells separated by pillars and arranged in circular combs

whereas the smallest species do not build combs but instead make loose chains of cells or clusters. Most species are known to envelope brood combs and storage pots by a series of membranes of cerumen mainly for protection and thermoregulation (Pereboom and Biesmeijer, 2003; Posey and Camargo, 1985).

2.1.3 Colony cycle and life history of meliponine bees

A meliponine bee colony typically comprises of three castes, namely, a single reproductive female (queen), a few hundred drones and about 40,000 to 60,000 worker bees. The number of bees in a colony differs from species to species but generally ranges from several hundreds to more than a hundred thousand bees (Eardley *et al*., 2004). Meliponine bees cannot easily migrate except under exceptional circumstances, they mate only once as they cannot freely swarm to reproduce, while the gravid queens cannot fly (Alves *et al*., 2011). They do not use water to cool their nest neither do they use pure wax to build it. Egg-laying queens are much larger than most workers and distinct forms of division of labor and task specialization occur among the members of a meliponine bee colony (Ribeiro *et al*., 2006; Wiseman, 2009). While honey bees are progressive provisioners, meliponine bees have a system of mass-provisioning their brood cells (Maia-Silva *et al*., 2016). Colony maintenance and defense, foraging activity, reproduction, and community ecology of meliponine bees are intimately related to nesting biology of each meliponine bee species. Although they are found within the tropical and Neotropical regions, they are believed to be native to Africa. They represent one of the most vital insect pollinators in tropical rain forests (Roubik, 2006). The life cycle of meliponine bees is different from that of honey bees. In meliponine bees, there can be two or more queens laying eggs in the same nest because new queens are produced regularly but eventually killed or imprisoned in special cells as reserves. Replacement of the egg laying queen does not take place annually because some queens can live as long as 3-7 years (Eardley, 2006), and meliponine colonies have been reported to survive for as long as 15-25 years (Roubik, 1989).

2.2 Pollination and its importance as an ecosystem service

Pollination is an essential ecosystem service required by most flowering plants, with an estimated 87.5% of angiosperms requiring some form of biotic pollination (Kajobe, 2007). This biological process involves pollen transfer from the anther (male part) to the stigma (female part) of either the same flower or another flower. It can be achieved through abiotic means (water or wind) or biotic means by agents such as animals or insects that visit the flowers. A wide variety of animals can act as pollinators; these include insects, birds, bats and other mammals (Potts *et al.*, 2005; Winfree and Williams, 2008).

Insects comprises majority of the pollinator population and of these insects, eusocial bees are known to be the most important pollinators both in natural and agricultural systems due to their foraging behaviour on floral products (nectar and pollen) for the most part of their life cycle. Crop pollination is often attributed to the honeybee, *Apis mellifera* as the major pollinator, but other native pollinators do often carry out the majority of crop pollination, such as other bees of the same family Apidae (Williams *et al.,* 1991; Breeze *et al.,* 2011; Ollerton *et al.,* 2011a; Rader *et al.,* 2012). In fact, native bees alone were discovered to provide the majority of pollination services on farms in USA (Winfree *et al.,* 2007; 2008). Pollinators are not only responsible for the reproduction of wild plant species, but also for the pollination of a high number of food and non-food crops for humans (Rodger *et al.*, 2004; Senapathi *et al.*, 2015). An estimated 30 out of the 350 leading world food crops have greater yields with pollination, with about 35% of the world's food supply coming from insect pollinated crops (Klein *et al.,* 2007). The value of pollination in agriculture has been estimated to be netting about €153 billion per year *(*Winfree *et al.*, 2007; Giannini *et al.*, 2015), but these estimates could be potentially threatened by the global decline in pollinator populations, especially the honey bee.

2.2.1 Meliponine bees as alternative pollinators

Many similar features of meliponine bees resemble those of honey bees and these essential characteristics that influence the ability of meliponine bees to be used as pollinators are: polylecty and adaptability, which enable them to pollinate multiple plant

species and adapt to new ones; floral constancy: whereby a forager on a trip usually pollinates only one plant species (Ramalho *et al*., 1990; Silva *et al*., 2013). Meliponine bees are generalist flower visitors and pollinate a broad range of plant species. For example, *Hypotrigona pothieri* pollinates 54 species in 28 families (Cousins and Eriksson, 2002; Minckley, 2008), *Melipona marginata* pollinates 173 species in 38 families (Jaffé *et al*., 2014; Giannini *et al*., 2015), and *Melipona favosa* pollinates 38 species in 26 families (Kerr *et al*., 2004; Maia-Silva *et al*., 2015). The number of plant species pollinated while searching for nectar may be higher than the number of plant species pollinated while searching for pollen (Ramalho *et al*., 1994). Despite their generalized flower selection behaviour, meliponine bees are effective and important pollinators of nine economically important crops, and that they contribute to pollination in about 60 other species out of approximately 90 crop species they were found visiting (Heard,1994, 2001).

2.2.2 Crop pollination with meliponine bees

Meliponine bees have been reported to be just as effective pollinators as honey bees (Heard,1999), but such information as crop pollinators for most plant species is lacking in Africa, as the concept of maintaining colonies of meliponine bees as pollinators to increase crop yield is relatively new (Roubik,1995; Slaa *et al*., 2006). There is renewed interest in members of the genus Meliponula because of their ability to perform buzz pollination, which is a more effective pollination mechanism than the contact pollination performed by honey bees (Kerr *et al*., 2004; Maia-Silva *et al*., 2015). Buzz pollination, also referred to as sonication, is a resonant vibration technique that is used by some bees such as bumblebees and many solitary bees, to release pollen which is firmly held by the anthers (Adriaensen *et al*., 2006; Githiru *et al*., 2011). These bees grab onto the flower and by moving their flight muscles rapidly, they cause the flower and anthers to vibrate thereby dislodging pollen.

Pollination trials using meliponine bees species alongside honey bees in Japan, demonstrated that they are just as effective in pollinating crops such as tomatoes, cucumbers, eggplants, and bell peppers as honey bees (Amano, 2004). Santos *et al.,*

(2004) also studied the effectiveness of pollinating tomatoes by *Meliponula quadrifasciata* alongside *Apis mellifera,* it was seen that tomatoes pollinated by *former* were much bigger in size and heavier in weight than those pollinated by *Apis mellifera*. Malagodi-Braga and Kleinert (2004) also demonstrated through pollination experiments that *Tetragonisca angustula* was an effective pollinator of a variety of strawberry. *Vanilla planifolia* in parts of Uganda has been found to be naturally pollinated by *Meliponula spp* (Martins, 2008). With the growing pressure on the environment and global decline of honey bees, focus has shifted to readily available pollinators such as the African meliponine bee species as potential alternative pollinators and their domestication (meliponiculture) as an eco-friendly agro-based venture.

2.2.3 Potential pests of African meliponine bee species

Only of recent have some pests been observed to infest African meliponine bee species such as the phorid fly (*Melaloncha puchella*) (Brown, 1996; Core *et al*., 2012) and the small hive beetle (*Aethina tumida*) (Neumann *et al*., 2015) and causing considerable damage. The phorid fly feeds on both stored pollen and brood as they readily invade a bee colony to lay their very numerous eggs inside pollen pots (Core *et al*., 2012). The larval stage causes damage by feeding on the stored pollen, while simultaneously defecating causing fermentation of pollen and other hive products. This putrefies the hive and causes the premature worker mortality and the colony to rapidly breakdown. The small hive beetle causes damage in similar pattern, but causes considerable damage by re-infesting colonies, since they pupate at close range from the infested hive, resulting in a continuous cycle of infestation (Ellis and Hepburn, 2006; Eyer *et al*., 2009; Hoffmann *et al*., 2008; Neumann and Elzen, 2004; Pirk and Neumann, 2013).

2.2.4 Foraged resources of meliponine bees

Foraging is one of the most complex tasks performed by a social insect colony, as a fraction of the colony's inhabitants have to collect food for all the members of the colony (Jarau *et al*., 2003). Pollen and nectar are the main resources collected by meliponine bees but they also collect a variety of other resources such as sap, oil, water, gums and plant resins for nutrition or nest construction (Roubik, 1989). The main protein source for

bee larvae and adults is pollen which is added to brood cells, while adults exchange it via trophallaxis (Biesmeijer *et al*., 1999; Robroek *et al*., 2003; Slaa *et al*., 2003) as nectar provides energy for adult bees. Resin is a sticky aromatic plant product that the bees mix with wax to produce cerumen, which is the main construction material for building their nests and defending their colonies against predators and this substance is constantly used as its thought to possess components that aid chemical defenses against microbial pathogens and infections (Duangphakdee *et al*., 2009; Gastauer *et al*., 2013; Leonhardt *et al*., 2009; 2010; Simone-Finstrom and Spivak, 2010). Due to their obligate dependency on floral resources,(Hardman *et al*., 2016; Nagamitsu and Inoue, 2005) concluded that pollinators such as meliponine bees are highly opportunistic foragers and visit a broad range of plants for pollen, nectar and resin collection (Aleixo *et al*., 2016; Hardman *et al*., 2016; Sánchez *et al*., 2004; Sharma *et al*., 2011).

2.2.5 Land use change: Pollinator survival in unpredictable ecosystems.

Global changes to natural habitats such as forests are being driven by the need to provide food, fiber, water, and shelter for an ever increasing human population, resulting in expanded croplands, pastures, plantations, and urban areas in recent decades, thereby negatively impacting a considerable amount of biodiversity (flora and fauna) (Foley *et al*., 2005; Hatfield and LeBuhn, 2007; Hendrickx and Maelfait, 2007; De Palma *et al*., 2015). Negative impacts can be seen through the loss, modification, and fragmentation of habitats making both flora and fauna species to become vulnerable, threatened or extinct (Cane, 2001; Hadley and Betts, 2012; Hargis *et al*., 1999; Kearns *et al*., 1998). Ecosystems are now rapidly shaped by anthropogenic activities (clearing of native habitats for agricultural uses, pesticide application, tree felling for charcoal production) which drastically alter the biological, chemical and geological functioning of these habitats (Kearns *et al*., 1998). These sequential changes in land use and landscape structure negatively influence pollinators at individual, population and community scales and make it difficult for bees of nearly any guild to continually persist in such habitats (De Palma *et al*., 2015; Hatfield and LeBuhn, 2007; Potts *et al*., 2006). The impact of these anthropogenic activities on the disturbance of the indigenous forest habitats have

been reported in most regions of the world (Hadley and Betts, 2012; Morris, 2010; Winfree *et al*., 2009; 2011).

Pollination provided by wild bees in unpredictable ecosystems is likely to be reduced, resulting in increased pollination-related problems within natural and agricultural ecosystems (Buchmann and Nabhan, 1996; Martins *et al*., 2015). These have further raised concerns about the loss of pollinators and the services they potentially provide (Antonini *et al*., 2013; Bjerknes *et al*., 2007; Goulson *et al*., 2012; Holzschuh *et al*., 2010; Sharma *et al*., 2011; Winfree *et al*., 2009). Recent studies have elucidated the effects of land-use changes on pollinator survival, but only a few studies have been published for Kenya (Gikungu, 2006; Mwangi *et al*., 2012; Ogol *et al*., 2013). These concerns are warranted based on recent evidence of pollinator declines (Biesmeijer *et al*., 2006, Kleijn *et al*., 2015) in both natural habitats and agro-ecosystems which may produce more severe consequences in biodiversity hotspots such as Taita hills due to habitat fragmentation and rapid isolations of its forests, thereby pushing both flora and fauna to the brink of extinction. The current knowledge on meliponine bee's community is largely based on studies carried in the Neotropical regions (Roubik, 1989, 2002; Ricketts, 2008). These studies have considered the impact of natural forest habitat disturbances on meliponine bees community (Ewers and Didham, 2006; Hadley and Betts, 2012; Williams *et al*., 2011; Winfree *et al*., 2009, 2011) as well as the impact of anthropogenic land use on the conservation of wild bee species (Winfree *et al*., 2007; Costa *et al*., 2014). Significant studies have also been made in the Neotropical region on the importance of native eusocial meliponine bee species as pollinators of flowers on wild and cultivated plants (Slaa *et al*., 2006; Alexio *et al*., 2016) and their nest structure, nesting habit and foraging behaviour also reported. The ability to produce honey has also been studied for different meliponine bee species in Neotropical regions (Amano, 2004; Antonini *et al*., 2013; Dos Santos *et al*., 2016; Zanette *et al*., 2005).

According to Kajobe (2008) and Nkoba (2012), natural forest systems are vital to the survival of the bee species. A review on pollination service providers and their potential for income generation in different ecosystems of Kenya revealed the presence of a

diverse number of meliponine bee species including *Meliponula bocandei*, *Meliponula ferruginea* (reddish brown), *Meliponula ferruginea* (black), *Hypotrigona gribodoi* and *Meliponula lendliana* (Nkoba 2012; 2016). Other studies have largely focused on aspects of taxonomy (Eardley, 2004), nest structure description (Darchen, 1981) as well as biology of some African *Trigona* species (Darchen, 1972), but the extent to which fragmented habitat affects the diversity and shape behaviours in these species has not been profoundly investigated. With little attention given to meliponine bee's biology in Africa in the last decade, there is still paucity of information on their diversity, abundance and ecology. It is evident that the African continent, Kenya inclusive, lacks in-depth information on the effects and impacts of varied landscapes and anthropogenic activities within natural habitats on the distribution of native meliponine bee species (Kajobe and Echazarreta, 2005; Macharia and Raina, 2010; Kajobe, 2007, 2008; Karikari and Kwapong, 2007; Kwapong *et al*., 2010; Ogol *et al.*, 2013). By bridging this gap, the process of domestication of these species for pollination and conservation purposes can be realized for better food security.

Table 2.1 Geographic distribution of meliponine bee species in Kenya

Species	Locality	Reference
Meliponula bocandei *Meliponula ferruginea* (reddish brown) *Meliponula ferruginea* (black) *Meliponula lendiliana* *Hypotrigona gribodoi* *Plebeina hildebrandti*	Kakamega forest	Raina *et al*., 2006
Hypotrigona gribodoi *Meliponula ferruginea* (black and brown)	Arabuka Sokoke forest	Raina *et al*., 2006 Macharia *et al*., 2007
Hypotrigona araujo	Mwingi	Macharia *et al*., 2007 Raina *et al*., 2006

2.3 Communication systems in social insects

The ecological success of social insects relies on their ability to carry out tasks to ensure their survival (Slaa *et al*., 2003; Hrncir, 2009; Jarau, 2009; Jarau and Hrncir, 2009; Slaa and Hughes, 2009; Verheggen *et al*., 2010). One of the organizational principles common in social insects is the use of the nest environment as a collective information pool with the insect colony efficiently functioning as an information center (Slaa *et al*., 2003;

Biesmeijer and Slaa, 2004; Besmeijer and Laa, 2004; Hendrickx *et al*., 2007), from where each individual forager collects information and make its decisions based on the information collected by other nest-mates. This solely depends on precise communication systems; but it can also rely on the fact that the actions of individuals can be perceived by others who react appropriately. Such changes, if they are by-products of other behaviors, are called cues, as opposed to signals that have specifically evolved for the purpose of communication (Akino and Yamamura, 2004; Barth *et al*., 2008; Leonhardt *et al*., 2010; Nieh and Vandame, 2011). However, most social insects communicate differently, exhibiting behaviors such as antennation, trophallaxis, and tasting, stroking, biting, tapping, stridulation, grasping, and nudging to convey information. All these can predict behavioral responses ranging from recognition, recruitment and defense (Hölldobler and Wilson, 2009). Olfaction is deemed to be a universal form of detection that permits all animals to locate food sources, identify con-specific mating partners, and most importantly to avoid predators (Bergström, 2008; Hartlieb and Anderson,1999; Leonhardt *et al*., 2010; Mc Cabe and Farina, 2010; Wang and Tan, 2014).

The use of volatile chemical compounds to communicate is daunting as the external atmosphere contains diverse mixtures of millions of these volatile compounds, which could potentially make it difficult for insects to understand when to distinguish, discriminate and react to certain odors to convey task related information during foraging, nest mate's recognition, and resource and nest site marking and, for defense against predators amidst others (Halcroft, 2007; Greco *et al*., 2010; Halcroft *et al*., 2011; Schorkopf and Hrncir, 2009).

These compounds used in communication by any insect society contain a diverse and complex mixture of substances which differ in relative proportions, as these multi-component signals are either produced in single exocrine glands or blends composed of secretions from several glands and thereafter released into the environment (Billen, 2004; Stangler *et al*., 2009; Jarau *et al*., 2012). They range from hydrocarbons, aldehydes, terpenes, esters, ketones among other volatile compounds (Jarau *et al*., 2004; Schorkopf *et al*., 2007; Reichle *et al*., 2010; Reichle *et al*., 2013). These organic volatile compounds

vary in structure and are believed to govern essential stimuli that facilitate the various forms of behaviour that necessitate the successful functioning of any insect caste system (Jarau *et al*., 2003; 2004; Reichle *et al*., 2013).

Such chemical signals can be combined with cues of other sensory origin, such as vibrational or tactile stimuli (Hölldobler and Wilson, 2009) to elicit complex behavior. Studies have confirmed that quantitative disparities between these compound groups connote differences in ages, gender and castes in the reproductive status of individuals (D'Ettorre and Heinze, 2004; Howard and Blomquist, 2005; Nielsen *et al*., 2011; Nunes *et al*., 2014; Nunes *et al*., 2009).

2.3.1 Nature of cues and signals used by social insects

For effective communication between individuals in a colony, there must be a process of sending out signals or cues, in order to initiate a behavioral change in the receiver of such signal. This process can only be mediated through the release of pheromone compounds, which could induce the receiver to change behaviorally, exhibiting actions such as defensive or passive behavior, or even to exit the hive to commence foraging (Strangler *et al*., 2009). This pheromone emission can conclusively prevent robbing of stored food resources, successful marking and trailing of food locations and avoid colony invasion from various insect pests (Nieh 2004; Barth *et al*., 2008). A generally accepted terminology has evolved to categorize the functional chemical substances in insect communication. This semiochemical/ infochemical could be any chemical compound used in communication, either among species (hetero-specific) or between individuals of the same species (con-specific) (Hölldobler and Wilson, 1990; El-Sayed, 2012).

Such transmitted signals between individuals of different species are called allelo-chemicals, in contrast to those compounds mediating behavior between individuals of the same species is known as pheromones. Such transmitted signals between individuals of different species are called allelo-chemicals, in contrast to those compounds mediating behavior between individuals of the same species, which are known as pheromones. Pheromones are usually glandular secretions which when released by a singular

individual but trigger a behavioral response in other individuals upon detection (Bordereau and Pasteels, 2011; Mant *et al*., 2005; Verheggen *et al*., 2010; Wyatt, 2003).

2.3.1.1 Cues and signals governing recognition behavior in social insects

Nest mate recognition cues are particularly crucial for colony survival by offering protection from social parasites during territorial interactions when defending their colonies and also during essential activities such as foraging (Stuart and Herbers, 2000; Gathmann and Tscharntke, 2002; Leonhard *et al*., 2007). The cognitive ability to identify and respond differently to either a nest mate or non-nest mate exists in many organisms and is vital for members of most social insect colonies (Wenseleers and Ratnieks, 2004; Couvillon *et al*., 2008; Hart and Ratnieks, 2002; Medina *et al*., 2009; Shackleton *et al*., 2014). The chemical identity of recognition cues in the honey bee *Apis mellifera* has been intensively studied (Getz and Page, 1991; Breed and Stiller 1992; Mann and Breed 1997; Bowden *et al*., 1998; Breed 1998a, b; Hepburn 1998; Downs *et al*., 2001; Stabentheiner *et al*., 2002; Akino *et al*., 2004) and their role at either individual and population levels has been studied in detail. Given that meliponine bees like honey bees are highly eusocial and belong to the same family, they should have the same behavioral patterns in the context of being able to recognize nest mates from non-nest mates. However, little is known about their recognition cue chemistry, the acquisition channels utilized and how such cues shape recognition behavior.

 Recognition behavior is mainly based on certain types of cues and members of a colony rely on the existence of a signature odor to fully carry out this function. Cuticular hydrocarbons, amongst other channels, may play a crucial role in these behavioral mechanisms functioning as contact pheromones, as surface hydrocarbons are essential cues for recognition in both solitary and social insects when they come in contact with each other either at an individual or colony levels. Besides serving as unique chemical signatures, these hydrocarbons also help to maintain the social structure of colonies by differentiating individuals according to caste and functions (Akino and Yamamura, 2004; Ferreira-Caliman and Nascimento, 2010; Mant *et al*., 2005; Martin and Drijfhout, 2009; Nunes *et al*., 2009). These odor cues further enhance the assessment of colony

membership, and subsequent recognition allows individuals to act non-aggressively towards nest mates and aggressively towards non-nest mates. In *A. mellifera*, adults emerge without any "signature odors" which could serve as recognition cues (Mc Cabe and Farina, 2010; Reichle *et al*., 2010). Hence, individual worker bees earn such "signature odors" comprising mainly alkenes and fatty acids, only after exposure to comb wax to acquire a distinctive template (Chen *et al*., 2009; Medina *et al*., 2009; Stanghellini *et al*., 2000). Aliphatic cuticular hydrocarbons (CHs) have been categorized to typically range from C_8 - C_{40} (Mant *et al*., 2005; Ferreira-Caliman and Nascimento, 2010; Leonhardt *et al*., 2009; Martin and Drijfhout, 2009) with 3 major structural classes: n-alkanes, n-alkenes and mono-, di- and tri- methyl- branched alkanes (Howard and Blomquist, 2005), with additional components in minute amounts such as fatty acids, glycerides, sterols, ketones, long chain alcohols and aldehydes (Akino and Yamamura, 2004; Nunes *et al*., 2008).

Three major structural classes of CHs are known namely, n-alkanes, n-alkenes and mono-, di- and tri-methyl-branched alkanes (Howard, 1993), with additional components in minute amounts such as fatty acids, glycerides, sterols, ketones, long chain alcohols and aldehydes. These main classes of chemical components have been speculated to play different physiological functions; alkanes forming impermeable layers on the insect's cuticle which help to form resistance against desiccation, while alkenes form permeable layers, that plays a vital role in chemical communication (Schulz, 2001; Bergström, 2008; Verheggen *et al*., 2010; Reichle *et al*., 2013). These hydrocarbons can be exchanged between individuals by means of trophallaxis, self and allo-grooming (Bergström, 2008; Guerrieri and d'Ettorre, 2008; Huang and Wang, 2008). The exact source and identity of CHCs that function as both individual and nest-specific identification signals remain largely unknown in Afro-tropical meliponine bees. Like honeybees, meliponine bees construct distinctive nests from a mixture of both endogenously produced wax and exogenously produced materials from the environment such as plant resin and floral oils (Moreno and Cardozo, 2003; Duangphakdee *et al*., 2009; Gastauer *et al*., 2013; Leonhardt *et al*., 2007; Leonhardt and Blüthgen, 2009; Simone-Finstrom and Spivak, 2010; Kaluza *et al*., 2016). Using the well-studied honeybee as a reference point, it's

imperative to know whether these African meliponine bee species uniquely utilize either endogenous derived cues (cuticular compounds) or a combination of 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. By establishing which compounds have been implicated in nest mate recognition systems of *Apis mellifera*, affect nest mate recognition in selected African meliponine bee species is a critical decision in answering the question of the possible cue sources of nest mate recognition in African meliponine bee's species. As all these confirmations in the honey bee, *Apis mellifera* are yet to be asserted in meliponine bee species of African origin.

2.3.1.2 Recruitment behavior and trail pheromones

An efficient communication amongst colony individuals can only be mediated via pheromones and other semiochemicals in order to recruit nest individuals to a particular food source (Wyatt, 2003), but even among closely related groups of the Apidae family, a large amount of variation can occur in the modes of communication. Recruitment is a certain form of behaviour which largely involves the aggregation of members of a colony to a particular direction or location, for the benefit of such group of individuals. Such recruitments usually serve to mobilize large numbers of colony members in response to an abundance of resources such as very rewarding food sources (nectar or pollen) or in defense to an immediate threat such as colony intruders Recruitment to food sources in honey bees is initiated through the waggle dance and is observed to be stereotyped, repetitive motor patterns (James, 2004; Sánchez *et al*., 2004; Barth *et al*., 2008). In the movements of this dance, the distance as well as the direction of a food source is coded (Farina, 1996; Jarau *et al*., 2000; Aguilar *et al*., 2005; Kajobe, 2007). Potential recruits receive food samples from the foragers in order to learn the scent of the food, and use this in addition to the dance to commence foraging. Additionally, the dancer deposits specific semiochemicals that serve to recruit more foragers to the food source it intends to communicate and these have been identified as the alkanes Tricosane and Pentacosane and alkenes (*Z*)-9-tricosene and (*Z*)-9-pentacosene (Hrncir *et al*., 2000; Nieh *et al*., 2003; Tereshko and Loengarov, 2005).

However, this "waggle dances" do not occur in meliponine bees (tribe Meliponini). Nevertheless, in some species of these bees, recruitment can be as efficient as in honey bees. In such cases, scent marks or even scent trails are used by foragers to guide recruits to the food source (Goulson *et al*., 2000; Schmidt *et al.*, 2003; Barth *et al.*, 2008). Other meliponine bee species seem to communicate distance of food sources using visual, tactile, acoustic and olfactory based signals in the nest (Nieh and Roubik 1995; Nieh, 2004; Jarau *et al*., 2009). In some species, however, the location of food sources is not communicated to recruits at all. Instead, foragers merely alert nest mates to the presence of food; these then exit the colony to search for the food source in all directions (Goulson *et al*., 2000; Jarau *et al.*, 2004). The communication systems used by meliponine bee species could differ in modalities, as well as in information content which could be dicey for recruits if they don't benefit from getting information on the exact location of food sources, just like honey bee recruits do. This observation points to a direction that has numerous unassuming theories with respect to foraging behavior in African meliponine bee species. With very little information available on the communication system employed by Afro-tropical bees (Henske *et al*., 2015), this represents a very promising study area for discoveries. The communication systems used by meliponine bee species differ in modalities, as well as in information content, and may be risky for recruits as they don't get information on the exact location of food sources, as is the case in honey bees. This observation points to a direction that has many theories with respect to foraging behavior in African meliponine bee species. This study seeks to understand how meliponine bees transmit information about food sources by investigating how the foragers transmit food odor and recruit fellow nest mates inside the nest via pheromones.

2.3.1.3 Origin of trail pheromone production

The first evidence that meliponine bee foragers transmit food odor to fellow nest mates inside the nest via footprint pheromones in the field came from an experiment conducted by Barth (2008). This chemical termed as "footprint pheromone' or "trail pheromone" (Jarau *et al*., 2006; Stangler *et al*., 2009; Reichle *et al.*, 2011) is certainly perceived by olfaction and possibly also by contact. Earlier studies by Kerr and Rocha (1988) hypothesized that volatiles used for trail marking food sources by foragers of *M.*

ruiventris and *M. compressipes* came from anal liquids which are excreted at sugar baited feeders after food uptake. However, this conclusion was only made on the observation of defecation behavior, without demonstrating if these bees are actually attracted by the same anal droplets. This seriously weakened the hypothesis that anal droplets function as attractive food-marking substances and consequently spurred greater interest to determine the origin of production of these trail pheromones.

Recent studies have revealed that meliponine bee foragers efficiently utilize 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 a trail following behavior in recruited workers (Reichle *et al*., 2011). 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 communication. This however raised question of whether any meliponine bee species could independently utilize labial gland secretions to lay pheromone trails and recruit other nest mates to a food source (Hrncir *et al*., 2006; Barth *et al*., 2008).

The other most obvious glands that could be implicated with strong evidence in the secretion of footprint pheromones are the tarsal (also called Arnhart) glands. This was inferred from studies carried out with *M. seminigra* by Hrncir *et al*., (2004). The tarsal (arnhart) gland is a flattened sac within each of the last tarsal segments of each leg (Goulson *et al*., 2000; Barth *et al*., 2008; 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 (Barth *et al*., 2008). These pheromones are then deposited by the terminal arolium between the tarsal claws as the bee walks on a surface bearing a food source. In addition to the feet, it is deposited by the tip of the abdomen, which often trails over any surface as the bee walks, as observed in

studies conducted on *M. seminigra* (Nieh and Roubik, 1995; Jarau *et al*., 2003b).This trail laying secretions was shown to affect the behavior 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 glands on the other hand was resolved by the discovery of a different system of glands within the bees' legs (Jarau *et al*., 2004b). This 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 *Meliponula seminigra* foragers, attracted foragers in the same pattern as feeders naturally marked by foragers themselves, thus providing strong evidence that the secretions of these glands account for the attraction of bees to a food source (Jarau *et al*., 2004b).This provided strong evidence that the secretions of these glands account for the attraction of bees to a food source.

To date, the chemical structures of compounds deposited by meliponine bees at food sources have been elucidated for only this one species (*Melipona seminigra*) and consist of 12 alkanes, eight alkenes, a methyl alkane and an aldehyde (Jarau *et al*., 2004b). The dominant alkanes, each constituting ≥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 an additional forty-one compounds, comprising mainly esters, acids, and methyl alkanes (Jarau *et al*., 2004b). These identified compounds from *Melipona seminigra* scent marks are somewhat similar to the compounds reported as bumble bee marker scents which direct foragers to food sources (Schmitt *et al*., 1991; Eltz 2006; Saleh *et al*., 2007). Therefore it seems, crucial to determine the actual site of production and or release of these trail pheromones inclusive of nasonov and tarsal gland secretions

so as to have a complete repertoire of potential glands involved in the recruitment behavior of African meliponine bee species.

2.4 Biodiversity of the Eastern Arc Mountains

The Eastern Arc Mountain is listed as one of the world's 34 biodiversity hotspots having some of the richest concentrations of endemic plants and animals on earth (Rogers *et al*., 2008; Maeda *et al*., 2010; Malonza *et al*., 2010; Omoro *et al*., 2010) which comprises of Taita hills amongst other hills, running from the southern part of Kenya to the northern part of Tanzania. Taita hills is one of the most degraded areas in the Eastern Arc Mountains, having lost about 99% of its original cloud forest during the past 50 years (Wilder *et al*.,1998; Salminen, 2004; Chege and Bytebier, 2005; Pellikka *et al*., 2005; Adriaensen *et al*., 2006; Clark and Pellikka, 2007; Maeda *et al*., 2010; Aerts *et al*., 2011). Some plant species unique to this region include, the African violet (*Streptocarpus teitensis*) is restricted to a small patch in Ngangao forest, *Ceropegia verticilliata, Chassalia discolor ssp taitensis*, *Coffea fadenii, Impatiens engleri spp taitensis, Impatiens teitemsis* and *Zimmermannia ovata*, also some endangered endemic bird species include the Taita thrush (*Turdus helleri*), Taita apalis (*Thoracica fuscigularis*), Taita white eye (Zosterops poliogaster). Some other notable endemic amphibians in this hill include the common reed frog (*Hyperolius viridiflavus*), and the forest gecko (*Cnemaspis dickersonii*) Sayer *et al*., (1992).

2.4.1 Taita Hills

The Taita Taveta County is one of Kenya's 47 counties, located in the coast province and lies approximately 200 km northwest of Mombasa and 360 km southeast of Nairobi (Adriaensen *et al*., 2006; Chege and Bytebier, 2005; Clark and Pellikka, 2007; Githiru and Lens, 2007; Lens and Van Dongen, 2002; Salminen, 2004). It covers an area of approximately 17,083.9 km^2 of which a bulk of 11,100 km^2 is within Tsavo East and Tsavo west National Parks. The county has four constituencies namely Voi, Mwatate, Wundayi and Taveta which are comprised of two distinct topographical areas: the Tsavo Plains, at an altitude of 400m in the east to 1000 m.a.s.l. in the west, and the mountainous Taita Hills at 1200-2200 m.a.s.l.

Located at an altitude of 700m to 2,208 m.a.s.l, Taita Hills cover an area of 1,000 km^2 forming the northernmost part of the Eastern Arc Mountains. The highest peak in the Taita Hills is Vuria at 2,208 m). Indigenous mountain rain forest fragments on the hills accommodate a variety of endemic and threatened flora and fauna species not found elsewhere in Africa, which are isolated from other mountainous areas to the southeast (Shimba Hills), south (Usambara Mountains), southwest (Mt. Kilimanjaro), Northsouth (Ngulia and Chyulu Hills) and northwest (Kenyan highlands) by the vast plains of Tsavo National Park (Tsavo plains). The mean annual rainfall ranges from 500 mm in the lowlands to over 1500 mm in the upper mountain zone (Clark and Pellikka, 2007; Malonza *et al*., 2010). There are two rainy seasons in the area: March-May/June and October-December. The variability of precipitation from year to year is high, especially at lower altitudes with a great number of ecological regions in the area based mainly on the different climatic conditions in the area.

CHAPTER THREE
LAND-USE CHANGES ALTER MELIPONINE BEE'S (HYMENOPTERA: APIDAE) ASSEMBLAGES IN AN AFRO-MONTANE BIODIVERSITY HOTSPOT.

3.1 Summary

Habitat degradation, together with other factors, has over the decades contributed significantly to dwindling populations of both fauna and flora by altering their habitats. Disturbances of natural habitats affect diversity of both vertebrates and invertebrates by altering their habitats, such as feeding and nesting sites. Little is known about the extent to which degraded habitats could shape the abundance or even diversity (ecological, taxonomic and genetic) of most indigent pollinators such as African meliponine bee species. This study was carried out to determine how habitat disturbance influences natural occurrence of African meliponine bee species in different ecological habitats of Taita hills and whether it gives rise to ecotypes evidenced by changes in their genetic and taxonomic diversity. Renyi diversity profile revealed a total of four meliponine bees species in five out of the six main habitat types surveyed whereas Shannon index revealed the highest species richness in a deciduous habitat type (H′ = 4.24). These meliponine bee species (*Hypotrigona gribodoi*, *Hypotrigona ruspolii*, *Meliponula ferruginea* (black) and *Plebeina hildebrandti*) were unevenly distributed across all habitats. Geometric morphometrics categorized all four meliponine bee species into two major clades, cluster 1(*H. gribodoi*, *H.ruspolii*, *M. ferruginea (*black)) and cluster 2 (*P.hildebrandti*) and further discriminated populations against the four potential habitats they are likely to persist or survive in. Each habitat appeared to consist of a cluster of sub-populations and may possibly reveal ecotypes within the four meliponine populations.

3.2 Introduction

The EAM forms a roughly crescent-shaped arc and consists of: Taita hills, north and south Pare, east and West Usambara, North and South Nguru, Ukaguru Mountains, Uluguru Mountains, Rubeho Mountains, Udzungwa Mountains, Mahenge escarpment,

Malunde hill, Uvidundwa Mountains. The unique habitats of the Eastern Arc Mountains and coastal forests are notably fragmented leading to rapid habitat loss with consequential effects on both flora and fauna species within key sites to become highly vulnerable. Agricultural encroachment and intensification, timber extraction and charcoal production are listed as the greatest threats to the survival of most flora and fauna species. Taita hills possesses a high level of endemic fauna and flora (Clark and Pellikka, 2007; Hermunen, 2004; Pellikka *et al*., 2013; Rowson and Lange, 2007; Wilder *et al*., 1998), but ironically it is one of the most degraded areas in the Eastern Arc Mountains, having lost about 99% of its original cloud forest during the past fifty years (Clark and Pellikka, 2007; Clark, *et al*., 2010; Omoro *et al*., 2010; Platts *et al.*, 2010; Pellikka *et al*., 2013). Some plant species unique to this region include, the African violet (*Streptocarpus teitensis)* which is restricted to a small patch in Ngangao forest, *Ceropegia verticilliata, Chassalia discolor spp taitensis*, *Coffea fadenii, Impatiens engleri spp teitensis, Impatiens teitensis* and *Zimmermannia ovata*. Also some endangered endemic bird species include the Taita thrush (*Turdus helleri*), Taita apalis (*Thoracica fuscigularis*), and Taita white eye (*Zosterops poliogaster*). Some other notable endemic amphibians in this hill include the common reed frog (*Hyperolius viridiflavus*) and the forest gecko (*Cnemaspis dickersonii*) (Lange, 2006; Martins, 2008; Mulwa *et al*., 2007; Rowson and Lange, 2007).

Ecologically, habitat features are important in regulating diversity of species and population size as plants and animals are highly dependent on the quality of their habitats (Munyuli, 2012; Rogers *et al*., 2008; Tscheulin *et al*., 2011). The fragmentation of natural and semi-natural habitats is regarded as a major threat to biodiversity (Fahrig, 2003; Kennedy *et al*., 2013) having negative effects on ecological processes such as primary productivity. This can have important ecological consequences at the population, community and ecosystem levels, and in most cases such effects are comparable in magnitude to the effects of species diversity. Disturbances of natural habitats affects diversity of both vertebrates and invertebrates by altering their habitats, such as feeding and nesting sites for which organisms are known to depend on for survival (Aarssen and Schamp, 2002; Bwong and John Measey, 2010; Jauker *et al*., 2009; Steffan-Dewenter, 2003;Williams and Kremen, 2007).

However, it is not clear how strongly these apply in nature, as studies to date have been biased towards manipulations of plant diversity in habitats, and little is known about the relative importance of other factors such as habitat type, forest age and its degree of fragmentation which may influence ecological processes for native bee species. Some recent studies on meliponine bee species have indicated that these bees are strongly associated with indigenous forested areas for both nesting and foraging requirements (Brosi *et al.*, 2008; Brown and Oliviera, 2014). African meliponine bees are reported to be one of the many invertebrates mostly affected by forest degeneration caused majorly by anthropogenic activities (Kajobe and Echazarreta, 2005; Kajobe, 2007; Karikari and Kwapong, 2007; Kajobe, 2008; Kwapong *et al.*, 2010; Eardley and Kwapong, 2013). Recent studies on the ecology of African meliponine bee species in countries such as Uganda (Kajobe 2008) and Kenya (Gikungu 2006; Nkoba, 2012) has mentioned the importance of intact and undisturbed habitats as a key driving factor for meliponine bees to thrive, but the extent to which these group of pollinators are affected by increasing habitat isolation in tropical regions have not been determined. Isolated habitats potentially serving as nesting sites for these bees may possibly cause speciation among many bee taxa; subsequently increasing bee biodiversity.

Differentiating between possible ecotypes arising from this can prove to be quite challenging when relying on traditional taxonomy. However, these traditional identification methods for large data sets are often expensive, cumbersome and demanding a high level of taxonomic training. It was therefore imperative to utilize improved taxonomic techniques which are less expensive, highly effective with simpler application and could effectively discriminate bee species (Francoy *et al.*, 2008, 2009, 2011, 2012, 2016; Jaffé *et al.*, 2014). Presently, geometric morphometric analyses of bee wing vennation have provided an effective and efficient means of identification among various bee taxa (Francoy *et al.*, 2006; 2008; 2009; 2011). By further combining this with molecular markers such as the mitochondrial DNA of animals whose high substitution rate and its non-involvement on nucleic acid hybridizations will effectively resolve the setbacks of traditional taxonomy (Schroder *et al.*, 2002; Koch, 2010; Meulemeester and Michez, 2012; Eardley and Kwapong, 2013) to successfully characterize any closely

related species. Detailed research on mitochondrial DNA identified a 650bp segment close to the 5' end of the mitochondrial cytochrome c oxidase subunit I(mtCOI) gene as a suitable target with sections of both variable and conserved regions in its sequence to fully segregate between a majority of animal species (May-Itzá *et al*., 2010; Nogueira *et al.*, 2014; Ramírez *et al*., 2010). This region has been proposed as the core barcode region for the animal kingdom (Koch, 2010; May-Itzá *et al*., 2010). With the utilization of this tool, clarifications regarding morphometric relatedness could be easily resolved, as much recent studies today have successfully separated the genera of some meliponine bee species using molecular studies (Cameron and Mardulyn 2001; 2003; Lockhart and Cameron 2001; Thompson and Oldroyd 2004).

