# STUDIES ON THE ECOLOGY OF HIVE BEETLES IN KENYA

BY

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## DECLARATION

**Candidate**

I, **AYUKA TEBOH FOMBONG**, declare that this thesis is my original work and, to the best of my knowledge, it has not been presented for award of a degree at any other University

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## DEDICATION

This thesis is dedicated to my mother Mrs. Fombong Mary T. and my siblings Enongonwei, Formuyah, Forkwa, Tenyim and Ndaya, for their love, care and concern during moments of joy and sorrow which have been an integral part of my life.

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## ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| BOLD | Barcode of Life Data systems |
| DCM | Dichloromethane |
| FID | Flame Ionisation Detector |
| GC | Gas Chromatograph |
| GC-EAD | Gas Chromatography-Electroantennographic Detection |
| GC-MS | Gas Chromatography-Mass Spectrometry |
| *icipe* | International Centre of Insect Physiology and Ecology |
| MS | Mass Spectrometry |
| NIST | National Institute of Standards and Technology |
| PCR | Polymerase Chain Reaction |

## ABSTRACT

Although beekeeping has become an important economic venture in Kenya, and is being used to alleviate poverty and promote environmental conservation, very little information exists on arthropods associated with honey bees in the country. This is especially true for hive beetles which cause great damage to honey bees and hive products. With knowledge on the biology of these beetles and their chemical ecological interaction with honey bees virtually non-existent, this study was carried out to close this knowledge gap with the goal of providing key data towards further development into potential management tools for these beetles.

Nation-wide surveys in Kenya covering major beekeeping areas identified three beetle pests of honey bees viz *Aethina tumida* Murray, *Oplostomus haroldi* Witte and *Oplostomus* sp. *Aethina tumida* was cosmopolitan whereas *O. haroldi* and *Oplostomus* sp. are confined to the eastern and coastal parts. Beetle infestations differed across sites being higher in areas with high precipitation and those close to water bodies suggesting that climatic factors influence their distribution. Inside the hive, *A. tumida* occurred mainly on the bottom board and the scarabs on the frames. Adult scarabs caused damage by feeding on bee brood, pollen and honey with a greater preference for the brood. Discrimination of the scarab beetles was shown to be possible using *mt*COI gene and DNA barcodes.

*Oplostomus haroldi* was reared successfully on a diet of cow dung mixed with soil and it took 3-5 months to develop from egg to adult implying that these scarab pests can produce more than one generation per year depending on prevailing climatic conditions. Behavioural and electrophysiological assays together with coupled GC-MS analyses showed that both sexes of *O. haroldi* were significantly attracted to honey bee volatiles and mainly detected their esters. This may imply that this beetle uses honey bee odours as olfactory cues to locate its host.

Analysis of the mating behavior of *O. haroldi* showed five distinct stages namely, arrestment, alignment, mounting, copulation and post-copulatory mate guarding. Males distinguished other males from females using their maxillary palp tips and, a female contact sex pheromone composed of hydrocarbons. Of these hydrocarbons, *(Z)*-9-pentacosene, the dominant female component, elicited a partial mating behavior confirming its pheromonal role.

Behavioural assays also showed that volatiles from *Mangifera indica* L. and *Musa acuminata* x *Musa balbisiana* Colla significantly attracted both male and female beetles. In electrophysiological assays, components of the volatiles from both fruits were electrophysiologically active with components from *M. indica* being 2-3 times more active compared to *M. sapientum*. Coupled GC-MS analyses showed that the bio-active peaks were mostly terpenes, esters and ketones, with a number of them known to be produced by honey bees. Field trapping using *A. tumida* flight traps baited with ripe mango and banana fruits confirmed laboratory results which show that this beetle is attracted to ripe fruits in nature and can reproduce on them.

Overall, this study has revealed the diversity of beetles associated with honey bees in Kenya and gives a hitherto unknown account of the life history of the scarabs under laboratory conditions. It also demonstrates the use of olfactory and contact cues by *O. haroldi* for host location and mating, respectively. The possible use of alternate hosts by *A. tumida* is also demonstrated.

# CHAPTER ONE

## Introduction

### 1.1 Background

The honey bee, *Apis mellifera* L. (Hymenoptera: Apidae) ranks among the most economically important living organisms known to man. Knowledge about the usefulness of honey bees and their primary product, honey, dates back to ancient times (Strouhal and Werner, 1996; Strouhal, 1997; Ransome, 2004). Honey bees are the most important pollinators of flowering plants, including many crops. Through this pollination service, honey bees contribute significantly to agriculture (McGregor, 1976; Southwick and Southwick, 1992; Watanabe, 1994) by ensuring fruit or seed production besides perpetuating many flowering plant species (Klein *et al.*, 2007). Recent global estimates put the economic benefits of this pollination service to agriculture at USD 217 billion (HAGRC, 2008). Besides pollination benefits, valuable honey bee products such as honey, beeswax, propolis, royal jelly, bee venom (Crane, 1990; Jean-Prost, 1994) and bee brood (Crane 1990) have helped to improve the livelihood of beekeepers worldwide. Indeed, bee farming has been shown to improve household income and it is increasingly becoming popular in Kenya and many other African countries (Raina, 2006).

The raising and care of bees for commercial or agricultural purposes (apiculture) is constrained by a number of challenges that limit its exploitation. The most important of these constraints are challenges posed by arthropod pests and diseases (FAO, 2006). For example, the wax moth *Galleria mellonella* L. (FAO, 2006) and the small hive beetle *Aethina tumida* Murray (Elzen *et al.*, 1999; FAO, 2006) are a challenge to beekeepers in Kenya.

Recently, two large beetle species belonging to the genus *Oplostomus*, which feed on bee brood, pollen and honey, were recovered in bee hives in the coastal and eastern provinces of Kenya (Torto *et al.*, 2010a). Although the damage and losses caused locally by these beetle species have not yet been quantified, they live sympatrically in the same hives with *A. tumida* adults (Torto *et al.*, 2010a), which is a known bee hive pest. The permanent residence of adults of these large beetle species in honey bee hives, together with the damage they allegedly cause as reported by beekeepers, makes them a potential threat to beekeeping and the provision of pollination service, thus prompting their management in apiaries.

Four hive beetle species are indigenous to sub-Saharan Africa. These are *A. tumida* Murray, *Oplostomus haroldi* Witte, *Oplostomus fuligineus* Olivier and *Oplostomus* sp. Barclay. Until recently, all four were regarded as minor pests and attracted minimal attention from researchers. While *O. haroldi, O.* sp. and *O. fuligineus* have been reported to occur only within their native sub-Saharan range (Donaldson, 1989; Torto *et al.*, 2010a), *A. tumida* has become an invasive pest of European honey bee colonies in North America (Sanford, 1998) and Australia (Neumann and Elzen, 2004) where its larvae and adults cause severe damage to honey bee combs and other hive products (Elzen *et al.*, 1999).