3.3 Materials and methods

3.3.1 Study area and sampling method

This study was carried out in two locations namely the low lands and the high lands with three habitats selected in each locations. Lowlands (Mwatate, Msau and Mugama) and the high lands (Mwachora, Chawia and Kichuchenyi) in Taita hills.

Taita hills (Fig 3.1) is divided into two distinct locations which are the lowlands and the high lands respectively. The lowlands (Ll) is mostly characterized by dry and hot climatic conditions, with Mwatate, Msau and Mugama being habitats characterized by sparsely dispersed vegetations and indigenous tree species, while the highlands (Hl) is characterized by wet and cold climatic conditions, with Chawia forest, Mwachora forest and Kichuenyi forest patch is characterized by mixed indigenous and exotic forest. These habitats were chosen for this study based on features such as: habitat type, forest age and its degree of fragmentation/isolation. The lowlands lies along an altitude of approximately (600 – 1,000m.a.s.l) with severely disturbed forest fragments comprising of wood lands and agro forestry is practised on an extensive scale. The highlands lie along an altitude of approximately 1,200-2,200m.a.s.l with more relatively protected forest fragments patches comprising majorly of an uneven mixture of indigenous and exotic tree species. Both locations are unique as they represent a mixture of indigenous and exotic vegetation which provide potential nesting and foraging habitats for

meliponine bee species. In both study locations, meliponine bee species were sampled using standardized transect walk method (Westphal *et al*., 2008; Wilson *et al*., 2008; Nielsen *et al*., 2011; Silva *et al*., 2013; Munyuli, 2013) from the months of March to September, 2014 (combining both the long rainy season and dry season). In each of the two study locations, potential nesting sites of the four meliponine bee species were surveyed following a successive gradient. A total of 15 line transects of 250metres long and 20metres apart on a base line, were mapped out in a 25 ha area in each of the six successive habitats to investigate for nested colonies.

Field surveys were carried out during the sunny days in order to facilitate viewing of foraging bees exiting their colonies. Nest inspections were carried out on every substrate having the likelihood of accommodating nests such as trees, termite mounds and the ground (Sheffield *et al*., 2008; Roubik, 2006b; Hudewenz and Klein, 2013; Kennedy *et al*., 2013). When such nests were found the foraging bees were collected using an aspirator at their nest entrances and recorded. The specimens from different colonies were preserved in 70% alcohol in separate plastic vials for morphological identification and genetic characterization to confirm species identity. The number of meliponine bee species and their colonies observed per transect in the different habitats were recorded.
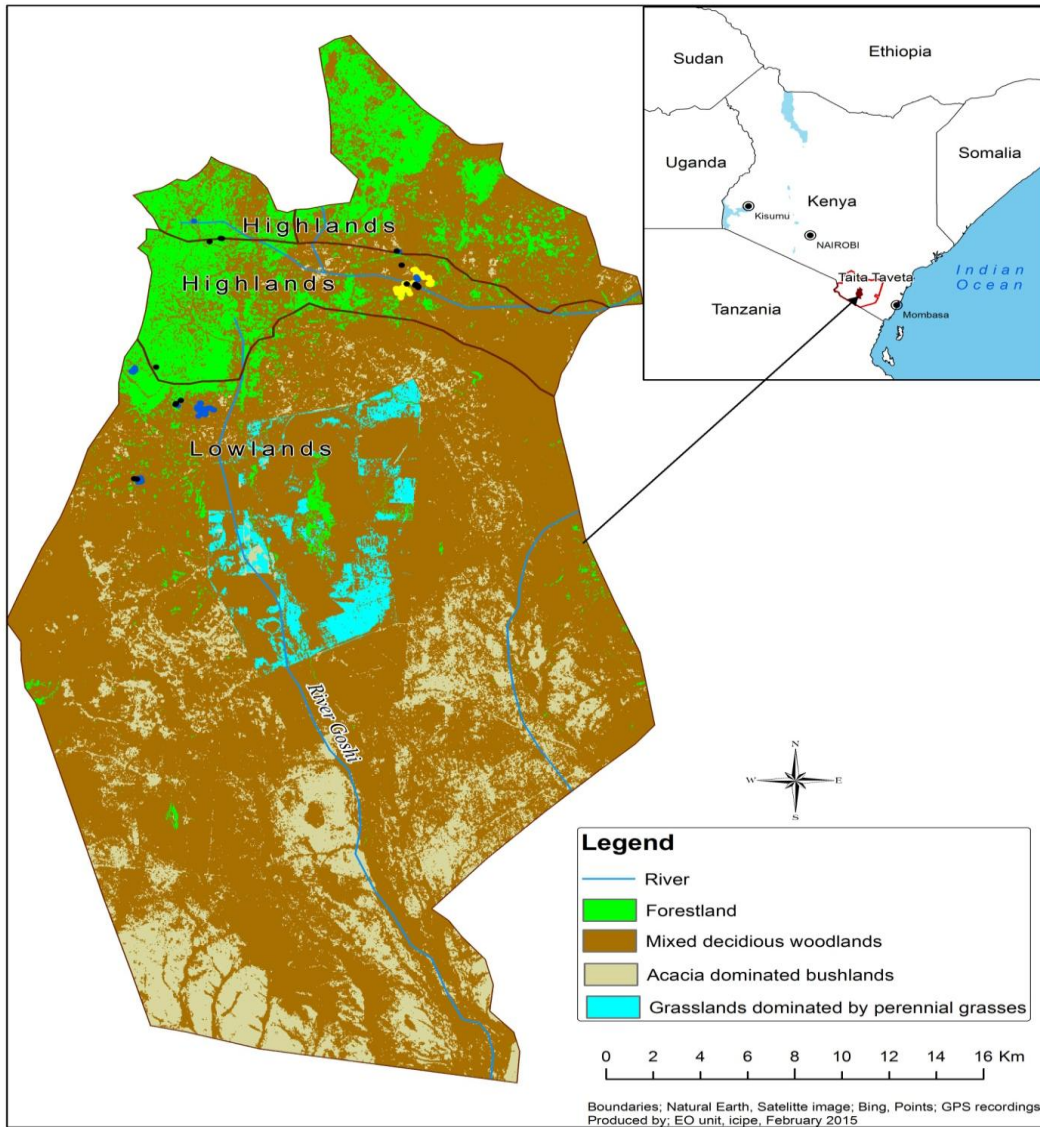
Figure 3.1: Map of Taita hills forests and surrounding areas

Table 3.1: Details of sampling sites surveyed for meliponine bees in Taita hills, Kenya, in March-September 2014.

Sampling site	Habitat type	Location	Habitat code	Coordinates
Msau	Woodland	Lowlands	MDW	3.26086^{o}S/38.26525^{o}E
Mwatate	Grassland	Lowlands	GR	3.46000^{o}S/38.36528^{o}E
Mugama	Bush land	Lowlands	ADBL	3.37269^{o}S/38.42814^{o}E
Mwachora	Indigenous forest	Highlands	IMF	3.41875^{o}S/38.36939^{o}E
Kichuchenyi	Exotic forest	Highlands	EFP	3.36208^{o}S/38.33072^{o}E
Chawia	Mixed forest	Highlands	HCH	3.46612^{o}S/38.35899^{o}E

3.3.2 Sampling procedure

In each study habitat, 20 linear transect each measuring 250 m x 20 m each was established using a GPS receiver to mark coordinates. Meliponine bees were sampled using the conventional complementary method, belt transect (direct observation of nesting colonies synonymous to a visual census), and data such as nesting site/substrate, GPS coordinates of nest and names of nesting trees were recorded. Belt transect counts are the traditional (with standardized protocols) methods of sampling bees from existing vegetation (Potts *et al*., 2005). 2375 specimens constituting four species were collected from 147 feral colonies within six different habitats.

3.3.2.1 Specimen identification using wing morphometrics and DNA barcoding.

Representative specimens (n=20) from each of the 147 feral colonies were examined by the biosystematics unit of the international center of insect physiology and ecology (*icipe*), Nairobi, Kenya and tentatively identified as *Hypotrigona gribodoi*, *Hypotrigona ruspolii*, *Meliponula ferruginea* (black) and *Plebeina hildebrandti* based on external morphology (Fig 3.2a). The right forewing of each forager was removed and placed between a 35mm microscope glass slide and cover slip. Each individual wing was

captured with a digital camera connected to a stereomicroscope (Hartfelder and Makert, 2006; Rohlf, 2004, 2010). The morphometric character (right forewing) was chosen in accordance with Hartfelder and Makert (2006). Wing images were captured and further created in JPEG format, with one TPS file created from the image files using tpsUtil software (version 1.49). Approximately eight homologous points of correspondence (Fig 3.2b) were plotted at specified junctions of the wing venation using tpsDig2 software version (Hartfelder and Engels, 1992) with one single TPS file grouping each of the processed wings. The remaining collected specimens were deposited at the biometrics unit of *icipe*, duduville campus, Nairobi.

A total of 36 individuals were selected from this pool of previously morphologically identified specimens and their genomic DNA extracted using a guided protocol (Cameron and Mardulyn, 2003; Franck *et al*., 2004; Rasmussen and Cameron, 2007, 2010) CO1 region was selected and used based on their demonstrated ability in resolving generic relationships within arthropod–species (Cameron and Mardulyn, 2001; Franck *et al*., 2004).
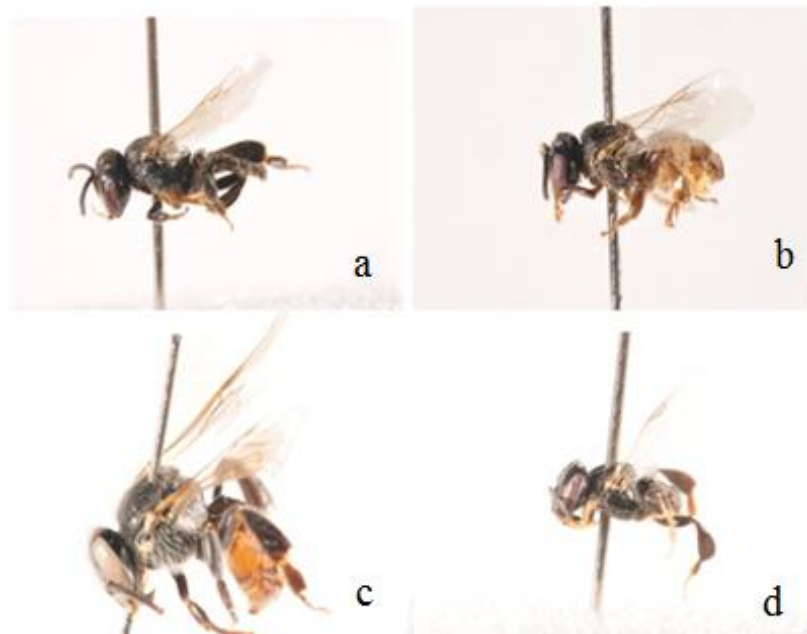
Figure 3.2a: Meliponine bee species occurring within Taita hills, Kenya. a: *Hypotrigona gribodoi*; b: *Meliponula ferruginea* (black); c: *Plebeina hildebrandti*; d: *Hypotrigona ruspolii*.
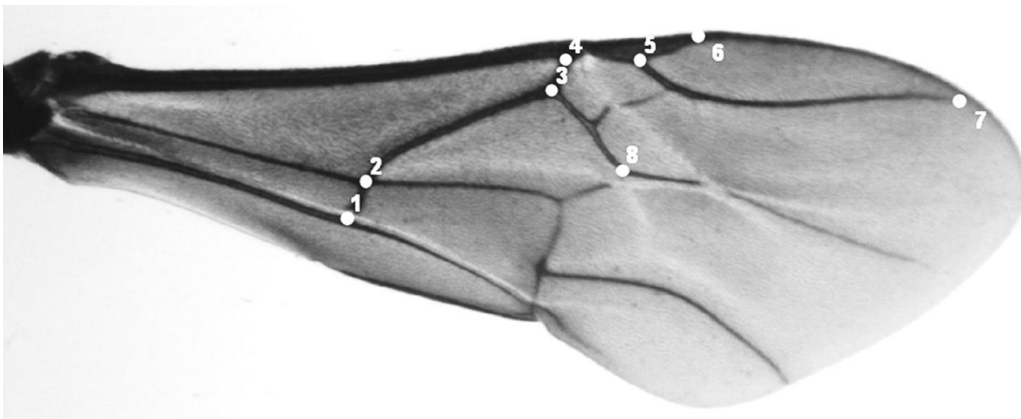
Figure 3.2b: A representative meliponine bee forewing with vennation landmarks used for segregation. 1: wing length; 2: wing width; 3: Costal cell; 4: radial cell (a); 5: radial cell (b); 6: radial cell (c); 7: 1st cubital cell (a); 8: 1st cubital cell (**b).**

PCR conditions were optimized and followed with an initial denaturation step @ 96 °C for 2 minutes, followed by 35 cycles @ 96 °C of denaturation for 30 secs. An annealing cycle @ 50 °C for 30 secs and elongation step @ 72 °C for 1 min followed with an initial and final extension step @ 72 °C for 10mins. A pre-stained agarose gel (1.5%) with ethidium bromide was used to visualize the PCR amplified products. A run time of 45 minutes was used to fully separate the bands and then visualized with a UV trans-illuminator**.** A total volume of 10ul of PCR product was digested with exonuclease II and shrimp alkaline phosphatase for 15 minutes @ 37 °C prior to sequencing, essentially to remove any residual primers and dNTPs. Bidirectional sequencing of the PCR products was outsourced to Inqaba biotech, South Africa.

Specimen sequences for CO I gene were aligned using Geneious v8.1 software program (Kearse *et al*., 2012; Masters *et al*., 2011) and an appropriate model of sequence evolution was determined using the model with the lowest information criterion. A maximum likelihood phylogenetic tree was then generated using a GTR model in phyML and Gamma model in Mr Bayes (Huelsenbeck and Ronquist, 2000, 2005; Huelsenbeck *et al*., 2003; Ronquist and Mark, 2009). Assessment of branch support was done with 1000 bootstrap replicates to generate a neighbor joining tree and estimate the confidence relations in the NJ tree (Kumar *et al*., 2012; Tamura *et al*., 2011). The comparisons of nucleotide sequences of *Hypotrigona gribodoi, Hypotrigona ruspolii, Meliponula*

ferruginea (black) and *Plebeina hildebrandti* were performed by alignment with *Liotrigona madecassa* (**Accession number: HQ012823**) which served as the closest related out-group, using the BLASTX (NCBI). A maximum likelihood phylogenetic tree was then generated using a GTR model in phyML and Gamma model in Mr Bayes (Huelsenbeck and Ronquist, 2005; Ronquist *et al*., 2012).

3.3.3 Statistical analysis

Analysis of variance (ANOVA) using R statistical package was used to compute the significant effect of habitat type on species abundance. A non-linear regression model such as the species accumulation curve was used to estimate the number of meliponine bee species represented in the whole surveyed area (Ugland *et al*., 2003). The species accumulation was used to estimate species richness and rank abundance of meliponine bee species across varying habitat types (Colwell *et al*., 2004). Biodiversity indices (species richness, abundance and Shannon index) were computed using the Biodiversity R package (Kindt and Coe, 2010) installed in R software. Species richness, species diversity (using Shannon index and Renyi diversity profiles), and the proportion of habitat type with most abundant meliponine bee species were computed using Renyi diversity profiles (Tóthmérész, 1995). Similarity index was also used to derive dendograms that establish similarities between habitats types in terms of species composition(Legendre and Gallagher, 2001).

MorphoJ software (version 1.03) (Klingenberg, 2014) was used to create Cartesian coordinates of the eight landmarks which were then procrustes aligned to determine existing shape variations among the different species. The data points were subjected to principal component analysis (PCA); canonical variate analyses (CVA), discriminant function analyses (DFA), Procrustes ANOVA and Regression analyses were carried out to further delineate the bee species. After all characters were measured, they were compared between the two study sites (high lands and lowlands) using ANOVA and tukey's test for a posteriori comparison among means. Differences in wing venation between the two locations by means of a contingency G test was carried out, then a principal component analysis using a correlation matrix was performed on all log-

transformed metric characters (Krauss, 2009). Colony principal component scores (PCs) were obtained by multiplying the character coefficients for the four components by their mean value for each colony. Colony PCs from both sites were compared by means of ANOVA and were plotted orthogonally against the axes of components to obtain a comparative spatial distribution of all species within the two habitats.

3.4 Results

3.4.1 Meliponine bee species and native names

In most communities situated in both lowlands and highlands in Taita hills, meliponine bees are locally called "Mbuche" and are commonly categorized according to external body morphology such as body size, preferred nesting sites and body color (**Table 3.2**).

The species which exhibited the highest form of nesting plasticity, *Hypotrigona gribodoi* is also generally termed as "mbuche" because of their availability in most environs; hence categorized as the bee with the smallest body size, black color and commonly found in wall and roof crevices of homesteads. *Meliponula ferruginea* (black) is commonly called "wesu" and is known to be a prolific honey producer; it's generally black in color, of medium size and majorly found nesting in open tree cavities in the forest. *Plebeina hildebrandti* is also a medium sized bee which nests only in certain peculiar areas (underground and termite mounds), they are also regarded as prolific honey producers.

Table 3.2: Local names (Taita language) of various meliponine bee species.

Species	Local name[§]	Proportion caught (%)	Colonies recorded	Bee color	Nesting site
Hypotrigona gribodoi	Mbuche	58.3(1385)	87	Black and brown	Wall cavity, Tree trunk
Hypotrigona ruspolii	Mbuche	7.4 (175)	13	Black/ brown	Wall cavity; Tree trunk
Meliponula ferruginea (Black)	Mvusi	32.0 (760)	41	Black and brown abdomen.	Tree trunk
Plebeina hildebrandti	Wesu	2.3 (55)	6	Black, brownish thorax	Underground

Local name in Taita language. Number in parenthesis indicates total number of bees caught.

3.4.2 Overall species richness of Meliponine bees.

The four species were unevenly distributed among all habitats (mixed deciduous woodlands, grasslands and *Acacia* dominated bush lands) in lowlands and highlands (Indigenous mixed forests, Exotic forest). *Hypotrigona gribodoi* had highest species (4), followed by *Meliponula ferruginea (black)* (3) *Hypotrigona ruspolii* (2) while *Plebeina hildebrandti* had the lowest (1) (Fig. 3.3). Species richness signified a high number of four in every 80 line transects surveyed in both habitats.
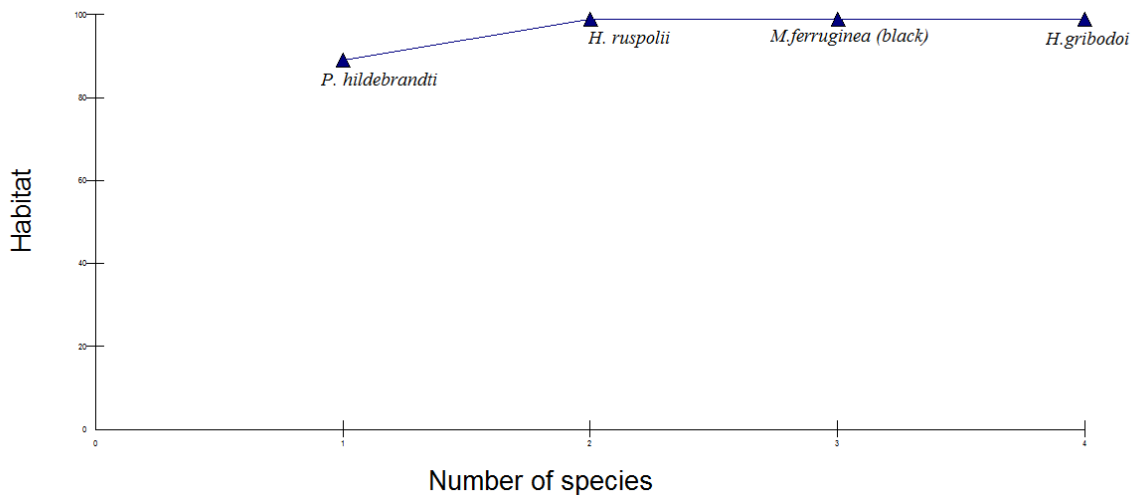
Figure 3.3 Overall species richness of meliponine bees in all pooled habitat types in the highlands and lowlands of Taita hills area**.**

Varying distribution ranges of meliponine bee species within the four habitat types (forests, grasslands, woodlands and bush lands) revealed an unequal range of distribution of nests (Fig. 3.4). Habitats were ranked according to the abundance of nests in all habitats of the lowlands (mixed deciduous tree woodlands, Acacia dominated bush lands, and grasslands), signifying a normal distribution pattern for every 60 transects surveyed. Mixed deciduous woodlands curve (MDW) was skewed to the farthest right indicating the highest diversity occurring in this habitat compared to the two habitats (IMF, EFP) whose curves steeped to the farthest left for these bee species to occur (Fig. 3.5). However, analysis of variance (ANOVA) indicated that the level of disturbance

(fragmented and unfragmented) had significant effect (P=0.003) on nest abundance thus revealing a distinct preference amongst the five main habitat types.
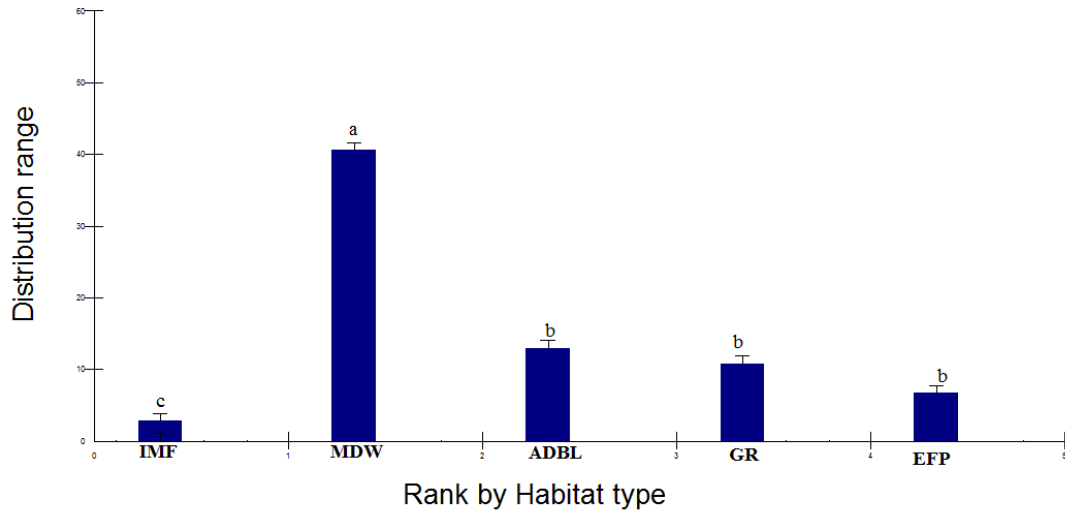
Figure 3.4: Distribution range of meliponine bees nest abundance within specific habitats types in the highlands and lowlands of Taita hills area.

IMF, Indigenous mist forest (**H**); **ADBL**, Acacia dominated bush lands (**L**); **EFP**, Exotic forest patches (**H**); **GR**, Grasslands (**L**); **MDW**, Mixed deciduous woodlands (**L**); MF, **Mixed highland forest**. (H), Highlands; (L), Lowlands.
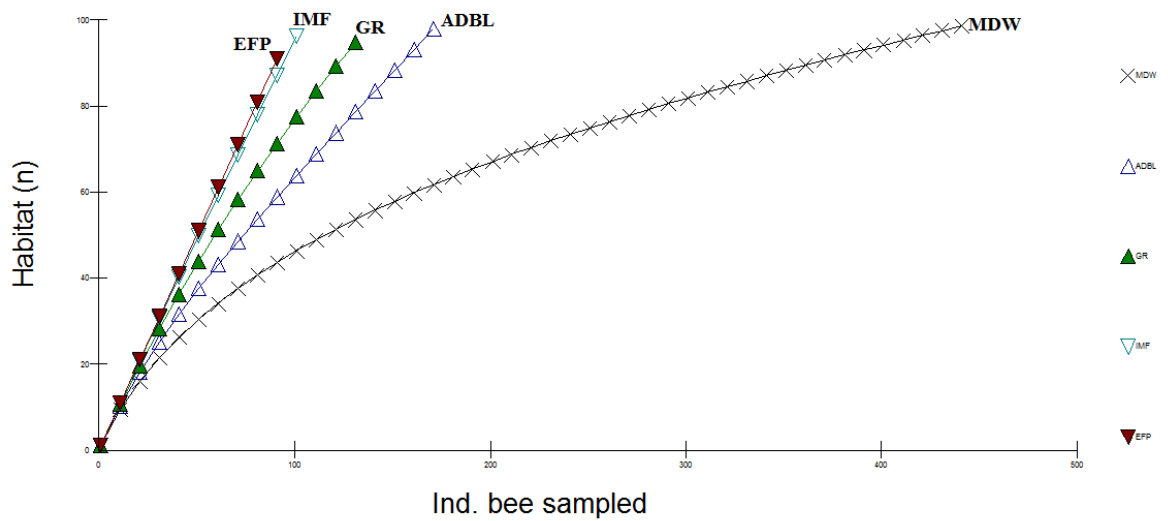
Figure 3.5: Species accumulation curve indicating bee abundance in all sampled habitat types.

IMF, Indigenous mist forest (**H**); **ADBL**, Acacia dominated bush lands (**L**); EFP, Exotic forest patches (**H**); GR, Grasslands (**L**); MDW, Mixed deciduous woodlands (**L**); MF, Mixed highland forest. (H), Highlands; (L), Lowlands.
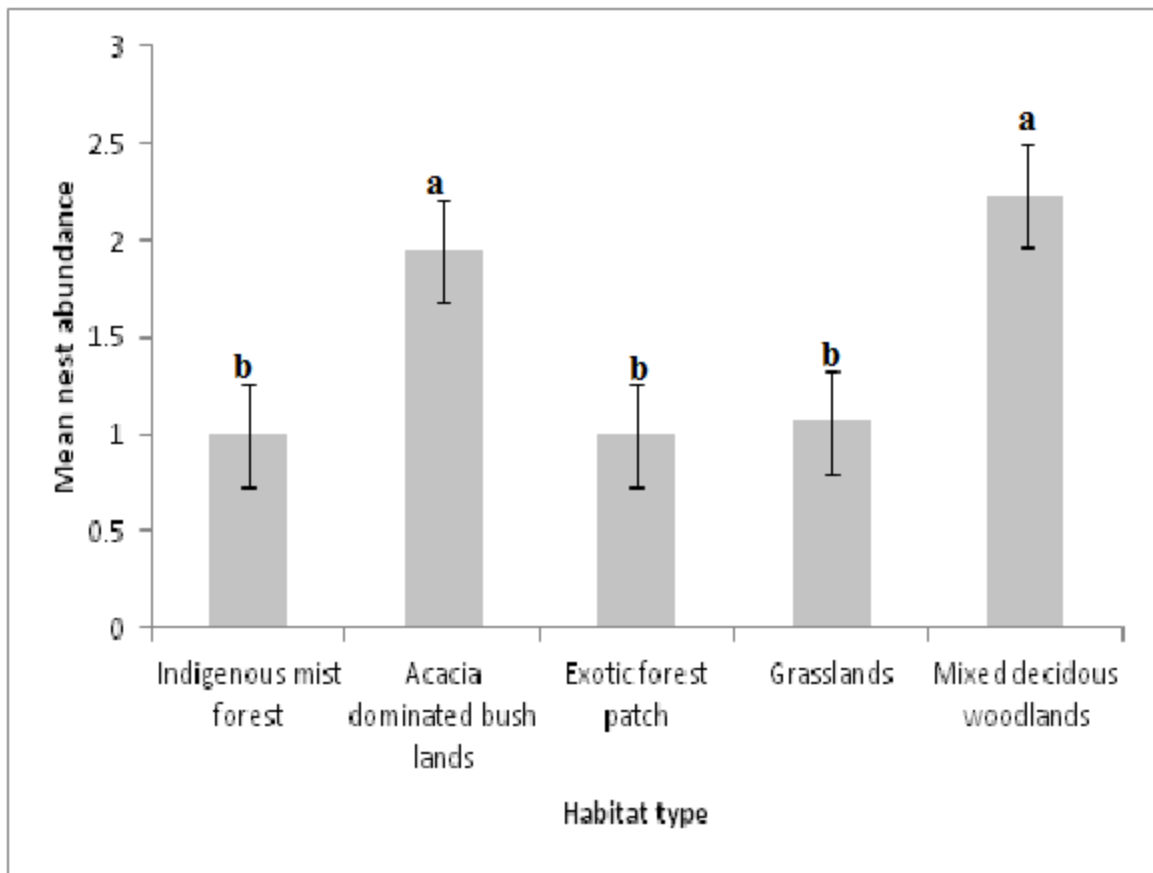
Figure 3.6: Mean (±SE) nests abundance of meliponine bee species in the highlands and lowlands habitat types of Taita hills area, March-September 2014**.**

IMF, Indigenous mist forest (**H**); **ADBL**, Acacia dominated bush lands (**L**); EFP, Exotic forest patches (**H**); GR, Grasslands (**L**); MDW, Mixed deciduous woodlands (**L**); MF, Mixed highland forest. (H), Highlands; (L), Lowlands.

3.4.3 Species richness and diversity of meliponine bees

Renyi's diversity profile was used to provide information on species diversity. A total of four species was recorded in five out of the six main habitat types surveyed (Fig.3.7) and a further extrapolation with Shannon index (Eeveness) also predicted the highest species richness of 4.24 in the mixed deciduous woodlands habitat type (Table 3.3). The species accumulation profiles peaked at a plateau level (H-alpha = 0.5) for MDW while EFP and IMF habitats overlapped at (H-alpha= 0.0) (Fig.3.8). A comparison of species richness for individual habitats showed no significant difference (P=0.08). The profiles indicated that MDW habitat is more diverse than GR, ADBL, EFP and IMF habitats in descending order. The EFP and IMF could not be adequately ordered, as their profile curves overlapped. At α=0 scale, IMF habitat overlapped with EFP habitat (Fig 3.9), whereas at α=1 (Shannon index), species diversity ranked the habitats in a sequential descending order: MDW>GR>ADBL> EFP>IMF. At α=2 (Simpson index). Shannon diversity extrapolation for each habitat clearly predicted more species in MDW and GR than for other surveyed habitats.
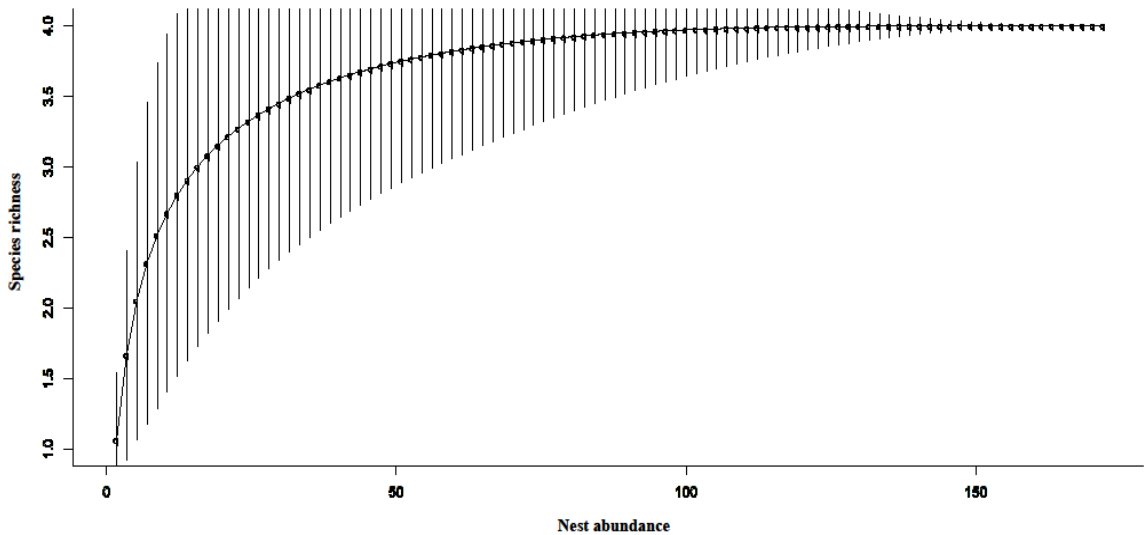
Figure 3.7: Species accumulation curve indicating meliponine bee species richness of pooled nests surveyed in both locations of Taita hills.

Table 3.3: Diversity indices and its associated Evenness for each habitat type surveyed.

Habitat	Sampled points	Total Richness	Shannon diversity	Total Abundance	Evenness
IMF	2	1	0	2	1
EFP	2	1	0	4	1
ADBL	19	3	0.723	32	0.687
GR	31	3	0.965	41	0.875
MDW	44	4	1.010	96	0.686

IMF, Indigenous mist forest (**H**); **ADBL**, Acacia dominated bush lands (**L**); EFP, Exotic forest patches (**H**); GR, Grasslands (**L**); MDW, Mixed deciduous woodlands (**L**); MF, Mixed highland forest. (H), Highlands; (L), Lowlands.

Habitat types and nesting substrates were grouped according to similarity for all meliponine bee species. Three distinct groups were recognized based on habitat type: Group A comprised of EFP and IMF habitats, Group B comprised of MDW and GL habitats, Group C consisted of only ADBL habitats. Similarly, three categories of nesting substrates were recognized: tree (T), ground (G) and homestead (H) (Fig. 3.10).
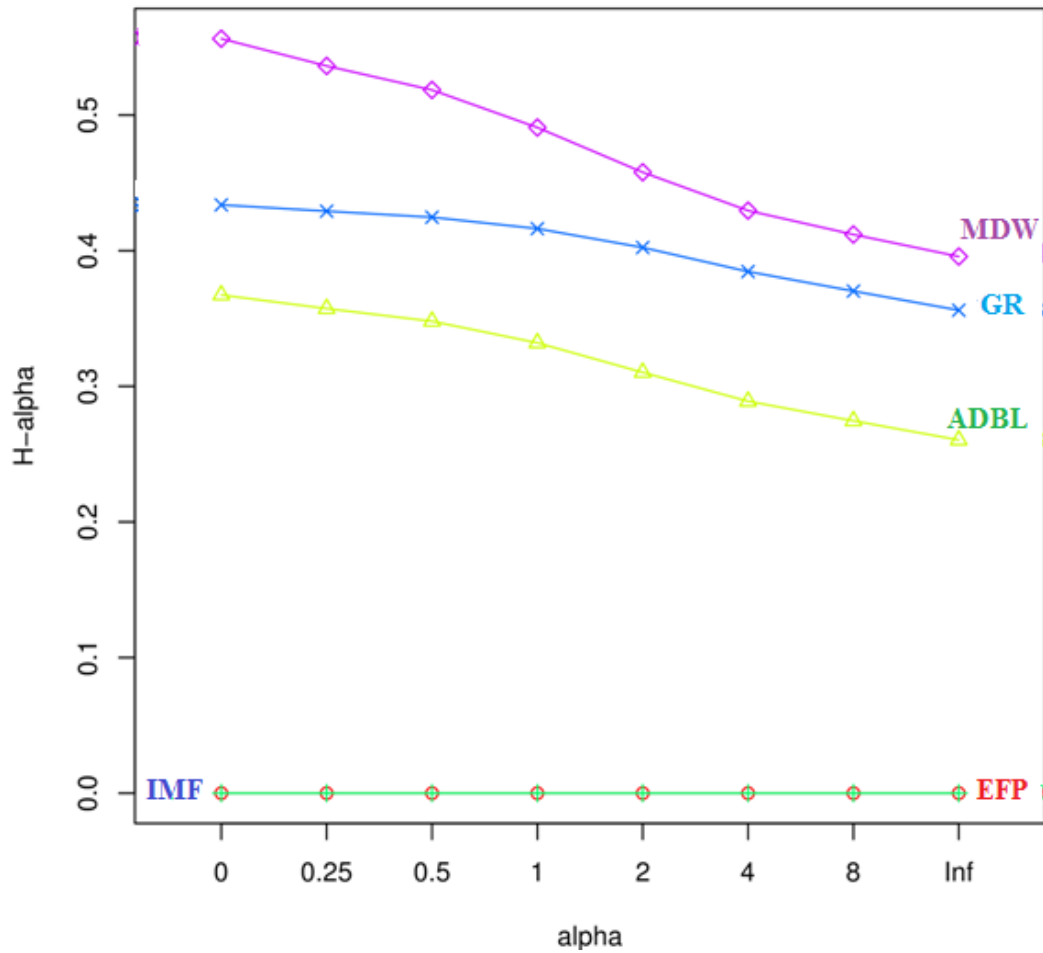
Figure 3.8: Renyi diversity profile indicating the diversity across all habitat types

IMF, Indigenous mist forest (H); **ADBL**, Acacia dominated bush lands (L); **EFP**, Exotic forest patches (H); **GR**, Grasslands (L); **MDW**, Mixed deciduous woodlands (L); (H), Highlands; (L), Lowlands.
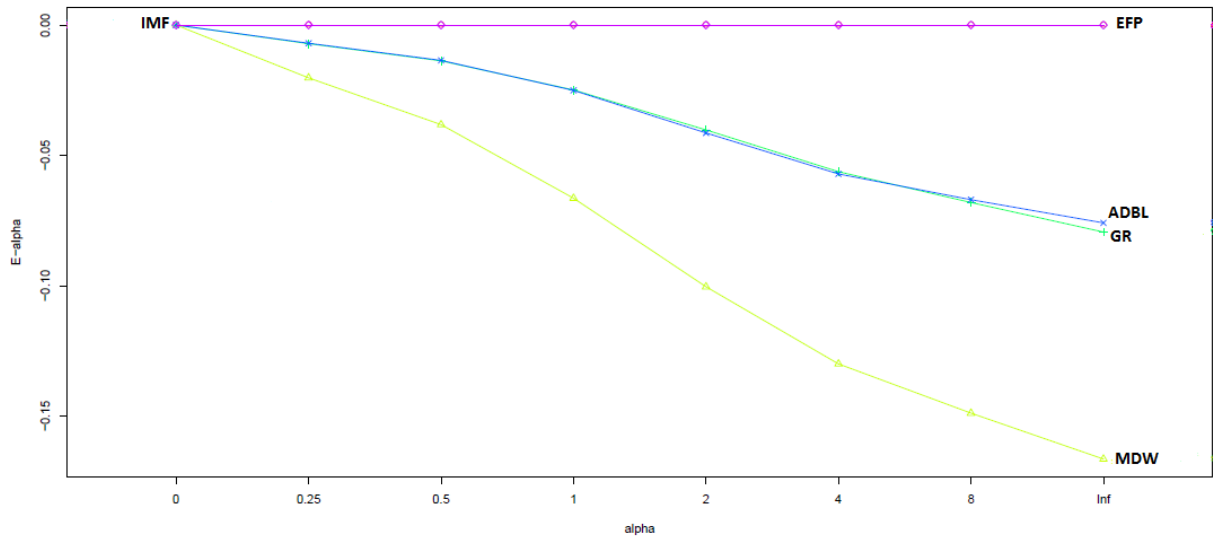
Figure 3.9: Renyi diversity profile indicating the Evenness across all habitat types

IMF, Indigenous mist forest (H); **ADBL**, Acacia dominated bush lands (L); **EFP**, Exotic forest patches (H); **GR**, Grasslands (L); **MDW**, Mixed deciduous woodlands (L); (H), Highlands; (L), Lowlands.
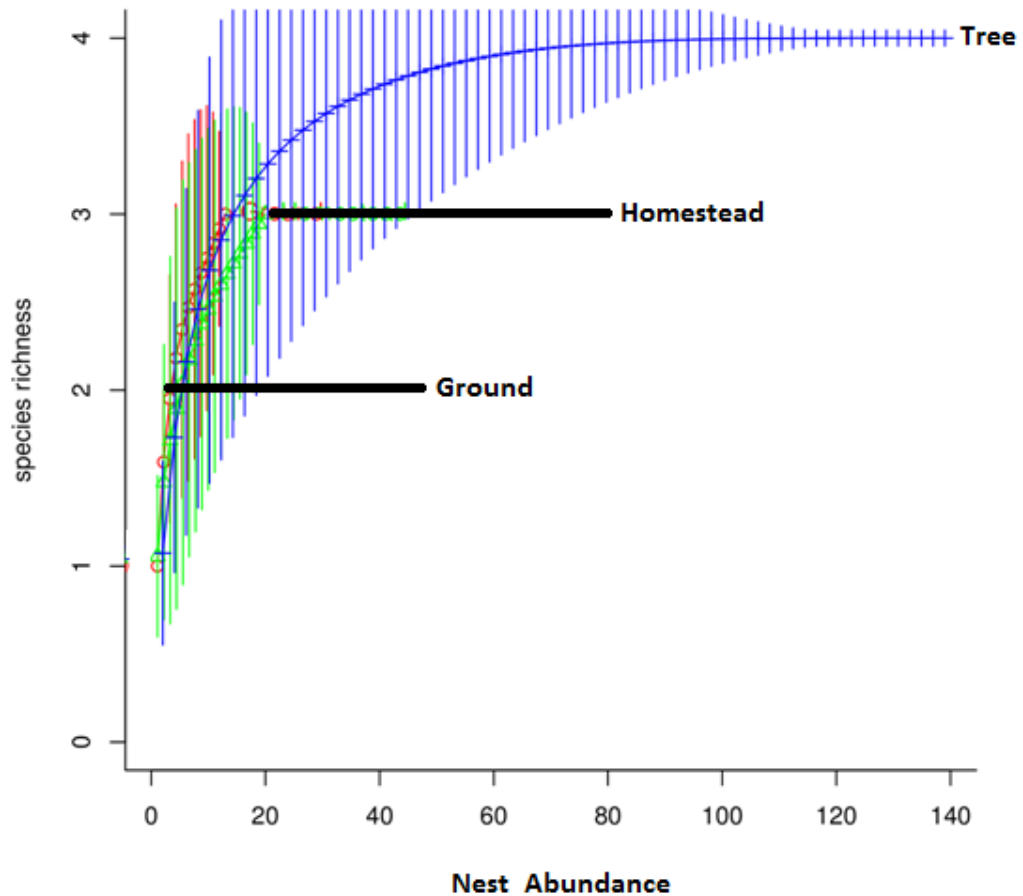
Figure 3.10: Species accumulation curve with respect to preferred nesting substrates (Tree (T), Ground (G), and Homestead (H).