Due to the huge losses caused by *A. tumida* outside its native geographic range, there has been a lot of effort in understanding various aspects of its biology (Torto *et al.*, 2010b), with the overall aim of developing environmentally friendly and sustainable ways of its management. Although this beetle has been shown to survive on fruits as alternate hosts under laboratory conditions, evidence to support its occurrence and utilization of such hosts in the natural settings is currently lacking. The large number of adult infestations reported by Torto *et al.* (2010a) with no evidence of reproduction in the hives strongly suggests that alternate hosts

play a role in survival of this beetle in its native home range. Management of these beetles will therefore require proper monitoring and timely application of control agents in and around managed honey bee colonies.

Adult *A. tumida* can be successfully monitored using in-hive bottom board and flight traps baited with pollen dough inoculated with the yeast *Kodamaea ohmeri* (Torto *et al.*, 2007b; Arbogast *et al.*, 2009a). Successful detection and control of *A. tumida* in managed honey bee colonies in the USA has been demonstrated using CheckMite + strips™ containing 10% Coumaphos (an organophosphate insecticide) (Elzen *et al.*, 1999; Neumann and Hoffmann, 2008). However, all these tools are laborious to deploy and monitor, the bait is not affordable to small scale beekeepers in the tropics while the organophosphate Coumaphos poses health risks to both beekeepers and consumers. In Kenya, management tools for *A. tumida* are lacking. Furthermore, none of these tools have resulted in total elimination of the beetle hence, the need for the development of affordable and more effective management tools.

*Oplostomus fuligineus, O. haroldi* and *Oplostomus* sp are the only scarabs that show a strong association with honey bees. These large hive beetles are seldom found on vegetation or fruits unlike other opportunist scarabs that feed on fruits but occasionally infest honey bee colonies (Donaldson, 1989; Torto *et al.*, 2010a). The management of large hive beetles in southern Africa has been mainly by physical methods that prevent the beetle from accessing the bee hives. For example, placing a 9-mm mesh-wire around the hive entrance (200 mm x 25 mm) or reducing the hive entrance or even handpicking of beetles already in the hive are some of the commonly used methods (Johannsmeier, 2001). Nevertheless, these methods are not only laborious and time-consuming but are also inefficient for long term monitoring and control.

Semiochemicals, commonly referred to as 'message bearing chemicals' or 'infochemicals' mediate many interactions between insects and their hosts (Cork, 2004). These chemical substances elicit a behavioural or physiological response in individuals of the same or different species (Chapman, 1998; Cranston and Gullan, 2005). They are increasingly being used to lure insects in pest management programmes (Taschenberg *et al.*, 1974; Schlyter *et al.*, 2001; Alpizar *et al.*, 2002; Cork, 2004; Allou *et al.*, 2006; Witzgall *et al.*, 2010). Since Torto *et al.* (2007b) and Arbogast *et al.* (2009a) have showed that small hive beetles can indeed be monitored and managed using such a strategy, using odour-baited traps, it may also be possible to use a similar strategy for the large hive beetles. However, to make this possible, it is imperative to have a thorough understanding of the life cycle and the chemically mediated behavioural interactions these beetles have with their honey bee host.

### 1.2 Problem Statement

In Kenya, beekeeping is increasingly becoming popular as a source of income for many small scale farmers to supplement their livelihoods. One of the most important emerging constraints in the industry is the challenge posed by arthropod pests and diseases. For example, the wax moth (*Galleria mellonella)* and the small hive beetle (*Aethina tumida)* are well-known honey bee hive pests. Recent reports of large hive beetles (*Oplostomus* spp.) in the eastern and coastal provinces further exacerbate this challenge. Several unconfirmed reports by beekeepers have linked decline in honey production in their hives to presence of hive beetles. Moreover, beekeepers at various regional workshops organised by *icipe* have identified beetle infestations as key to colony loss besides wax moths and honey badgers. Preliminary field observations and laboratory studies have confirmed that hive beetles feed on pollen and bee brood.

In Kenya, migratory beekeeping for pollination of crops is non-existent. Honey bee hives remain in one location for years, providing a continuous pollination service to the surrounding ecosystems. In such hives, population of beetles may build up quickly and the honey bee colonies may collapse or the bees may abscond due to the increasing pest pressure from the hive beetles leading to loss or decline of a critical ecosystem service as well as honey and other hive products.

Since honey bee hives are specially confined environments, they pose many challenges to the use of conventional pest management options. For example, the use of pesticides poses health risks to the bees, beekeeper and the environment, and also may contaminate beehive products. As such, management of these pests will require development of alternative monitoring and management strategies which are sustainable and environmentally benign. One such strategy would be to develop a lure and kill system based on a good understanding of hive beetles semiochemicals. To develop such a strategy, knowledge of the biology and chemical ecology of these beetle pests is imperative. This thesis addresses this knowledge gaps and therefore reports the diversity and distribution, life cycle and chemoecological interactions between these beetles and their primary host, honey bees.

### 1.3 Objectives

The overall objective of this study was to study the biology and chemical ecology of hive beetles with a view to document their diversity, distribution and provide details of their life cycle, and chemoecological interactions between these beetles and the honey bee host. The specific objectives of this study were:

1. To document the occurrence, diversity and damage pattern of large hive beetles in Kenya

2. To establish the life history of *Oplostomus haroldi* and *Oplostomus* sp. under laboratory conditions
3. To identify semiochemicals mediating intra-specific and inter-specific communication in *Oplostomus haroldi* using bioassays and analytical chemistry methods
4. To evaluate the potential of *Musa* sp (Banana) and *Mangifera indica* (Mango) as alternate host of the small hive beetle *A. tumida*

**1.4 Justification**

With apiculture becoming an increasingly important activity in Kenya, there is need to document arthropods associated with honey bee colonies and establish their relationship with the bees. This study advances our understanding of the interactions between honey bees and hive beetles. Current global concerns over insect pollinators decline calls for adequate research on pollinators so as to unearth possible causes of their decline. With the small hive beetle now an invasive pest in the western hemisphere, an understanding of its interaction within its native geographic range will provide in-depth information on its ecology which could be vital for its management locally and elsewhere where its has established as an invasive pest. In Kenya, hive beetles infestations have been reported to cause absconding of honey bee colonies thereby leading to huge economic loss to beekeepers. Presently, no methods are available to reduce beetle populations in honey bee colonies in Kenya. This study which investigates the chemoecological interactions between hive beetles and their host might lead to results that can be exploited for developing management options for these beetles.

# CHAPTER TWO

## Literature Review

### 2. 1 Honey bees

#### 2.1.1 Honey bees, their species diversity and geographical distribution

Honey bees are a subset of bees known globally for their honey production and the pollination services they render while foraging on plants for food. They belong to the insect order Hymenoptera; suborder, Apocrita; superfamily, Apoidea; family, Apidae and the genus, *Apis* (Ruttner, 1986, 1988). They are social insects and familiar for their ability to inflict pain on hive intruders with their stings. Besides honey bees, stingless bees which belong to the family Meliponidae also produce honey (Crane, 1999).

Four major species belong to the genus *Apis* and these are *A. mellifera, A. dorsata, A. florea* and *A. cerana* (Ruttner, 1988, Crane, 1990, Jean-Prost, 1994). Some of these species are further composed of races scattered within the tropics and temperate regions of the world where they tend to occupy specific geographical locations (Crane, 1990). The European honey bee, *A. mellifera,* is the most studied of all honey bees and its early domestication permitted its wide dispersal by man and it is virtually present on all the continents (Crane, 1990; Michener, 2000). Its continuous evolution and adaptation in the areas of introduction gave rise to the many subspecies of this species (Table 2.1)

**Table 2.1**: The known subspecies of *Apis mellifera* and their geographical distribution

| | **Subspecies** | **Geographical distribution** |
|---|---|---|
| *1* | *Apis mellifera mellifera* | North and West Europe, Russia, North America and the Caribbean islands |
| *2* | *A. m. yementica* | Chad and Sudan |
| *3* | *A. m. litorea* | Tanzania, Mozambique and Kenya |
| *4* | *A. m. lamarckii* | North Africa |
| *5* | *A. m. adansonii* | West Africa |
| *6* | *A. m. capensis* | South Africa |
| *7* | *A. m. scutellata* | Ethiopia, Eastern and Southern Africa |
| *8* | *A. m. unicolor* | Madagascar, Réunion and Mauritius |
| *9* | *A. m. sahariensis* | North Africa |
| *10* | *A. m. syriaca* | Syria and Palestine |
| *11* | *A. m. monticola* | Kenya, Tanzania, Burundi and Ethiopia |
| *12* | *A. m. media* | Iran |
| *13* | *A. m. cypria* | Cyprus |
| *14* | *A. m. iran* | Iran |
| *15* | *A. m. anatoliaca* | Anatolia-Turkey |
| *16* | *A. m. sicula* | Sicily |
| *17* | *A. m. adami* | Crete |
| *18* | *A.m. caucasica* | Georgia and the Caucasus |
| *19* | *A. m. intermissa* | North Africa |
| *20* | *A. m. iberica* | Iberian Peninsula |
| *21* | *A. m. ligustica* | Italy |
| *22* | *A. m. carnica* | Carniola-Yugoslavia and Austria |
| *23* | *A. m. cecropia* | Greece |

Adapted from Crane (1990)

Due to advances in biosystematics through discovery of more useful morphological and molecular taxonomic characters, the classification of honey bees is being revised continuously. Moreover, the increase in global honey bee trade has led to a greater distribution of most bee species, increased hybridisation and evolution necessitating a continuous revision and development of diagnostic keys for species and subspecies identification.

### 2.1.2 Life history of *Apis mellifera*

*Apis* species undergo a developmental cycle with four life stages, namely, egg, larva, pupa and imago or adult. The duration of the cycle varies among castes of the same species and differs between species and races (Crane, 1990).

The egg is small, whitish, rod-shaped, about 1.5 mm in length and 0.3 mm in diameter (Jean-Prost, 1994). It has a narrow end and a broad end. It is positioned in the comb cell such that its narrow end almost makes contact with the comb's midrib. At the broad end is a tiny opening called the micropyle through which the male sperm passes to fertilise the egg (Jean-Prost, 1994). Three days post-oviposition, a larva hatches out of the egg and develops into a worker, queen or drone as intended by the colony.

The larva is whitish, segmented, curved and possesses no eyes or legs. It is smaller than the egg initially but grows rapidly within a few days and changes in form, curving its body until the anterior and posterior ends touch each other. The larva is fed on 'worker's jelly' by nurse bees (young adult worker bees) until it is about 5 days old, after which feeding ceases and the workers build a wax capping to confine the larva within the cell (Crane, 1990). Prior to pupation, the larva spins a cocoon within which its pupa develops in the cell. During its larval

stage, it moults 5 times, with the last moult taking place within the capped cell (capped brood) (Jean-Prost, 1994). The larval stage lasts 4 – 7 days depending on the honey bee species (Crane, 1990).

Pupation usually lasts 10 – 14 days after which the desired adult emerges. During this stage, the organ systems of the larva are completely reorganized, the three characteristic regions of insects develop, eyes are formed, and the legs, wings and antennae also develop (Crane, 1990; Jean-Prost, 1994). Following its complete development, the young adult chews away the wax cap of the cell and emerges as a soft, greyish bee (Crane, 1990). Queen bees have the shortest developmental time followed by the worker bees while drones take the longest time to develop (Crane, 1990; Jean-Prost, 1994).

### 2.1.3 History of Honey bee keeping

Hunting of honey from feral nests of bees by humans has existed throughout mankind's existence and started from ancient times with the oldest piece of evidence of this activity dating back to 6000 BC (Before Christ) (Crane, 1990; 1999). Besides honey, beeswax also found use in ancient paints as depicted by pictorial evidence from the ancient Egyptian civilization (Crane, 1990, 1999; Strouhal and Forman, 1996; Strouhal, 1997; Ransome, 2004). The ancient practice of honey hunting from wild nests which is still being practiced today in some parts of the world, involves honey bees and stingless bees (Crane, 1990; 1999). Besides humans, other animals such as insects, bears, honey badgers, primates like the monkeys, baboons and chimpanzees, and birds like the honey buzzard and the honeyguide also exploit honey bees and their nests facultatively, especially during seasons of abundance of honey (Crane, 1999). Only the honeyguide feeds on beeswax as it is the only bird capable of digesting wax (Crane, 1999).

Prior to modern honey bee farming known as apiculture, traditional beekeeping practices using horizontal hives made out of logs of wood, woven materials, mud, clay and hollowed rocks in different archaic human cultures are explicitly documented and date back to about 2500 BC (Crane, 1990; 1999; Graham 2003). The different descriptions of traditional hives reveal great similarity in the structure and design of ancient and modern bee hives (Crane, 1990; 1999). Improvements in the nature of comb constructions, facilitation of honey harvesting and increased mobility of hives, among others, greatly account for modifications which have taken place across millennia (Crane, 1990). These different types of horizontal hives were used for beekeeping until around 1600 which saw the beginning of forest beekeeping using vertical tree trunks (Crane, 1999). These tree trunks were later designed into vertical hives with both ends closed with flat circular pieces of wood and a vertical door created for easy access to the hive combs. Other hive types designed included; the leaf hive (Huber, 1792), the box hive with movable frames (Prokopovich, 1806 cited in Crane, 1999), the tiered hive (Munn, 1844 cited in Crane, 1999) and the practical movable-frame hive (Langstroth hive) (Langstroth, 1851 cited in Crane, 1999). The Langstroth hive has now become the most popular and widely used hive type for beekeeping owing to its unique versatility which enables its easy manipulation and transportation. Most modern hives are based on the Langstroth hive design with modifications.

Besides the improvement of hive types used for beekeeping, there was also the growth and accumulation of knowledge about honey bees especially with relevance to beekeeping (Crane, 1990; 1999). Such technological improvements and the continuous accrual of knowledge about bees made beekeeping become a very profitable commercial activity.

### 2.1.4 Economic Importance of Honey bees (*Apis* species)

Benefits derived from honey bees can be grouped into direct and indirect, with direct benefits referring to immediate profits derived from the honey bee products and, indirect benefits being profits resulting from the activities of honey bees.