3.4.4 Sequence Analysis

All Eigen values were found to be less than one which accounted for 99.17% of data variability. Graphical representation of CVA scores showed a clear differentiation of species within all habitats sampled (Figure 3.12). The discriminant function analyses (DFA) also revealed significant differences within populations from the different habitats with P-values of <0.001. In general, 99.59% of all specimens were correctly classified according to the respective habitats; with *H. gribodoi* populations accounting for 93.96%, *Meliponula ferruginea* (black) accounting for 3.57%, *Hypotrigona ruspolii* 2.05%, while *Plebeina hildbrandti* recorded the least DFA 0.40%. Geometric morphometrics correctly grouped all four meliponine bee species into two clusters,

cluster 1 (*H.gribodoi*, *H.ruspolii*, *M. ferruginea (*black)) and cluster 2 (*P.hildebrandti*) and further discriminated populations against the four habitats they could potentially persist in. However, each habitat appeared to consist of a cluster of sub-populations and may possibly reveal ecotypes within the four meliponine populations.
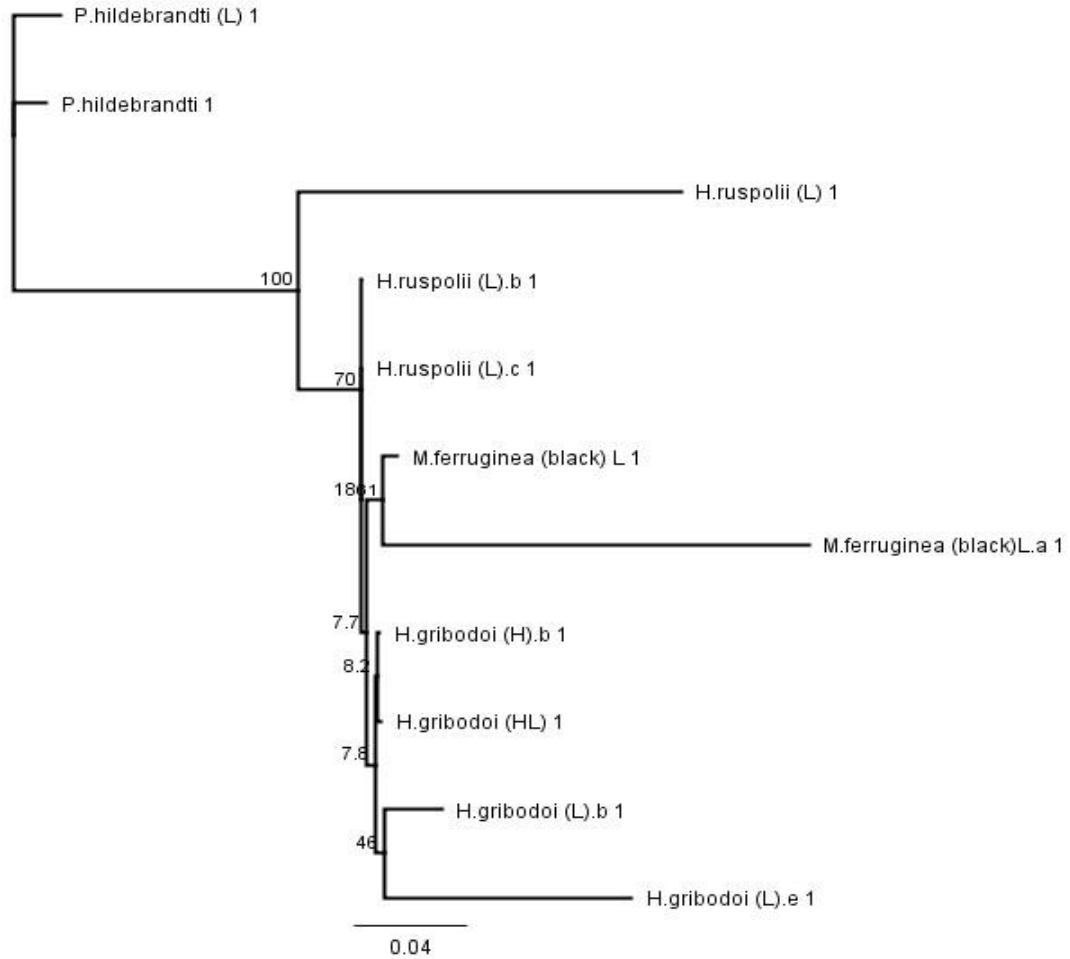
Figure 3.11: Dendogram obtained by cluster analysis based on mtCO1 region of four bee species found in Taita hills**.**
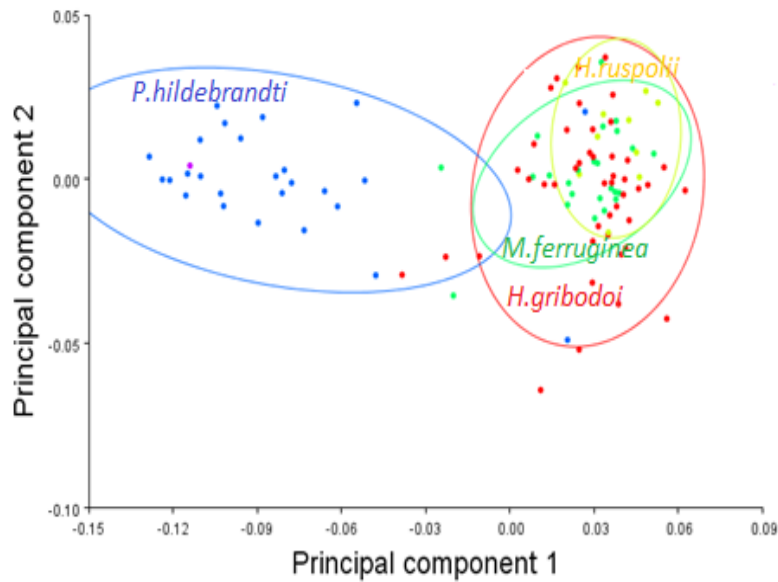
Figure 3.12: Wing morpho-metrics PCA grouping all four species found across all sampled habitats.

3.5 Discussion

Habitat loss and fragmentation arising from human activity are two key factors driving declines of native species worldwide (Bommarco and Biesmeijer, 2010; Cane, 2001; Ewers and Didham, 2006; Taki *et al*., 2008). The synergistic effect of fragmentation reveals how bee communities could potentially respond to isolated habitats as some empirical studies reveal a range of responses to fragment size (Aerts *et al*., 2011; Brent *et al*., 2001; Cane, 2001; Ingolf Steffan-Dewenter & Tscharntke, 2002). In this current study, species richness of meliponine bee species correlated with relation to habitat type (Fig 3.5) and preferred nesting substrates (Fig 3.10) denoting the clear evidence of low distribution and diversity in the highlands compared to the lowlands ,this may have potentially resulted from the high conversion of natural habitats to agriculture which is the primary form of land-use change and the largest cause of native habitat loss and fragmentation (Winfree *et al*., 2009; Bartomeus *et al*., 2010; Jaff *et al*., 2016). The dominance of agro-ecosystems worldwide means that increasing bee populations exist at the interface of agricultural and natural habitats or within agricultural areas, as is currently observed from results of this study. Low bee abundance and species richness

with increasing agricultural intensity have been reported from a wide variety of agro-ecosystems(Hendrickx *et al*., 2007; Jauker *et al*., 2009; Carvalheiro *et al*., 2010; Diekötter and Crist, 2013). Species number recorded in both locations indicates the uneven composition of meliponine bee species within this hotspot, though this is comparatively less than the species recorded in Kakamega forest (Macharia and Raina, 2010), it unmistakably signifies the negative effects of habitat fragmentation in predicting the diversity of bee species within an ecosystem. *Hypotrigona gribodoi* species was more dominant and featured in all habitat types but at variable proportions, which may be attributed to its plasticity in nesting in varying habitat types.

This corroborates with studies on pollinators abundance in agro-ecosystems that contain a mixture of semi-natural habitats throughout any particular landscape, can successfully maintain significant levels of bee diversity and abundance (Tscharntke *et al*., 2005; Winfree *et al*., 2011), even at regional scales (Connelly *et al*., 2015; Frund *et al*., 2013; Jha and Kremen, 2013). The profiles indicates that MDW (mixed deciduous wood lands) (Fig 3.5) presented itself as a much preferred habitat for nesting and trees as a preferred nesting substrate (Fig 3.10) as profile curves indicated that more species could be identified with increased sampling sites and on more tree nesting substrates. Other studies, Hendrickx *et al*., (2007) affirm that land-use intensity and proximity to semi-natural habitats best explained bee species richness across landscapes, but loss of bee species richness was not solely the result of declines within habitats, but also increased homogenization of community composition between habitats could be a contributing factor acting in synergy with land-use intensity, as also confirmed in this study. The four species recorded directly from sampling are close to the Evenness extrapolated predicted value of 4.24. The species accumulation curve indicated approximately 80 sampling points as adequate to recover at least four species. Geometric morphometrics analyses showed that all four meliponine bee species at Taita hills could be grouped into two clusters, cluster 1 (*H. gribodoi*, *H. ruspolii*, *M. ferruginea (*black)) and cluster 2 (*P.hildebrandti*) and successfully discriminated populations against four different habitats in Taita hills. Each habitat appeared to consist of a cluster of sub-populations and may possibly reveal ecotypes within the four meliponine populations.

A major reason for this clustering of species would be the superficial resemblance of the three species belonging to cluster 1(*H. gribodoi*, *H. ruspolii* and *M. ferruginea* (black)) with regards to similarities in forewing characters (open sub marginal cells, anterior region of the sub marginal cross vein faintly visible, and non-distinct veins) and cluster 2 (*P. hildebrandti*) which has distinct marginal cells, closed sub marginal cells and distinct veins. Also characteristic type of vegetation and climatic conditions each habitat appeared to have; may have ultimately altered morphological characters for greater survival in such habitats.

The results of a principal component analysis on the morphological measurements corroborated with molecular analysis, revealing the specimens clustering in four different clades (*Hypotrigona gribodoi, Hypotrigona ruspolii, Meliponula ferruginea* (black) and *Plebeina hildebrandti* respectively. This shows that integrating DNA bar-coding with morpho-metrics can help in segregating species that have high levels of similarities, i.e. *Hypotrigona spp*.

3.5.1 Conclusion

The similarity index clearly distinguished habitat types that share similarity in species diversity. The habitat types with IMF and EFP were in the same group, while ADBL and GR formed a slightly divergent group from MDW, both indicating similarities and close proximity to each other (dispersed habitats in lowlands) in the same group. Similar trends were noted with respect to habitat type (dispersed lowland habitats) showing higher variation in lowland habitats with minimal habitat disturbance than in patchy or isolated forested landscapes of the highlands where greater land use change occurs, implying greater heterogeneity in dispersed vegetation of the lowlands than in patchy forested landscapes in highlands. The study has shown greater species diversity in mixed deciduous woodland habitats characterized with deciduous tree species that are indigenous to this habitat and could thereby predict the diversity of meliponine bee species.

CHAPTER FOUR

FLORAL RESOURCES SUSTAINING AFRICAN MELIPONINE BEE SPECIES (APIDAE: MELIPONINI) IN A FRAGILE HABITAT OF KENYA[1].

4.1 Summary

A vast majority of insects visit flowers for food, generally termed as floral rewards. Detailed insights of flowering phenology of plants could give a hint of any habitat status and the extent to which such landscapes could support insect pollinators to render both direct and indirect ecosystem services. This study monitored flowering plants which could potentially provide pollen and nectar to four African meliponine bees species (Apidae: Meliponini) naturally occurring in six diverse habitat gradients of the eastern arc mountains (Taita hills) of Kenya. Blooming sequences of identified flowering plants overlapped across seasons with approximately 80 different plant species belonging to 34 families recorded, with the highest proportions from Fabaceae and Asteraceae families dominating flowering plants that were visited (67 % of the visits) in both the lowland and highland habitats. A flowering calendar is presented to indicate the phenological pattern of all identified floral resources. *Hypotrigona gribodoi* was the most abundant meliponine bee species, and had the highest visitation rates of the plants belonging to the Fabaceae and Asteraceae families, followed by *Meliponula ferruginea* (black), *Plebeina hildebrandti* and *Hypotrigona ruspolii*. This indicates that such diverse vegetation may invariably sustain nutritional requirements essential for the survival of insect pollinators such as native meliponine bee species, but can still be affected from the drastic environmental changes that limit the availability and quality of nectar or pollen resources which could invariably alter pollinator foraging behaviour.

4.2 Introduction

Pollinators are known to play key roles in delivering various forms of ecosystem services such as pollination and seed dispersal which benefits most plant populations (Hatfield

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[1] Bobadoye *et al*., (2017). Floral Resources Sustaining African Meliponine Bee Species (Hymenoptera: Meliponini) in a Fragile Habitat of Kenya. *Journal of Biology & Life Sciences* 8, 42-58.

and LeBuhn, 2007) and to a certain degree predict the community structure of plants in most habitats (Steffan-Dewenter and Tscharntke, 2002; 2003; Klein *et al*., 2003;Westphal *et al*., 2003; Klein *et al*., 2007; Ricketts *et al*., 2008; Holzschuh *et al*., 2010; Kleijn *et al*., 2015). A vast majority of angiosperms, including agricultural crops are insect pollinated (Gill *et al*., 2016; Senapathi *et al*., 2015; Sheffield *et al*., 2008) with almost 25% of tropical crops depending mostly on bees for pollination (Heard, 1999). Insect groups such as moths, wasps, bees, beetles, butterflies and bats are essential for providing effective pollination of both cultivated and uncultivated plants (Hadley and Betts, 2012; Kleijn *et al*., 2015) facilitating symbiotic relationships in plant communities between blooming flowers and any random visiting insect.

These floral resources usually mediate mutualisms between flowers and potential visitors. As such, flowering plants benefit from the pollinator by being pollinated while the insect pollinator obtains floral rewards. Floral rewards are of two major forms (pollen and nectar) and they can be considered to be any component of a flower or an inflorescence utilized by invertebrates, and this ensures repeated visitation which leads to pollination. Pollen is a vital food and source of protein for a majority of insects, it contains essential amino acids and lipids which is known to be an essential resource for foraging bees and a vital component in plant reproduction providing dual function interchangeably (Aleixo *et al*., 2016; Campos *et al*., 2008;Leonhardt *et al*., 2007; Nicolson 2011), while nectar is a simple sugar solution consisting of a variety of chemicals suspended in aqueous solution (glucose, sucrose and fructose) to even more complex sugar solutions or mixtures of sugars, vitamins, lipids and other compounds (Kajobe and Echazarreta, 2005; Nicolson, 2011; Tereshko and Loengarov, 2005).

The most abundant bees in the tropics are members of the diverse group of meliponine bees (Apidae, Meliponinae) (Couvillon *et al*., 2008; Eltz *et al*., 2003; Engel and Michener, 2013; Leonhardt, 2010; Reyes-González *et al*., 2014). African meliponine bees (Hymenoptera: Apidae) belong to the tribe *Meliponini* of which more than 30 species are native to Africa, 14 of which are found in Kenya (Carroll, 2006; Macharia and Raina, 2010; Mwangi *et al*., 2012). They are important indicators of biodiversity as they have

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co- evolutionary relationships with plants and therefore their services are inevitable for the maintenance of the life cycles of many plant species (Carvalheiro *et al*., 2010; Dauber, 2003; Fahrig, 2003; Kovács-Hostyánszkia *et al*., 2013; Tscharntke *et al*., 2005). It is widely accepted that habitat loss has negative effects on biodiversity and that the amount of suitable habitat in any landscape influences species distribution of any organism (Frankie *et al*., 1998) and its abundance (Turner *et al*., 1999).

The structure of bee populations is closely related to the floral communities they forage upon with several other key drivers such as floral diversity (Tepedino and Stanton, 1981) floral abundance (Frankie *et al*., 1998) and seasonal availability of these resources (Ricketts *et al*., 2008) shaping their distribution and diversity. While forage rewards provided by floral communities are generally accepted as the primary determinant of pollinator community structure, there is an increasing body of evidence suggesting that the extent to which a habitat has been disturbed may also play an important role for the occurrence of bees within any habitat (Westphal *et al*., 2008). Increasing isolation from naturally ideal habitats can be associated with either a decline or an increase in species composition, richness and diversity (Ewers and Didham, 2006) which is yet to be determined in fragile habitats found within Taita hills, a very likely place to suffer plant and animal extinction due to drastic loss of its habitat. The aim of this study was to monitor and describe the blooming sequence of dominant plants in Taita hills over seasons while associating it with the natural occurrence of feral meliponine bee colonies.

4.3 Materials and methods

4.3.1 Study sites

Taita hills is the northernmost part of the Eastern Arc mountains (Maeda *et al*., 2010; Pellikka, *et al*., 2010; Maeda, 2012; Pellikka *et al*., 2013) and categorized into both highlands and lowlands, respectively. The area has the status of a global biodiversity hot-spot (Adriaensen *et al*., 2006). Taita hills lies in south- eastern Kenya at $03^{o}20$ S, $38^{o}15$ E, about 150km inland from the coast and covering an area of about 250km^2 (Brooks *et al*., 1998) (Figure 4.1). The hills are isolated from other mountainous areas to the south-east (Shimba Hills), south (Usambara Mountains), south-west (Mt Kilimanjaro), west

(Ngulia and Chyulu Hills) and north-west (Kenyan highlands) by the vast plains of Tsavo (Maeda *et al.*, 2010). Annual rainfall is received during two major seasons (March- May, September – October) and varies between 480 - 1200mm in the highlands (Reitalu *et al.*, 2012), but much less rain (~400mm) received on the surrounding plains of the lowlands (Pellikka *et al.*, 2005).

4.3.1.1 Lowlands

The lowlands are characterized by highly dispersed vegetation and fragmented patches of habitats dominated by grassland plains. Three different geographically detached communities (Msau, Mwatate and Mugama) make up a large percentage of the lowlands. Mean rainfall in the lowlands ranges around 400mm with annual rainfall peaks in April and November.

In the Msau community, vegetation is characterized by abundant *Commiphora myrrha* deciduous woodlands which are widely dispersed, but a considerable number of other deciduous tree species are persistently reduced to shrubs by extensive grazing and deforestation (Omoro *et al.*, 2010). Common deciduous trees such as *Albizia gummifera, Haplocoelum foliolosum, Comiphora schimperi, Balanites pedicellaris, Tamarindus indica, Sterculia africana, Ficus sycomorus, and Cordia sinensis* are mainly found along streams (Pfeifer *et al.*, 2012).

The Mwatate community is a grassland habitat consisting mainly of perennial grasses such as *Chloris roxburghiana*, *Cenchrus ciliaris, Erythrococca bongensis*, *Pennisetum menzianum* and *Setaria sphacelata*. Undergrowth is minimal with bare soil surface exposure clearly evident, and with increased regional slope, large gully systems are rapidly formed rendering the land unproductive for agriculture. Such large-scale gullies effectively transport organic soils from hilly slopes to river Voi which runs from Msau through Mwatate and Mugama.

The Mugama community is dominated by *Acacia* bush lands characterized by very poor vegetation cover caused by overgrazing of small ruminants aiding the removal of topsoil

which leads to friable red soil patches and silting of local water supplies. Rocky outcrops are quite common. *Acacia* tree species interspersed with perennial grasses dominate this habitat. *Acacia geradii* was more dominant and closely associated with *A. nilotica L*, and *A. tortilis*. Grasses predominant in this area included *Cynodon dactylon*, *Themeda triandra*,*Cenchrus ciliaris*, *Chloris roxburghiana* and *Pennisetum menzianum* (Omoro *et al*., 2010).

4.3.1.2 Highlands

A total of seven forest fragments are found in this region, and characterized by continuous forest landscapes. The highlands is composed of several communal forests such as Mwachora forest, Kichuchenyi forest, Chawia forest which are considered as fertile areas suitable for agriculture. However, a very small area is available for agricultural purposes due to steep slopes and shallow soils occurring at high altitudes. Mwachora forest (03°25'S, 38°22'E) is an indigenous forest habitat situated at an altitude of 1,400 m^2 measuring approximately 2 ha (Wilder *et al*.,1998) and is regarded as part of remnants of the original afromontane forest, receiving 1700–2400 mm of annual precipitation. Tree species such as *Lobelia gibberoa*, *Phoenix sylvestis*, *Dracaena steudneri* and *Cyathea manniana* are characteristic to this forest. Chawia forest (03°28'S, 38°28'E) is a mixed forest habitat comprising of both indigenous and exotic tree species forming dense and continuous canopies, this forest has the status of being the most disturbed forest fragment out of the seven forest fragments found in Taita hills. Exotic tree species predominantly found in the highlands are cypress trees (*Cupressus lusitanica*), eucalyptus trees (*Eucalyptus saligna*), pines (*Pinus caribaea*), *Maesopsis eminii*, grevillea trees (*Grevillea robusta*) and some species of acacia such as *Acacia mearnsii*.

4.3.2 Sampling Procedure
4.3.2.1 Flowering Phenology and Floral Resources

Flowering phenology was monitored within each study site. A total of 20 linear transect measuring 250m x 20m each was established using a GPS to mark coordinates with relation to each habitat type. All flowering plants were surveyed using the conventional

belt transect method (direct observation of blooming flowers via visual census) (Potts *et al*., 2005) for flowering peaks. The time duration in which a relatively large number of flowers were in anthesis were regarded as the flowering peaks(Eltz *et al*., 2001; Frankie *et al*., 1998; Rader and Howlett, 2009; Sharma *et al*., 2011; Westphal *et al*., 2003). The blooming duration of most flowering plant species were followed on a daily basis throughout the study period from May-December, 2014. Data on the type of flower reward obtained over months were recorded. Flowering stages in each species were classified into four groups: initial stage (when plants have started producing flower buds - stage A); Peak stage (when plants have opened flowers - stage B); late stage (when flowers retain their bloom after peak flowering - stage C) and terminal stage (when most flowers have passed blooming - stage D). For the purpose of this study, blooming periods have been defined as the time from actual senescence to the end of each bloom. Floral resources were expressed quantitatively based on the number of overlapping flowering species across both seasons (May-December), because major plant species flowering during this period largely represent the persistent plants in full bloom at any sampling period. To test the validity of using the number of overlapping flowering species as an index of floral resource level, the Shannon index (H) between the number of flowering species and floral density was measured in all transects that were surveyed from the lowlands (< 900m elevation) to the highlands (<1,400m elevation). Flowering plant species were counted on a daily basis throughout this period and floral density estimations were made based on the average number of open flowers within every measured transect. Samples of all flowering plants in the study area were collected and identified at the East African Herbarium in the Botany Department of National Museums of Kenya.

4.3.2.2 Meliponine bee Monitoring and Visitation Rates
Sampling of bees to determine fauna diversity, floral resource use and overlap was carried out using net-trapping and visual observation of bees at flowers which provided the main sources of data. Within each transect, feral nests of meliponine bee species were carefully and systematically searched for in the possible nesting sites from 08.00 - 17:00hrs daily. The bees were sought on all flowering plants at reachable heights. When

one was spotted, it was caught with an entomological sweep net. The floral resource (pollen and nectar) collected by the bee was identified by observing the corbicula. Visit frequency was recorded by counting the number of times foragers of any Meliponine bee species were seen on each visited plant.

A representative sample of approximately five bees was taken from each feral nest and deposited in the biosystematics unit of the International Center for Insect Physiology and Ecology (*icipe*), Kenya. Observations were not conducted on rainy or cloudy days. Only data recorded in the measured transects were used for analysis in this study because the focus of the research was to monitor plant phenology and the occurrence of meliponine bee species.

4.3.3 Data Analysis

Flowering phenology of individual plant species were compared between habitats (fragmented and non-fragmented) while the differences in resource availability (richness of flowering plants), and frequency of visits were evaluated by the chi-square test using Sigma plot v11.0 statistical software (Systat Software, San Jose CA, 2011). Spearman's correlation was used to determine if number of visits and richness of flowering plants correlated. Richness of the actual plant-species trophic niches was determined by recording both the number of visited and non-visited flowering species. Trophic niche breadth was calculated by using the Shannon-Weiner Diversity Index (Pielou, 1969). Nest abundance of feral colonies was compared in both sites by carrying out logarithmic transformation on the data and further subjecting it to a Pearson's correlation test throughout the entire sampling period using the Sigma plot v11.0 statistical software.
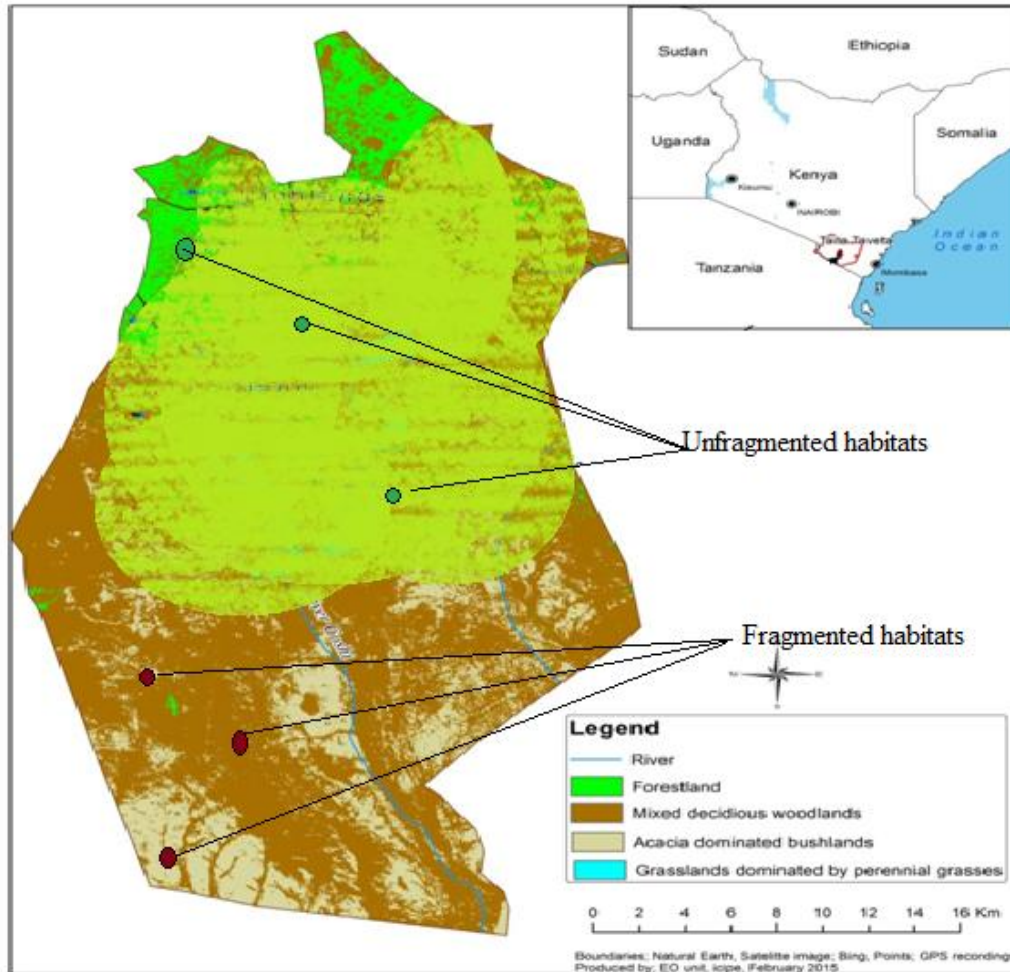
Figure 4.1: Map of Taita hills forests and surrounding areas further indicating fragmented and unfragmented habitats

4.3.4 Results

4.3.4.1 Flowering Phenology

A total of 80 plant species belonging to 34 families were found to be constantly flowering in both sites, with plants of the Asteraceae and Fabaceae families forming bulk of this proportion (80%) (Table 4.1). Flowering commenced earlier in the lowlands (Msau, Mwatate and Mugama) at an altitude of < 900m than in the highlands (Mwachora forest, Chawia forest and Kichucheyni) standing at an altitude peaking at 1,800m. Major periods (stages A-C) of flowering plants sampled in the lowlands lasted approximately 240 days compared to the highlands which had a flowering period of approximately 190 days.

All four species visited 54 species (48%) from 8 families. 36% of 192 visits were to twelve species of Asteraceae and ten species of Fabaceae. Fabaceae (40%) and Asteraceae (33%) were the most visited families. However, we found a high variation in the number of flowering plants belonging to both families during the study period ($\chi^2 = 67$; $df = 2$; P < 0.001). The highest numbers of flowering species were observed to bloom at the commencement of the short rain months of September (Figure. 4.2a). 78% of *H. gribodoi* bee species visits were to ten species of Malvaceae. Vernonia *species* and *Bidens pilosa* were the most visited species of Asteraceae and Fabaceae visited by *Meliponula ferruginea* (black) and *Plebeina hildebrandti* respectively. Peak flowering period expressed as flowering overlap of more than half of the identified plant species, occurred from May in the lowlands and sharply peaking in September, however low peak periods were observed from May -June in the highlands with further declines in the month of October (Figure 4.2a). During the monthly sampling of feral bee colonies, approximately three colonies could be found naturally occurring in each sampled transect of the lowlands while an estimated one colony would naturally occur in each habitat of the highlands. A total of 147 colonies was recorded, which comprises of four species namely; *Hypotrigona gribodoi, Hypotrigona ruspolii*, *Plebeina hildebrandti* and *Meliponula ferruginea* (black). The number of visited species significantly changed across both habitats during the study period ($\chi^2 = 92$; $df = 2$; P < 0.001). However, no correlation was found between monthly richness of flowering and visited plants. The Asteraceae*,* Malvaceae, Fabaceae, Meliaceae and Apocynaceae species were the main pollen sources, accounting for 32% of 71 visits. But the main floral resource collected was nectar, accounting for 66% of 121 visits, while pollen collection accounted for only 34%. (Table 4.1).
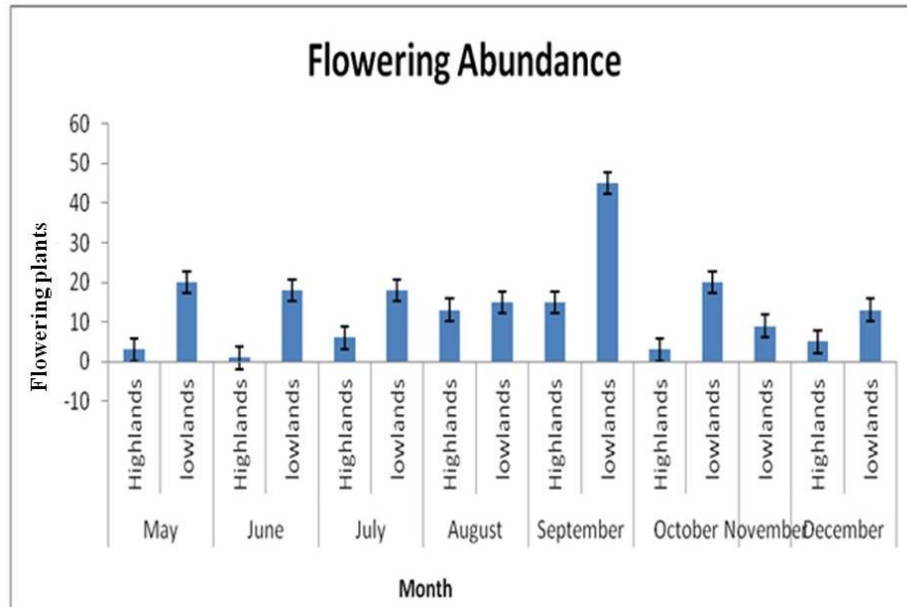
Figure 4.2a: Flowering abundance across months comprising two seasons.
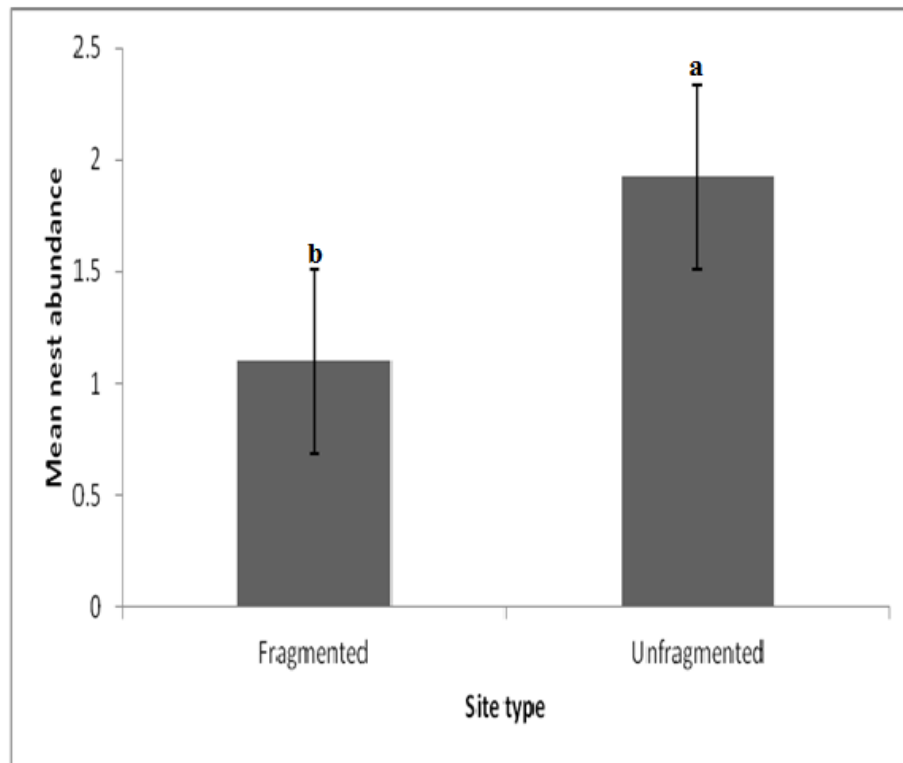
Figure 4.2b: Mean nests abundance of meliponine bee colonies across two habitat forms.