Direct benefits of honey bees include honey, beeswax, propolis, pollen, royal jelly, bee venom and bee brood (Crane, 1990; Michener, 2000), all of which are known as hive products as they are obtained directly from the hive. These may further be categorized based on how long these products have and are being utilised into traditional hive products which are honey and beeswax and the newer hive products which are pollen, propolis, royal jelly, bee venom and bee brood (Crane, 1990). Indirect benefits of honey bees are the pollination services they render to flowering plants and the ecological balance they maintain through this ecosystem service (Michener, 2000; Klein *et al.*, 2008).

#### 2.1.4.1 Direct Benefits of Honey bees

**Honey**

Honey is a substance produced by bees and some other social insects from nectar or honeydew that they collect from plants, which they transform by evaporating water and by the action of enzymes they secrete and seal the finished honey in cells of their comb (Crane, 1990).

Honey is widely used as food and serves as a sweetener and a sweet spread. It is also used in baking, and the production of confections and alcoholic drinks (Crane, 1990; 1999). Due to its nutrient content, it also contributes to the dietary requirements of humans. It is also used in medicine and pharmacy as an antiseptic for dressing wounds, cuts and burns, treating chronic

and infected wounds, bed sores, skin and varicose ulcers and also in surgery (Crane, 1990; 1999). Another use of honey is its formulation into balms with additional components used for cough, and sore throat. Honey is also used in the preservation of bull semen and human corneas for transplants, as food for pigs and cattle (Crane, 1990) and in the cosmetics industry (Crane, 1980; Krochmal, 1985).

**Beeswax**

Beeswax, which refers to wax produced by honey bees and stingless bees has a huge commercial value due to its wide range of uses. Beeswax is used by bees in constructing their combs and cell caps (Crane, 1990; Jean-Prost, 1994). In modern apiaries, the fitting of comb foundations made out of beeswax in hive frames to facilitate the commencement of comb building by bees has become a major use of beeswax (Crane, 1990). Another use of beeswax is in the making of candles used for domestic lighting and religious ceremonies. Beeswax has a long historical use in models, casting and etching of objects (Crane, 1990). It is also used traditionally in dying cloths. It is used in the production of ointments, soothing skin creams and lotions used for cosmetic and pharmaceutical purposes. Other uses of beeswax include its utilization in the production of furniture and shoe polishes, furniture varnishes, water-resistant preparation for walls, air-permeable waterproofing compositions, finishes for leather, textiles, wood and paper. It is also used in anticorrosion preparations for terminals of lead accumulators, lubricants and electrets (Crane, 1990).

**Pollen**

Pollen exists as grains enclosed within pollen sacs of the anthers of stamen (male part of a flower) and contain cells that develop into the male sex cells or sperm of the plant. Pollen produced commercially is used for a number of specific purposes, some of which include;

plant breeding programmes, fruit pollination, as bees feed solely or with additional material, as a dietary supplement for humans and domestic animals, in cosmetics, in the study and treatment of allergic conditions, environmental pollution monitoring and mineral prospecting (Crane, 1990; Jean-Prost, 1994).

**Propolis**

Propolis refers to resins that honey bees and other bees collect from living plants, and use alone or with beeswax in the construction and adaptation of their nest (Crane, 1990). Like the other hive products, propolis shows great variation in its composition based on its different plant sources. It has the most pronounced antibiotic properties among all hive products (Crane, 1990; Jean-Prost, 1994) and, its production and commercialisation has been along this line. It is used in the pharmaceutical and cosmetic industries for the production of medications and body creams respectively (Crane, 1990; Jean-Prost, 1994)

**Royal Jelly**

Another hive product of global importance is royal jelly, which is a secretion of the hypopharyngeal or brood food gland in the head of young worker bees (Crane, 1990). It consists of a mixture of compounds and its composition is comparatively constant with respect to other hive products (Crane, 1990). Due to its antibacterial properties, it is used in pharmaceutic and cosmetic industries, and as a specialized diet supplement for human consumption (Crane, 1990).

**Bee Venom**

Bee venom is derived from the venom gland linked to the sting. Bee venoms of different species vary in their toxicity (Benton and Morse, 1968), protein pattern (Benton and Morse,

1968; Mello, 1970) and melittin content (Kreil, 1975). Some components of bee venom have noteworthy pharmacological properties and have been used in the natural treatment of arthritis and rheumatism (Crane, 1990; Graham 2003).

**Bee Brood**

The newest of all hive products is bee brood which consist of immature (larvae and pupae) bees (Crane, 1990). Although not popular among world trade commodities, the potential of bee brood as a food source especially for poorer people in various parts of the world where it is consumed can not be undervalued (Crane, 1990). It is used as feed in rearing insects used in biological pest control and domestic animals (Crane, 1990).

**2.1.4.2 Indirect benefits of Honey bees**

Benefits which accrue from the services of pollination rendered by bees can not be overemphasized. Such indirect profits are portrayed in the crop production figures listed by Klein *et al.* (2008). The production of 39 of the leading 57 single crops which account for 35 % global food production increases with pollination. Although insect pollination does not play a dominant role in global agriculture (contributes only 9.5% to global food production), its importance in ensuring an ecological balance cannot be ignored. Estimates of the economic value of insect pollination services to world food production stood at € 153 billion (about USD 217 billion) in 2005 (HAGRC, 2008). Similar annual estimates of the value of honey bee pollination in the United States and Canada were USD 9 billion and 443 million, respectively (Delaplane and Mayer, 2000). It has become evident globally that honey bees have an essential role to play in the ecosystem.

### 2.1.5 Pests of Honey bees, *Apis* species

As a result of the enormous benefits derived from honey bee species in the past, beekeeping has undergone tremendous changes which have modernized and facilitated the practice for man's direct and indirect benefits. This has led to large scale beekeeping in many parts of the world (Crane, 1999). Apiculture has however not gone unchallenged by pests and diseases (Crane, 1990). Records of honey bee pests and diseases date back to Roman times and have received a great deal of attention since mid 1900s to present as evidenced from modern documentations listed by Crane (1990). These pests include pathogens, arthropods, primates, mammals, reptiles, amphibians and birds, with their economic importance varying from one geographical location to another (Crane, 1990). Crane (1990) further grouped pests of bees according to the damage they caused and the life stage of honey bees to which such damage was inflicted. Based on these criteria, pests of honey bees worldwide can be grouped into vectors of pathogens of honey bee brood, adult honey bees, parasites of honey bees, predators of honey bees and pests of honey bee hives (referring to organisms which go after hive contents other than the honey bees) (Crane, 1990).

Diseases of honey bee brood include the American foul brood (AFB) caused by *Paenibacillus larvae*, the European foul brood (EFB) caused *Melissococcus pluten* (White), sac brood caused the virus *Morator aetotulas*, chalk brood caused by the fungus *Ascosphaera apis* Maassen ex Claussen (Olive and Spiltoir), stone brood by the fungus *Aspergillus flavus* L. and the black queen cell viral disease caused by the black queen cell virus (BQCV) (Crane, 1990; FAO, 2006).

Other diseases which affect adult honey bees are nosema, amoeba and gregarine diseases caused by the protozoans *Nosema apis* Zander, *Malphiamoeba mellificae* and gregarinids

respectively; septicaemia caused by the bacterium *Pseudomonas apiseptica*; melanosis caused by the fungus *Torulopsis* species and viral diseases caused by viruses (Crane, 1990; FAO, 2006).

Honey bees are mostly parasitised by mites and insects. Mites known to parasitise honey bees are *Varroa destructor* Anderson and Trueman, *Euvarroa sinhai* Delfinado and Baker, *Tropilaelaps clareae* Delfinado and Baker, *Acarapsis woodi* (Rennie) and *Neocypholaelaps* species which are phoretic (Crane, 1990; FAO, 2006). Bee lice such as *Braula coeca* Nitzsch, *Braula kohli* Schmitz, *Braula pretoriensis* Orosi Pal, *Braula schmitzi* Orosi Pal, *Braula orientalis* Orosi Pal, *Braula angulata* Orosi Pal and *Braula hansruttneri* Huttinger (Crane, 1990; Bisby *et al.*, 2008) are among insects known to parasitize honey bees. Other insects include the internal flies such as *Setontainia tricuspis* (Meigen) and *Stylops* species (Crane, 1990), the blister beetles *Meloe* species (Crane, 1990), the small hive beetle *A. tumida* Murray (Lundie, 1940; Schmolke, 1974) and the large hive beetles *O. fuligineus* Olivier (Donaldson, 1989) and *O. haroldi* Witte (Torto *et al*, 2010a).

Ants and wasps (both social and solitary) prey on honey bees sometimes but can be a serious threat to apiaries in locations where they are abundant. Ants such as *Oecophylla smaragdina* Fabricius, *Monomorium indicum* Forel, *Monomorium destructor* (Jerdon), *Oligomyrmex* species, *Dorylus* species, *Solenopsis* species and *Formica* species have been cited to attack hive colonies (Crane, 1990; FAO, 2006). Also, the vespid wasps, *Vespa mandarinia* Smith, *Vespa orientalis* L., *Vespa crabro* L., *Vespa cincta* (L.), *Vespa affinis* L., *Vespa tropica* (L.), *Vespa velutina* Lepeletier, *Vespa Mongolica* (L.), *Vespula flaviceps* (Smith), and *Vespula vulgaris* L. and the sphecid wasps, *Philanthus triangulum* (Fabricius) and *Palarus latifrons*

Kohl have been recorded to prey on honey bees within their hives and when they visit flowers for nectar and pollen (Crane, 1990; FAO, 2006).

Insects which cause damage to the honey bee comb and its contents include the greater wax moth *Galleria mellonella*, the lesser wax moth *Achroia grisella* (Fabricius) and the small hive beetles, *A. tumida* (Crane, 1990). The pollen mould *Bettsia alvei* (Betts) Skou can also be a serious problem in the northern part of temperate zone (Crane, 1990).

In addition to arthropods and micro-organisms, mammals such as bears and skunks in the temperate zones and badgers and primates in the tropics have been observed to occasionally cause significant damage to honey bee hives and their products. Besides mammals, birds like the bee-eaters *Merops* species, the honey-buzzard *Pernis apivorus* L., swifts, kingbirds, woodpeckers and the honeyguides are known predators of honey bees (Crane, 1990). The economic importance of these pests varies with locations and seasons with only a small proportion known to be of cosmopolitan occurrence and importance (FAO, 2006). In Kenya, major constraints to apiculture are mites and insect pests, with the hive beetles and ants being the most damaging (Torto *et al.*, Unpublished data).

**2.2 Hive beetles**

Several beetles have been observed in hives within the tropics. These include *Diplognatha gagates* Forster, *Coeonochilus bicolor* Nonfried, *Rhizoplatys trituberculatus*, *Oplostomus* sp., *O. fuligineus*, *O. haroldi* and *A. tumida* (all in Africa) and *Platybolium alvaerium* and *Dermestes vulpinus* (Fabricius) in Asia (Crane, 1990; Johannsmeier, 2001; Torto *et al.*, 2010a).

### 2.2.1 The large hive beetles

Several large beetle species have been observed in honey bee colonies where they cause varied damage by feeding on bee brood, pollen and honey (Donaldson, 1989; Crane, 1990; Johannsmeier, 2001). Reported species of economic importance are *Oplostomus* sp. in Kenya (specific name not yet confirmed), the black predacious scarab beetle *O. fuligineus* in southern Africa (Donaldson, 1989) and *O. haroldi* in East Africa (Torto *et al.*, 2010a).

#### 2.2.1.1 Taxonomy, species composition and distribution of the genus *Oplostomus*

The genus *Oplostomus* belongs to the order Coleoptera, superfamily Scarabaeiodea, family Scarabaeidae, subfamily Cetoniinae and tribe Cremastochelini (Donaldson, 1989). Within different parts of Africa, 10 beetle species have been identified as members of this genus (Table 2.2). Adults can be differentiated from one another using the differences in shape of the clypeus, colour patterns on the lateral sides of the dorsal surface and ventral side, density of small pits present on the head and location and colour of hair on their bodies (Bisby *et al.*, 2008).

Adults of *O. fuligineus* and *O. haroldi* can readily be distinguished from each other by the shape of the clypeus which is rectangular and curved upwards in the latter species while it has round ends and is relatively flat in the former (Torto, pers. communication) and, the presence of portions of brown-coloured exoskeleton on the lateral sides of the dorsal surface of *O. haroldi* which are absent in *O. fuligineus* and *Oplostomus sp.* The presence of a central grove on the 2$^{nd}$, 3$^{rd}$ and sometimes the 4$^{th}$ abdominal sternites of males of both species clearly distinguishes them from their respective females (Donaldson, 1989; Torto *et al.*, 2010a).

**Table 2.2**: Known beetle species belonging to the genus *Oplostomus*

| | Species name | Taxonomic ranking | Geographical Distribution |
|---|---|---|---|
| 1 | *Oplostomus abdominalis* | Species | East Africa |
| 2 | *O. bicolor* | Species | Angola |
| 3 | *O. frontalis* | Species | East Africa |
| 4 | *O. fuligineus* | Species | Mali, Kenya, Senegal, South Africa |
| 5 | *O. haroldi* | Species | Kenya, Tanzania |
| 6 | *O. meyeri* | Species | East Africa |
| 7 | *O. nigerrimus* | Species | Usambara-Namibia |
| 8 | *O. pectoralis* | Species | Eritrea |
| 9 | *O. rotundiceps* | Species | East Africa |
| 10 | *O. rufiventris* | Species | Congo, Angola, Usambara |
| 11 | *O. pectoralis morettoi* | Infraspecies | Ivory Coast |

Adapted from Bisby *et al.* (2008)

### 2.2.1.2 Life cycle of *Oplostomus haroldi*

*Oplostomus haroldi* has a life cycle similar to that of *O. fuligineus* described by Donaldson (1989). Its life cycle consists of four stages namely egg, larva, pupa and adult with its entire life history completed in moist soil and dung pads. With no existing documentation of its biology, providing such information is one of the objectives of this study.