Table 4.1: Floral blooming sequences of dominant plants found in habitats of Taita hills

Plant species	Family	Form	Nectar source	Pollen source	High lands	Low lands	May	June	July	Aug	Sep	Oct	Nov	Dec
Helianthus annus	Asteraceae	Shrub		■		■	A-D							
Calendula spp	Asteraceae	Shrub		■		■	A-D							
Bridelia micrantha	Phyllanthaceae	Shrub	■			■	A-D							
Bidens pilosa	Asteraceae	herb		■		■	A,B	A,B	B, C	C	C,D	D	D	
Vernonia brachycalyx	Asteraceae	Shrub		■		■	A	B						
Vernonia gamalensis	Asteraceae	Shrub		■		■			A	B	C-D			
Rhus natalensis	Anacardiaceace	Shrub	■			■	A-D				A-D			
Persea americana	Lauraceae	Tree	■			■	A	C						
Grewia bicolor	Malvaceae	Shrub		■		■	A-D							
Tamarindus indica	Fabaceae	Tree		■		■	A	B	C					
Carica papaya	Caricaceae	Tree	■			■	A-D							
Cajanus cajan	Fabaceae	Legume	■			■	A-D		A-D					
Lantana camara	Verbenaceae	Shrub	■		■	■	A	A	A,B	B	B,C	C	D	
Thevetia thevetoides	Apocynaceae	Tree	■		■	■	A	B	C	D				
Melia volkensii	Meliaceae	Tree		■		■	A	B	B,C				A-D	
Albizia amara	Fabaceae	Tree		■		■	A		B					

Species	Family	Habit	Col1	Col2	Col3	Col4	Data
Bougainvillea spp	Nyctaginaceae	Shruby vine	green			light blue	A B C,D C,D D
Alstonia boonei	Apocynaceae	Tree		yellow		light blue	A B C C,D
Gravillea robusta	Proteaceae	Tree		yellow	dark blue	light blue	A-D … A-D A-D
Acacia mellifera	Fabaceae	Tree		yellow		light blue	A B,C … A B C-D
Acacia tortilis	Fabaceae	Tree		yellow		light blue	A B,C B,C … A B,C C-D
Acacia nilotica	Fabaceae	Tree		yellow		light blue	A B,C B,C … A B,C C,D
Acacia gerrardii	Fabaceae	Tree		yellow		light blue	A B,C B,C … A-D
Acacia mearnsii	Fabaceae	Tree		yellow	dark blue		A B C-D … A-D
Cactus spp	Cactaceae	Shrub	green			light blue	A B C D … A B C-D
Euphobia spp	Euphobiaceae	Shrub	green			light blue	A B C C C D
Ipomea batatas	Euphobiaceae	Annual plant	green		dark blue		A B,C C D … A-D
Tithonia diversifolia	Asteraceae	Perennial herb		yellow	dark blue	light blue	A B CD D
Acyranthes aspera	Amaranthaceae	Perennial herb	green		dark blue	light blue	A BC CD D
Musa acuminata	Musaceae	Tree	green			light blue	A-D
Fragaria anannassa	Rosaceae	Shrub	green		dark blue	light blue	A BC CD … A-D
Thurbegia alata	Acanthaceae	Shruby vine	green			light blue	A-D
Commiphora myrrha	Burseraceae	Tree		yellow		light blue	AB CD
Erythrina abysinica	Fabaceae	Tree	green		dark blue		A BC D

Species	Family	Type	Codes
Commelina benghalensis	Commelinaceae	plant	AB B CD D
Dalbergia latifolia	Fabaceae	Tree	A B-D
Mangifera indica	Anacardiaceae	Tree	A BC D
Tagetes lucida	Asteraceae	Perennial herb	AB C D
Aspilia spp	Asteraceae	Semi-woody herb	A-D
Cesalpinia decapetala	Fabaceae	Shrub	A-D
Ocimum gratissimum	Lamiaceae	Shrub	A B C CD D
Piper capensis	Piperaceae	Shruby vine	A-D
Culcasia scandens	Araceae	Shruby vine	A-D
Pentas lanceolata	Rubiaceae	Shrub	AB CD
Dombeya burgessiae	Malvaceae	Shrub	A-D
Impatiens balsamina	Balsaminaceae	plant	A-D
Pentaisia angustifolia	Rubiaceae	Perennial herb	A-D
Tarchonanthus camphoratus	Asteraceae	Tree	A-D
Psycotia domingensis	Rubiaceae	Shrub	A-D AB CD
Abutilon hirtum	Malvaceae	Shrub	A-D
Lactuca innermis	Compositae	Shrub	AB CD

Species	Family	Type												
Vigna unguiculata	Fabaceae	Plant							A-D					
Maerua kirkii	Capparaceae	Shrub							AB	C	CD			
Calotropis procera	Apocyanceae	Shrub							AB	CD				
Senna didymobotyra	Fabaceae	Shrub							AB	CD				
Psaidia punctulata	Asteraceae	Perennial herb							AB	CD				
Crotolaria agatiflora	Fabaceae	Shrub								A-D				
Dodonea viscosa	Sapindaceae	Tree								A-D				
Aspilia mossambicensis	Asteraceae	plant								A-D				
Santalum album	Santalaceae	Tree								A-D				
Phytolacca dodecandra	Phytolaccaceae	Trailing shrub							A	BC	CD			
Solanum incanum	Solanaceae	Shrub							A	BC	CD			
Tribulus terrestris	Zygophyllaceae	Annual plant								A-D				
Plectranthus amboinicus	Lamiaceae	Perennial plant								A-D				
Adenium arabicum	Apocynaceae	Perrenial plant							A	BC	C	CD		
Tridax procumbens	Asteraceae	Annual plant							AB	CD				
Gardenia manni	Rubiaceae	Tree								A-D				
Brugmansia spp	Solanaceae	Shrub					A-D		AB	CD				
Lantana trifolia	Verbenaceae	Shrub								A-D				

Species	Family	Habit									Stage
Phoenix dactylifera	Arecaceae	Tree	green		dark blue						A-D
Galinsoga parviflora	Asteraceae	Herbacious plant		yellow		blue					A-D
Sansevieria trifasciata	Asparagaceae	Perennial plant		yellow		blue					A-D
Megalochlamys violacea	Acanthaceae	Shrub		yellow		blue					AB CD
Asystasia gangetica	Acanthaceae	Perennial plant	green			blue					A-D
Acalypha indica	Euphobiaceae	Perennial plant	green			blue				AB CD	
Rudbeckia fulgida	Asteraceae	Perennial plant		yellow		blue					A-D
Ficus sur	Moraceae	Tree	green			blue					A-D
Ficus sycomorus	Moraceae	Tree	green			blue					AB CD
Adansonia digitata	Malvaceae	Tree		yellow		blue					A-D
Aloe barbadensis	Asphodelaceae	Annual plant	green			blue			AB BC CD		
Capsicum annum	Solanaceae	Perennial plant	green			blue					A-D

*: Initial stage (plant produce flower buds) Stage A; Peak stage (plant have opened flowers) Stage B; Late stage (plants retain their bloom after peak flowering) Stage C; terminal stage (flowers have passed blooming stage) Stage D.

Figure 4.3a: *Adenium arabicum* "desert rose" in full bloom in the lowland areas.

Figure 4.3b: "Unidentified plant" entering senescence in the lowland areas of Taita hills.

4.3.5 Discussion

In this vulnerable habitat, it was revealed that as many as 80 different plant species of 34 different families could still sequentially flower with overlapping blooming periods through the two seasons. Specific plant families including *Asteraceae, phyllanthaceae, Anacardiaceae, Lauraceae, Malvaceae, Fabaceae, Caricaceae, Verbenaceae, Apocynaceae, Moraceae, Meliaceae, Nyctaginaceae, Proteaceae, Cactaceae, Euphorbiaceae, Amaranthaceae, Musaceae, Rosaceae, Burseraceae, Arecaceae, Commelinaceae, Lamiaceae, Piperaceae, Rubiaceae, Balsaminaceae, Capparaceae, Sapindaceae, Santalaceae, Phytolaccaceae, Solanaceae and Zygophyllaceae* comprised of forest trees, shrubs, grasses and weeds. (Table 4.1). However, only a small proportion of plants of the *Verbenaceae*, *Apocynaceae*, *Proteaceae*, *Fabaceae*, *Euphorbiaceae*, *Asteraceae*, *Rosaceae* and *Commelineceae* families were found to bloom at the same time in both highlands and lowlands sites. This study revealed that indigent pollinators such as African meliponine bee species can constantly visit different kind of flowers from these families, thereby benefiting from a diverse mix of resources of both pollen and nectar produced by flowers from this wide array of plants (Table 4.1). Despite harsh environmental conditions experienced in the lowlands, habitats could still support a wide range of plant species, but only within unfragmented and undisturbed sample sites (Figure 4.2b), with higher feral bee nesting abundance. This is in agreement with Tscharntke *et al.*, (2005) who revealed that no clear ontogenetic sequence for floral resource availability is an indicator of ecological mutualisms (Tylianakis *et al.*, 2008), where flowering resources through seasons functions to satisfy foraging requirements of pollinators, especially native bee species. It was revealed that contemporaneous floral resource availability in unfragmented habitats and phenological resources could interact to explain the higher mean nest abundance in unfragmented habitats. (Figure 4.2b).

The researcher showed that blooming sequences overlapped sequentially in both habitats but plant composition differed over months as they represented different combinations of floral resources. Floral phenology in such habitats is largely determined by a combination of both climatic factors and level of anthropogenic disturbance (Nagamitsu and Inoue, 2005; Roubik 2006a; Roubik *et al*., 2005) which influence sequential flowering of

available plants at any point in time (Table 4.1). We speculate that the context of flowering phenology available at a sampling time could impact on how bees may exploit available food resources for optimum survival.

At the individual scale, management and land-use practices determine the community composition of both pollinators and plants, and the extent to which biotic factors affect both groups (Kremen *et al*., 2007). In relation to floral resources, it can be observed that flower abundance and species richness are positively associated (Wcislo and Cane, 1996; Steffan-Dewenter and Tscharnke, 2001; Potts *et al*., 2003; Holzschuh *et al*., 2007). Ultimately, increasing floral diversity provides a wider array of foraging niches for these bee species (Fenster *et al*., 2004).

The availability of nesting resources could also play a key role in structuring native bee communities (Cane, 1991; Eltz *et al*., 2002; Potts *et al*., 2005) as seen in the case of meliponine bees naturally occurring in taita hills. In parallel with floral resources, the temporal and spatial distribution of nesting resources may determine natural occurring bee community composition in any given location. Eltz *et al*. (2002) found that the abundance, size and species of trees in tropical forests of Southeast Asia influenced the density of stingless bee nests. Similarly, in a diverse Mediterranean bee assemblage, the amount of exposed soil, number of sloped surfaces and number of cavities available as nest sites accounted for a high percentage of the variation in community composition (Potts *et al*., 2005). Pollinators (meliponine bee species) which are more generalized in their requirements for mutualistic relationships with plants could be highly successful in such fragile habitats, such as Taita hills of Kenya, but could still be affected by drastic environmental changes that could limit the availability and quality of nectar or pollen resources, thereby invariably altering pollinator foraging behaviour. Future studies are needed to investigate possible mechanisms driving these patterns for dispersal and foraging efficiency in these bee species, particularly, how they exploit food resources depending on the context of resource needs. Combinations of protected area networks and bee-friendly habitats within agriculture will become increasingly important for bee conservation as the impacts of global environmental change work in synergy with other

contributing factors (Tylianakis *et al*., 2008). Currently, high quality habitats for bees may become unavailable as bee life cycles gradually shift with changing climatic conditions and/or as habitats become degraded.

4.3.6 Conclusion

It has been revealed that fragile habitats could modify microclimates and the availability of biotic resources, which may directly or indirectly change the patterns of plant reproduction and further altering floral resource availability for native pollinators (Holzschuh *et al*., 2008; Kennedy *et al*., 2013). In summary, in response to global environmental changes, adopting a wider landscape approach and linking up fragments of (semi-) natural landscapes possessing essential foraging and nesting features, such as hedgerows and field margins, will make it possible to increase landscape connectivity and allow bees to forage and disperse to more suitable areas (Gilbert *et al*., 1998; Tewksbury *et al*., 2002).

Combinations of protected area networks and bee-friendly habitats within agro-ecosystems will become increasingly important for bee conservation as the impacts of global environmental change work in synergy with other contributing factors (Tylianakis *et al*., 2008). Currently, high quality habitats for bees may become unavailable as bee life cycles gradually shift with changing climatic conditions and/or habitats become degraded. By taking a wider landscape approach and linking up fragments of (semi-) natural landscape with essential foraging and nesting features, such as hedgerows and field margins, it will be possible to increase landscape connectivity and allow bees to forage and disperse to more suitable areas under global change (Gilbert *et al*., 1998; Tewksbury *et al*., 2002).

CHAPTER FIVE

POTENTIAL CUES SIGNALING NEST MATE RECOGNITION BEHAVIOUR IN AFRICAN MELIPONINE BEE SPECIES (HYMENOPTERA: MELIPONINI).

5.1 Summary

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 (Martin, 2008; Potts *et al*., 2005; Stuart and Herbers, 2000). 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 (P=0.661), with the species *M. ferruginea (black)* (76.01%, N= 25) exhibiting the most aggression, followed by *H. ruspolii* (69.3%, N= 22), and *P.hildebrandti* (66.7%, N= 25), while the least aggressive was *H. gribodoi* (62.7%, N= 24). 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 (Wald's χ^2=128.3, df = 2, P<0.001). 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.

5.1.1 Introduction

Social insects, especially honey bees 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 (Evans *et al*., 2007; Goulson *et al*., 2000). The use of certain mechanisms to transfer information between individuals to initiate certain behaviors has long been confirmed in honeybees (Biesmeijer and Slaa, 2004; Nieh, 2004; Schorkopf *et al*., 2007; Slaa and Hughes, 2009). 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 they come 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 (Leonhardt *et al*., 2009; Nunes *et al*., 2008; Yusuf *et al*., 2010; Zweden and d'Ettorre, 2010a). The cuticle of most insects is coated with a lipid layer, with hydrocarbons forming a dominant group of chemical components of this layer(Leonhardt *et al*., 2009; Gastauer *et al*., 2013).

Cuticular hydrocarbons (CHs) have been categorized to typically range from C8- C40 (Akino, 2005) with 3 major structural classes: n-alkanes, n-alkenes and mono-, di-, tri-methyl- branched alkanes (Zweden and d'Ettorre, 2010a) with additional components found in trace amounts such as fatty acids, glycerides, sterols, ketones, long chain alcohols and aldehydes (Katzerke *et al*., 2006; Yusuf *et al*., 2010; Zweden and d'Ettorre, 2010a). 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 (Gibbs and Stanton, 2001; Foley *et al*., 2005). These hydrocarbons can be exchanged between individuals by means of trophallaxis, self and allo-grooming (Huang and Wang, 2008). 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 (Nunes *et al*., 2009; Zweden and d'Ettorre, 2010a). They also function as an attractant or repellant during courtship (Torto *et al*., 2007; Barth *et al*., 2008; Chen *et al*., 2009) 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 (Jungnickel and Costa, 2004; Schorkopf and Hrncir, 2009; Simone-Finstrom and Spivak, 2010).

The chemical identity of recognition cues in the honey bee *Apis mellifera* has been intensively studied (Aguilar *et al*., 2005; Akino and Yamamura, 2004; Akino *et al*., 2004; Goulson *et al*., 2000; Nunes *et al*., 2008; Zweden and d'Ettorre, 2010b) 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 (Bowden *et al*.,1998; Breed and Bennett, 1987; Breed *et al*., 2004; Breed *et al*.,1988). 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" (Bowden *et al*.,1998; Breed *et al*., 2004). 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 (Burger *et al*., 2010; Nunes *et al*., 2008; Zweden and d'Ettorre, 2010a) 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 (Potts *et al*., 2005; Roubik, 2006b). 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) (Simone-Finstrom and Spivak, 2010) 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* (Human and Nicolson, 2006; Leonhardt and Kaltenpoth, 2014) and environmentally derived odors playing an important role in nest mate recognition in some ants (Hölldobler and Wilson, 1990; Jackson and Morgan, 1993; Stuart and Herbers, 2000), some species of social wasps largely depend on methyl-branched alkanes (Akino *et al*., 2004; Goulson *et al*., 2000), however, floral odors seem to be relatively unimportant in honey bee nest mate recognition (Nieh *et al*., 2004; Katzerke *et al*., 2006; Huang and Wang, 2008; Reichle *et al*., 2010). 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 (Bowden *et al*., 1998; Stuart and Herbers, 2000; Dos Santos and Antonini, 2008). Bowden *et al*., (1998) 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* (Suka and Inoue, 1993), and *Hypotrigona gribodoi* (Kirchner and Friebe, 1999) 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.

5.2 Materials and Methods

5.2.1 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 ruspolii 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.

5.2.2 Extraction of CHCs for Bioassays

Cuticular hydrocarbons from five nurse bees of each species were sourced from colonies and extracted (Guerrieri and d'Ettorre, 2008) 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°C 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) (Guerrieri and D'Ettorre, 2008) and nest entrance defense bioassays (Shackleton *et al*., 2014).

5.2.2.1 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 (Guerrieri and D'Ettore, 2008) 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 5.1). 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 ruspolii* 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 5.2a) while non-aggressive behavior was scored as (0) when the test bee repeatedly shook its antennae (Fig 5.2b). 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.
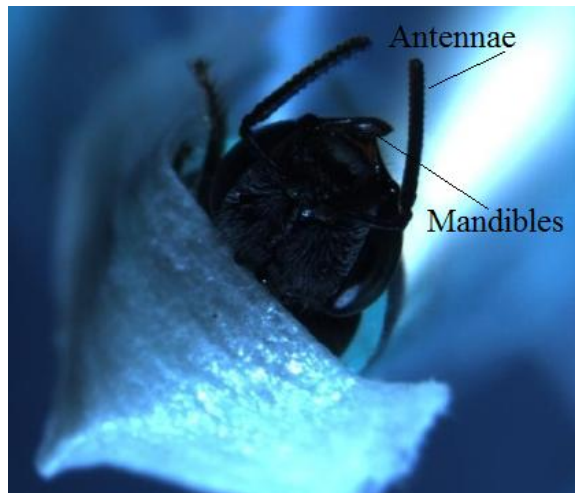
Figure 5.1: Harnessing set-up showing an individual bee, *Hypotrigona ruspolii* harnessed and conditioned prior to the mandible opening response bioassay (MOR).

5.2.2.2 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 ruspolii* 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.
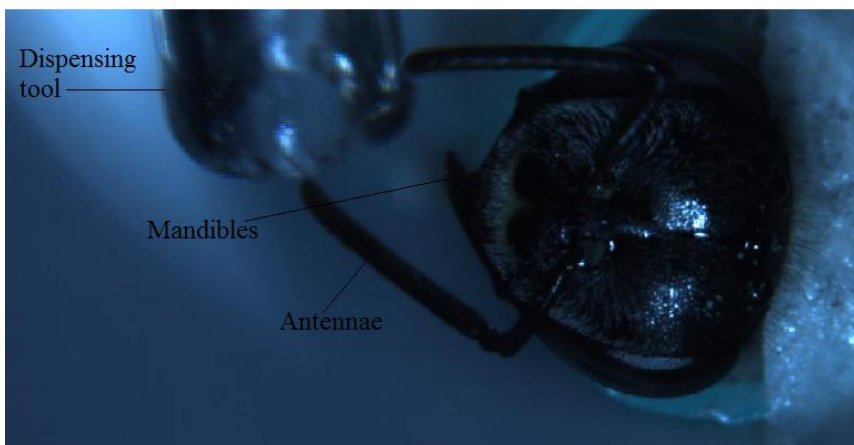
Figure 5.2a: A harnessed bee showing aggressive response (continuous opening of mandibles) when presented with a hetero-specific non-nest mate extract from another bee species.

Figure 5.2b: A harnessed bee exhibiting non- aggressive response (continous antennation) when presented with a con-specific nest mate extract from another colony.

5.2.2.3 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 ruspolii* 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.

5.2.2.4 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 Guerrieri and D'Ettore (2008), 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°C 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 (Guerrieri and D'Ettore, 2008).

5.2.2.5 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 (El-Sayed, 2009). For compound quantification, peak areas were compared to an external standard corresponding to 5ng/μl of Eicosane (C20).

5.2.2.6 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,6E*) 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).

5.2.2.7 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 Breed *et al.,* (2004).

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.

5.2.3 **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).

5.2.4 Results

5.2.4.1 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 5.3). 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 ruspolii* (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).
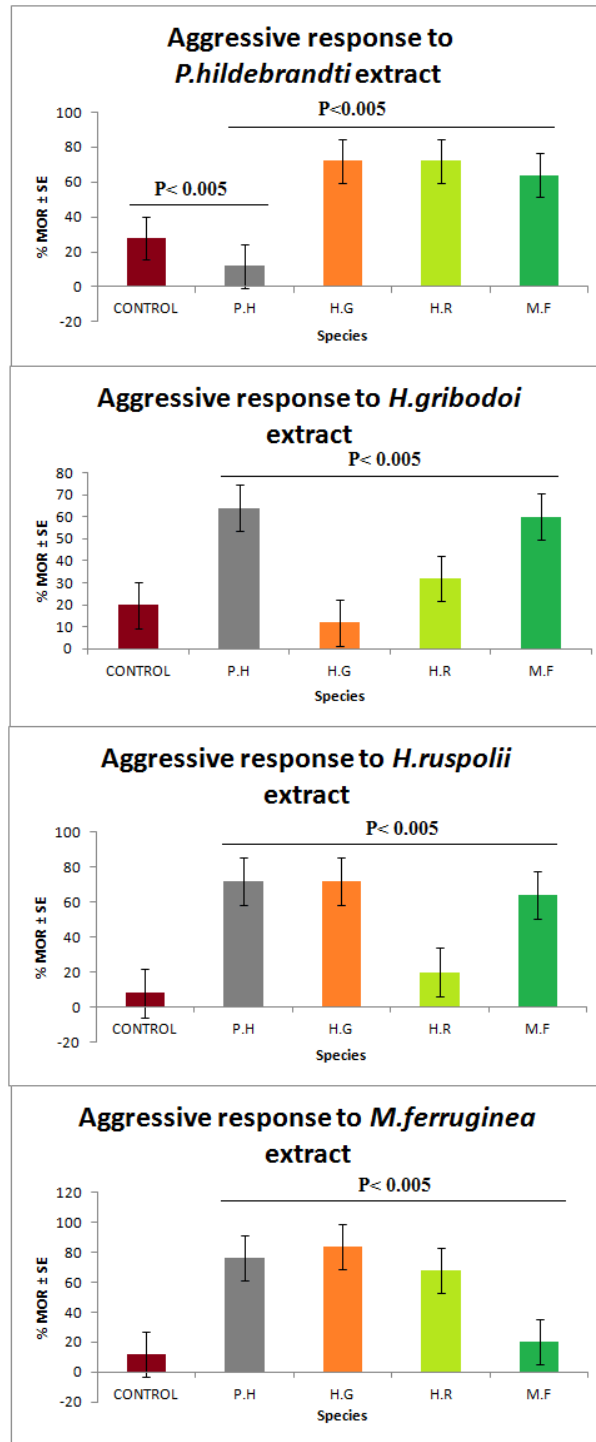
Figure 5.3a: Aggressive responses exhibited by four meliponine bee species during the mandible opening response bioassay when presented with both con/hetero-specific stimuli (cuticular hydrocarbons).

5.2.4.2 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 ruspolii* (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).
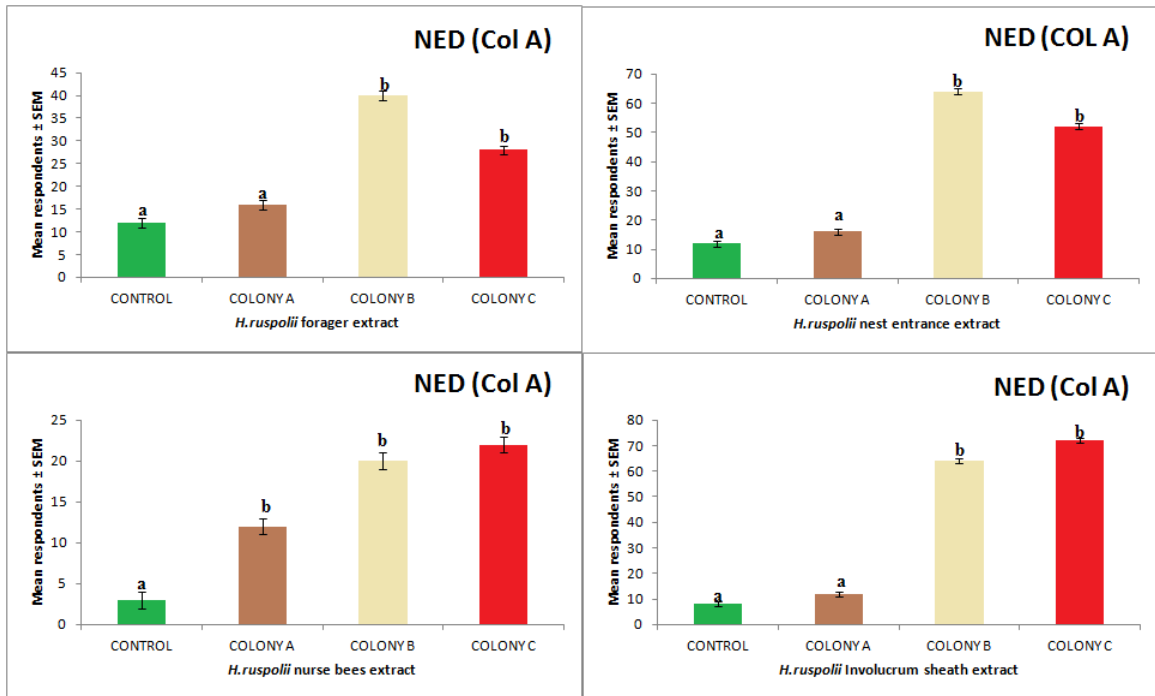
Figure 5.3b: Aggressive responses exhibited by the meliponine bee species, *Hypotrigona ruspolii* during the nest entrance defense bioassay when presented with respective con-specific stimulus (between nests).

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 5.4 a-f) 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 5.4g). 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.
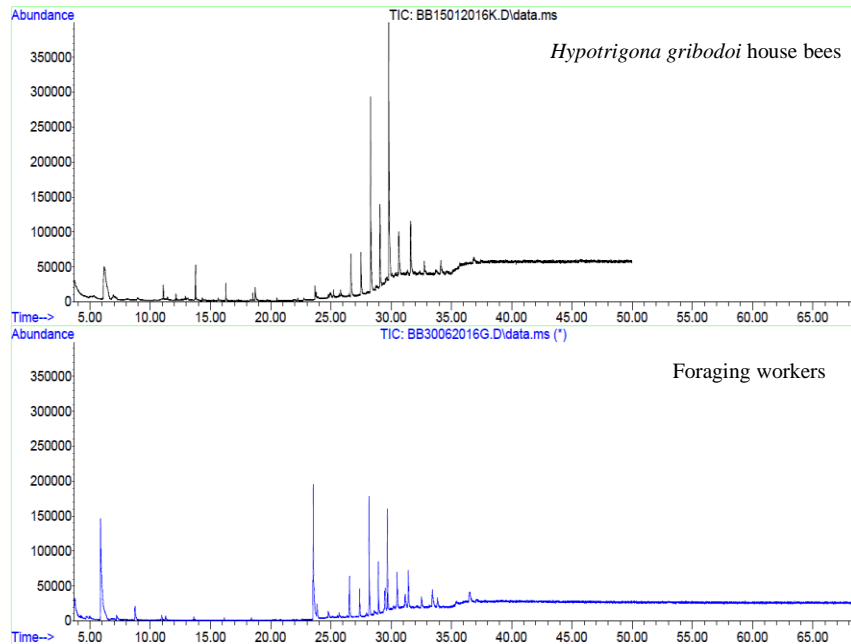
Figure 5.4a: Cuticular hydrocarbon profile of *H. gribodoi* house bees and foragers.
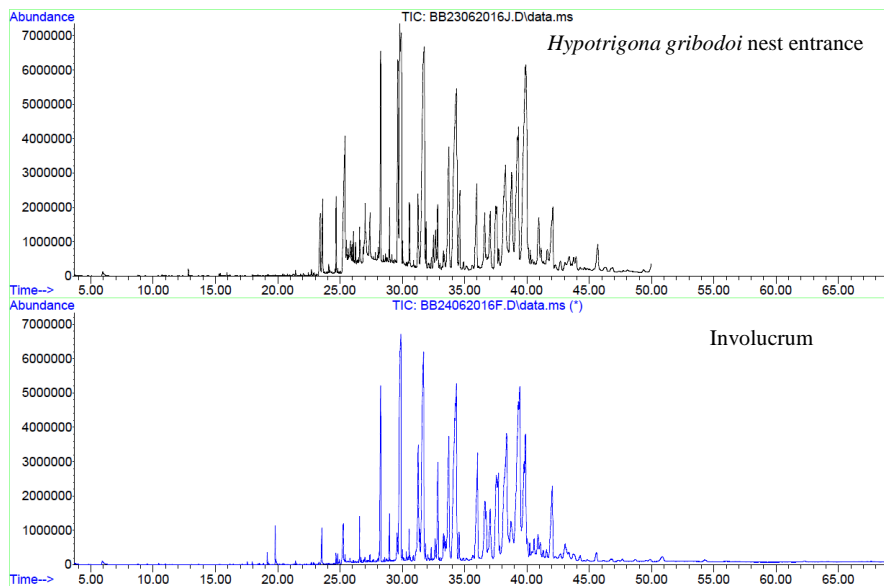
Figure 5.4b: Cuticular hydrocarbon profile of *H. gribodoi* nest entrance and involucrum.

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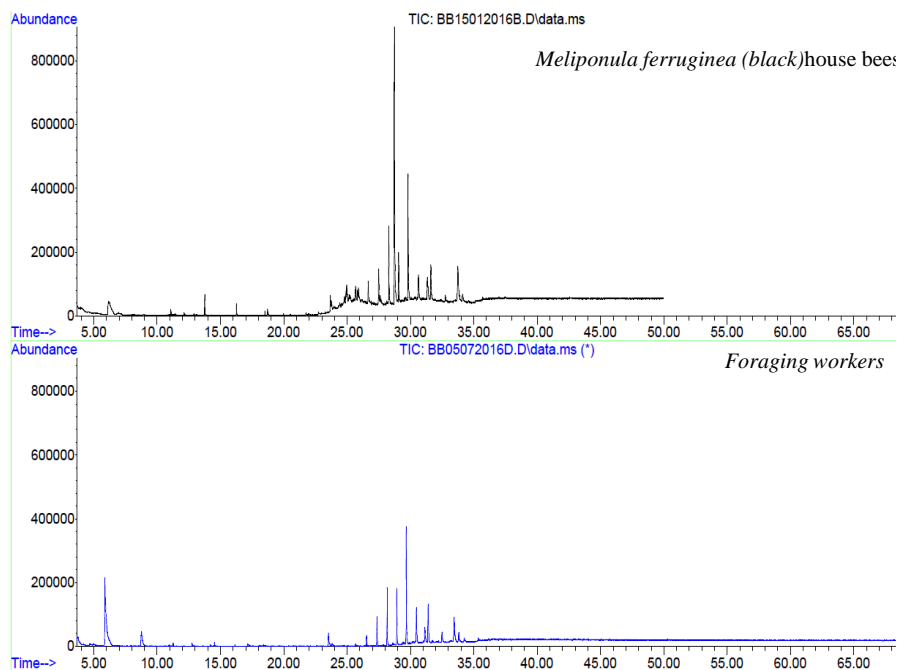
Figure 5.4c: Cuticular hydrocarbon profile of *M. ferruginea* (black) house bees and foragers.
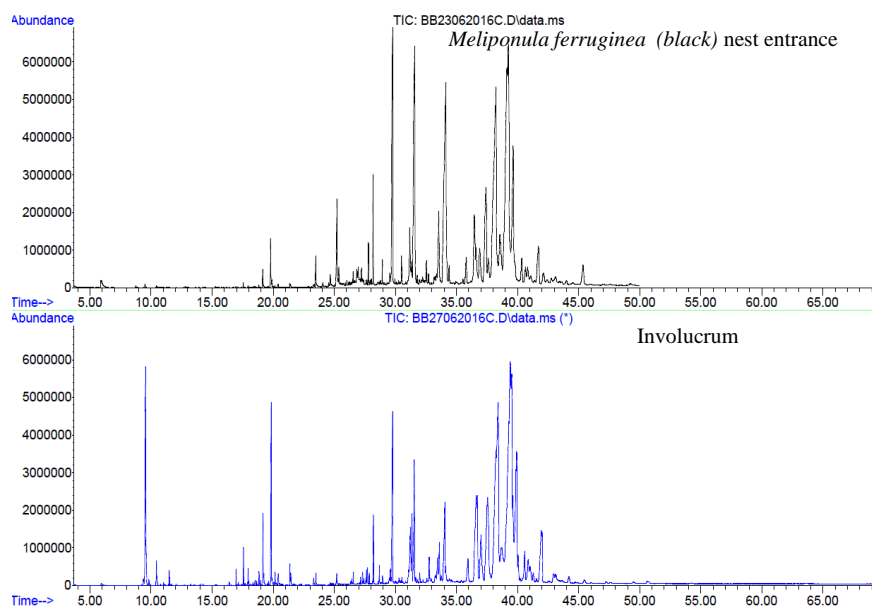
Figure 5.4d: Cuticular hydrocarbon profile of *M. ferruginea* (black) nest entrance and involucrum.
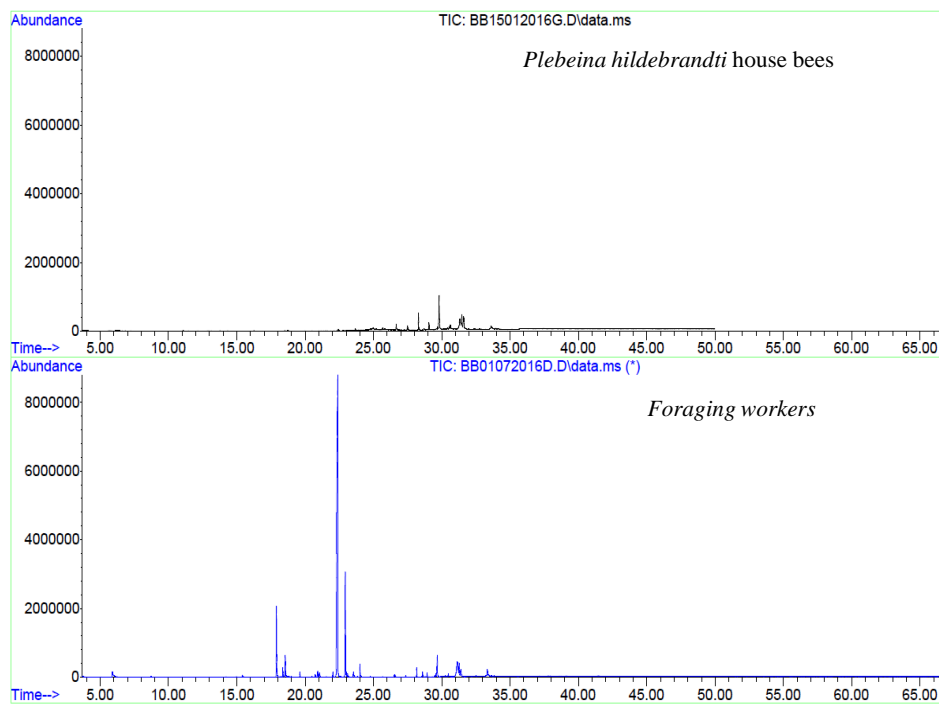
Figure 5.4e: Cuticular hydrocarbon profile of *Plebeina hildebrandti h*ouse bees and foragers.
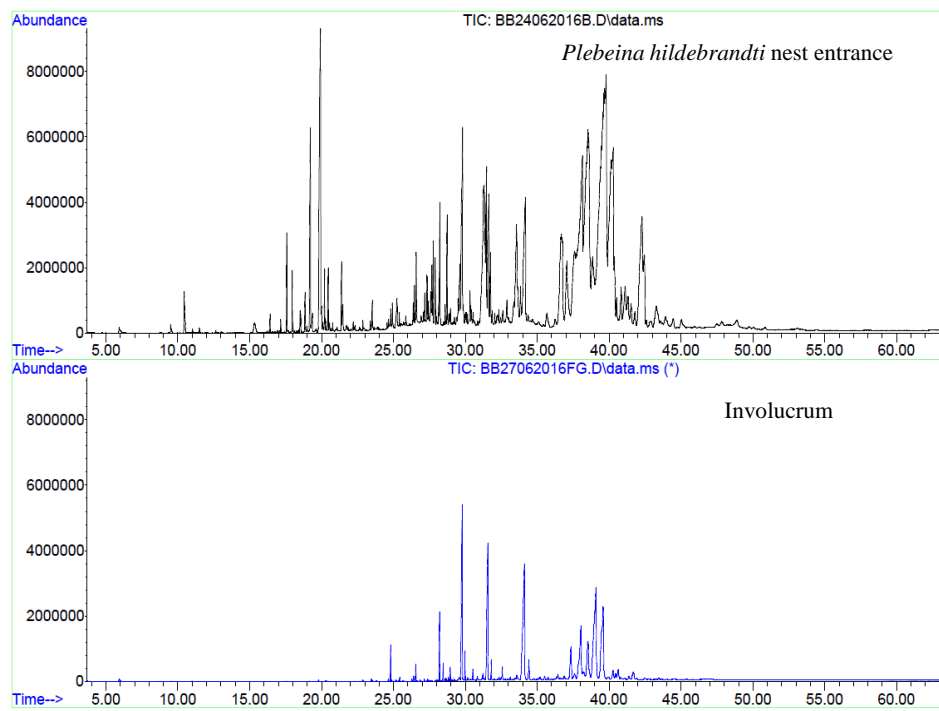
Figure 5.4f: Cuticular hydrocarbon profile of *Plebeina hildebrandti* nest entrance and involucrum.
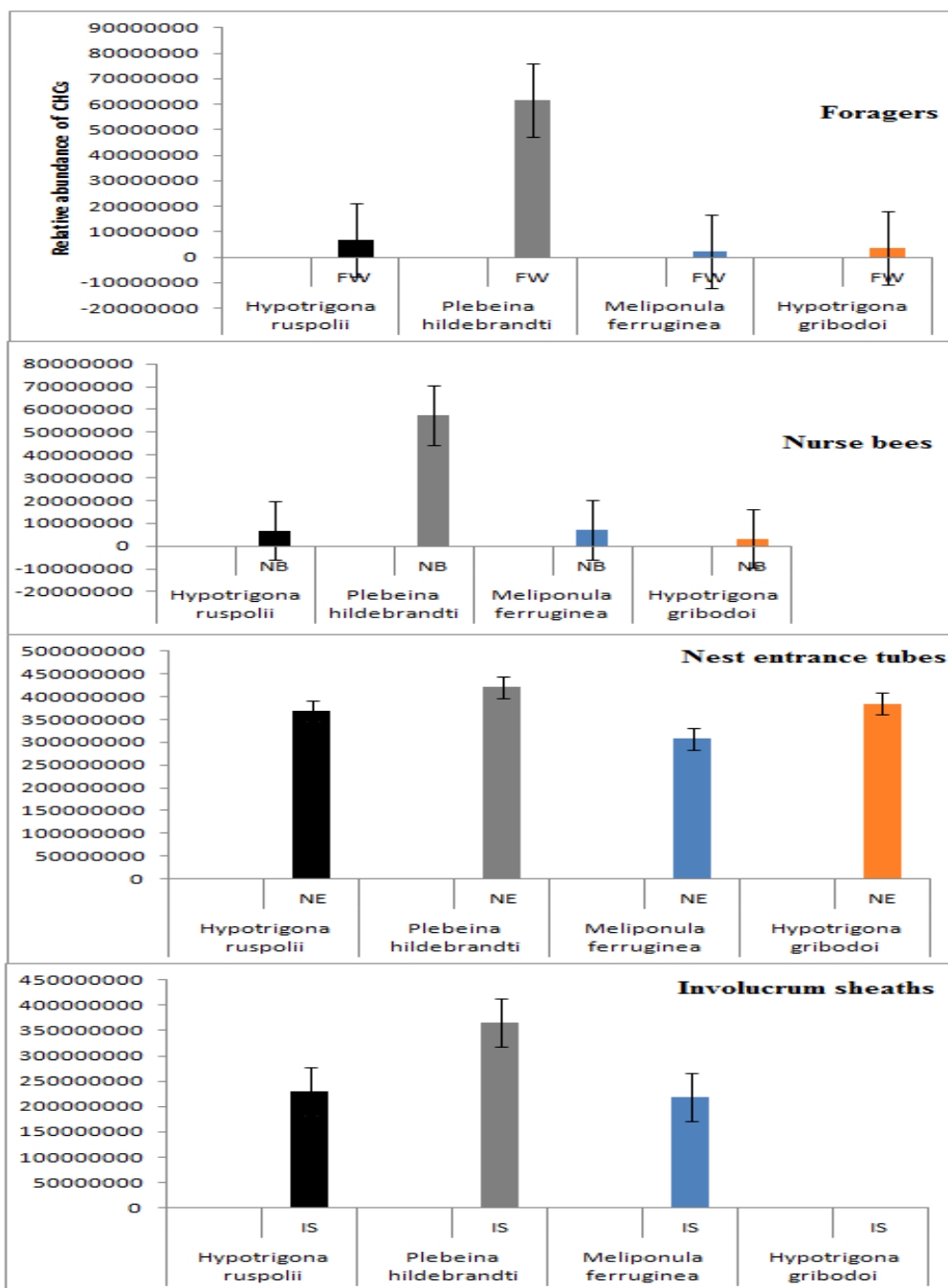
Figure 5.4g: Relative abundance of cuticular hydrocarbons (alkenes and methyl-branched alkanes) from the different stimulus (foragers, nurse bees, nest entrance tubes and involucrum sheaths) of the four meliponine bee species.

5.2.4.4 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 5.5 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).
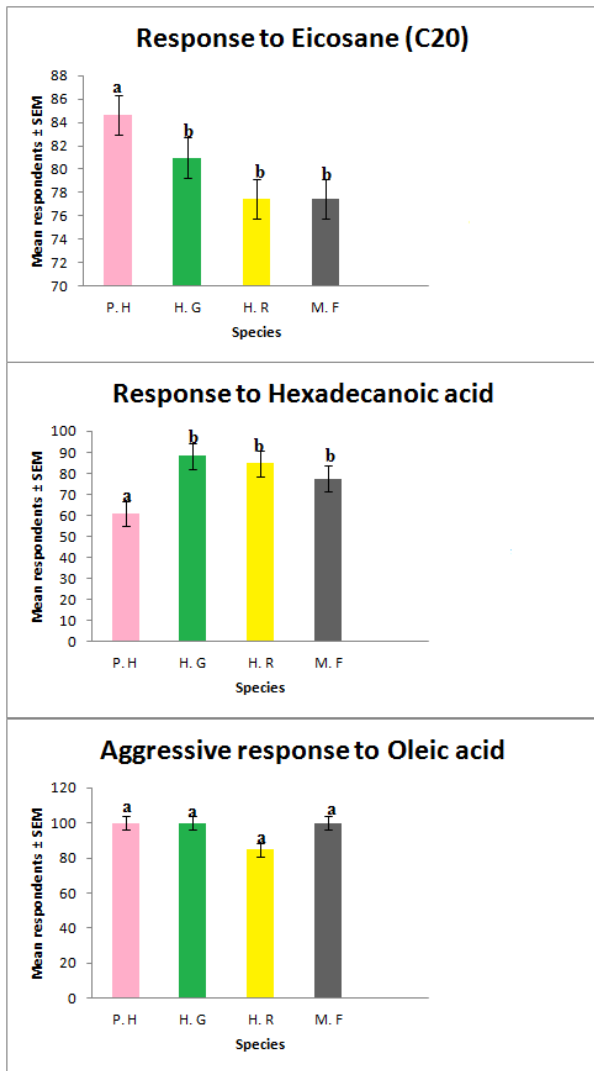
Figure 5.5: Aggressive responses exhibited by four meliponine bee species during the (MOR) mandible opening response bioassay when presented with selected synthetic compound stimuli found to dominate their cuticular profiles. *M.F: *Meliponula ferruginea*, H.R: *Hypotrigona ruspolii*, H.G: *Hypotrigona gribodoi*, P.H: *Plebeina hildebrandti*.

5.2.5 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 ruspolii* all positively responded to a group of compounds similar to responses elicited from *Apis mellifera* (Breed, 1998b). 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* (Breed *et al*., 1995; Breed, 1998b) 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 (Breed *et al*., 2004a, b). 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 ruspolii* 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 (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 (Johnson *et al*., 1985; Nieh *et al*., 2004; Slaa *et al*., 1998). Koedam *et al*., (2002) 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 ruspolii* (11%), *Plebeina hildebrandti* (18%). Koedam *et al*., (2002) 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 (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 (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 ruspolii* 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 (Blomquist *et al*., 1985; Milborrow *et al*., 1987; Koedam *et al*., 2002), 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 (Patricio et al. 2002).

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 (Downs *et al*., 2000) 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 (Eltz *et al*., 2003) 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.

5.2.6 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.

CHAPTER SIX
HOST POTENTIAL OF AFRICAN MELIPONINE BEES (Hymenoptera: Apidae) FOR THE SMALL HIVE BEETLE PEST, *Aethina tumida* Murray (Coleoptera: Nititulidae).

6.1 Summary

Recent studies have shown that honey, bumble and some meliponine bee species of *Trigona*, *Meliponula* and *Dactylurina* genera are hosts of the small hive beetle (SHB) *Aethina tumida*, a known pest of honeybee colonies in various regions of the world with olfactory cues implicated in beetle infestations of honey and bumble bees. However, the mechanism by which the beetle locates meliponine bee colonies is not known. In this study, dual-choice olfactometer bioassays were used to investigate the preferences of adult male and female SHBs to intact colony odors of *Apis mellifera* and colony matrices (pot honey, pot pollen, cerumen and propolis) from the three meliponine bee species, *M. ferruginea* (black), *M. ruspolii* and *M. bocandei*. SHBs exhibited a significant preference of odors from *Apis mellifera* over the three tested meliponine bee species. Also, both beetle sexes showed substantially stronger preferences for pot honey, pot pollen and cerumen odors. Results suggest that honey bees are the native hosts of SHB from where they have spread to other similar hosts by exploiting the chemical similarity in their colony odor bouquets and are thus likely to pose a threat to commercial meliponiculture operations worldwide. These results may have implications in the context of domesticating African meliponine bees as alternative pollinators for agricultural crops.

6.1.1 Introduction

African meliponine bees (Hymenoptera: Apidae) belong to the tribe *Meliponini* of which more than 19 species are native to Africa (Eardley, 2004; Robert Kajobe, 2008; Karikari and Kwapong, 2007; Kwapong *et al*., 2010; Ogol *et al*., 2013) with 14 of these species found in Kenya (Nkoba, 2012). These bees are closely related to honey bees, carpenter bees, orchid bees and bumble bees (Imperatriz-fonseca *et al*., 2006) with over 300 species occurring worldwide (Heard, 2001; Moreno and Cardozo, 2003; Reyes-González *et al*., 2014).

Typically, a meliponine bee colony contains over 20,000 individuals, comprising of a single fertile queen, drones and workers. Meliponine bees are considered important pollinators of most indigenous flora in both tropical and subtropical parts of the world. (Heard, 1999). They also pollinate over 90 crop species worldwide (Slaa *et al.,* 2006; Abramson *et al*., 2007). The small hive beetle is a native parasite to sub-Saharan African honeybees on which it inflicts negligible damage. In the past two decades, it has become an invasive pest of European honeybees in the Americas, Australia, Asia (Ellis *et al*., 2002; Ellis and Hepburn, 2006; Neumann and Elzen, 2004; Spiewok and Neumann, 2006; Graham *et al*., 2011; Pirk and Neumann, 2013; Mustafa *et al*., 2014) and most recently Europe (Palmeri *et al*., 2015). Aside from honeybees, it has also been found in the nests of other Apidae members such as bumble bees, *Bombus impatiens* (Mustafa *et al*., 2014; Spiewok and Neumann, 2006; Stanghellini *et al*., 2000) and some neo-tropical meliponine bee species of *Trigona* and *Dactylurina* genera in various parts of the world (Greco *et al*., 2010; Halcroft *et al*., 2011; Neumann *et al.,* 2016; Nkoba, 2012).

Adults of the small hive beetle typically live up to six months (Torto *et al*., 2005; Mustafa *et al*., 2014; Neumann *et al*., 2016) and typically are found at the rear portion of the bottom board of a hive, where the females locate cracks and crevices to lay massive numbers of eggs after mating (Hoffmann, Pettis, and Neumann 2008). Upon hatching, the larvae feed on pollen and honey, putrefying hive products as they simultaneously defecate leading to honey contamination and sometimes colony collapse in severe cases.

In honeybees, colony location by the beetle has been demonstrated to be mainly mediated by air-borne volatiles released by a host which serve as kairomones (Arbogast *et al*., 2007, 2010; Suazo *et al*., 2003; Torto *et al*., 2005, 2007). The SHB is known to show differential preference for air-borne volatiles from different hive components of both honeybees and bumble bees (Suazo *et al*., 2003; Spiewok and Neumann, 2006; Graham *et al*., 2011) suggesting that odors from specific colony matrices play key roles in host colony choice prior to infestation. This dependency on olfaction is further supported by a large portion of neurons in the beetle's brain being tuned to olfactory detection (Kollmann *et al*., 2016). Besides olfaction, additional stimuli such as light (visual cues)

have been reported to orient both adults and larvae (wandering stage) towards host colonies (Arbogast *et al*., 2009) and pupation sites respectively (Duehl *et al*., 2012). Similarly, colony odors of bumble bees were demonstrated to serve as a kairomone (Graham *et al*., 2011; Spiewok and Neumann, 2006) and shared similar chemical components with the odor bouquet of honey bees (Graham *et al*., 2011), suggesting that colony odor plasticity may facilitate host expansion of the beetle among eusocial bees in the Apidae family.

With increasing global losses of honeybee colonies worldwide (IPBES, 2016), the need to understand host-pest interactions is crucial as meliponine bees are increasingly being promoted as alternative pollinators because commercial operations of meliponiculture could potentially serve as reservoir for cross beetle infestation (Slaa *et al*., 2003; 2006), and (2) Cross-over of honey bee pathogens to meliponine bees and vice-versa via the beetle as a vector (Genersch 2010; Vit, Roubik, and Pedro 2012). Since the SHB is known to infest honey, bumble and meliponine bee colonies, with the mechanism of host colony location in the former two bee species known (Graham *et al*., 2011; Spiewok and Neumann, 2006; Stanghellini *et al*., 2000), it is important to establish whether infestation follows the same pattern in meliponine bees. Thus, investigating the mechanism through which the SHB is attracted to and locates meliponine bee colonies as potential host is vital towards understanding its ecology and developing semiochemical-based tools for its management in meliponiculture. It was therefore hypothesized that the SHB locates meliponine bee colonies using air-borne colony volatiles through olfaction and are able to discriminate odors from various colony matrices.
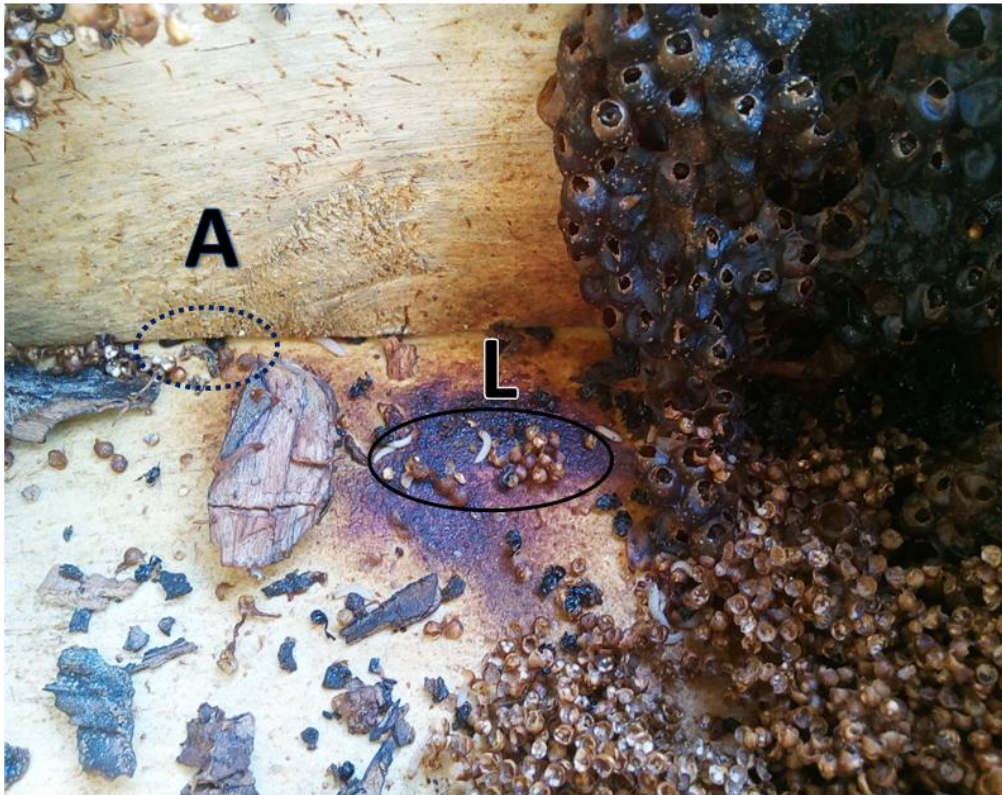
Figure 6.1. Small hive beetle (SHB) larvae (L) and adults (A) in a domesticated colony of *Meliponula ferruginea* (black). *Punctured honey and pollen pots are indication of SHB larval feeding**.**

6.2 Materials and Methods

6.2.1 Experimental colonies

Between July and December 2014, a total of 14 colonies of *Meliponula bocandei*, *Meliponula ferruginea* (black) and *M. ferruginea* (reddish brown) were transferred from a meliponary in Kakamega (Nkoba *et al.,* 2012), western Kenya (0º 30′N 34º 35′E) to an already existing meliponary comprising of 10 colonies at the International Centre of Insect Physiology and Ecology (*icipe*), Duduville campus (1º 17′S, 36º 49′E) in Nairobi and left to forage freely. All 24 meliponine colonies and three honeybee colonies served as treatment sources for the experiments (intact colony, pot honey, pollen, propolis and cerumen odors) used in all experiments.

Sexually mature (14 days old) *Aethina tumida* adult populations used in this study, were mass reared in the laboratory as described by Suazo *et al*., (2003) and Torto *et al.,* (2010). Prior to each behavioral assay, beetles were separated based on sex by following laboratory procedures (Ellis and Hepburn, 2006), thereafter the beetles were starved of food and water for 24 h and only individuals showing no signs of physical injury such as broken legs and antennae were used in the assays.

6.2.1.1 Odor sources

Odor sources were collected from three meliponine bee species previously reported to harbor SHBs infestation, namely *Meliponula ferruginea* (black), *Meliponula ferruginea* (reddish-brown) and *Meliponula bocandei* intact colonies and their separate hive components; pot honey, pot pollen, cerumen and propolis which differed in both quality and quantity, due to varied colony sizes. All colonies were queen-right colonies and each contained workers, brood, pot honey, pot pollen, propolis, and involucrum sheaths (cerumen). A representation of an intact colony from honeybees and meliponine bee were collected (50 bees), 10g of brood, pot honey, pot pollen, propolis and involucrum sheaths. Pot honey, pot pollen, propolis and cerumen (10 g) were collected separately from each of the meliponine bee colonies. Pot pollen was collected from each colony of the three species by gently opening pollen pots and extracting the stored pollen with an autoclaved spatula. All separate portions were weighed on a Whitman filter paper and then transferred directly into the bioassay platform. Pot honey was collected from completely sealed but ripened pots by scraping honey out of the pots using an autoclaved spoon. The honey then was transferred into a perspex Petri dish and weighed. Propolis was collected by scrapping internal linings of the hive which had been sealed with propolis using an autoclaved hive tool. The involucrum sheaths (cerumen) was peeled off the food pots and brood cells and weighed on a Whitman filter paper and transferred onto the bioassay platform prior to the bioassay.

6.2.1.2 Dual choice olfactometer assays

The behavioral responses of sexually mature male and female SHBs (14 days old) to meliponine bee odors were studied using a dual choice olfactometer mounted on to a Perspex platform (19.5cm length and 9.5 cm width). This single odor source bioassay was conducted to investigate SHBs attraction to meliponine bee colony component volatiles in the absence of other air-borne volatiles. The olfactometer consisted of a large Perspex Petri dish (9 cm in diameter) glued between two small Perspex Petri dishes (6 cm in diameter). The Petri-dishes had holes (1cm in diameter) drilled at the point of connection and the opposite ends of the smaller dishes which were connected to Teflon tubing to serve as entry/exit points for the SHBs. A 1-cm wide hole drilled into the centre of the lid of the large dish connected the olfactometer to a vacuum pump (Fig. 6.2). The vacuum pump (parts assembled at the USDA/ARS, Gainesville, FL, USA) pushed and pulled charcoal-purified air through the olfactometer at 0.5l/min into two quick fit glass chambers (22.5cm length and 7.5 cm width). One chamber held the test odor (~10g of each hive component), while the second chamber into which purified air only was passed to serve as the blank (control). Pair-wise choice bioassays were similarly carried out to confirm preferences to either honey bee or meliponine bee meliponine intact colony odors under the same conditions. Experiments were conducted in a bioassay room maintained at 26 $^{\circ}$C and 70 % relative humidity. A red 25 W bulb placed 50 cm above the olfactometer area evenly illuminated the experimental arena. Each starved beetle was used only once in the assays. The behavioral responses of both sexes of the SHBs to matrix component odors from each bee species were studied between 16:00 - 20:00 h to coincide with optimal activity of the beetles (Suazo *et al.,* 2003), as both sexes are known to differ in flight activity (Ellis *et al.*, 2003). Individuals of both sexes of the beetle (N=25) were introduced into the olfactometer and the time spent to make a hit (choice) to stay in either arm of the olfactometer during a 10 min period was recorded. To minimize positional bias, either of the treatments and blank olfactometer chambers was interchanged after five replicates.
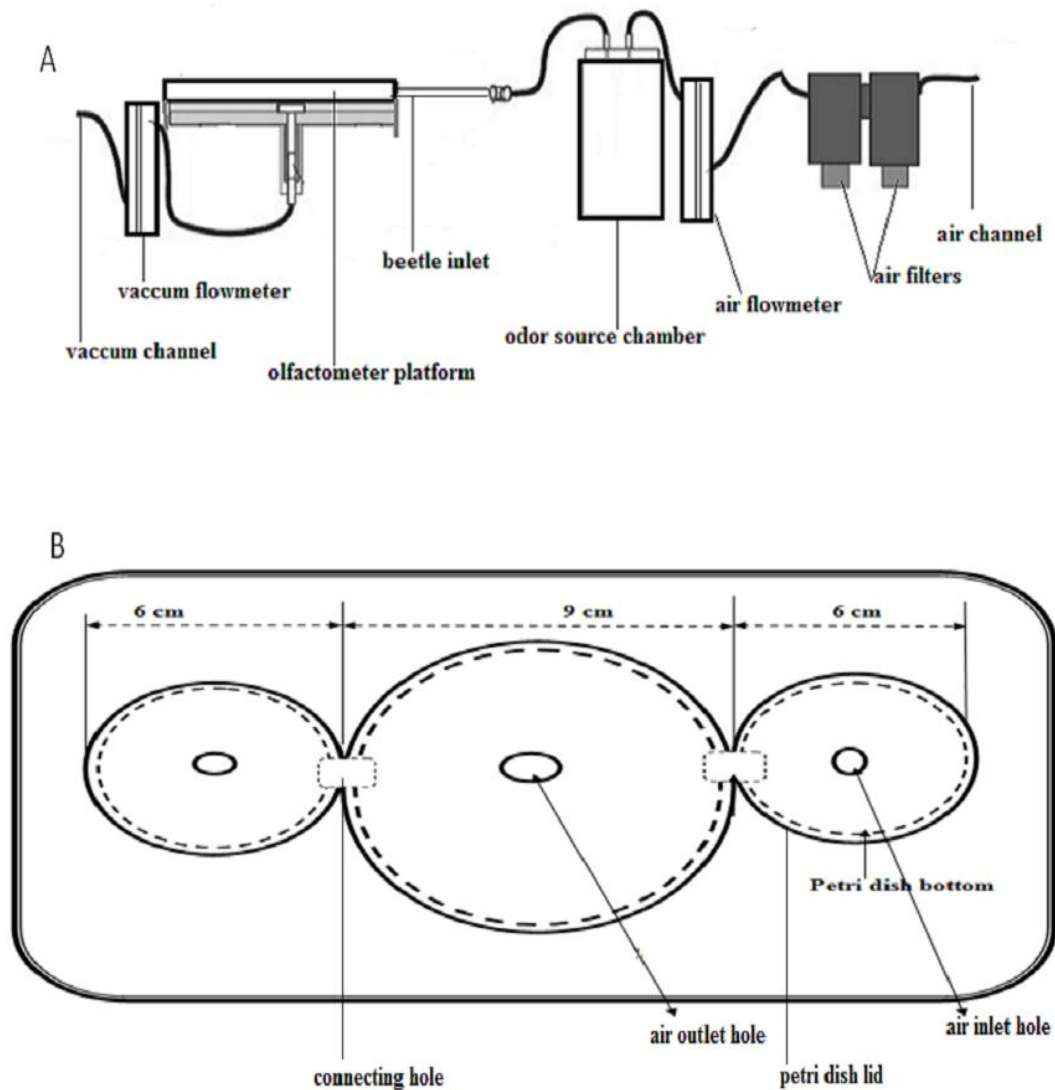
Figure 6.2: Olfactometer bioassay platform. **A:** Dorsal view of the bioassay platform with structural dimensions; **B:** Ventral view of the bioassay platform

6.2.1.3 Statistical Analyses

The preference indexes of male and female beetles to either honeybee (positive control) or meliponine bee (main effect) odors were calculated using the formula:

$$PI = (TT - TC)/(TT + TC)$$

Where *PI* = preference index, *TT* = time spent in treatment odor zone, *TC* = time spent in control odor zone. However, non-respondent beetles (individuals which did not move from the release zone throughout the 10 min observation period) were not included in the statistical analysis. The calculated *PI*s for each responding beetle were used to separately compare male and female beetle preference to the same treatment odor from the different meliponine bee species compared to honeybee odors (positive control). Further analyses were carried out using a one way ANOVA with means separation by the student Newman- Keuls (SNK) test for each beetle sex and each odor type using Sigma Plot statistical software v 10.0 (San Jose CA, 2011).

6.3 Results

6.3.1 SHBs response to intact colony and separate component odors of *Meliponula ferruginea* (black).

Pair-wise comparisons demonstrated higher preference index by both male and female SHBs for honey bee intact colony odors over odors from an intact colony of *Meliponula ferruginea* (black) (Fig.6. 3). In response to *M. ferruginea* (black) odors, females showed a significant difference in the mean time spent in intact colony odors zones (t_{46}= 86.28, P< 0.001); and those of pot honey (t_{46}= 47.98, P<0.001); pot pollen (t_{38}= 71.77, P<0.001); cerumen odors (t_{48}= 25.27, P<0.001) with the exception of propolis (t_{36}= 0.05, P=4.129) (Fig 6.4A-B). Males displayed similar attraction patterns to the same odor sets with a significant difference in the mean time spent in the treatment over control odor zones (clean air) for intact colony odors (*M. ferruginea* (black) t_{42}= 113.16, P< 0.001), pot honey odors (t_{44}= 21.24, P<0.001); pot pollen odors (t_{46}= 25.93, P<0.001) and cerumen odors (t_{40}= 4.73, P= 0.036). A non-significant preference was observed for propolis odors (t_{38}= 4.056, P=0.05).
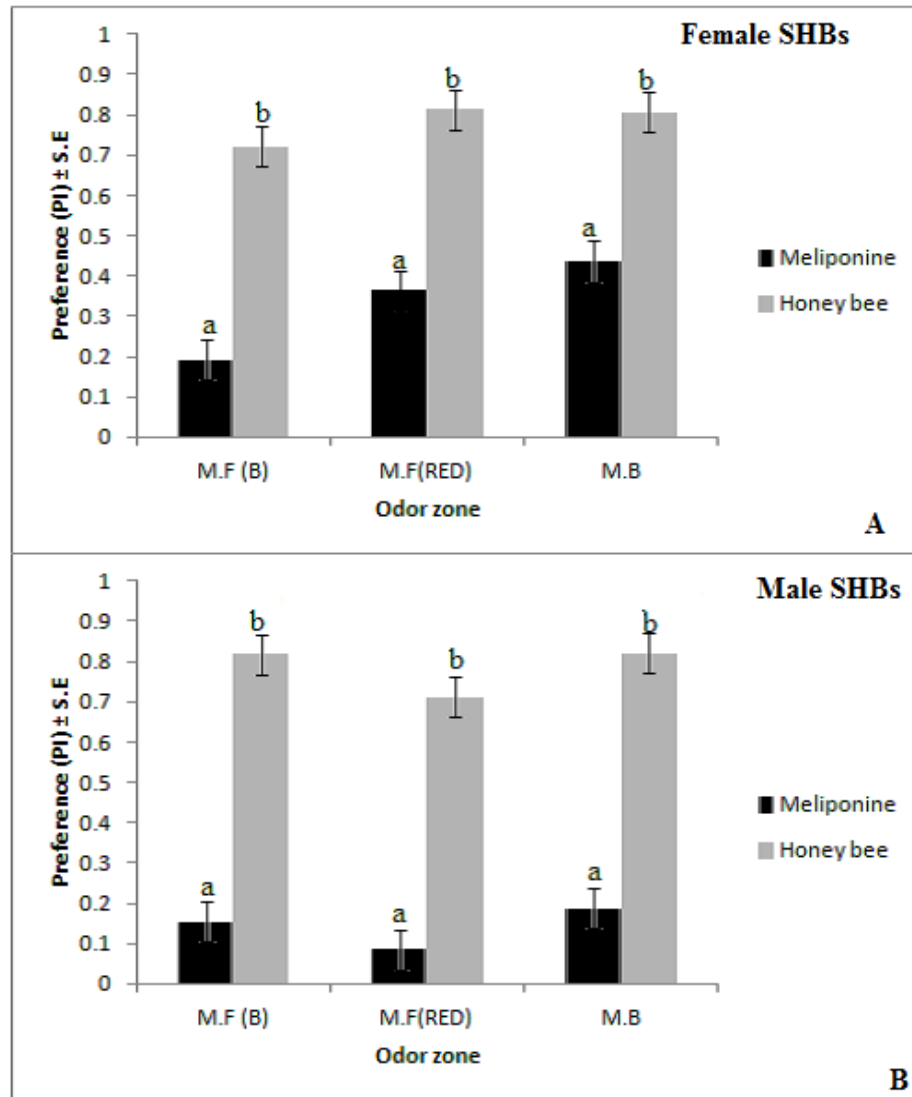
Figure 6.3. Behavioral preferences of male and female Aethina tumida to intact hives (expressed as preference indices) of honey bees and three Afro tropical meliponine species A: Females B: Males. *M.F: *Meliponula ferruginea* (black), *Meliponula ferruginea* (red), *Melipona bocandei.*

6.3.2 SHBs response to intact colony and separate component odors of *Meliponula ferruginea* (reddish brown).

Both male and female SHBs showed significant preference for intact honeybee colony odors over those from intact colonies of *Meliponula ferruginea* (reddish brown) (Fig.6. 4). Similarly, female SHBs showed significant differences in the mean time spent in the treatment odors over control odor zones for *M. ferruginea* (reddish brown), intact colony

odors (t_{42}= 264.35, P < 0.001), pot honey odors (t_{40}= 13.79, P<0.001), pot pollen odors (t_{38}= 71.78, P<0.001), cerumen odors (t_{42}= 28.77, P<0.001). A non-significant attraction to propolis odors was observed (t_{42}= -0.026, P= 0.927) (Fig 6.4C-D). Like-wise, males displayed a similar response pattern for *M. ferruginea* (reddish brown) intact colony odors (t_{48}= 36.11, P < 0.001), pot honey odors (t_{46}= 14.28, P<0.001), pot pollen odors (t_{46}= 21.03, P<0.001) and cerumen odors (t_{44}= 26.48, P<0.001). A non-significant preference was also observed for propolis odors (t_{42}= 0.042, P= 0.837).

Female shbs response to *M.ferruginea* (black)

M.ferruginea (red)

M.bocandei
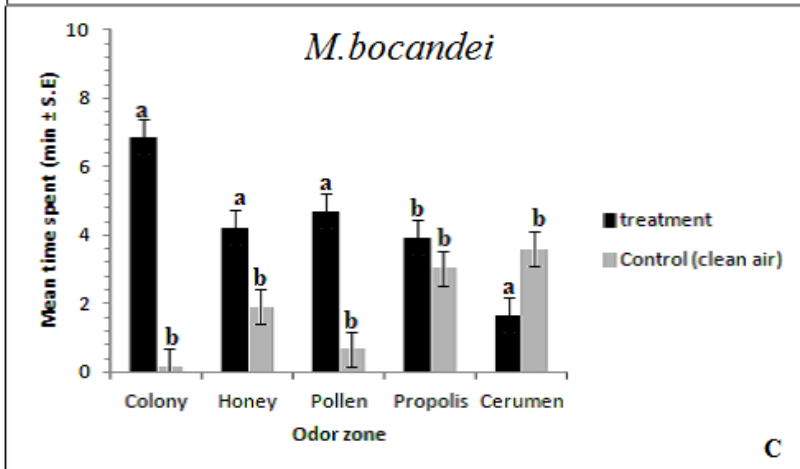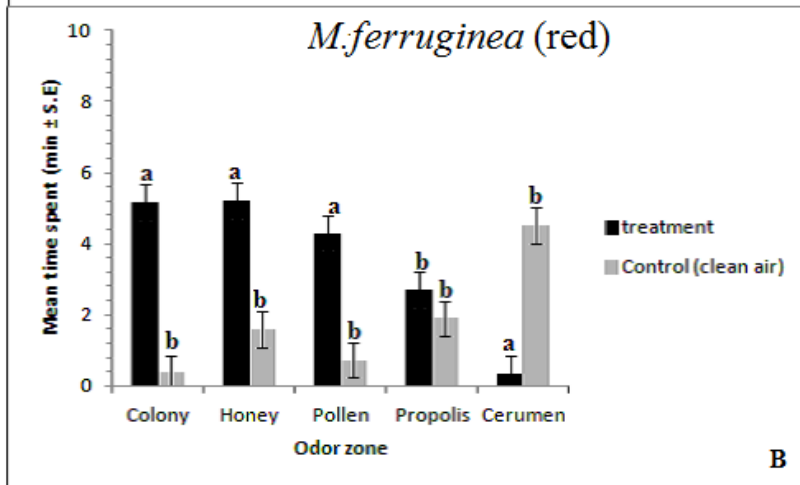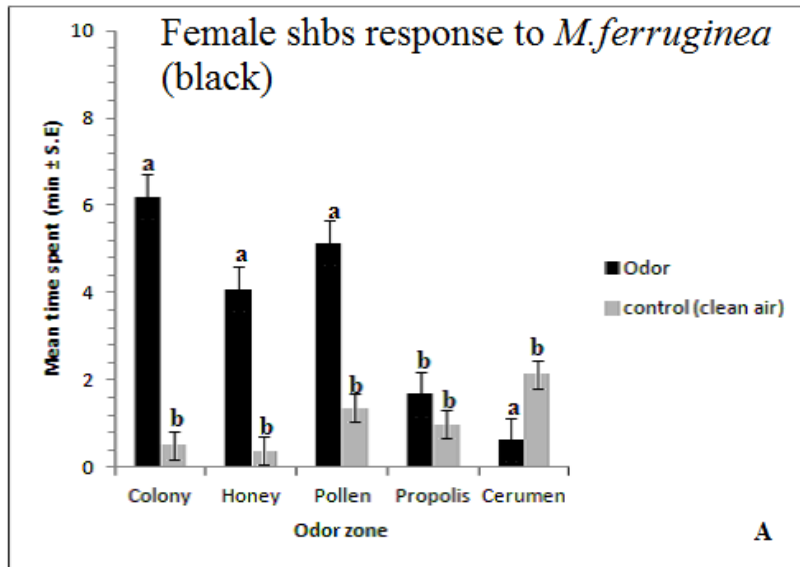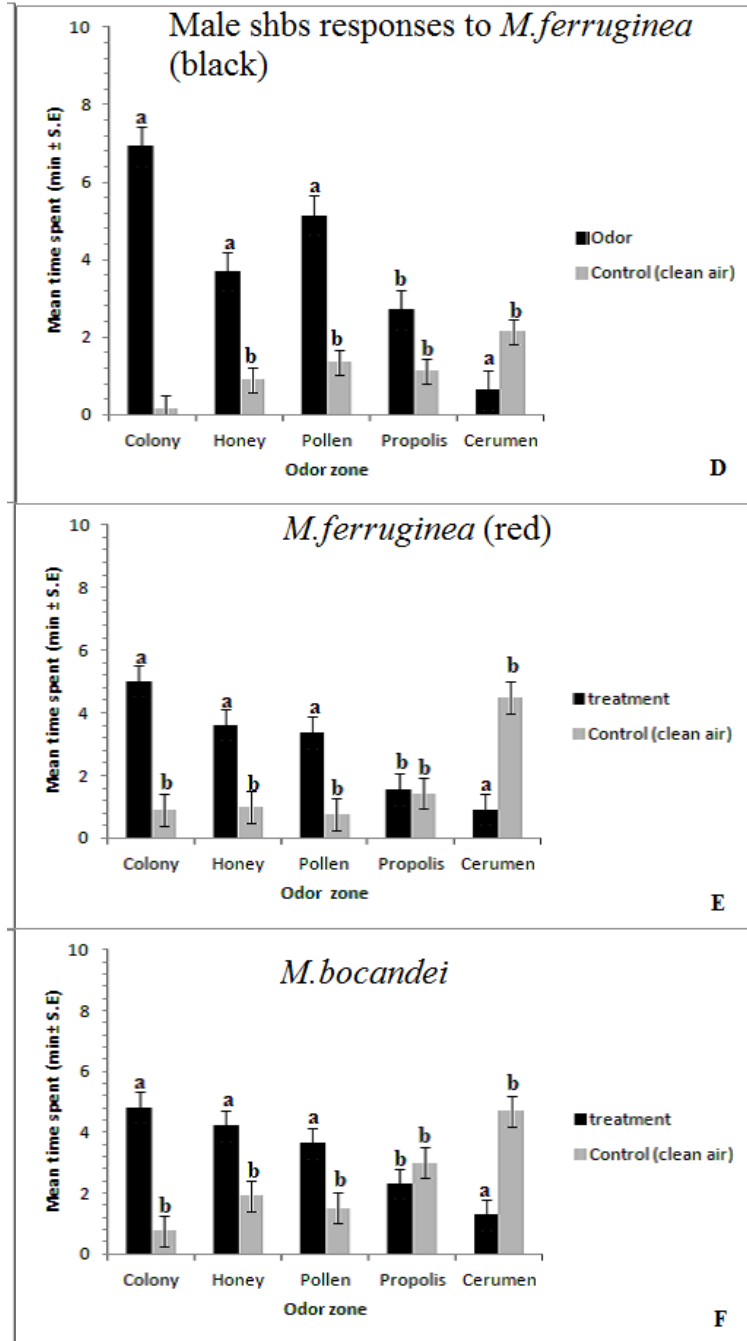
Figure 6.4 (A-F): Behavioral responses of *Aethina tumida* females (A-C) and males (D-F) to intact colony and colony matrix odors (expressed as mean time spent in odor zones) of three Afro tropical meliponine bee species: *Meliponula ferruginea* (black), *Meliponula ferruginea* (reddish brown), *Meliponula bocandei*. *Pair of black and grey bars with different letters represents statistically different responses.

6.3.3 SHBs response to intact colony and separate component odors of Meliponula bocandei

Pair-wise comparisons of all separate component odors showed significant preferences by both male and female SHBs for intact honey bee colony odors over *Meliponula bocandei* odors (Fig 6.4). Females spent significantly more time in intact colony odor zone of *M. ferruginea* (black) (t_{46}= 242.63, P< 0.001), pot honey (t_{46}= 5.863, P=0.02), pot pollen (t_{46}= 34.06, P<0.001), cerumen (t_{44}= 12.27, P= 0.001) with the exception of propolis odors (t_{48}= 0.363, P=0.55) (Fig 6.4E-F). Males exhibited a similar response pattern to intact colony odors of *M. ferruginea* (black) (t_{46}= 26.73, P< 0.001), pot honey odors (t_{44}= 32.77, P<0.001), pot pollen odors (t_{38}= 6.12, P=0.018), cerumen odors (t_{36}= 16.53, P<0.001) and propolis odors (t_{38}= 0.388, P=0.537).

6.4 Discussion

In this study, responses of SHBs to odors released by three African meliponine bee species of the *meliponula* genera; including *Meliponula ferruginea* (black), *M. ferruginea* (reddish-brown) and *M. bocandei* and their hive matrix components; pot honey, pot pollen, cerumen and propolis were investigated. Small hive beetles elicited significant olfactory responses to meliponine bee volatiles which varied with the sex of the beetle, bee species and type of hive matrix components. All these suggest that the composition of odor cues of meliponine bee species may serve as a predictor for attraction by these free-flying SHBs. Previous studies had shown that meliponine bee colonies that were infested by the SHB were predominantly from the *Trigona* and *Dactylurina* genera (Pino *et al*., 2006; Reichle *et al*., 2011; Halcroft *et al*., 2011). This study provides the first behavioral evidence of laboratory-based SHB attraction to meliponine bee species of the *meliponula* genera that are native to Africa. This finding is consistent with similar investigations on the honey bee and bumble bee (Spiewok and Neumann, 2006; Graham *et al*., 2011; Palmeri *et al*., 2015; Neumann *et al*., 2016) indicating that eusocial bee species colony odors provide critical cues for host-searching SHBs.

The findings of this study may suggest a number of reasons for the varied responses by both sexes of the beetles to odors of the different meliponine bee species and their matrix components. Firstly, sexual variations in detection and processing of meliponine bee odors could facilitate successful biological processes such as feeding and reproduction (Mustafa *et al*., 2015). Secondly, airborne volatiles from an intact colony readily offers short range olfactory cues for orientation to locate food resources and thirdly, whole hive matrix component quantity and quality present in a colony at the time of assays and environmental season would all contribute to the quality of the odor signal detected and behavioral response elicited in both sexes of the beetle.

These are consistent with responses to the different odor sources, especially pollen odors, as observed in a previous study, which showed that females SHBs exhibited a stronger dose-dependent response than males in wind tunnel assays to odors of fresh pollen obtained from honeybee colonies (Graham *et al*., 2011; Spiewok and Neumann, 2006; Suazo *et al*., 2003). However, further studies are required to investigate the chemical basis for which attraction is mediated by SHBs to intact meliponine bee colonies as previous work on meliponine bees have mainly focused on pheromones within and between species (Smith and Roubik, 1983; Johnson *et al*., 1985; Engels *et al*., 1987; Cruz López *et al*., 2002; Jarau *et al*., 2004; Reichle *et al*., 2011;).

The findings of this study also suggests that olfaction plays a key role in the location of the four meliponine bees' colonies by SHBs as previously reported for honeybees' colonies by Suazo *et al*., (2003) and Torto *et al*., (2005); (2007a). Finally, it is important to consider the several challenges that may occur when meliponine bees are domesticated. Key among these is their health which may be compromised by exposure to pests and pathogens.