### 2.2.1.3 Economic importance of *Oplostomus haroldi* and *Oplostomus* sp.

Like the small hive beetle, the large hive beetles have been observed to feed on pollen, honey and bee brood (Torto *et al.*, Unpublished). Aggregation of large hive beetles in honey bee colonies suggests that like small hive beetles, they might also respond to chemical cues released by the bees (Torto *et al.*, Unpublished). Unlike the small hive beetle, these beetles breed in cow dung pads and decaying plant material and only their adult stages cause damage in honey bee colonies (Donaldson, 1989; Crane, 1990). Although the extent of their damage has not been quantified, they together with small hive beetles have been observed to cause the absconding of bee colonies and therefore constitute a threat to apiculture in Kenya.

### 2.2.1.4 Management of *Oplostomus* species

Being identified only recently as pests of honey bees in Kenya, these beetles have received virtually no attention, a reason for the existence of only cultural management tools against them. These tools aimed at preventing beetles from gaining access into the hives by minimising the hive entrance (200 mm x 25 mm) by making them either smaller (5-7 mm in diameter) or fitting a 9 mm square wire gauze (queen excluder) at standard hive entrances. In cases where beetles finally gain access into the hives, their removal by handpicking becomes the last option (Johannsmeier, 2001). These management tools/options are laborious, time-consuming and only feasible on a small scale. These limitations call for the development of more cost effective control tools.

## 2.2.2 The small hive beetle *Aethina tumida* Murray

### 2.2.2.1 Taxonomy, description and world distribution of *Aethina tumida*

The small hive beetle belongs to the coleopteran family Nitidulidae. The presence of transverse procoxal cavities, grooved metacoxae, dilated tarsal segments, small forward tarsi and 3-segmented clubbed antennae clearly distinguishes *A. tumida* from other nitidulids (Neumann and Elzen, 2004).

*Aethina tumida* is native to Africa where it occurs south of the Sahara desert and has been identified in 18 countries (Neumann and Elzen, 2004). Its introduction and occurrence in Australia, North America (USA and Canada) and Europe (Portugal) (Neumann and Ellis, 2008) now indicates that it occurs in 4 continents. While it is considered a minor pest of honey bee colonies in sub-Saharan Africa (Hepburn and Radloff, 1998), and its damage effect not yet pronounced in Australia (PIRSA, 2006), it causes a great deal of damage to honey bee colonies in the United States (Elzen *et al.*, 1999; Hood, 2000).

Within its different regions of occurrence, *A. tumida* has been recorded within coastal zones, forest areas and savannahs, with arid and very cold conditions significantly limiting its distribution within the 4 continents (Neumann and Elzen, 2004).

### 2.2.2.2 Life cycle of *Aethina tumida*

*Aethina tumida* has a holometabolous life cycle (egg, larva, pupa and adult) that is completed in 4 to 6 weeks in moderate climatic conditions. The eggs are small, whitish, rod-shaped and about 1.4 mm long and 0.26 mm wide and show remarkable similarity to that of honey bees except for their smaller size (PIRSA, 2006). They are laid in clusters of 10–30 in open or capped brood cells or cracks within the hive, with females known to lay over 1000 eggs during their entire life. The eggs usually take 2–4 days to hatch into young larvae (Schmolke, 1974; Elzen *et al.*, 1999; PIRSA, 2006). The larval stage has over 5 instars and lasts 10–14 days before entering the 'wandering' stage where they stop feeding and wander about in search of moist soil for pupation (Schmolke, 1974; Elzen *et al.*, 1999; PIRSA, 2006). The larva is cigar-shaped, pale yellow in colour with a light brown head and the presence of a pair of distinct dorsal spines on each body segment which is used for distinguishing small hive beetle larvae from larvae of other insects within the hive (Elzen *et al.*, 1999; PIRSA, 2006). A mature larva measures about 11.1 mm in length and 1.6 mm in width, with body size varying based on availability of food (Elzen *et al.*, 1999; PIRSA, 2006). Following the arrestment of feeding, mature larvae enter the wandering stage, and being attracted to light, they move out of the hives at dusk in search of a suitable pupation substrate (Elzen *et al.*, 1999; PIRSA, 2006). Larvae burrow 10–20 cm and sometimes up to 30 cm into a suitable substrate usually moist soil and construct a pupation chamber where pupation and development takes place (Torto *et al.*, 2010b). The pupa appears whitish in colour and gradually becomes brown before

emergence (Elzen *et al.*, 1999; PIRSA, 2006). Adult usually emerges 15–60 days post-pupation with most adults emerging after 21 to 28 days (Elzen *et al.*, 1999; PIRSA, 2006).

Newly emerged adults are brown-black in colour on the dorsal side and reddish brown to black on the ventral side (Schmolke, 1974; Elzen *et al.*, 1999; PIRSA, 2006). They are oval in shape and approximately 5–7 mm long and 2.5–3.5 mm wide. Adults have been reported to successfully reproduce on a variety of diets which include bee brood, pollen, honey, fresh and rotten fruits, empty bee combs (Elzen *et al.*, 2000; Ellis *et al.*, 2002; Buchholtz *et al.*, 2008; Arbogast *et al.*, 2009a) thereby increasing their chances of survival as their parasitisation of honey bee colonies appears facultative.

**2.2.2.3 Economic importance and management of *A. tumida***

Both larval and adult stages of the small hive beetle cause damage to bee hives, the larva causing the most damage. In the USA, this exotic pest is known to feed on bee brood, pollen, honey and bee combs causing severe damage within the hives (Hood, 2000). Similar but minor or negligible damage patterns have been observed for this insect within its African host (Lundie, 1940; Schmolke, 1974; Hepburn and Radloff, 1998; Elzen *et al.*, 1999) and several studies have attributed these observed difference in infestation levels between African and European honey bee colonies to the greater hygienic behaviour of the former compared to the latter (Elzen *et al.*, 2001; Neumann and Härtel, 2004). The huge losses caused by this pest in the Western hemisphere and its occasionally severe damage to African apiaries have demanded the development of tools and strategies to monitor and manage its damage.

Recent evidence has shown that the small hive beetle can occasionally infest and damage bumble bees (Graham *et al.*, 2011) as well as stingless bees (Nkoba, pers. comm.). This is not

surprising as both insect species belong to the same genus Apidae and share similar biology to that of honey bees. However, these observations raise critical concerns on whether social bees are the true host of this beetle as several studies have shown that it can survive on different fruits types (Buchholz *et al.* 2007; Arbogast *et al.*, 2009) in the presence or absence of the yeast *Kodamaea ohmeri*, shown to be associated with it (Benda *et al.*, 2008). Furthermore, Elzen and Neumann (1999) postulated that in the absence of honey bees, the beetle can survive on alternate host in a natural system but no records of such observations have been documented. One of the objectives of this study is to determine potential alternate hosts of the beetle in its native host range.