Consequently, it is strongly recommended here that domestication of meliponine bee species for pollination services currently going on in several African countries should ensure the use of better constructed hives, free of crevices and cracks, which are known to facilitate easy entry by the SHB (Elzen *et al*., 1999) by making concise modifications

to currently used hive designs (UTOB hive, *Nogueira –neto* hive, *icipe* 1, *icipe* 2) (Sommet, 1999). This will help to reduce unnecessary infestations, rapid expansions of potential host ranges and dispersal of SHBs into new landscapes. In summary, the study has shown that olfaction plays a key role in the attraction of SHBs to African meliponine bees and that the SHB has the potential to expand its host range to include various species of meliponine bees of the *meliponula* genera.

CHAPTER SEVEN
RECRUITMENT BEHAVIOUR IN AN AFRICAN MELIPONINE BEE SPECIES (HYMENOPTERA, APIDAE: MELIPONINI): GLANDULAR ORIGIN AND CHEMICAL COMPONENTS OF ITS TRAIL PHEROMONES.

7.1 Summary

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 social bees. This study was carried out to test the hypothesis that a) African meliponine bees carry out scent marking behaviour at food sources and effectively recruit other foragers b) pheromones responsible for scent marking behaviour originate from the nasonov gland but maybe deposited by the tarsal glands. 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 carry out scent marking and recruitment behavior at food sources. Secondly we tested if either nasonov or tarsal gland secretions elicited trail-following behavior in newly recruited bees by means of chemical and electrophysiological 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) between the hours of 11:00–14:00 hours, *Meliponula ferruginea* (black)($F_{3,116}$ =5.61, P<0.001), *Hypotrigona gribodoi* ($F_{3,116}$ =6.46, P<0.001), *Hypotrigona ruspolii* ($F_{3,116}$ =2.81, P=0.042) and *Plebeina hildebrandti* ($F_{3,116}$ =4.19, P=0.007). A significantly higher proportion of foragers from the four species were attracted and recruited additional foragers to food resources baited with natural extracts from both their own nasonov and tarsal glands.

7.1.1 Introduction

Chemical compounds play a vital role 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 chemotactically. 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 transmit food odors to fellow nest mates inside the nest via footprint pheromones came from an experiment conducted by Lindauer (1956), 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 a clear deterrent effect at food sources and play a vital role in alarm communication and defense (Jarau *et al*., 2003b, 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*., 2003b), *Trigona spinipes* (Schorkopf, 2007), *Geotrigona mombuca* (Stangler *et al*., 2009) and *Scaptotrigona pectoralis* (Sawaya 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 is the main component from labial gland secretions of *Trigona recursa* foragers and acts as a key compound for triggering trail-following behavior in newly recruited workers of this species (Jarau *et al*., 2003b). 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 behavior as the complete labial gland extract (Schorkopf *et al*., 2007).

Stangler *et al*., (2009) identified a series of terpene- and wax-type esters from labial gland secretions of *Geotrigona mombuca*, with farnesyl butanoate as major component. Thus, the trail pheromone of *Geotrigona mombuca* is composed of esters, but the specific role 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 communication.

Kerr and Rocha (1988) raised another parallel hypothesis that volatiles 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 behavioral 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 spurned 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 utilizes 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 (Dahl 1885; 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 behavior 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 was resolved by the discovery of a different system of glands within the bees' legs (Jarau *et al*., 2004b) 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*., 2004b). 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 of 12 alkanes, eight alkenes, one methyl alkane, and one aldehyde (*Jarau et al*., 2003b). The dominant compounds, each constituting ≥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 an additional forty-one compounds, comprising mainly esters, acids, and methyl branched alkanes (Jarau *et al*., 2003b; 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 a similar effect on the behavior of foragers. This study was intended to confirm if African meliponine bees scent mark at food sources and identify the components of both nasonov and tarsal gland secretions and elucidate its effects on the recruitment behavior of four species of African meliponine bee species.

7.2 Materials and Methods

7.2.1 Experimental colonies

Behavioral experiments were conducted between April and September, 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. In February, 2014 colonies which had been sourced from Taita taveta county (03° 20' S, 38º 15' E) were transported to the meliponary section of the International center for insect physiology and ecology (*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 and estimated to be approximately similar in size and fitness, having similar numbers of workers (> 500) individuals. They were placed at a distance of 1m from each other and left to forage freely on nearby vegetation throughout the experimental period.

7.2.1.2 Behavioral experiment 1: Scent marking behaviour on food resources

On the day when each bioassay was to be conducted, a total of 12 marked artificial feeders were randomly baited with different artificially made food sources (nectar, pollen and water) respectively. These food resources had no deposits of scent marks on them (un-baited). The experimental setup and procedure followed the method for scent trail

bioassays described in 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 bioassays. 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 observation of scent marking behaviour was 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) (Fig 7.2) after landing on the feeders.

7.2.1.3 Extraction of glands for Bioassays

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 a source of contamination were excised. Gland extraction procedure and concentration of gland extracts was 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) (Fig 7.1). For all extracts, the amount of pentane was adjusted to 100µl per pair of glands (e.g., 10 nasonov/tarsal glands in 500 µL pentane). This is to ensure that 100µl of the pooled extracts corresponded to the gland content of one individual bee (one bee equivalent). 12 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 bioassays.
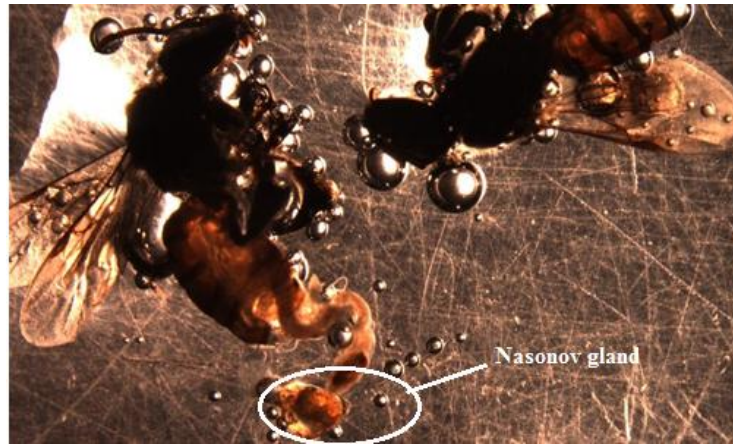
Figure 7.1: Excised abdominal region containing the nasonov gland (glandular epithelia) from *H. ruspolii* prior to solvent extraction.

7.2.1.4 **Behavioral 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 glands respectively from the four species. Approximately 10µl 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. Throughout all observations, the species identity, number of bees landing on each baited feeder and time of collection was recorded.

Most importantly the observation of scent marking behaviour was 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) (Fig 7.2) after landing on the feeders.

Figure 7.2: A *H. ruspolii* forager exhibiting scent marking behaviour on an unscented feeder provisioned with nectar**.**

7.2.2 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 in both nasonov and tarsal gland extracts. Excised antennae (Olsson *et al.*, 2013) 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, 3µl gland extract was injected in split less mode at 50°C onto the column. The Flame Ionization Detector (FID) was heated to 300 °C to detect all compounds. 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 electro-antennogram (EAG) system was connected to the GC system with a custom, 40 cm heated (250 °C) transfer line. Separate recordings of both EAD and FID signals were done. We replicated EADs with three individual foragers from each of the four species. A peak was classified as electro-physiologically active when it coincided with an EAD baseline deflection.

7.2.3 Extraction of headspace volatiles (nasonov and tarsal glands) for chemical analyses

Headspace 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 lml of pentane for 24 hours at room temperature (24°C), thereafter evaporating the solvent under a gentle stream of nitrogen gas to adjust 100µl per pair of glands (e.g., 10 nasonov/tarsal glands in 500 µL pentane), thus 100µl of the pooled extracts corresponded to the gland content of one individual bee (one bee equivalent). Extracts were stored in -20°C until ready to use for chemical analyses. A pure pentane control was subjected to similar evaporation process.

7.2.3.1 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 gland extracts from different species was injected in split less mode (Inlet temperature = 250 °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 °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 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, 2009). For compound quantification, peak areas were compared to an external standard corresponding to 5ng/µl of 2-heptanol.

7.2.3.2 Chemicals

Authentic chemical standards (>95 % purity by GC) (E)-β Farnesene, (Z)-β Farnesene, Nerolidyl acetate <E>, Bergamotene<α-trans>, Sesquisabinene, Humulene<alpha>, Myrcene, Limonene, Longipinene, Bisabolene<(Z)-alpha, Sinensal <beta>, Funebrene<beta>, Caryophyllene(E), Sesquilavandulol <E>, Butanoate<3-methyl-2-butenyl 2-met>, Sesquiphellandrene<beta->, Ocimene<(Z)-beta->, Clovene<alpha-neo->, Himachalene<alpha->, Farnesol<2Z, 6Z) were purchased from Sigma Aldrich (St. Louis, MO, USA)**.**

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

Bioassays 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. Exposure to food sources baited with a synthetic form of the dominant compound identified from the nasonov and the tarsal glands: (*E*)-*β* Farnesene was carried out respectively. Initiation of scent marking behaviour in response to the synthetic compound were conducted in a dual choice test bioassay Perspex platform measuring 13x5.7cm and sealed with a glass lid (Fig 7.3). An aliquot of this synthetic pheromone (25µl) was dispensed round a food source placed onto a filter paper (Whatman No.1) which was placed on one side of the bioassay chamber while the other chamber was provisioned with an untreated food resource (positive control).

Figure 7.3: Dual choice test bioassay Perspex platform provisioned with both baited (treatment) and un-baited food resource (positive control)**.**

7.2.5 Statistical Analyses

The foraging pattern of individual foragers on each food resource was also analyzed using descriptive statistics. Student Newman Keuls 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 was 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 *Hypotrigona gribodoi*, *Hypotrigona ruspolii*, *Meliponula ferruginea* (black) and *Plebeina hildebrandti* were calculated, then log-transformed and data subjected to Kruskal-Wallis ANOVA test. All statistical analyses were carried out using Sigmaplot V 11.0 statistical software (Systat Software, San Jose, CA 2011).

7.2.6 Results

7.2.6.1 Behavioral 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; *Meliponula ferruginea* (black) ($F_{3,116}$ =5.61, P<0.001), *Hypotrigona gribodoi* ($F_{3,116}$ =6.46, P<0.001), *Hypotrigona ruspolii* ($F_{3,116}$ =2.81, P=0.042) and *Plebeina hildebrandti* ($F_{3,116}$ =4.19, P=0.007). In all four species, the total number of bees landing and initiating scent marking progressed with increasing foraging hours. Foraging activity peaked between 11:00 hours and 14:00 hours as 70% of all foraging bouts gradually declined after this observation period. *Meliponula ferruginea* (black) species showed the highest foraging activity on both baited and un-baited nectar sources, as workers began landing on the feeders as from 11:05hours and peaked at 13:00hours, while *Hypotrigona gribodoi*, *Hypotrigona ruspolii* and *Plebeina hildebrandti* all foraged till much later, signifying similar commencement of foraging but having peak periods which lasted until 15:00hours. In general, the collection of nectar started to decrease after this peak period until cessation. Nectar was always the most collected resource *Meliponula ferruginea* (black) (N=220), *Hypotrigona gribodoi* (N=117), *Hypotrigona ruspolii* (N=124), *Plebeina hildebrandti* (N=109, throughout the whole observational period, while water was the second most collected resource: *Meliponula ferruginea* (black) (N=101), *Hypotrigona gribodoi* (N=97), *Hypotrigona ruspolii* (N=94), *Plebeina hildebrandti* (N=71, followed lastly by pollen: *Meliponula ferruginea* (black) (N=84), *Hypotrigona gribodoi* (N=60), *Hypotrigona ruspolii* (N=61), *Plebeina hildebrandti* (N=73). The foraging activity for pollen followed the same sequence across all 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; *Hypotrigona gribodoi* and *Hypotrigona 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 *Meliponula ferruginea* (black) and *Plebeina hildebrandti*. Notable however was the foraging pattern for water which was observed to be more regular after 13:00hours.
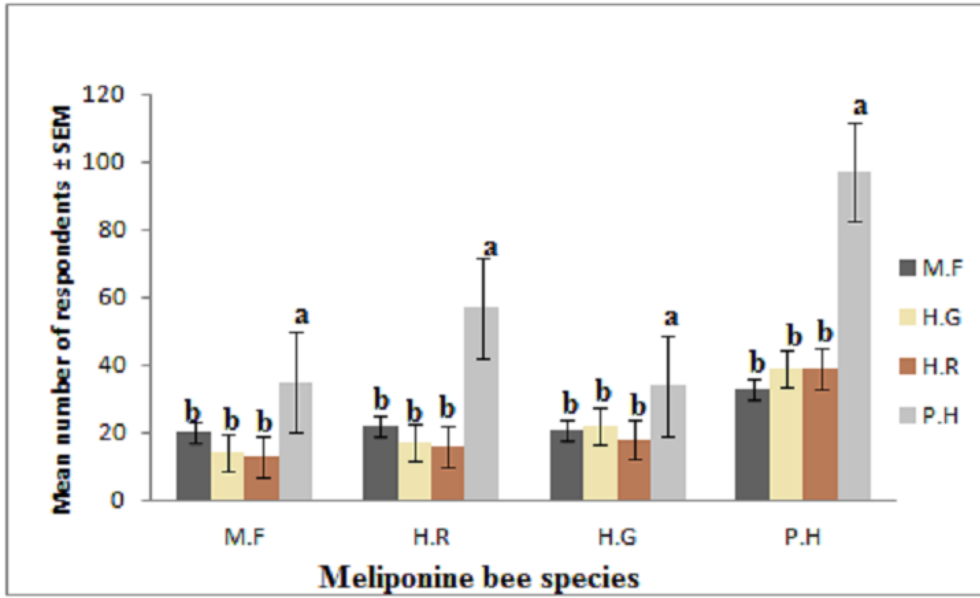
Figure 7.4a: Mean number of individuals recruited to food sites which have been baited with nasonov gland extracts from the respective species. *M.F: *Meliponula ferruginea*, H.R: *Hypotrigona ruspolii*, H.G: *Hypotrigona gribodoi*, P.H: *Plebeina hildebrandti*.
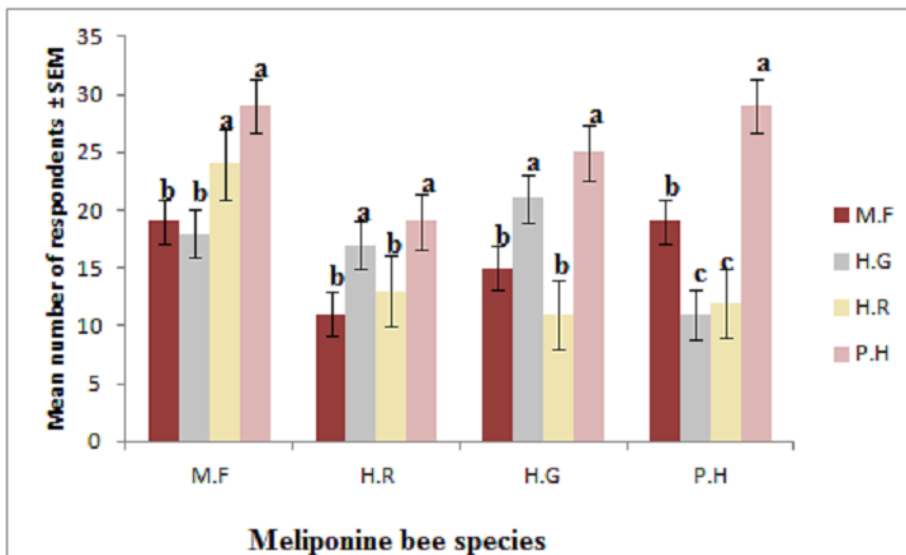
Figure 7.4b: Mean number of individuals recruited to food sites which have been baited with tarsal gland extracts from the respective species*M.F: *Meliponula ferruginea*, H.R: *Hypotrigona ruspolii*, H.G: *Hypotrigona gribodoi*, P.H: *Plebeina hildebrandti*.

7.2.6.2 Chemical and electrophysiological analyses

Chemical analyses of both nasonov and tarsal gland extracts demonstrated that the trail pheromone of *Plebeina hildebrandti*, *Hypotrigona gribodoi*, *Hypotrigona ruspolii* and *Meliponula 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 7.5a) and two dominant compounds from the tarsal gland extract (Figure 7.5b) which are sesquiterpenes.

GC-EAD analyses done with 30 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.
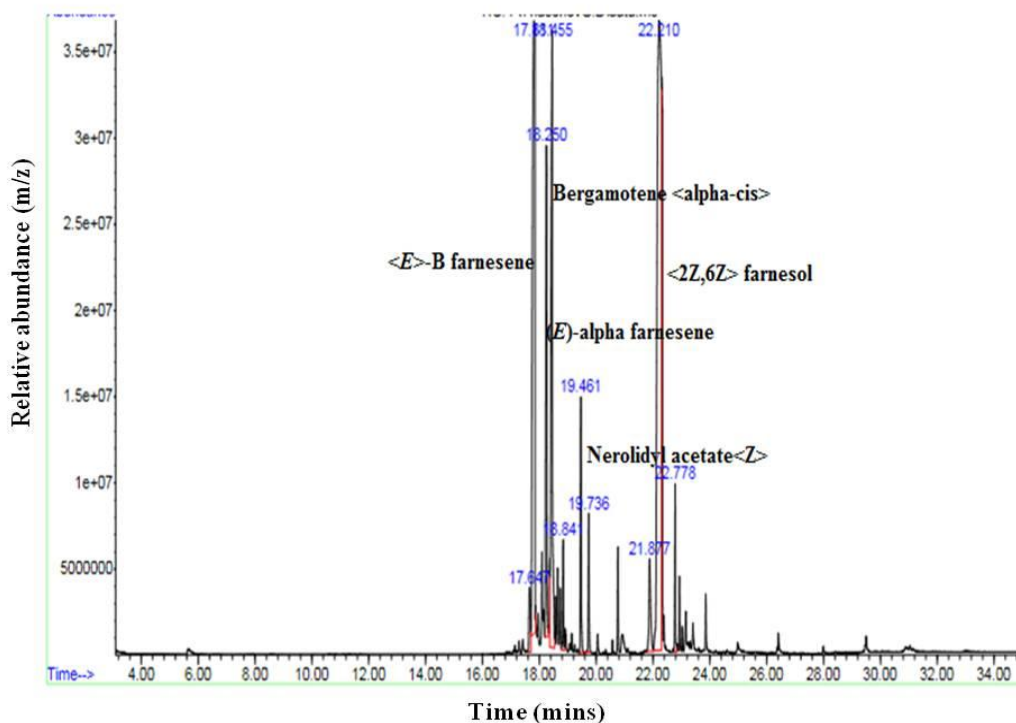
Figure 7.5a: Mass spectrum showing dominant compounds identified from the nasonov epithelial gland extract of a meliponine bee species, *Hypotrigona ruspolii*.
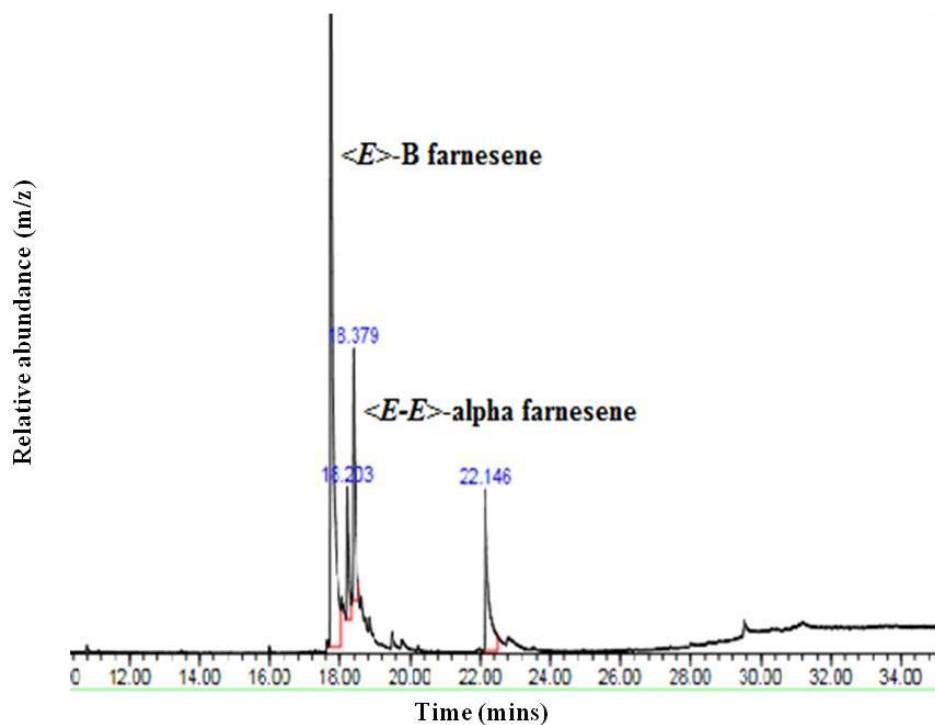
Figure 7.5b: Mass spectrum showing dominant compounds identified from the tarsal gland extract of a meliponine bee species, *Hypotrigona ruspolii*.

7.2.6.3 Bioassays with synthetic compounds

Scent trail bioassays with synthetics

To test whether the physiologically active compounds from both nasonov and tarsal glands constitute the behaviorally active trail pheromone of these species, a further set of trail bioassays was conducted. Experimental trails were baited with (*E*)-β Farnesene which was the dominant peak in the nasonov and tarsal gland secretions of foragers collected from the four species of *Hypotrigona gribodoi*, *Hypotrigona ruspolii*, *Meliponula ferruginea* (black) and *Plebeina hildebrandti* respectively.

A significantly higher proportion 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), *Hypotrigona ruspolii* (t = 0.633, df = 58, P = 0.005), *Hypotrigona gribodoi* (t = 2.64, df = 58, P= 0.004) and

Plebeina hildbrandti (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 significance preference for any species: *M. ferruginea* (black) (t = 2.41, df = 58, P= 0.011), *Hypotrigona ruspolii* (t = 2.49, df = 58, P = 0.015), *Hypotrigona gribodoi* (t = 2.52, df = 58, P = 0.014) and *Plebeina 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 was observed between respective treatments. The synthetic compound, (*E*) - β Farnesene was significantly as attractive to foragers of the four species 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).

7.2.6.4 Discussion

The results of our bioassays 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, 2003b; 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 compound 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 analysis 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 in-complete as a synthetic pheromone trail bouquet, which has been shown to contain

varied amounts of geraniol and citral in some studies (Jarau *et al*., 2003b; Stangler *et al*., 2009; Hrncir *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 (Bergstrom, 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 behavior. This conclusive 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, and 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 behavior originally was believed to be based on active deposition of lipid "scent-marks" by bees, two recent studies suggested that these chemicals are deposited wherever the bees walk, and were used as footprint cues rather than pheromonal signals. 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 behavioral context (Nieh and Roubik, 1995; Schmidt *et al*., 2003; Hrncir *et al*., 2004). Recently, Holldobler *et al*., (2004) 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. A colony-specific effect of abdominal extracts in releasing trail following behavior was demonstrated in *Lasius neoniger* (Traniello, 1980) but by contrast, the actual trail pheromone extracted from workers abdominal region was not specific in initiating scent marking behavior in other closely related species, *Lasius japonicus* and *Lasius nipponensis.* 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).

7.2.6.5 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 scent 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.

When foragers of different species meet at food sites, they become entangled in fierce fights, which usually lead to the death of many individuals (Johnson and Hubbell, 1974; Hncrir *et al*., 2004). Hence, by limiting aggressive encounters between foragers of different species, the loss of large numbers of workers could be avoided and the colonies' fitness maintained. This could potentially be a mechanism for resource partitioning and competition avoidance between con-specific and hetero-specific foragers, which occurs when foragers from different colonies have been domesticated and scout for food sources in overlapping foraging areas such as green houses.

CHAPTER EIGHT
GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

8.1 General Discussion

This study was carried out with the general aim of understanding in detail the ecology and communication mechanisms governing defense and foraging behaviors in these bee species. Cues that influence decision making in an unpredictable environment when scouting for nesting sites and food sources could provide information on how these African meliponine bee's species potentially survive when searching overlapping foraging regions with other species of their own tribe. It's been known that for bees of these species to potentially be used as alternative pollinators, their ecology and behaviour must be clearly understood and in this context, the diversity of these bee species, pattern of communication during defense at both individual and colony levels; and during foraging at potential food sites were studied.

Surveys conducted in two location sites of Taita- Taveta County revealed that as many as 80 different plant species of 34 different families could still sequentially flower with overlapping blooming periods across two main seasons in this fragile but unpredictable habitat. Despite the natural occurrence of four meliponine bee species: *Hypotrigona gribodoi*, *Hypotrigona ruspolii*, *Meliponula ferruginea* (black) and *Plebeina hildebrandti* (Hymenoptera: Apidae), they can regularly visit different kinds of flowers from these families, thereby benefiting from a diverse mix of resources of both pollen and nectar produced by these flowers. Differences in the nest abundance of meliponine bee species with relation to habitat type indicated a clear evidence of both high distribution and diversity in the lowlands compared to the highlands which has possibly resulted from the conversion of native habitats to agriculture landscapes, and has been implicated as the primary form of land-use change and the largest cause of indigenous habitat loss and fragmentation (Tilman *et al*., 2001; DeFries *et al*., 2004). The results indicate that mixed deciduous wood lands presented itself as a much preferred habitat for nesting and trees as

a preferred nesting substrate as profile curves indicated that more species could be identified with increased sampling sites and on more tree nesting substrates.

Geometric morphometrics analyses showed that all four stingless bee species at Taita hills could be grouped into two clusters, cluster 1 (*Hypotrigona gribodoi*, *Hypotrigona ruspolii*, *Meliponula ferruginea (*black) and cluster 2 (*Plebeina hildebrandti*) by successfully discriminating populations from the four different habitats surveyed in Taita hills. Each habitat appeared to consist of a cluster of sub-populations and revealed ecotypes within the four meliponine populations. A possible reason for this clustering of these species could be linked to superficial resemblance of the three species belonging to cluster 1(*Hypotrigona gribodoi*, *Hypotrigona ruspolii* and *Meliponula ferruginea* (black)) with regards to similarities in forewing characters (open sub marginal cells, anterior region of the sub marginal cross vein and non-distinct veins) and cluster 2 (*Plebeina hildbrandti*) which had more distinct marginal cells, closed sub marginal cells and pronounced distinct veins. Also, the characteristic type of vegetation and climatic conditions each habitat appeared to have; ultimately altered morphological characters for higher probability of survival in such habitats. DNA bar-coding clearly confirmed the distinctness of these four species from each other. A BLASTN search further confirmed their identities as members of the families Meliponinae for *Meliponula ferruginea* (black) (accession number: GU245578), *Hypotrigona gribodoi* (accession number: AY945189), *Hypotrigona ruspolii* (accession number: EU980053), and Meliplebeia for *Plebeina hildebrandti* (accession number: GU245413) respectively. The results of a principal component analysis on the morphological measurements corroborated with molecular analysis, revealing the species clustering in four different species clades (*Hypotrigona gribodoi, Hypotrigona ruspolii, Meliponula ferruginea* (black) and *Plebeina hildebrandti* respectively. This has invariably demonstrated that integrating DNA bar-coding with morpho-metrics can solve taxonomic bottlenecks and may further segregate sub-species that share similar ecotypes and have high levels of similarities, i.e. *Hypotrigona spp*.

One vital cue these meliponine bee species depend on to detect and discriminate nest mates from non-nest mates during defensive behaviour are principally olfactory based which has demonstrated to also be similarly utilized by honey bees, *Apis mellifera*. Nest

mate recognition system of these four meliponine bee species operates remarkably similar to that of *Apis mellifera*, and this further confirms that individuals can predict the difference between a nest mate, con-specific non-nest mate and hetero-specific non nest-mate encountered during certain ecological interactions which has already been reported for species like *Melipona seminigra* (Breed *et al*. 2004a, b).

This suggests that olfactory cues alone can singularly trigger cognitive defensive behaviour in meliponine bees as aggression levels observed in these four bee species could have resulted from differing olfactory perception for compounds or an insignificant effect of some compounds found in trace amounts in making nest mate/non-nest mate recognition decisions. This small differences between an expected and an actual odor would definitely take longer to detect and process compared to compounds which make up a large proportion resulting in rapid assessments and hence a shorter time to exhibit aggression. Environmental odors are known to affect nest mate recognition in many eusocial insect species (Downs *et al*., 2000) and the use of odors derived from the environment gives a complexity to nest mate recognition templates, making room for more precise recognitions compared to the limited range of compounds such as straight-chained hydrocarbons found only in their cuticular profiles.

Unlike honeybees, meliponine bees utilize additional plant materials during nest construction, such as resins in addition to wax (Eltz *et al*., 2003) which may contribute to a more complex blend of recognition cues, as seen in the case of these four meliponine bee species. The use of a wider range of cues unlike *Apis mellifera* in nest mate 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 of bees. Exogenous volatiles, such as those found in resins, when brought into the nest during construction and maintenance of nest entrances and the involucrum sheaths may also serve as cue sources, which may further point to the use of exogenous derived cues to predict nest mates from non-nest mates. These signals, even if released in quantities well below the amount easily detected by individual foragers, is still

capable of releasing aggressive behaviour, which is vital during territorial defense and foraging at food sources.

Regarding foraging, chemical signals facilitate coordinated resource utilization outside colonies in instances where pheromone trails are laid down by recruiting foragers (e.g. genera *Scaptotrigona* and *Trigona*). However, the dependence on chemical signals to effectively mark profitable foraging sites, recruit additional foragers and successfully orientate them towards these food sites in the field could also lead to some inertia in their foraging decision making process. The findings presented in this thesis show many similarities to cues and signal mechanisms already described in other well studied social insects, such as honey bees, ants or termite species (Hölldobler and Wilson 2009).

8.2 Conclusion

The current study focused on specific areas in the ecology of African meliponine bee species with the overall aim of providing more detailed insight into chemical communication influencing behaviors (defense and foraging) which is essential for the survival of these species. These four bee species are *Hypotrigona gribodoi*, *Hypotrigona ruspolii*, *Meliponula ferruginea* (black) and *Plebeina hildebrandti*. The ecological aspects investigated are the occurrence of these bee species in diverse habitats, some aspects of flowering phenology of plants potentially able to support the existence of these bee species, and the types of chemical communication mechanisms utilized during defense and foraging.

Surveys to determine the pattern of natural occurrence of African meliponine bee species in diverse habitats were carried out to understand how vulnerable habitats could shape their diversity. The findings showed that:

1. Each habitat appeared to consist of a cluster of sub-populations and revealed ecotypes within the four meliponine populations of *Hypotrigona gribodoi*, *Hypotrigona ruspolii*, *Meliponula ferruginea* (black) and *Plebeina hildebrandti*.
2. Mixed deciduous wood lands and *Acacia* dominated bush-lands presented itself as preferred habitats for nesting and trees as a preferred nesting substrate as profile

curves indicated that an increasing number of species could be identified with additional sampling sites and tree nesting substrates.

3. Geometric morpho-metrics analyses showed that all four stingless bee species at Taita hills could be grouped into two clusters, cluster 1 (*H. gribodoi*, *H. ruspolii*, *M. ferruginea (*black)) and cluster 2 (*P.hildebrandti*) and they successfully discriminated populations against the four different habitats surveyed in Taita hills.

4. DNA bar-coding clearly confirmed the distinctness of these four species from each other, revealing the species clustering in four different clades (*Hypotrigona gribodoi, Hypotrigona ruspolii, Meliponula ferruginea* (black) and *Plebeina hildebrandti* respectively.

   The diversity of these bee species was studied to obtain relevant data of their occurrence in vulnerable habitats. This information is vital for monitoring various landscapes that can be used to predict their distribution as vigorous domestication programs are ongoing in Kenya and Africa at large. The findings in this study support the conclusion that African meliponine bee species preferred unfragmented habitats that possess structural features that are indigenous to such habitat.

Additional surveys to investigate flowering phenology of plant species in these diverse habitats were carried out to determine potential food sources (pollen and nectar) that could support their survival. The findings showed that:

1. As many as 80 different plant species of 34 different families sequentially flower with overlapping blooming periods in this hotspot.

2. A clear evidence of higher distribution and diversity in the lowlands compared to the highlands which may have possibly resulted from the conversion of natural habitats to agricultural landscapes, is the primary form of land-use change and the largest cause of habitat loss and fragmentation in this region.

Recognition cue chemistry, what acquisition channels are utilized and how such cues shape recognition behaviour in these African bee species were studied. This information

is important in ascertaining if the same mechanism of nest mate recognition in honey bees, *Apis mellifera* also applies to meliponine bees of Afro-tropical origin. The findings of this study support the conclusion that recognition cue compounds utilized by Afro-tropical meliponine bee species were similar to the honeybee.

From both bioassay results and GC-EAD/GC-MS analyses it can be concluded that:

1. Nest mate recognition system in these four African meliponine bee species operates remarkably similar to that of *Apis mellifera*.

2. Meliponine bee species depend on short range olfactory cues to detect and discriminate nest mates from non nest mates, which have also demonstrated to be utilized by honey bees, *Apis mellifera*. This suggests that olfactory cues alone can singularly trigger cognitive defense or aggressive behaviour in meliponine bees as observed in these four bee species.

3. The use of exogenous odors derived from the environment gives a complexity to nest mate recognition templates, making room for more precise recognitions /predictions compared to the limited range of compounds found only in their cuticular profiles.

4. Unlike honeybees, meliponine bees utilize additional plant materials during nest construction, such as resins in addition to wax, which may contribute to a more complex blend of recognition cues dominated by terpenes, as seen in the case of cuticular profiles of these four meliponine bee species.

5. The use of a differing range of cues like *Apis mellifera* in nest mate recognition by all four bee species reveals that signals' originating from endogenously produced cuticular hydrocarbons is not the only acquisition channel of recognition cues in these species of bees.

Similar bioassays, coupled GC-EAD and GC-MS analysis were focused at confirming the origin of trail pheromone production and its components in African meliponine bee species shaping their foraging behaviour, and resulted in the following findings:

1. African meliponine bees are observed to initiate trail laying behavior on food sources. The origin of production of trail pheromones in these African stingless

bees' species could possibly originate from the nasonov gland with the tarsal gland facilitating the deposition of such trail pheromones at potential food sites.

2. Though there seemed to be a generality in the scent marking components of these bee species, it was observed that terpenoids dominated the glands of larger sized bees, *Plebeina hildebrandti*, compared to smaller sized bee species, *Hypotrigona gribodoi*, *Hypotrigona ruspolii* and *Meliponula ferruginea* (black).

3. The conclusive finding, that foragers are significantly attracted to food sources baited with nasonov gland extracts secreted by their nest-mates, compared to foragers of a foreign colony, can be explained by the demonstrated differences in the relative proportions of trail pheromone components.

8.3 Recommendations

Although there seems to be some level of similarity on defense mechanisms and recruitment behaviour between meliponine bee species and the honey bee, *Apis mellifera* presented in this study, it is pertinent to make recommendations within this context. These include:

1. Estimation and identification of indigenous bee species can contribute towards the monitoring of pollinators in habitats that are rapidly converted into agricultural landscapes.

2. Collation of information on plant pollinator interactions/pollination services can readily help to assess pollinator status and implement conservation strategies.

3. Evaluation of communication mechanisms which can provide a better understanding of their behaviour as they are rapidly domesticated for pollination services.

REFERENCES

Aarssen, L. W., & Schamp, B. S. (2002). Predicting distributions of species richness and species size in regional floras: Applying the species pool hypothesis to the habitat templet model. *Perspectives in Plant Ecology, Evolution and Systematics*, *5*(1), pp 3–12.

Abrol, D. P. (2012). Non Bee Pollinators-Plant Interaction. In *Pollination Biology* ,pp. 265–310. Dordrecht: Springer Netherlands. http://doi.org/10.1007/978-94-007-1942-2_9

Adriaensen, F., Githiru, M., Mwang'ombe, J., Matthysen, E., & Lens, L. (2006). Restoration and increase of connectivity among fragmented forest patches in the Taita Hills, Southeast Kenya. *Report of Critical Ecosystem Partnership Fund Project*, *1095347968*, pp1–149.

Aerts, R., Thijs, K. W., Lehouck, V., Beentje, H., Bytebier, B., Matthysen, E., Muys, B. (2011). Woody plant communities of isolated Afromontane cloud forests in Taita Hills, Kenya. *Plant Ecology*, *212*(4), pp 639–649.

Aguilar, I., Fonseca, A., & Biesmeijer, J. C. (2005). Recruitment and communication of food source location in three species of stingless bees (Hymenoptera, Apidae, Meliponini). *Apidologie*, *36*(3), pp 313–324.

Akino, T., Yamamura, K., Wakamura, S., & Yamaoka, R. (2004). Direct behavioral evidence for hydrocarbons as nestmate recognition cues in *Formica japonica* (Hymenoptera: Formicidae). *Applied Entomology and Zoology*, *39*(3), pp 381–387. http://doi.org/10.1303/aez.2004.381

Aleixo, K. P., Menezes, C., Imperatriz Fonseca, V. L., & da Silva, C. I. (2016). Seasonal availability of floral resources and ambient temperature shape stingless bee foraging behavior (*Scaptotrigona* aff. *depilis*). *Apidologie*. http://doi.org/10.1007/s13592-016-0456-4.

Alves, D. A., Imperatriz-Fonseca, V. L., Francoy, T. M., Santos-Filho, P. S., Billen, J., & Wenseleers, T. (2011). Successful maintenance of a stingless bee population despite a severe genetic bottleneck. *Conservation Genetics*, *12*(3), pp 647–658.

Amano, K. (2004). Attempts to introduce stingless bees for the pollination of crops under greenhouse. *Food and Fertilizer Technology Center*, pp 1–9.

Antonini, Y., & Martins, R. P. (2003). The value of a tree species (Caryocar brasiliense) for a stingless bee Melipona quadrifasciata quadrifasciata. *Journal of Insect Conservation*, *7*(3), pp 167–174.

Antonini, Y., Martins, R. P., Aguiar, L. M., & Loyola, R. D. (2013). Richness, composition and trophic niche of stingless bee assemblages in urban forest remnants. *Urban Ecosystems* *16*(3), pp 527–541. http://doi.org/10.1007/s11252-012-0281-0.

Araújo, E. D., Costa, M., Chaud-Netto, J., & Fowler, H. G. (2004). Body size and flight distance in stingless bees (Hymenoptera: Meliponini): inference of flight range and possible ecological implications. *Brazilian Journal of Biology (Revista Brasleira de Biologia)*, *64*(3B), pp 563–568.

Arbogast, R. T., Torto, B., & Teal, P. E. A. (2010). Potential for Population Growth of the Small Hive Beetle Aethina tumida (Coleoptera: Nitidulidae) on Diets of Pollen Dough and Oranges. *Florida Entomologist*, *93*(2), pp 224–230.

Arbogast, R. T., Torto, B., Van Engelsdorp, D., & Teal, P. E. A. (2007). An effective trap and bait combination for monitoring the small hive beetle, A*ethina tumida* (Coleoptera: nitidulidae). *Florida Entomologist, 22*(8), pp 25–31.