Although a wide variety of management options already exist for numerous insect pests, the very unique nature of honey bee colonies and their products imposes great constraints on the adoption of already available control options. Within the native geographical range of *A. tumida*, its occasional pest status has not called for the development of control measures. However, the devastation it causes in its introduced geographical range necessitates the tracking and monitoring of its spread as well as the development of tools to manage it.

The new geographical range of this beetle straddles different agroclimatic zones some of which are suitable for its survival while others are not. This makes it compelling to track the origin and spread of this beetle so as to understand factors which either enhance or limit its survival. In this regard, Evans *et al.* (2003) demonstrated the suitability of a 1080 base-pair fragment of the mitochondrial cytochrome oxidase c subunit I (*mtCOI*) gene as molecular marker for tracking small hive beetle populations. The aptness of the mtCOI and microsatellite markers was further confirmed by Lounsberry *et al.* (2010). These studies

clearly portrayed mtCOI and microsatellite markers as cheap, fast and efficient ways of tracking the global spread of this beetle.

Besides tracking the spread of *A. tumida*, monitoring for its presence is vital for the detection and management of infestations. A corrugated bottom-board trap which provides hideouts for the beetle was shown by Neumann and Hoffmann (2008) to be a simple and effective way of detecting the presence of small hive beetle. Suazo *et al.*, (2003) and Torto *et al.*, (2005) showed that the small hive beetle located honey bee colonies by detecting their odours. However, these authors have been unable to successfully develop synthetic lures for the beetles based on these odours. Following the detection of a yeast *Kodamaea ohmeri* associated with the small hive beetle (Torto *et al.*, 2007c; Benda *et al.*, 2008), an odour bait composed of a mixture of this yeast and pollen dough placed in an in-hive bottom board trap, formulated by Torto *et al.* (2007b) showed potential as a monitoring tool. The potential of this bait was confirmed by Arbogast *et al*, (2007) and Torto *et al.*, (2010b) who used them in flight and bottom-board trap types, respectively.

The organophosphate, coumaphos (Elzen *et al.*, 1999) and entomopathogenic fungi recovered from *A. tumida* in Southern Africa (Muerrle *et al.*, 2006) have been demonstrated as possible control tools for the beetle. However, only coumaphos in combination with CheckMite and the corrugated bottom board traps have shown practical applications in beetle detection and management (Neumann and Hoffmann, 2008). Recently, Mutyamba (2010) showed that the baited in-hive bottom-board trap was capable of significantly reducing small hive beetle infestations over a 7 month period to near zero. However, the enormous amount of labour involved in deploying and removing these traps indicates that there is still the need for more cost-effective tools for managing *A. tumida*.

## CHAPTER THREE

## Occurrence, diversity and pattern of damage of two *Oplostomus* species (Coleoptera: Scarabaeidae), pests of honey bees in Kenya

### 3.1 Summary

Honey bees (*Apis mellifera* L.) contribute significantly both directly and indirectly to the livelihood of man by producing useful hive products and providing ecosystems services. Although beekeeping is globally popular and arthropods associated with European honey bees well documented, not much is known about the arthropods associated with honey bees in African. This study was carried out to document hive beetles associated with honey bees of Kenya. Managed honey bee colonies kept in Langstroth hives across the country were inspected for beetle infestations. Two scarabs, *O. haroldi* and *Oplostomus* sp. were recovered from colonies in two out of five agroecological zones in Kenya. The numbers recovered were dissimilar across locations, with *O. species* confined to the Eastern province and 3-fold more *O. haroldi* in the Coast than Eastern province. Samples of these beetles from different locations were studied using sequence analysis of their mitochondrial cytochrome oxidase 1 (mtCOI) gene. There was sufficient sequence divergence to separate beetle samples according to their species and location of origin for *O. haroldi.* The damage inflicted by mixed sexes of these beetles on honey bees was investigated in assays using honey combs containing brood, pollen and honey but void of adult worker bees in an observation hive. Beetles damaged brood (80.5% for *O. haroldi* and 100% for *Oplostomus* species), honey (16.1% for *O. haroldi*) and pollen (3.4%) by feeding on them and showed a significant preference for brood.

### 3.1 Introduction

Globally, beekeeping is an important economic activity with enormous direct and indirect benefits to both beekeepers and the environment (Graham, 2003). The realisation of the immense potential of honey bees has led to an ever growing interest to fully comprehend its biology as evidenced from the vast amount of scholastic and non-scholastic literature on bees which continues to grow at an exponential pace. Studies and investigations spurred by this interest have led to breakthroughs and inventions which have shaped the history of beekeeping and bee research. Notably, is the invention of the Langstroth box hive (Crane, 1999; Graham, 2003) currently regarded as the best hive type due to its worldwide adoption and usage by most beekeepers and bee researchers. The ever-increasing popularity of apiculture globally can in part be attributed to this hive type and its local modifications. In Kenya, apiculture is becoming a major economic activity being used to combat poverty, unemployment, gender disparity and to promote biodiversity conservation in rural areas, with a growing preference for Langstroth box hives over traditional ones (Raina, 2006). However, the continuous growth of apiculture has been constrained by pests (Crane, 1999; FAO, 2006) among which arthropod pests are the most economically important (FAO, 2006; Torto *et al.*, 2010a).

Recent field studies conducted in several parts of Kenya, as part of a project aimed at enacting guidelines for beekeeping, reported the presence of the large hive beetle *O. haroldi* along with *A. tumida* (Torto *et al.*, 2010a) and *V. destructor* (Frazier *et al.*, 2011) as the main arthropod pests of honey bee, among others. This large hive beetle is predominantly found at the coastal part of Kenya and occurs more on the frames than any other section of the hive (Torto *et al.*, 2010a). Its large size and huge infestations coupled with reports of low honey production from bee keepers in areas with high infestation undeniably makes it an economically

important pest. However, information on the damage it causes is currently lacking and needs to be established to appreciate the full extent of its economic importance. With the potential of *O. haroldi* to cause huge damage on honey bees and negatively affect honey production, there is the need to expand field sampling so as to fully document the distribution of this beetle. An earlier study by Njau *et al.* (2009) also reported the occurrence of *O. haroldi* in Tanzania as a pest of honey bees. Another member of this genus, *O. fuligineus,* occurs in several countries within the southern part of the continent and has been reported as a major pest of honey bees and preys on their brood (larvae and pupae) and sometimes wasp brood (Donaldson, 1989).