Barth, F. G., Hrncir, M., & Jarau, S. (2008). Signals and cues in the recruitment behavior of stingless bees (*Meliponini*). *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology, 52*(3), pp 45–51.

Bartomeus, I., Vilà, M., & Steffan-Dewenter, I. (2010). Combined effects of Impatiens glandulifera invasion and landscape structure on native plant pollination. *Journal of Ecology*, *98*(2), pp 440–450.

Bergström, L. (2008). Chemical communication by behaviour-guiding olfactory signals. *Chemical Communications*. Retrieved from http://pubs.rsc.org/en/content/articlehtml/2008/cc/b712681f

Biesmeijer, J. C., Giurfa, M., Koedam, D., Potts, S. G., Joel, D. M., & Dafni, A. (2005). Convergent evolution: Floral guides, stingless bee nest entrances, and insectivorous pitchers. *Naturwissenschaften*, *92*(9), pp 444–450.

Biesmeijer, J. C., Smeets, M. J. A. P. A. P., Richter, J. A. P. P., & Sommeijer, M. J. (1999). Nectar foraging by stingless bees in Costa Rica : botanical and climatological influences sugar concentration of nectar collected by Melipona. *Apidologie*, *30*, pp 43–55.

Biesmeijer, J., & Slaa, E. (2004). Information flow and organization of stingless bee foraging. *Apidologie*. Retrieved from http://www.apidologie.org/index.php?option=com_article&access=standard&Itemid=129&url=/articles/apido/pdf/2004/02/M4203.pdf

Billen, J. (2004). Morphology of exocrine glands in social insects with special emphasis on the contributions by Italian researchers. *Insect Soc. Life*. Retrieved from http://bio.kuleuven.be/ento/pdfs/billen_inssoclife_2004_morphology_exocrine_glands_social_insects_italian.pdf

Bjerknes, A. L., Totland, Ø., Hegland, S. J., & Nielsen, A. (2007). Do alien plant invasions really affect pollination success in native plant species? *Biological Conservation*.

Bommarco, R., & Biesmeijer, J. (2010). Dispersal capacity and diet breadth modify the response of wild bees to habitat loss. http://rspb.royalsocietypublishing.org/content/early/2010/03/05/rspb.2009.2221.

Bordereau, C., & Pasteels, J. (2011). Pheromones and chemical ecology of dispersal and foraging in termites. *Biology of Termites: A Modern Synthesis*. Retrieved from http://link.springer.com/chapter/10.1007/978-90-481-3977-4_11.

Bowden, R., Williamson, S., & Breed, M. (1998). Floral oils: their effect on nestmate recognition in the honey bee, *Apis mellifera*. *Insectes Sociaux*.

Bradbear, N. (2009). Bees and their role in forest livelihoods: a guide to the services provided by bees and the sustainable harvesting, processing and marketing of their products. *Non-Wood Forest Products*. Retrieved from http://www.cabdirect.org/abstracts/20093225520.html

Breed, M., & Bennett, B. (1987). Kin recognition in highly eusocial insects. *Kin Recognition in Animals/edited by David JC.*. Retrieved from http://agris.fao.org/agris-search/search.do?recordID=US201302067801.

Breed, M., Perry, S., & Bjostad, L. (2004). Testing the blank slate hypothesis: why honey

bee colonies accept young bees. *Insectes Sociaux*. Retrieved from http://link.springer.com/article/10.1007/s00040-003-0698-9.

Breed, M., Stiller, T., & Moor, M. (1988). The ontogeny of kin discrimination cues in the honey bee, Apis mellifera. *Behavior Genetics*. Retrieved from http://www.springerlink.com/index/j825772052wx92w7.pdf

Brent Gurd, D., Nudds, T. D., & Rivard, D. H. (2001). Conservation of mammals in eastern North American wildlife reserves: How small is too small? *Conservation Biology*, *15*(5), 1355–1363.

Brittain, C., Kremen, C., & Klein, A. M. (2013). Biodiversity buffers pollination from changes in environmental conditions. *Global Change Biology*, *19*(2). http://doi.org/10.1111/gcb.12043.

Brooks, T., Lens, L., Barnes, J., Barnes, R., Kihuria, J. K., & Wilder, C. (1998). The conservation status of the forest birds of the Taita Hills, Kenya. *Bird Conservation International*, *8*, 119–139. http://doi.org/10.1017/S0959270900003221.

Brown, J. C., & De Oliveira, M. L. (2014). The impact of agricultural colonization and deforestation on stingless bee (Apidae: Meliponini) composition and richness in Rondônia, Brazil. *Apidologie*, *45*(2). http://doi.org/10.1007/s13592-013-0236-3.

Brown, B. V. (1996). A further species of Apocephalus, subgenus Mesophora (Diptera, Phoridae) parasitic on stingless bees (Hymenoptera, Apidae, Meliponinae). *Studia Dipterologica*, *3*(2), 231–235.

Buchmann, S. L., & Nabhan, G. P. (1996). The Pollination Crisis. *The Sciences*, 22–27.

Burger, H., Dötterl, S., & Ayasse, M. (2010). Host-plant finding and recognition by visual and olfactory floral cues in an oligolectic bee. *Functional Ecology*, *24*(6), 1234–1240.

Bwong, B. A., & John Measey, G. (2010). Diet composition of Xenopus borealis in Taita Hills: Effects of habitat and predator size. *African Journal of Ecology*, *48*(2), 299–303.

Camargo, J. M. F., & Pedro, S. R. M. (2002). Mutualistic association between a tiny Amazonian stingless bee and a wax-producing scale insect. *Biotropica*, *34*(3), 446–451.

Cameron, S. A. (1993). Multiple origins of advanced eusociality in bees inferred from

mitochondrial DNA sequences. *Proceedings of the National Academy of Sciences of the United States of America*, *90*(18), 8687–8691.

Cameron, S., & Mardulyn, P. (2001). Multiple molecular data sets suggest independent origins of highly eusocial behavior in bees (Hymenoptera: Apinae). *Systematic Biology*. Retrieved from http://sysbio.oxfordjournals.org/content/50/2/194.

Cameron, S., & Mardulyn, P. (2003). The major opsin gene is useful for inferring higher level phylogenetic relationships of the corbiculate bees. *Molecular Phylogenetics and Evolution*.

Campos, M. G. R., Bogdanov, S., de Almeida-Muradian, L. B., Szczesna, T., Mancebo, Y., Frigerio, C., & Ferreira, F. (2008). Pollen composition and standardisation of analytical methods. *Journal of Apicultural Research*, *47*(2), 154–161. http://doi.org/10.3896/IBRA.1.47.2.12

Cane, J. H. (2001). Habitat fragmentation and native bees: A premature verdict? *Ecology and Society*, *5*(1).

Carroll, T. (2006). *A Beginner's Guide to Beekeeping in Kenya, 20*(7), 10–21.

Carvalheiro, L. G., Seymour, C. L., Veldtman, R., & Nicolson, S. W. (2010). Pollination services decline with distance from natural habitat even in biodiversity-rich areas. *Journal of Applied Ecology*, *47*(4), 810–820.

Chege, J., & Bytebier, B. (2005). Vegetation structure of four small forest fragments in taita hills, Kenya. *Journal of East African Natural History, 22*(10), 15–26.

Chen, L., Sharma, K. R., & Fadamiro, H. Y. (2009). Fire ant venom alkaloids act as key attractants for the parasitic phorid fly, *Pseudacteon tricuspis* (Diptera: Phoridae). *Naturwissenschaften*, *96*, 1421–1429.

Christopher Brown, J., & Albrecht, C. (2001). The effect of tropical deforestation on stingless bees of the genus Melipona (Insecta: Hymenoptera: Apidae: Meliponini) in central Rondonia, Brazil. *Journal of Biogeography*, *28*(5), 623–634.

Clark, B., & Pellikka, P. (2007). Mapping land cover change in the Taita Hills, Kenya, utilizing multi-scale segmentation and object-oriented classification of SPOT imagery. In *International Geoscience and Remote Sensing Symposium (IGARSS)* (pp. 1918–1921).

Colwell, R. K., Chang, X. M., & Chang, J. (2004). Interpolating, extrapolating, and

comparing incidence-based species accumulation curves. *Ecology*, *85*(10), 2717–2727.

Connelly, H., Poveda, K., & Loeb, G. (2015). Landscape simplification decreases wild bee pollination services to strawberry. *Agriculture, Ecosystems and Environment*, *211*. http://doi.org/10.1016/j.agee.2015.05.004.

Core, A., Runckel, C., Ivers, J., Quock, C., Siapno, T., DeNault, S., & Hafernik, J. (2012). A new threat to honey bees, the parasitic phorid fly apocephalus borealis. *PLoS ONE*, *7*(1).

Corlett, R. (2004). Flower visitors and pollination in the Oriental (Indomalayan) Region. *Biological Reviews*. Retrieved from http://journals.cambridge.org/abstract_S1464793103006341.

Cousins, S. A. O., & Eriksson, O. (2002). The influence of management history and habitat on plant species richness in a rural hemiboreal landscape, Sweden. *Landscape Ecology*, *17*(6), 517–529.

Couvillon, M. J., Wenseleers, T., Imperatriz-Fonseca, V. L., Nogueira-Neto, P., & Ratnieks, F. L. W. (2008). Comparative study in stingless bees (Meliponini) demonstrates that nest entrance size predicts traffic and defensivity. *Journal of Evolutionary Biology*, *21*, 194–201. http://doi.org/10.1111/j.1420-9101.2007.01457.x

Cruz López, L., Patricio, E. F. L. R. A., Maile, R., & Morgan, E. D. (2002). Secretions of stingless bees: Cephalic secretions of two Frieseomelitta species. *Journal of Insect Physiology*, *48*(4), 453–458.

D'Ettorre, P., & Heinze, J. (2004). Does she smell like a queen? Chemoreception of a cuticular hydrocarbon signal in the ant Pachycondyla inversa. Retrieved from http://jeb.biologists.org/content/207/7/1085.

Dauber, J. (2003). Landscape structure as an indicator of biodiversity: matrix effects on species richness. *Agriculture, Ecosystems & Environment*, *98*(1–3), 321–329. http://doi.org/10.1016/S0167-8809(03)00092-6

De Menezes Pedro, S. R. (2014). The stingless bee fauna in Brazil (Hymenoptera: Apidae). *Sociobiology*, *61*(4). http://doi.org/10.13102/sociobiology.v61i4.348-354.

De Palma, A., Kuhlmann, M., Roberts, S. P. M., Potts, S. G., Börger, L., Hudson, L. N.,&

Purvis, A. (2015). Ecological traits affect the sensitivity of bees to land-use pressures in European agricultural landscapes. *Journal of Applied Ecology*, *52*(6). http://doi.org/10.1111/1365-2664.12524

Diekötter, T., & Crist, T. O. (2013). Quantifying habitat-specific contributions to insect diversity in agricultural mosaic landscapes. *Insect Conservation and Diversity*, *6*(5), 607–618.

Dos Santos, C. F., Acosta, A. L., Dorneles, A. L., Dos Santos, P. D. S., & Blochtein, B. (2016). Queens become workers: pesticides alter caste differentiation in bees. *Scientific Reports*, *6*. http://doi.org/10.1038/srep31605.

Dos Santos, G. M., & Antonini, Y. (2008). The traditional knowledge on stingless bees (Apidae: Meliponina) used by the Enawene-Nawe tribe in western Brazil. *Journal of Ethnobiology and Ethnomedicine*, (*4)*, pp19.

Duangphakdee, O., Koeniger, N., Deowanish, S., Hepburn, H. R., & Wongsiri, S. (2009). Ant repellent resins of honeybees and stingless bees. *Insectes Sociaux*, *56*(4), pp 333–339.

Eardley, C. D. (2004). Taxonomic revision of the African stingless bees (Apoidea : Apidae : Apinae : Meliponini). *African Plant Protection*, *10*(2), 63–69.

Eardley, C. D., Kuhlmann, M., & Pauly, a. (2010). The Bee Genera and Subgenera of sub-Saharan Africa. *ABC Taxa*, (*7)*,pp 1–138.

Eardley, C., & Kwapong, P. (2013). Taxonomy as a tool for conservation of African stingless bees and their honey. *Pot-Honey*. Retrieved from http://link.springer.com/chapter/10.1007/978-1-4614-4960-7_18

El-Sayed, A. (2012). The Pherobase: Database of Pheromones and Semiochemicals.

Ellis, J. D., & Hepburn, H. R. (2006). An ecological digest of the small hive beetle (*Aethina tumida*), a symbiont in honey bee colonies (*Apis mellifera*). *Insectes Sociaux, 89*(7), pp326–328.

Ellis, J., Pirk, C., Hepburn, H., Kastberger, G., & Elzen, P. (2002). Small hive beetles survive in honeybee prisons by behavioural mimicry. *Naturwissenschaften*, *22*(3), pp 46–58.

Eltz, T., Brühl, C. A., Imiyabir, Z., & Linsenmair, K. E. (2003). Nesting and nest trees of stingless bees (Apidae: Meliponini) in lowland dipterocarp forests in Sabah,

Malaysia, with implications for forest management. *Forest Ecology and Management*, *172*(2–3), pp.301–313.

Eltz, T., Brühl, C. A., van der Kaars, S., Chey, V. K., & Linsenmair, K. E. (2001). Pollen foraging and resource partitioning of stingless bees in relation to flowering dynamics in a Southeast Asian tropical rainforest. *Insectes Sociaux*, *21*(6), pp 65–73.

Engel, M. S., & Michener, C. D. (2013). Geological History of the Stingless Bees (Apidae: Meliponini). In *Stingless bees process honey and pollen in cerumen pots* pp. 1–7.

Engels, E., Engels, W., Schder, W., & Francke, W. (1987). Intranidal worker reactions to volatile compounds identified from cephalic secretions in the stingless bee,*Scaptotrigona postica* (Hymenoptera, Meliponinae). *Journal of Chemical Ecology*, *13*(2), pp.371–386. http://doi.org/10.1007/BF01025897

Evans, T., Inta, R., Lai, J., & Lenz, M. (2007). Foraging vibration signals attract foragers and identify food size in the drywood termite, Cryptotermes secundus. *Insectes Sociaux*. Retrieved from http://link.springer.com/article/10.1007/s00040-007-0958-1

Ewers, R. M., & Didham, R. K. (2006). Confounding factors in the detection of species responses to habitat fragmentation. *Biological Reviews of the Cambridge Philosophical Society*, *81*(1), pp.117–142.

Eyer, M., Chen, Y. P., Schafer, M. O., Pettis, J. S., & Neumann, P. (2009). Small hive beetle , Aethina tumida , as a potential biological vector of honeybee viruses. *Apidologie*, *40*, pp. 419–428.

Fahrig, L. (2003). Effects of Habitat Fragmentation on Biodiversity. *Annual Review of Ecology, Evolution, and Systematics*, *34*, pp. 487–515. Retrieved from http://www.jstor.org/stable/30033784

Farina, W. M. (1996). Food-exchange by foragers in the hive - a means of communication among honey bees? *Behavioral Ecology and Sociobiology*.

Ferreira-Caliman, M., & Nascimento, F. (2010). The cuticular hydrocarbons profiles in the stingless bee Melipona marginata reflect task-related differences. *Journal of Insect Biology,32 (3):21-29.* Retrieved from http://www.sciencedirect.com/science/article/pii/S0022191010000363

Foley, J. A., DeFries, R., Asner, G. P., Barford, C., Bonan, G., Carpenter, S. R, &

Snyder, P. K. (2005). Global Consequences of Land Use. *Science*, *309*(5734).

Franck, P., Cameron, E., Good, G., Rasplus, J. Y., & Oldroyd, B. P. (2004). Nest architecture and genetic differentiation in a species complex of Australian stingless bees. *Molecular Ecology*, *13*(8), pp. 2317–2331.

Francoy, T., Franco, F. de F., & Roubik, D. (2012). Integrated landmark and outline-based morphometric methods efficiently distinguish species of Euglossa (Hymenoptera, Apidae, Euglossini). *Apidologie*. Retrieved from http://link.springer.com/article/10.1007/s13592-012-0132-2

Francoy, T. M., Bonatti, V., Viraktamath, S., & Rajankar, B. R. (2016). Wing morphometrics indicates the existence of two distinct phenotypic clusters within population of Tetragonula iridipennis (Apidae: Meliponini) from India. *Insectes Sociaux*, *63*(1), pp.109–115. http://doi.org/10.1007/s00040-015-0442-2

Francoy, T. M., Grassi, M. L., Imperatriz-Fonseca, V. L., De Jesús May-Itzá, W., & Quezada-Euán, J. J. G. (2011). Geometric morphometrics of the wing as a tool for assigning genetic lineages and geographic origin to Melipona beecheii (Hymenoptera: Meliponini). *Apidologie*, *42*, pp. 499–507. http://doi.org/10.1007/s13592-011-0013-0

Francoy, T. M., Silva, R. A. O., Nunes-Silva, P., Menezes, C., & Imperatriz-Fonseca, V. L. (2009). Gender identification of five genera of stingless bees (Apidae, Meliponini) based on wing morphology. *Genetics and Molecular Research*, *8*(1), pp. 207–214.

Francoy, T., Wittmann, D., & Drauschke, M. (2008). Identification of Africanized honey bees through wing morphometrics: two fast and efficient procedures. *Apidologie*. Retrieved from http://link.springer.com/article/10.1051/apido:2008028

Frankie, G. W., Thorp, R. W., Newstrom-Lloyd, L. E., Rizzardi, M. A., Barthell, J. F., Griswold, T. L, Kappagoda, S. (1998). Monitoring solitary bees in modified wildland habitats: Implications for bee ecology and conservation . *Environmental Entomology*, *27*(5), pp. 1137–1147.

Frund, J., Dormann, C. F., Holzschuh, A., & Tscharntke, T. (2013). Bee diversity effects on pollination depend on functional complementarity and niche shifts. *Ecology*, *94*(9). http://doi.org/10.1890/12-1620.1

García, D., & Martínez, D. (2012). Species richness matters for the quality of ecosystem services: a test using seed dispersal by frugivorous birds. *Journal of the Royal Society of London, 37*(4), pp. 65–72.

Garibaldi, L. A., Bartomeus, I., Bommarco, R., Klein, A. M., Cunningham, S. A., Aizen, M. A., & Woyciechowski, M. (2015). Trait matching of flower visitors and crops predicts fruit set better than trait diversity. *Journal of Applied Ecology*. http://doi.org/10.1111/1365-2664.12530

Gastauer, M., Campos, L. A. O., & Wittmann, D. (2013). Handling sticky Resin by Stingless Bees : Adhesive Properties of Surface Structures, *85*, pp. 1189–1196.

Gathmann, A., & Tscharntke, T. (2002). Foraging ranges of solitary bees. *Journal of Animal Ecology*, *71*(5), pp. 757–764.

Genersch, E. (2010). Honey bee pathology: Current threats to honey bees and beekeeping. *Applied Microbiology and Biotechnology*.

Giannini, T. C., Cordeiro, G. D., Freitas, B. M., Saraiva, a. M., & Imperatriz-Fonseca, V. L. (2015). The Dependence of Crops for Pollinators and the Economic Value of Pollination in Brazil. *Journal of Economic Entomology*. http://doi.org/10.1093/jee/tov093.

Giannini, T. C., Garibaldi, L. A., Acosta, A. L., Silva, J. S., Maia, K. P., Saraiva, A. M., … Kleinert, A. M. P. (2015). Native and non-native supergeneralist bee species have different effects on plant-bee networks. *PLoS ONE*, *10*(9). http://doi.org/10.1371/journal.pone.0137198.

Gibbs, J. P., & Stanton, E. J. (2001). Habitat fragmentation and arthropod community change: Carrion beetles, phoretic mites, and flies. *Ecological Applications*, *11*(1), pp. 79–85.

Gikungu, M. W. (2006). Bee Diversity and some Aspects of their Ecological Interactions with Plants in a Successional Tropical Community, pp.1–201.

Gill, R. J., Baldock, K. C. R., Brown, M. J. F., Cresswell, J. E., Dicks, L. V., Fountain, M. T, Potts, S. G. (2016). Protecting an Ecosystem Service: Approaches to Understanding and Mitigating Threats to Wild Insect Pollinators. In *Advances in Ecological Research* (Vol. 54). http://doi.org/10.1016/bs.aecr.2015.10.007.

Githiru, M., & Lens, L. (2007). Application of fragmentation research to conservation

planning for multiple stakeholders: An example from the Taita Hills, southeast Kenya. *Biological Conservation*, *134*(2), 271–278.

Githiru, M., Lens, L., Adriaensen, F., Mwang'ombe, J., & Matthysen, E. (2011). Using science to guide conservation: From landscape modelling to increased connectivity in the Taita Hills, SE Kenya. *Journal for Nature Conservation*, *19*(5), 263–268.

Goulson, D., Hughes, W. O. H., Derwent, L. C., & Stout, J. C. (2002). Colony growth of the bumblebee, Bombus terrestris, in improved and conventional agricultural and suburban habitats. *Oecologia*, *130*(2), 267–273.

Goulson, D., Lye, G. C., & Darvill, B. (2008). Decline and conservation of bumble bees. *Annual Review of Entomology*, *53*, 191–208.

Goulson, D., Nicholls, E., Botías, C., & Rotheray, E. L. (2015). Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science*, *347*(6229). http://doi.org/10.1126/science.1255957.

Goulson, D., Stout, J. C., Langley, J., & Hughes, W. O. H. (2000). Identity and function of scent marks deposited by foraging bumblebees. *Journal of Chemical Ecology*, *26*(12).

Graham, J. R., Ellis, J. D., Carroll, M. J., & Teal, P. E. A. (2011). Aethina tumida (Coleoptera: Nitidulidae) attraction to volatiles produced by *Apis mellifera* (Hymenoptera: Apidae) and *Bombus impatiens* (Hymenoptera: Apidae) colonies. *Apidologie*, *42*(3), 326–336. http://doi.org/10.1007/s13592-011-0017-9.

Greco, M. K., Hoffmann, D., Dollin, A., Duncan, M., Spooner-Hart, R., & Neumann, P. (2010). The alternative Pharaoh approach: Stingless bees mummify beetle parasites alive. *Naturwissenschaften*, *97*(3), pp. 319–323.

Guerrieri, F., & d'Ettorre, P. (2008). The mandible opening response: quantifying aggression elicited by chemical cues in ants. *Journal of Experimental Biology*. Retrieved from http://jeb.biologists.org/content/211/7/1109.

Hadley, A. S., & Betts, M. G. (2012). The effects of landscape fragmentation on pollination dynamics: Absence of evidence not evidence of absence. *Biological Reviews*, *87*, pp. 526–544. http://doi.org/10.1111/j.1469-185X.2011.00205.

Halcroft, M., Spooner-Hart, R., & Neumann, P. (2011). Behavioral defense strategies of the stingless bee, Austroplebeia australis, against the small hive beetle, *Aethina*

tumida. *Insectes Sociaux*, *58*(2), 245–253.

Halcroft, M. T. (2007). Investigations on the behaviour of the Australian native bee , *Austroplebeia australis* , with particular reference to its defence strategies against the African small hive beetle , *Aethina tumida*, pp.14-23.

Hardman, C. J., Norris, K., Nevard, T. D., Hughes, B., & Potts, S. G. (2016). Delivery of floral resources and pollination services on farmland under three different wildlife-friendly schemes. *Agriculture, Ecosystems and Environment*, *220*. http://doi.org/10.1016/j.agee.2016.01.015

Hargis, C. D., Bissonette, J. A., & Turner, D. L. (1999). The influence of forest fragmentation and landscape pattern on American martens. *Journal of Applied Ecology*, *36*(1), pp. 157–172.

Harrison, D. J., Katnik, D. D., & Elowe, K. D. (1999). Habitat selection by martens: landscape implications for a forest specialist. *NCASI Technical Bulletin*, 224.

Hart, A. G., & Ratnieks, F. L. W. (2002). Task-partitioned nectar transfer in stingless bees: Work organisation in a phylogenetic context. *Ecological Entomology*, *27*(2), pp. 163–168.

Hartfelder, K., & Makert, G. R.(2006). Physiological and genetic mechanisms underlying caste development , reproduction and division of labor in stingless bees. *Apidologie*, *37*, pp. 144–163.

Hartlieb, E., & Anderson, P. (1999). Olfactory-Released Behaviours. In *Insect Olfaction* (pp.315–349). Berlin, Heidelberg: Springer Berlin Heidelberg. http://doi.org/10.1007/978-3-662-07911-9_12

Hatfield, R. G., & LeBuhn, G. (2007). Patch and landscape factors shape community assemblage of bumble bees, *Bombus spp*. (Hymenoptera: Apidae), in montane meadows. *Biological Conservation*, *139*(1–2), pp. 150–158.

Heard, T. (1999). The role of stingless bees in crop pollination. *Annual Review of Entomology*. Retrieved from http://www.annualreviews.org/doi/abs/10.1146/annurev.ento.44.1.183

Heard, T. (2000). Could Aussie stingless bees pollinate avocado? Secrets from the jungles of Central America. *Aussie Bee*, pp. 14-16.

Heard, T. (2001). Stingless bees and crop pollination. *Bee World*, *82*(2), pp.110–112.

Heard, T. A. (1994). Behaviour and pollinator efficiency of stingless bees and honey bees on macadamia flowers. *Journal Of Apicultural Research*, *33*(4), pp.191–198.

Heard, T. A. (1999). The role of stingless bees in crop pollination. *Annu. Rev. Entomol*, *44*, pp.183–206.

Hendrickx, F., Maelfait, J. P., Van Wingerden, W., Schweiger, O., Speelmans, M., Aviron, S., Bugter, R. (2007). How landscape structure, land-use intensity and habitat diversity affect components of total arthropod diversity in agricultural landscapes. *Journal of Applied Ecology*, *44*(2), pp. 340–351.

Henske, J., Krausa, K., Hager, F. A., Nkoba, K., & Kirchner, W. H. (2015). Olfactory associative learning in two African stingless bee species (Meliponula ferruginea and M. bocandei, Meliponini). *Insectes Sociaux*, *62*(4), pp.507–516. http://doi.org/10.1007/s00040-015-0430-6

Hermunen, T. (2004). *Land use policy in Kenya–experiences from Taita Taveta district*. *University of Helsinki, Department of Geography*. University of Helsinki. Retrieved from http://www.helsinki.fi/geo/research/publications/Taita_Hills_publications/Reports _and_papers/Expedition Reports of Department of Geography 2004/Hermunen_Land_use_policy.

Hoffmann, D., Pettis, J. S., & Neumann, P. (2008). Potential host shift of the small hive beetle (Aethina tumida) to bumblebee colonies (Bombus impatiens). *Insectes Sociaux*, *55*(2), pp. 153–162.

Hölldobler, B., & Palmer, J. M. (1989). Footprint glands in *Amblyopone australis* (Formicidae, Ponerinae). *Psyche*, *96*, pp. 111–122. http://doi.org/10.1109/IEMBS.2010.5627469

Hölldobler, B., & Wilson, E. (1990). *The ants*. Retrieved from https://books.google.com/books.

Holzschuh, A., Steffan-Dewenter, I., & Tscharntke, T. (2010). How do landscape composition and configuration, organic farming and fallow strips affect the diversity of bees, wasps and their parasitoids? *Journal of Animal Ecology*, *79*(2), pp. 491–500.

Howard, R., & Blomquist, G. (2005). Ecological, behavioral, and biochemical aspects of

insect hydrocarbons. *Annual Review of Entomology* Retrieved from http://www.annualreviews.org/doi/abs/10.1146/annurev.ento.50.071803.130359.

Hrncir, M. (2009). Mobilizing the foraging force: mechanical signals in stingless bee recruitment. *Food Exploitation by Social Insects. Journal of Ecological Science, 11*(3), pp. 72–80.

Hrncir, M., Jarau, S., & Barth, F. G. (2016). Stingless bees (Meliponini): senses and behavior. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology*. http://doi.org/10.1007/s00359-016-1117-9.

Hrncir, M., Jarau, S., Zucchi, R., & Barth, F. (2004). On the origin and properties of scent marks deposited at the food source by a stingless bee, *Melipona seminigra*. *Apidologie*. Retrieved from https://hal.archives-ouvertes.fr/hal-00891875/

Hrncir, M., Jarau, S., Zucchi, R., & Barth, F. G. (2000). Recruitment behavior in stingless bees, *Melipona scutellaris* and *M. quadrifasciata* . II. Possible mechanisms of communication. *Apidologie, 31*(1), pp. 93–113. Retrieved from https://hal.archives-ouvertes.fr/hal-00891700/

Huang, Q., & Wang, W. (2008). Studies on feeding and trophallaxis in the subterranean termite Odontotermes formosanus using rubidium chloride. Retrieved from http://onlinelibrary.wiley.com/doi/10.1111/j.1570-7458.2008.00764.x/full

Hudewenz, A., & Klein, A. M. (2013). Competition between honey bees and wild bees and the role of nesting resources in a nature reserve. *Journal of Insect Conservation, 17*(6), pp. 1275–1283.

Huelsenbeck, J., & Ronquist, F. (2000). MrBayes: Bayesian inferences of phylogeny (software). *University of Rochester, New York*.

Huelsenbeck, J., & Ronquist, F. (2005). Bayesian analysis of molecular evolution using MrBayes. *Statistical Methods in Molecular Evolution*. Retrieved from http://link.springer.com/chapter/10.1007/0-387-27733-1_7.

Huelsenbeck, J., Ronquist, F., & Hall, B. (2003). MrBayes: a program for the Bayesian inference of phylogeny. Version 3.0.

Human, H., & Nicolson, S. W. (2006). Nutritional content of fresh, bee-collected and stored pollen of Aloe greatheadii var. davyana (Asphodelaceae). *Phytochemistry, 67*, pp. 1486–1492. http://doi.org/10.1016/j.phytochem.2006.05.023

Hurtado-Burillo, M., Ruiz, C., De Jesús May-Itzá, W., Quezada-Eúan, J. J. G., & De La Rúa, P. (2013). Barcoding stingless bees: Genetic diversity of the economically important genus Scaptotrigona in Mesoamerica. *Apidologie*, *44*(1), pp. 1–10.

Iesmeijer, J. C. B., & Laa, E. J. S. (2004). Review article Information flow and organization of stingless bee foraging. *Apidologie*, *35*(2), pp.143–157. Retrieved from http://www.edpsciences.org/10.1051/apido:2004003

Imperatriz-fonseca, V. L., Saraiva, A. M., & Jong, D. De. (2006). *Bees as pollinators in Brazil. Ribeirão Preto*. Retrieved from http://scholar.google.com/scholar.

Jackson, B. D., & Morgan, E. D. (1993). Insect chemical communication: Pheromones and exocrine glands of ants. *Chemoecology*, *4*(3), pp.125–144. http://doi.org/10.1007/BF01256548

Jaff, R., Castilla, A., Pope, N., Imperatriz-Fonseca, V. L., Metzger, J. P., Arias, M. C., & Jha, S. (2016). Landscape genetics of a tropical rescue pollinator. *Conservation Genetics*, *17*(2). http://doi.org/10.1007/s10592-015-0779-0.

Jaffé, R., Pioker-Hara, F. C., Dos Santos, C. F., Santiago, L. R., Alves, D. A., De M. P. Kleinert, A., & Imperatriz-Fonseca, V. L. (2014). Monogamy in large bee societies: A stingless paradox. *Naturwissenschaften*, *101*(3). http://doi.org/10.1007/s00114-014-1149-3.

James, C. (2004). Recruitment communication in stingless bees (Hymenoptera, Apidae, Meliponini). *Apidologie*. Retrieved from http://www.apidologie.org/index.php?option=com_article&access=standard&Itemid=129&url=/articles/apido/pdf/2004/02/M4207.pdf

Jarau, S. (2009). Chemical communication during food exploitation in stingless bees. *Food Exploitation by Social Insects: Journal of Chemical Ecology*, *20*(4), 11–23.

Jarau, S., Dambacher, J., & Twele, R. (2010). The trail pheromone of a stingless bee, *Trigona corvina* (Hymenoptera, Apidae, Meliponini), varies between populations. Retrieved from http://chemse.oxfordjournals.org/content/early/2010/06/09/chemse.bjq057.

Jarau, S., Hrncir, M., Ayasse, M., Schulz, C., Francke, W., Zucchi, R., & Barth, F. G. (2004). A Stingless Bee (Melipona seminigra) Marks Food Sources with a Pheromone from Its Claw Retractor Tendons. *Journal of Chemical Ecology*, *30*(4),

pp. 793–804. http://doi.org/10.1023/B:JOEC.0000028432.29759.ed

Jarau, S., Hrncir, M., Schmidt, V. M., Zucchi, R., & Barth, F. G. (2003a). Effectiveness of recruitment behavior in stingless bees (Apidae, Meliponini). *Insectes Sociaux*, *50*(4), pp. 365–374. http://doi.org/10.1007/s00040-003-0684-2.

Jarau, S., Hrncir, M., Schmidt, V. M., Zucchi, R., & Barth, F. G. (2003b). Effectiveness of recruitment behavior in stingless bees (Apidae, Meliponini). *Insectes Sociaux*, *50*(4), 365–374.

Jarau, S., Hrncir, M., Schmidt, V., Zucchi, R., & Barth, F. (2003). Effectiveness of recruitment behavior in stingless bees (Apidae, Meliponini). *Insectes Sociaux*. Retrieved from http://link.springer.com/article/10.1007/s00040-003-0684-2.

Jarau, S., Hrncir, M., Zucchi, R., & Barth, F. (2004). A stingless bee uses labial gland secretions for scent trail communication (Trigona recursa Smith 1863). *Journal of Comparative Physiology*. Retrieved from http://link.springer.com/article/10.1007/s00359-003-0489-9.

Jarau, S., Hrncir, M., Zucchi, R., & Barth, F. G. (2000). Recruitment behavior in stingless bees, *Melipona scutellaris* and *M . quadrifasciata*. Foraging at food sources differing in direction and distance. *Apidologie*, *31*, pp.81–91.

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: Neuroethology, Sensory, Neural, and Behavioral Physiology*, *190*(3), pp. 233–239.

Jarau, S., Schulz, C. M., Hrncir, M., Francke, W., Zucchi, R., Barth, F. G.,and Zucchi, R. (2006). Hexyl Decanoate, the First Trail Pheromone Compound Identified in a Stingless Bee, *Trigona recursa*. *J Chem Ecol*, *32*, pp. 1555–1564. http://doi.org/10.1007/s10886-006-9069-0

Jarau, S., Žáček, P., Šobotník, J., Vrkoslav, V., Hadravová, R., Coppée, A., and Valterová, I. (2012). Leg tendon glands in male bumblebees (Bombus terrestris): Structure, secretion chemistry, and possible functions. *Naturwissenschaften*, *99*(12), pp. 1039–1049.

Jauker, F., Diekötter, T., Schwarzbach, F., & Wolters, V. (2009). Pollinator dispersal in an agricultural matrix: Opposing responses of wild bees and hoverflies to landscape

structure and distance from main habitat. *Landscape Ecology*, *24*(4), pp. 547–555.

Jha, S., & Kremen, C. (2013). Resource diversity and landscape-level homogeneity drive native bee foraging. *Proceedings of the National Academy of Sciences*, *110*(2). http://doi.org/10.1073/pnas.1208682110.

Johnson, L. K., Haynes, L. W., Carlson, M. A., Fortnum, H. A., & Gorgas, D. L. (1985). Alarm substances of the stingless bee,*Trigona silvestriana*. *Journal of Chemical Ecology*, *11*(4), 409–416. http://doi.org/10.1007/BF00989552.

Joseph K. Macharia, Suresh Kumar Raina, and E. M. M. (2010). Stingless beekeeping: an incentive for rain forest conservation in Kenya. *Ecosystem Based Management: Beyond Boundaries. Proceedings of the Sixth International Conference of Science and the Management of Protected Areas, 21–26 May 2007, Acadia University, Wolfville, Nova Scotia.* (pp. 514–518).

Judith Slaa, E., Alejandro Sánchez Chaves, L., Sampaio Malagodi-Braga, K., & Elisabeth Hofstede, F. (2006). Stingless bees in applied pollination: practice and perspectives. *Apidologie*, *37*(2), 293–315. Retrieved from https://hal.archives-ouvertes.fr/hal-00892203.

Jungnickel, H., & Costa, A. Da. (2004). Chemical basis for inter-colonial aggression in the stingless bee *Scaptotrigona bipunctata* (Hymenoptera: Apidae). Retrieved from http://www.sciencedirect.com/science/article/pii/S0022191004000988.

Kajobe, R. (2007). Pollen foraging by *Apis mellifera* and stingless bees *Meliponula bocandei* and *Meliponula nebulata* in Bwindi Impenetrable National Park, Uganda. *African Journal of Ecology*, *45*(3), 265–274.

Kajobe, R. (2008). Foraging Behaviour of Equatorial Afrotropical Stingless Bees: Habitat Selection and Competition for Resources. Retrieved from http://igitur-archive.library.uu.nl/dissertations/2008-0605-200535/UUindex.html.

Kajobe, R., & Echazarreta, C. M. (2005). Temporal resource partitioning and climatological influences on colony flight and foraging of stingless bees (Apidae; Meliponini) in Ugandan tropical forests. In *African Journal of Ecology* (Vol. 43, pp. 267–275).

Kaluza, B. F., Wallace, H., Heard, T. A., Klein, A. M., & Leonhardt, S. D. (2016). Urban gardens promote bee foraging over natural habitats and plantations. *Ecology and Evolution*, *6*(5). http://doi.org/10.1002/ece3.1941.

Karikari, A., & Kwapong, P. (2007). A survey of indigenous knowledge of stingless bees (Apidae meliponini) in the Central region of Ghana. *Journal of the Ghana Science Association*. Retrieved from http://www.africabib.org/rec.php?RID=Q00046283

Katzerke, A., Neumann, P., & Pirk, C. (2006). Seasonal nestmate recognition in the ant *Formica exsecta*. Retrieved from http://link.springer.com/article/10.1007/s00265-006-0245-6.

Kearns, C., Inouye, D., & Waser, N. (1998). Endangered mutualisms: the conservation of plant-pollinator interactions. *Annual Review of Ecology and*. Retrieved from http://www.jstor.org/stable/221703.

Kearse, M., Moir, R., Wilson, A., & Stones-Havas, S. (2012). Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Retrieved from http://bioinformatics.oxfordjournals.org/content/28/12/1647.

Keith N. Slessor, Mark L. Winston, and Conte, Y. (2005). Pheromone communication in the honeybee (Apis mellifera L.). *Journal of Chemical Ecology*, *31(11),pp.2731-2745.* http://doi.org/DOI: 10.1007/s10886-005-7623-9.

Kelly, N., Farisya, M. S. N., Kumara, T. K., & Marcela, P. (2014). Species diversity and external nest characteristics of stingless bees in meliponiculture. *Pertanika Journal of Tropical Agricultural Science*, *37*(3), pp. 293–298.

Kennedy, C. M., Lonsdorf, E., Neel, M. C., Williams, N. M., Ricketts, T. H., Winfree, R., … Kremen, C. (2013). A global quantitative synthesis of local and landscape effects on wild bee pollinators in agroecosystems. *Ecology Letters*, *16*(5). http://doi.org/10.1111/ele.12082.

Kerr, W. (1969). Some aspects of the evolution of social bees (Apidae), *Journal of Tropical Agricultural Science*, *21*(6), 19–32.

Kerr, W., Ferreira, A., & Mattos, N.S.D (1963). Communication among stingless bees-additional data (Hymenoptera: Apidae). *Journal of the New York Entomological Society, pp. 80-90.* Retrieved from http://www.jstor.org/stable/25005852.

Kerr, W., Jungnickel, H., & Morgan, E.D (2004). Workers of the stingless bee *Melipona scutellaris* are more similar to males than to queens in their cuticular compounds. *Apidologie*, 35(6), pp. 611-618.

Kindt, R., & Coe, R. (2010). Tree diversity analysis. *Training*, *18*, 18598, pp. 614. http://doi.org/10.1198/tas.2008.s264

Kleijn, D., Winfree, R., Bartomeus, I., Carvalheiro, L. G., Henry, M., Isaacs, R., Potts, S. G. (2015). Delivery of crop pollination services is an insufficient argument for wild pollinator conservation. *Nature Communications*, http://doi.org/10.1038/ncomms8414.

Klein, A.-M., Steffan-Dewenter, I., & Tscharntke, T. (2003). Fruit set of highland coffee increases with the diversity of pollinating bees. *Proceedings. Biological Sciences / The Royal Society*, *270*, pp. 955–961. http://doi.org/10.1098/rspb.2002.2306.

Klein, A.-M., Vaissière, B. E., Cane, J. H., Steffan-Dewenter, I., Cunningham, S. A., Kremen, C., & Tscharntke, T. (2007). Importance of pollinators in changing landscapes for world crops. *Proceedings. Biological Sciences / The Royal Society*, *274*(1608),pp. 303–313.

Klein, A. M., Steffan-Dewenter, I., Buchori, D., & Tscharntke, T. (2002). Effects of land-use intensity in tropical agroforestry systems on coffee flower-visiting and trap-nesting bees and wasps. *Conservation Biology*, *16*(4), pp.1003–1014.

Koch, H. (2010). Combining morphology and DNA barcoding resolves the taxonomy of western Malagasy *Liotrigona Moure*, 1961 (Hymenoptera: Apidae: Meliponini). *African Invertebrates, 112*(7), 47–52.

Kollmann, M., Rupenthal, A., & Neumann, P. (2016). Novel antennal lobe substructures revealed in the small hive beetle Aethina tumida. *Cell and Tissue*. Retrieved from http://link.springer.com/article/10.1007/s00441-015-2282-9

Kovács-Hostyánszkia, A., Elek, Z., Balázs, K., Centeri, C., Falusi, E., Jeanneret, P., & Báldi, A. (2013). Earthworms, spiders and bees as indicators of habitat quality and management in a low-input farming region - A whole farm approach. *Ecological Indicators*, *33*, 111–120.

Krauss, J. (2009). Habitat area but not habitat age determines wild bee richness in limestone quarries.*Journal of Applied Ecology*. Retrieved from

http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2664.2008.01582.x/pdf

Kremen, C., & M'Gonigle, L. K. (2015). EDITOR'S CHOICE: Small-scale restoration in intensive agricultural landscapes supports more specialized and less mobile pollinator species. *Journal of Applied Ecology*, *52*(3). http://doi.org/10.1111/1365-2664.12418.

Kremen, C., Williams, N., & Aizen, M. (2007). Pollination and other ecosystem services produced by mobile organisms: a conceptual framework for the effects of land-use change. *Ecology*. Retrieved from http://onlinelibrary.wiley.com/doi/10.1111/j.1461-0248.2007.01018.x/full.

Kuhn-Neto, B., Contrera, F. A. L., Castro, M. S., & Nieh, J. C. (2009). Long distance foraging and recruitment by a stingless bee, Melipona mandacaia*. *Apidologie*, *40*, 472–480. http://doi.org/10.1051/apido/2009007.

Kwaiser, K. S., & Hendrix, S. D. (2008). Diversity and abundance of bees (Hymenoptera: Apiformes) in native and ruderal grasslands of agriculturally dominated landscapes. *Agriculture, Ecosystems and Environment*, *124*(3–4), pp. 200–204.

Kwapong, P., Aidoo, K., Combey, R., & Karikari, A. (2010). Stingless bees: importance, management and utilization. *Unimax Macmillan Ltd. Ghana: Accra*

Lange, C. N. (2006). The endemic land snail *Gulella taitensis* of the Taita Hills forests, Kenya: on the brink of extinction. *Oryx*, *40*(3), pp. 362-364.

Legendre, P., & Gallagher, E. D. (2001). Ecologically meaningful transformations for ordination of species data. *Oecologia*, *129*(2), pp. 271–280.

Lens, L., & Van Dongen, S. (2002). Fluctuating asymmetry as a bio-indicator in isolated populations of the Taita thrush: A Bayesian perspective. *Journal of Biogeography*, *29*(5–6), pp. 809–819.

Leonhardt, S. D. (2010). Resin collection and use in stingless bees. Retrieved from opus.bibliothek.uni-wuerzburg.de

Leonhardt, S. D., & Blüthgen, N. (2009). A Sticky Affair: Resin Collection by Bornean Stingless Bees. *Biotropica*, *41*(6), pp. 730–736.

Leonhardt, S. D., Blüthgen, N., & Schmitt, T. (2009). Smelling like resin: Terpenoids account for species-specific cuticular profiles in Southeast-Asian stingless bees. *Insectes Sociaux*, *56*(2), pp.157–170.

Leonhardt, S. D., & Kaltenpoth, M. (2014). Microbial communities of three sympatric Australian stingless bee species. *PloS One*, *9*(8), pp. 105-118.

Leonhardt, S. D., Zeilhofer, S., Blüthgen, N., & Schmitt, T. (2010). Stingless Bees use terpenes as olfactory cues to find resin sources. *Chemical Senses*, *35*(7), pp. 603–611.

Leonhardt, S., Dworschak, K., Eltz, T., & Blüthgen, N. (2007). Foraging loads of stingless bees and utilisation of stored nectar for pollen harvesting. *Apidologie*, *38*, pp. 125–135. http://doi.org/10.1051/apido:2006059

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*(4), pp. 763–774.

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*(1), pp. 105–113.

Lindauer, M., & Kerr, W. (1958). Die gegenseitige Verständigung bei den stachellosen Bienen. *Zeitschrift Für Vergleichende Physiologie*. Retrieved from http://link.springer.com/article/10.1007/BF00344263

Lindauer, M., & Kerr, W. (1960). Communication between the workers of stingless bees. *Bee World*. Retrieved from http://www.tandfonline.com/doi/pdf/10.1080/0005772X.1960.11095309.

Lucas, C., & Fresneau, D. (2002). A multidisciplinary approach to discriminating different taxa in the species complex Pachycondyla villosa (Formicidae). Retrieved from http://onlinelibrary.wiley.com/doi/10.1046/j.1095-8312.2002.00017.

Maeda, E. E. (2012). The future of environmental sustainability in the Taita Hills, Kenya: Assessing potential impacts of agricultural expansion and climate change. *Fennia*, *190*(1), pp. 41–59.

Maeda, E. E., Clark, B., Pellikka, P., Siljander, M., & Vi, P. T. (2010). Driving forces of land-use change in the Taita Hills , Kenya.

Maeda, E. E., Pellikka, P. K. E., Siljander, M., & Clark, B. J. F. (2010). Potential impacts of agricultural expansion and climate change on soil erosion in the Eastern Arc Mountains of Kenya. *Geomorphology*, *123*(3–4), pp. 279–289.

Maia-Silva, C., Hrncir, M., da Silva, C. I., & Imperatriz-Fonseca, V. L. (2015). Survival strategies of stingless bees (Melipona subnitida) in an unpredictable environment, the Brazilian tropical dry forest. *Apidologie*, *46*(5). http://doi.org/10.1007/s13592-015-0354-1

Maia-Silva, C., Hrncir, M., Imperatriz-Fonseca, V. L., & Schorkopf, D. L. P. (2016). Stingless bees (*Melipona subnitida*) adjust brood production rather than foraging activity in response to changes in pollen stores. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology*, *202*, pp. 9–10. http://doi.org/10.1007/s00359-016-1095.

Malonza, P. K., Lötters, S., & Measey, G. J. (2010). The montane forest associated amphibian species of the Taita Hills, Kenya. *Journal of East African Natural History*, *99*(1), pp. 47-63.

Mant, J., Brändli, C., & Vereecken, N. (2005). Cuticular hydrocarbons as sex pheromone of the bee Colletes cunicularius and the key to its mimicry by the sexually deceptive orchid, Ophrys exaltata. *Journal of Chemical ecology.* Retrieved from http://link.springer.com/article/10.1007/s10886-005-5926-5.

Martin, S. J., Vitikainen, E., Helanterä, H., & Drijfhout, F. P. (2008). Chemical basis of nest-mate discrimination in the ant *Formica exsecta*. *Proceedings of the Royal Society of London B: Biological Sciences*, *275*(1640), pp. 1271-1278.

Martin, S., & Drijfhout, F. (2009). Nestmate and task cues are influenced and encoded differently within ant cuticular hydrocarbon profiles. *Journal of Chemical Ecology*. Retrieved from http://link.springer.com/article/10.1007/s10886-009-9612.

Martins, D. J. (2008). Pollination observations of the African violet in the Taita Hills, Kenya. *Journal of East African Natural History*, *97*(1), pp.33-42.

Martins, K. T., Gonzalez, A., & Lechowicz, M. J. (2015). Pollination services are mediated by bee functional diversity and landscape context. *Agriculture, Ecosystems and Environment*, *200*. http://doi.org/10.1016/j.agee.2014.10.018.

Masters, B., Fan, V., & Ross, H. (2011). Species delimitation–a geneious plugin for the exploration of species boundaries. *Molecular Ecology Resources*. Retrieved from http://onlinelibrary.wiley.com/doi/10.1111/j.1755-0998.2010.02896.

May-Itzá, W. de J., Quezada-Euán, J. J. G., Medina, L. A. M., Enríquez, E., & de la Rúa,

P. (2010). Morphometric and genetic differentiation in isolated populations of the endangered Mesoamerican stingless bee Melipona yucatanica (Hymenoptera: Apoidea) suggest the existence of a two species complex. *Conservation Genetics*, *11*(5), pp. 2079–2084.

Mc Cabe, S. I. M., & Farina, W. M. (2010). Olfactory learning in the stingless bee *Tetragonisca angustula* (Hymenoptera, Apidae, Meliponini). *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology*, *196*(7), pp. 481–490.

Medina, L. M., Hart, A. G., & Ratnieks, F. L. W. (2009). Hygienic behavior in the stingless bees *Melipona beecheii* and *Scaptotrigona pectoralis* (Hymenoptera: Meliponini). *Genetics and Molecular Research*, *8*(2), pp. 571–576.

Meulemeester, T. De, & Michez, D. (2012). Taxonomic affinity of halictid bee fossils (Hymenoptera: Anthophila) based on geometric morphometrics analyses of wing shape.Retrieved from http://www.tandfonline.com/doi/abs/10.1080/14772019.2011.628701.

Minckley, R. (2008). Faunal composition and species richness differences of bees (Hymenoptera : Apiformes) from two north American regions. *Apidologie*, *39*(1), pp.176-134.

Moreno, F. A., & Cardozo, A. F. (2003). Field procedures for the location and identification of stingless bees (Meliponinae). *Livestock Research for Rural Development*, *15*(2), pp. 27–30.

Morris, R. (2010). Anthropogenic impacts on tropical forest biodiversity: a network structure and ecosystem functioning perspective. Retrieved from http://rstb.royalsocietypublishing.org/content/365/1558/3709.short

Mulwa, R. K., Bennun, L. A., Ogol, C. K., & Lens, L. (2007). Population status and distribution of Taita White-eye *Zosterops silvanus* in the fragmented forests of Taita Hills and Mount Kasigau, Kenya. *Bird Conservation International*, *17*(2), pp. 141-150.

Munyuli, T. (2010). Pollinator Biodiversity and Economic Value of Pollination Services in Uganda, pp. 431.

Munyuli, T. (2012). Diversity of life-history traits, functional groups and indicator

species of bee communities from farmlands of central Uganda. *Jordan Journal of Biological Sciences, 68*(3), pp. 42–50.

Mustafa, S. G., Spiewok, S., Duncan, M., Spooner-Hart, R., & Rosenkranz, P. (2014). Susceptibility of small honey bee colonies to invasion by the small hive beetle, Aethina tumida (Coleoptera, Nitidulidae). *Journal of Applied Entomology*, *138*(7), pp. 547–550.

Mwangi, D., Kasina, M., Nderit, J., Hagen, M., Gikungu, M., & Kraemer, M. (2012). Diversity and abundance of native bees foraging on hedgerow plants in the Kakamega farmlands, western Kenya. *Journal of Apicultural Research, 13*(2), pp. 7–15.

Nagamitsu, T., & Inoue, T. (1997). Aggressive foraging of social bees as a mechanism of floral resource partitioning in an asian tropical rainforest. *Oecologia*, *110*(3), pp. 432–439.

Nagamitsu, T., & Inoue, T. (2005). Floral resource utilization by stingless bees (Apidae, Meliponini). *Pollination Ecology and the Rain Forest*, pp. 73–88.

Neumann, P., & Elzen, P. (2004). The biology of the small hive beetle (*Aethina tumida*, Coleoptera: Nitidulidae): Gaps in our knowledge of an invasive species. *Apidologie*, *35*, pp. 229–247.

Nicolson, S. W. (2011). Bee Food: The Chemistry and Nutritional Value of Nectar, Pollen and Mixtures of the Two. *African Zoology*, (*46*), pp. 197–204. http://doi.org/10.3377/004.046.0201

Nieh, J. C. (2004). Recruitment communication in stingless bees (Hymenoptera, Apidae, Meliponini). *Apidologie*, (*35)*, pp.159–182.

Nieh, J. C., Barreto, L. S., Contrera, F. A. L., & Imperatriz-Fonseca, V. L. (2004). Olfactory eavesdropping by a competitively foraging stingless bee, Trigona spinipes. *Proceedings. Biological Sciences / The Royal Society*, (*271)*,pp. 1633–1640.

Nieh, J. C., Contrera, F. A. L., & Nogueira-Neto, P. (2003). Pulsed mass recruitment by a stingless bee, Trigona hyalinata. *Proceedings. Biological Sciences / The Royal Society*, *270*(1529), pp. 2191–2196.

Nieh, J. C., Contrera, F. A. L., Ramírez, S., & Imperatriz–Fonseca, V. L. (2003).

Variation in the ability to communicate three-dimensional resource location by stingless bees from different habitats. *Animal Behaviour*, *66*(6), pp. 1129–1139.

Nieh, J. C., & Roubik, D. W. (1995). A stingless bee (*Melipona panamica*) indicates food location without using a scent trail. *Behavioral Ecology and Sociobiology*, *37*(1), pp. 63–70.

Nieh, J. C., Contrera, F. A., Yoon, R. R., Barreto, L. S., & Imperatriz-Fonseca, V. L. (2004). Polarized short odor-trail recruitment communication by a stingless bee, *Trigona spinipes*. *Behavioral Ecology and Sociobiology*, *56*(5), pp. 435-448.

Nielsen, A., Steffan-Dewenter, I., Westphal, C., Messinger, O., Potts, S. G., Roberts, S. P. M., & Petanidou, T. (2011). Assessing bee species richness in two Mediterranean communities: Importance of habitat type and sampling techniques. *Ecological Research*, *26*(5), pp. 969–983.

Nogueira, J., Ramos, J. de C., Benevenuto, J., Fernandes-Salomão, T. M., Resende, H. C., Campos, L. A. de O., & Tavares, M. G. (2014). Conservation study of an endangered stingless bee (Melipona capixaba-Hymenoptera: Apidae) with restricted distribution in Brazil. *Journal of Insect Conservation*, *18*(3). http://doi.org/10.1007/s10841-014-9639-3.

Nunes, T. M., Mateus, S., Favaris, A. P., Amaral, M. F. Z. J., von Zuben, L. G., Clososki, G. C., Lopes, N. P. (2014). Queen signals in a stingless bee: suppression of worker ovary activation and spatial distribution of active compounds. *Scientific Reports*, (*4*), pp. 74-81.

Nunes, T. M., Nascimento, F. S., Turatti, I. C., Lopes, N. P., & Zucchi, R. (2008). Nest-mate recognition in a stingless bee: does the similarity of chemical cues determine guard acceptance? *Animal Behaviour*, *75*(3), pp. 1165-1171.

Nunes, T., Turatti, I., & Mateus, S. (2009). Cuticular hydrocarbons in the stingless bee *Schwarziana quadripunctata* (Hymenoptera, Apidae, Meliponini): differences between colonies, castes and age. *Genet. Mol. 17*(5), pp.55–61.

Ogol, C., Kioko, E., Gikungu, M., & Museums, N. (2013). Bee diversity and floral resources along a disturbance gradient, coastal Kenya.

Omoro, L. M. A., Pellikka, P. K. E., & Rogers, P. C. (2010). Tree species diversity, richness, and similarity between exotic and indigenous forests in the cloud forests of

Eastern Arc Mountains, Taita Hills, Kenya. *Journal of Forestry Research*, *21*(3), pp. 255–264.

Patricio, E. F. L. R. A., Cruz-López, L., Maile, R., Tentschert, J., Jones, G. R., & Morgan, E. D. (2002). The propolis of stingless bees: Terpenes from the tibia of three Frieseomelitta species. *Journal of Insect Physiology*, *48*(2), pp. 249–254.

Pellikka, P. K. E., Clark, B. J. F., Gosa, A. G., Himberg, N., Hurskainen, P., Maeda, E., … Siljander, M. (2013). Agricultural Expansion and Its Consequences in the Taita Hills, Kenya. *Developments in Earth Surface Processes*, *16*, pp. 165–179.

Pellikka, P. K. E., Clark, B. J. F., Sirviö, T., & Masalin, K. (2005). Environmental change monitoring applying satellite and airborne remote sensing data in the Taita Hills , Kenya. *Area*, pp. 223–232.

Pereboom, J. J. M., & Biesmeijer, J. C. (2003). Thermal constraints for stingless bee foragers: The importance of body size and coloration. *Oecologia*, *137*(1),pp. 42–50.

Pfeifer, M., Burgess, N. D., Swetnam, R. D., Platts, P. J., Willcock, S., & Marchant, R. (2012). Protected areas: Mixed success in conserving East Africa's evergreen forests. *PLoS ONE*, *7*(6), pp. 1–10. http://doi.org/10.1371/journal.pone.0039337

Pino, J. A., Marbot, R., Delgado, A., Zumarraga, C., & Sauri, E. (2006). Volatile constituents of propolis from honey bees and stingless bees from Yucatan. *Journal of Essential Oil Research*, *18*(1), pp. 53–56.

Pirk, C. W. W., & Neumann, P. (2013). Small Hive Beetles are Facultative Predators of Adult Honey Bees. *Journal of Insect Behavior*, *26*(6), pp. 796–803.

Platts, P. J., Ahrends, A., Gereau, R. E., McClean, C. J., Lovett, J. C., Marshall, A. R., Marchant, R. (2010). Can distribution models help refine inventory-based estimates of conservation priority? A case study in the Eastern Arc forests of Tanzania and Kenya. *Diversity and Distributions*, *16*, pp. 628–642. http://doi.org/10.1111/j.1472-4642.2010.00668.x

Posey, D. A., & Camargo, J. M. F. (1985). Additional notes on the classification and knowledge of stingless bees (Meliponinae, Apidae, Hymenoptera) by the Kayapó indians of Gorotire, Pará, Brazil. *Annals of Carnegie Museum*, *54*(8), pp. 247–274.

Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O., & Kunin, W. E. (2010). Global pollinator declines: trends, impacts and drivers. *Trends in ecology &*

evolution, *25*(6), pp. 345-353.

Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O., & Kunin, W. E. (2010b). Global pollinator declines: trends, impacts and drivers. *Trends in Ecology & Evolution*, *25*(6), pp. 345–353. http://doi.org/10.1016/j.tree.2010.01.007

Potts, S. G., Petanidou, T., Roberts, S., O'Toole, C., Hulbert, A., & Willmer, P. (2006). Plant-pollinator biodiversity and pollination services in a complex Mediterranean landscape. *Biological Conservation*, *129*(4), pp. 519–529.

Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O., & Kunin, W. E. (2010). Global pollinator declines: trends, impacts and drivers. *Trends in ecology & evolution*, *25*(6), pp. 345-353.

Rader, R., Bartomeus, I., Garibaldi, L. A., Garratt, M. P., Howlett, B. G., Winfree, R., & Bommarco, R. (2016). Non-bee insects are important contributors to global crop pollination. *Proceedings of the National Academy of Sciences*, *113*(1), pp. 146-151.

Rader, R., & Howlett, B. (2009). Alternative pollinator taxa are equally efficient but not as effective as the honeybee in a mass flowering crop.*Journal of Applied Ecology*. Retrieved from http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2664.2009.01700.

Ramalho, M., Kleinert-Giovannini, A., & Imperatriz-fonseca, V. L. (1990). Important bee plants for stingless bees (Melipona and Trigonini) and Africanized honeybees (Apis mellifera) in neotropical habitats: a review. *Apidologie*, (*21*),pp. 469–488.

Ramírez, S. R., Nieh, J. C., Quental, T. B., Roubik, D. W., Imperatriz-Fonseca, V. L., & Pierce, N. E. (2010). A molecular phylogeny of the stingless bee genus Melipona (Hymenoptera: Apidae). *Molecular Phylogenetics and Evolution*, *56*(2), pp. 519–525.

Rasmussen, C., & Cameron, S. A. (2007). A molecular phylogeny of the Old World stingless bees (Hymenoptera: Apidae: Meliponini) and the non-monophyly of the large genus *Trigona*. *Systematic Entomology*, *32*(1), pp. 26-39.

Rasmussen, C., & Cameron, S. (2010). Global stingless bee phylogeny supports ancient divergence, vicariance, and long distance dispersal. *Biological Journal of the Linnean conservation.* Retrieved from http://onlinelibrary.wiley.com/doi/10.1111/j.1095-8312.2009.01341.

Rathcke, B. J., & Jules, E. S. (1993). Habitat fragmentation and plant pollinator

interactions. *Current Science*, *65*(3), pp. 273–277.

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 Biology*. Retrieved from http://link.springer.com/article/10.1007/s00359-010-0605-6.

Reichle, C., Aguilar, I., Ayasse, M., Twele, R., Francke, W., & Jarau, S. (2013). Learnt information in species-specific "trail pheromone" communication in stingless bees. *Animal Behaviour*, *85*(1), pp. 225–232.

Reichle, C., Jarau, S., Aguilar, I., & Ayasse, M. (2010). Recruits of the stingless bee *Scaptotrigona pectoralis* learn food odors from the nest atmosphere. *Naturwissenschaften*. Retrieved from http://link.springer.com/article/10.1007/s00114-010-0662-2.

Reitalu, T., Purschke, O., Johansson, L. J., Hall, K., Sykes, M. T., & Prentice, H. C. (2012). Responses of grassland species richness to local and landscape factors depend on spatial scale and habitat specialization. *Journal of Vegetation Science*, *23*(1), pp. 41–51.

Ribeiro, M. de F., Wenseleers, T., Santos Filho, P. D. S., & Alves, D. D. A. (2006). Miniature queens in stingless bees: basic facts and evolutionary hypotheses. *Apidologie*, *37*(2), 191–206. Retrieved from http://www.edpsciences.org/10.1051/apido:2006023

Ricketts, T. H., Regetz, J., Steffan-Dewenter, I., Cunningham, S. A., Kremen, C., Bogdanski, A., Viana, B. F. (2008). Landscape effects on crop pollination services: Are there general patterns? *Ecology Letters*, *11*(5), pp. 499–515.

Robroek, B. J. M., Jong, H. De, & Sommeijer, M. J. (2003). The behaviour of the kleptoparasite, *Pseudohypocera kerteszi* (Diptera, Phoridae), in hives of stingless bees (Hymenoptera , Apidae) in Central America. *Proceedings of the Section Experimental and Applied Entomology of the Netherlands Entomological Society*, *14*, pp. 65–70.

Rohlf, F. (2004). tpsUtil, file utility program. version 1.26.*, State University of New York at Stony Brook*.

Rohlf, F. (2010). tpsRelw, version 1.49. Department of Ecology and Evolution. *State*

University of New York, Stony Brook, NY, USA.

Ronquist, F., & Mark, P. van der. (2009). Bayesian phylogenetic analysis using MrBayes.

Ronquist, F., Teslenko, M., & Mark, P. Van Der. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Entomology 11(4), pp. 23-34.*

Roubik, D. (1989). Ecology andNatural History ofTropical Bees. *Cambridge, Cambridge University, 514p.*

Roubik, D. (1992). *Ecology and natural history of tropical bees*.

Roubik, D. W. (2006a). Review article stingless bee nesting biology. *Apidologie*, *37*, 124–143. http://doi.org/10.1051/apido.

Roubik, D. W. (2006b). Stingless bee nesting biology. *Apidologie*, *42*, 114–120. http://doi.org/14.1067/apido.

Rowson, B., & Lange, C. N. (2007). Two new species of Gulella (Mollusca: Pulmonata: Streptaxidae) from the Taita Hills, Kenya. *African Invertebrates*, *48*(2), pp. 21–32.

Salminen, H. (2004). A geographic overview of Taita Hills, Kenya. In *Taita Hills and Kenya, 2004 – seminar, reports and journal of a field excursion to Kenya. Expedition reports of the Department of Geography* (pp. 31–38).

Sánchez, D., Nieh, J. C., & Vandame, R. (2011). Visual and chemical cues provide redundant information in the multimodal recruitment system of the stingless bee Scaptotrigona mexicana (Apidae, Meliponini). *Insectes Sociaux*, *58*(4), pp. 575–579. http://doi.org/10.1007/s00040-011-0181-y.

Sawaya, a C. H. F. (2009). Composition and antioxidant activity of propolis from three species of Scaptotrigona stingless bees. *Journal of ApiProduct and ApiMedical Science*, *1*(2), 37–42. http://doi.org/10.3896/IBRA.4.01.2.03.

Schmidt, V., Zucchi, R., & Barth, F. (2003). A stingless bee marks the feeding site in addition to the scent path (Scaptotrigona aff. depilis). *Apidologie*. Retrieved from http://www.apidologie.org/index.php?option=com_article&access=standard&Itemid=129&url=/articles/apido/pdf/2003/03/M3307.

Schorkopf, D. L. P., Hrncir, M., Mateus, S., Zucchi, R., Schmidt, V. M., & Barth, F. G. (2009). Mandibular gland secretions of meliponine worker bees: further evidence for their role in interspecific and intraspecific defence and aggression and against their

role in food source signalling. *Journal of Experimental Biology*, *212*(8), pp. 1153-1162.

Schorkopf, D. L. P., Jarau, S., Francke, W., Twele, R., Zucchi, R., Hrncir, M.,& Barth, F. G. (2007). Spitting out information: Trigona bees deposit saliva to signal resource locations. *Proceedings. Biological Sciences / The Royal Society*, *274*(1611), 895–898. http://doi.org/10.1098/rspb.2006.3766.

Schroder, S., Wittmann, D., Drescher, W., Roth, V., Steinhage, V., & Cremers, A. B. (2002). THE NEW KEY TO BEES: AUTOMATED IDENTIFICATION BY IMAGE ANALYSIS OF WINGS Stefan Schroder, Dieter Wittmann, Wilhelm Drescher, Volker Roth, Volker Steinhage and Armin B. Cremers. *Pollinating Bees - The Conservation Linkage between Agriculture and Nature*, pp. 209–216.

Sharma, M. V., Uma Shaanker, R., Leather, S. R., Vasudeva, R., & Shivanna, K. R. (2011). Floral resources, pollinators and fruiting in a threatened tropical deciduous tree. *Journal of Plant Ecology*, *4*(4), pp. 259–267. http://doi.org/10.1093/jpe/rtq029

Sheffield, C. S., Kevan, P. G., Westby, S. M., & Smith, R. F. (2008). Diversity of cavity-nesting bees (Hymenoptera: Apoidea) within apple orchards and wild habitats in the Annapolis Valley, Nova Scotia, Canada. *The Canadian Entomologist*, *140*(2),pp. 235-249.

Silva, M. D. E., Ramalho, M., & Monteiro, D. (2013). Diversity and habitat use by stingless bees (Apidae) in the Brazilian Atlantic Forest. *Apidologie*, *44*(6). http://doi.org/10.1007/s13592-013-0218-5.

Simone-Finstrom, M., & Spivak, M. (2010). Propolis and bee health: the natural history and significance of resin use by honey bees. *Apidologie*, *41*(3), pp. 295–311. Retrieved from http://link.springer.com/10.1051/apido/2010016.

Slaa, E., & Hughes, W. (2009). Local enhancement, local inhibition, eavesdropping, and the parasitism of social insect communication. *Food Exploitation by Social Insects, 51*(6), pp. 90–101 .

Slaa, E. J., Cevaal, A., & Sommeijer, M. J. (1998). Floral constancy in Trigona stingless bees foraging on artificial flower patches: a comparative study. *Journal of Apicultural Research*, *37*(3), pp. 191–198.

Slaa, E. J., Tack, A. J. M., & Sommeijer, M. J. (2003). The effect of intrinsic and

extrinsic factors on flower constancy in stingless bees. *Apidologie*, *34*, pp. 457–468.

Slaa, E. J., Wassenberg, J., & Biesmeijer, J. C. (2003). The use of field-based social information in eusocial foragers: Local enhancement among nestmates and heterospecifics in stingless bees. *Ecological Entomology*, *28*(3), pp. 369–379.

Smith, B. H., & Roubik, D. W. (1983). Mandibular glands of stingless bees (Hymenoptera: Apidae): Chemical analysis of their contents and biological function in two species ofMelipona. *Journal of Chemical Ecology*, *9*(11), pp.1465–1472. http://doi.org/10.1007/BF00988512

Sommet, M. (1999). Beekeeping with stingless bees : a new type of hive. *Bee World*, *80*(2), pp. 70–79.

Spiewok, S., & Neumann, P. (2006). Infestation of commercial bumblebee (Bombus impatiens) field colonies by small hive beetles (*Aethina tumida*). *Ecological Entomology*, *31*(6), pp.623–628.

Stanghellini, M. S., Ambrose, J. T., & Hopkins, D. I. (2000). Bumble bee colonies as potential alternative hosts for the small hive beetle (Aethina tumida Murray). *American Bee Journal*, *140*(1), pp. 71–75.

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*(1), pp. 13–19. http://doi.org/10.1007/s00049-009-0003-0

Steffan-dewenter, I. (2003). Importance of Habitat Area and Landscape Context Fragmented Orchard Meadows. *Conservation Biology*, *17*(4), pp. 1036–1044. http://doi.org/10.1046/j.1523-1739.2003.01575.

Steffan-Dewenter, I., & Klein, A. (2006). Bee diversity and plant–pollinator interactions in fragmented landscapes, *Conservation Ecology*, *12*(9), pp. 36–48.

Steffan-Dewenter, I., & Tscharntke, T. (2002). Insect communities and biotic interactions on fragmented calcareous grasslands—a mini review. *Biological conservation*, *104*(3), pp. 275-284.

Suazo, A., Torto, B., Teal, P. E. A., & Tumlinson, J. H. (2003). Response of the small hive beetle (*Aethina tumida*) to honey bee (Apis mellifera) and beehive-produced volatiles. *Apidologie*, *34*, pp. 525–533.

Suresh Kumar, M., Ranjit Singh, A. J. A., & Alagumuthu, G. (2012). Traditional beekeeping of stingless bee (Trigona sp) by Kani tribes of Western Ghats, Tamil Nadu, India. *Indian Journal of Traditional Knowledge*, *11*(2), pp. 342–345.

Taki, H., Viana, B. F., Kevan, P. G., Silva, F. O., & Buck, M. (2008). Does forest loss affect the communities of trap-nesting wasps (Hymenoptera: Aculeata) in forests? Landscape vs. local habitat conditions. *Journal of Insect Conservation*, *12*(1), pp. 15–21.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular biology and evolution*, *28*(10), pp. 2731-2739.

Tepedino, V. J., & Stanton, N. L. (1981). Diversity and Competition in Bee-Plant Communities on Short-Grass Prairie. *Oikos*, *36*(1), pp. 35–44.

Théodore Munyuli, M. B. (2013). Is pan-trapping the most reliable sampling method for measuring and monitoring bee biodiversity in agroforestry systems in sub-Saharan Africa? *International Journal of Tropical Insect Science*, *33*(1), pp. 14–37.

Torto, B., Boucias, D. G., Arbogast, R. T., Tumlinson, J. H., & Teal, P. E. A. (2007). Multitrophic interaction facilitates parasite-host relationship between an invasive beetle and the honey bee. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(20), 8374–8378.

Torto, B., Suazo, A., Alborn, H., Tumlinson, J. H., & Teal, P. E. A. (2005). Response of the small hive beetle (Aethina tumida) to a blend of chemicals identified from honeybee (Apis mellifera) volatiles. *Apidologie*, *36*(4), pp. 523–532.

Tóthmérész, B. (1995). Comparison of different methods for diversity ordering. *Journal of Vegetation Science*, *6*(2), pp. 283–290. Retrieved from http://doi.wiley.com/10.2307/3236223

Tscharntke, T., Klein, A. M., Kruess, A., Steffan-Dewenter, I., & Thies, C. (2005). Landscape perspectives on agricultural intensification and biodiversity–ecosystem service management. *Ecology letters*, *8*(8), pp. 857-874.

Tscheulin, T., Neokosmidis, L., Petanidou, T., & Settele, J. (2011). Influence of landscape context on the abundance and diversity of bees in Mediterranean olive

groves. *Bulletin of Entomological Research*, *101*(5), pp. 557–564.

Ugland, K. I., Gray, J. S., & Ellingsen, K. E. (2003). The species–accumulation curve and estimation of species richness. *Journal of Animal Ecology*, *72*(5), pp. 888-897.

Velthuis, H. H. W., Koedamb, D., Imperatriz-Fonseca, V. L., Elthuisa, H. H. W. V, Oedamb, D. K., & Onsecab, V. L. I. M. (2005). The males of *Melipona* and other stingless bees, and their mothers. *Apidologie*, *36*, pp. 169–185.

Verheggen, F. J., Haubruge, E., & Mescher, M. C. (2010). *Alarm pheromones. Chemical signaling in response to danger*. *Vitamins and hormones* (Vol. 83). http://doi.org/10.1016/S0083-6729(10)83009-2.

Vilà, M., Bartomeus, I., Dietzsch, A. C., Petanidou, T., Steffan-Dewenter, I., Stout, J. C., & Tscheulin, T. (2009). Invasive plant integration into native plant-pollinator networks across Europe. *Proceedings. Biological Sciences / The Royal Society*, *276*(1674), pp. 3887–3893.

Vit, P., Roubik, D. W., & Pedro, S. R. M. (2012). *Pot-Honey: A legacy of stingless bees*. *Pot-Honey: A Legacy of Stingless Bees* (Vol. 9781461449). Springer New York.

Wang, Z., & Tan, K. (2014). Comparative analysis of olfactory learning of Apis cerana and *Apis mellifera*. *Apidologie*, *45*(1), 45–52. http://doi.org/10.1007/s13592-013-0228-3

Wenseleers, T., & Ratnieks, F. L. W. (2004). Tragedy of the commons in *Melipona* bees. *Proceedings. Biological Sciences / The Royal Society*, (*271), pp.*310–S312.

Westphal, C., Bommarco, R., Carré, G., Lamborn, E., Morison, N., Petanidou, T.,& Steffan-Dewenter, I. (2008). Measuring bee diversity in different European habitats and biogeographical regions. *Ecological Monographs*, *78*(4), pp.653–671.

Westphal, C., Steffan-Dewenter, I., & Tscharntke, T. (2003). Mass flowering crops enhance pollinator densities at a landscape scale. *Ecology Letters*, *6*(11), pp. 961–965.

Wilder, C., Brooks, T., & Lens, L. (1998). Vegetation Structure and Composition of the Taita Hills Forests. *Journal of East African Natural History*, *87*(1), pp. 181–187. http://doi.org/10.2982/0012-8317(1998)87[181:VSACOT]2.0.CO;2

Wille, A. (1983). Biology of the stingless bees. *Annual review of entomology*, *28*(1), pp. 41-64.

Williams, N. M., Cariveau, D., Winfree, R., & Kremen, C. (2011). Bees in disturbed habitats use, but do not prefer, alien plants. *Basic and Applied Ecology*, *12*(4), pp. 332–341.

Williams, N. M., & Kremen, C. (2007). Resource distributions among habitats determine solitary bee offspring production in a mosaic landscape. *Ecological Applications*, *17*(3), pp. 910–921.

Wilms, W., Imperatriz-Fonseca, V. L., & Engels, W. (1996). Resource partitioning between highly eusocial bees and possible impact of the introduced Africanized honey bee on native stingless bees in the Brazilian Atlantic rainforest. *Studies on Neotropical Fauna and Environment*, *31*(3), pp. 137–151.

Wilson, J. S., Griswold, T., & Messinger, O. J. (2008). Sampling bee communities (Hymenoptera: Apiformes) in a desert landscape: are pan traps sufficient?. *Journal of the Kansas Entomological Society*, *81*(3), pp. 288-300.

Winfree, R., Aguilar, R., Vázquez, D. P., Lebuhn, G., & Aizen, M. A. (2009). A meta-analysis of bees' responses to anthropogenic disturbance. *Ecology*, *90*(8), pp. 2068–2076.

Winfree, R., Bartomeus, I., & Cariveau, D. P. (2011). Native Pollinators in Anthropogenic Habitats. *Annual Review of Ecology, Evolution, and Systematics*.

Winfree, R., & Kremen, C. (2009). Are ecosystem services stabilized by differences among species? A test using crop pollination. *Proceedings of the Royal Society of London B: Biological Sciences*, *276*(1655), pp. 229-237.

Winfree, R., Williams, N. M., Gaines, H., Ascher, J. S., & Kremen, C. (2008). Wild bee pollinators provide the majority of crop visitation across land-use gradients in New Jersey and Pennsylvania, USA. *Journal of Applied Ecology*, *45*(3), pp. 793-802.

Winfree, R., Williams, N. M., Dushoff, J., & Kremen, C. (2007). Native bees provide insurance against ongoing honey bee losses. *Ecology Letters*, *10*(11), pp. 1105–1113.

Wyatt, T. (2003). *Pheromones and animal behaviour: communication by smell and taste, 31*(4), pp. 101–114.

Yusuf, A., Pirk, C., & Crewe, R. (2010). Nestmate recognition and the role of cuticular hydrocarbons in the African termite raiding ant Pachycondyla analis. *Journal of*

Chemical Ecology 4(11), pp. 56-67. Retrieved from http://link.springer.com/article/10.1007/s10886-010-9774-6.

Zanette, L. R. S., Martins, R. P., & Ribeiro, S. P. (2005). Effects of urbanization on Neotropical wasp and bee assemblages in a Brazilian metropolis. *Landscape and Urban Planning*, *71*(24),pp. 105–121.

Van Zweden, J. S., & d'Ettorre, P. (2010). Nestmate recognition in social insects and the role of hydrocarbons. *Insect hydrocarbons: biology, biochemistry and chemical ecology*, (*11*), pp. 222-243.