Mainstream identification of insects has relied on the fine study and understanding of their morphological features by a few expert taxonomists who carry out routine insect identification, an activity which can be quite challenging and time-consuming. This scenario was true for *O. haroldi,* a scarab whose identity was confirmed by two taxonomists (Torto *et al.*, 2010a) who took 8-12 weeks each to do so following sample delivery. In addition to these challenges, the identification of immature stages of many insects using conventional methods is still not possible (Raupach *et al.*, 2010). These shortcomings generated an interest in the search for rapid and effective alternative tools, which led to the development of molecular markers as tools for identification. One of such markers is the mitochondrial DNA of animals whose high substitution rate, the nearly exclusive maternal origin and the non-involvement on nucleic acid hybridisations has made it a prime candidate for molecular taxonomy (Raupach *et al.*, 2010). In-depth studies on mitochondrial DNA identified a 650 bp segment close to the 5′ end of the mitochondrial cytochrome *c* oxidase subunit I (mtCOI) gene as a suitable target with sections of conserved and variable regions in its sequence sufficient to discriminate between most animal species (Hebert *et al.*, 2003a; Hajibabaei *et al.* 2006). This region has

been proposed as the core barcode region for the animal kingdom (Hebert and Gregory, 2005; Hebert *et al.*, 2003b). With different *Oplostomus* species sharing great similarities in body size, adult external morphology, habitats and seasonal occurrence coupled with the possibility of recovering only dead propolised specimens at certain times of the year poses a challenge to their accurate identification and study of their distribution. The generation of DNA barcodes for several tropical scarabs by Monaghan *et al.* (2011) suggest that this marker could also be used to distinguish the *Oplostomus* species singly or in a DNA-assisted taxonomy approach. This study was carried out to investigate the current distribution of scarab pests of honey bees within Kenya so as to obtain baseline data necessary for drawing-up regulations for the apicultural industry. The study also sought to characterise and quantify the damage caused by these beetles on honey bee and, to explore the potential use of a 650 base pair DNA barcode region to distinguish between these scarabs.

## 3.3 Materials and methods

### 3.3.1 Survey sites and collection of specimens

Twenty-one beekeeping sites in 10 districts in five (North-eastern, Eastern, Central, Western and Coast provinces) of the eight provinces in Kenya were surveyed for the presence of beetles in honey bee colonies (Table 3.1). These sites were chosen to provide a representation of the major agroecological zones (humid, humid to sub-humid, semi-arid and arid zones) in Kenya (Reynolds, 2004). Only honey bee colonies kept in Langstroth box hives were inspected for beetle infestation as this permitted comparison of beetle infestation levels across locations.

At all the sites, only colonies of honey bees without crossed combs were chosen at random for inspection within each apiary. Depending on the number of bee colonies available at each

apiary, 60 – 100% of the hives were randomly selected for inspection. Scarab beetles found at the top board, frames, inside walls of the hive box and its bottom board were handpicked and counted using a procedure similar to that described by Torto *et al.* (2010a) and pooled for each hive as some had their bottoms fixed to the hive box. All scarab beetles collected from the hives during the survey were tentatively identified using the external morphology of their mouth parts, head, legs and abdomen. After preliminary identification, the insects were maintained on moist sterile cotton wool in rectangular plastic bowls with perforated lids and brought back to the *icipe* laboratory. Their identities were later confirmed by comparing them to *O. haroldi* type specimens kept at *icipe*'s insect collection. The insects were then kept on substrate composed of sterilised cow dung and soil (mixed in the ratio 1:1 v/v). Besides *O. haroldi*, another scarab, *Oplostomus* sp. (identified by Max Barclay, Natural History Museum, London, who is currently contacting other museums to confirm if this is a new species in this genus), was also recovered from honey bee colonies. The different sexes of both *Oplostomus* species were distinguished using the morphology of their abdominal sterna, as described by Torto *et al.* (2010a) for *O. haroldi.*

**Table 3.1**. Summary of locations surveyed between February 2010 and May 2011

| Locality | District | Agroecological zone | Location coordinates | Sampling date | No. colonies and apiaries inspected (in parentheses) | Nitidulid species present | Scarab species present |
|---|---|---|---|---|---|---|---|
| Mtwapa | Kilifi | Sub-humid zone | 03°20'21.1"S, 39°59'15.8"E | Feb. 2011 | 7 (1) | *Aethina tumida* | *Oplostomus haroldi* |
| Muhaka | Kwale | Sub-humid zone | 04°19'43.4"S, 39°31'4.9"E | Jun. 2010 | 2 (1) | *Aethina tumida* | *Oplostomus haroldi* |
| Kitui | Kitui | Semi-arid zone | 01°17'40.5"S, 37°59'15.8"E | Feb. 2010 | 4 (1) | *Aethina tumida* | *Oplostomus haroldi* |
| Kina | Isiolo | Semi-arid zone | 00°19'09.1"N, 38°12'19.4"E | Aug. 2010 | 13 (2) | *Aethina tumida* | *Oplostomus species* |
| Isiolo Central | | Semi-arid zone | 00°21'2.1"N, 37°34'54.1"E | Aug. 2010 | 17 (4) | *Aethina tumida* | *Oplostomus species* |
| Merti | | Arid zone | 01°03'40.5"N, 38°39'53.1"E | Aug. 2010 | 4 (2) | *Aethina tumida* | None |
| Sericho | | Arid zone | 01°07'59.9"N, 39°06'20.8"E | Aug. 2010 | 4 (1) | *Aethina tumida* | None |
| Il Parua | | Semi-arid zone | 00°17'50.9"N, 37°26'5.0"E | Aug. 2010 | 8 (2) | *Aethina tumida* | *Oplostomus species* |
| Oreteti | | Semi-arid zone | 00°17'46.6"N, 37°16'42.8"E | Aug. 2010 | 5 (1) | *Aethina tumida* | None |
| Oldoniro | | Semi-arid zone | 00°37'21.5"N, 36°59'13.3"E | Aug. 2010 | 10 (2) | *Aethina tumida* | None |
| Kakamega | Kakamega | Humid to sub-humid zone | 00°09'40.2"N, 34°51'27.5"E | Dec. 2010 | 8 (2) | *Aethina tumida* | None |
| Elgon | Mount Elgon | Humid zone | 00°48'7.8"N, 34°27'15.8"E | Oct. 2010 | 4 (4) | *Aethina tumida* | None |
| Busia | Busia | Humid to sub- | 00°22'23.3"N, | Oct. 2010 | 2 (2) | *Aethina tumida* | None |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | humid zone | 34°09'29.2"E | | | | |
| Mandera | Mandera | Arid zone | 03°56'12.3"N, 41°52'5.4"E | Aug. 2010 | 2 (2) | *Aethina tumida* | None |
| Mandera West | | Arid zone | 03°56'11.8"N, 41°52'4.9"E | Aug. 2010 | 1 (1) | *Aethina tumida* | None |
| Malewa | Nyadarua | Humid zone | 00°31'30.1"S, 36°21'11.8"E | Jun. 2010 | 5 (1) | *Aethina tumida* | None |
| Nadasa-Aberdare | | Humid zone | 00°31'13.0"S, 36°37'20.1"E | Jun. 2010 | 2 (1) | None | None |
| Ngeta-Aberdare | | Humid zone | 00°34'41.6"S, 36°37'47.5"E | Jun. 2010 | 1 (1) | None | None |
| Kamuieti | Maragwa | Humid zone | 00°21'8.9"S, 37°18'57.9"E | Jun. 2010 | 3 (2) | None | None |
| Nji-ini | | Humid zone | 00°31'8.2"S, 37°25'14.1"E | Jun. 2010 | 2 (1) | None | None |
| Ichiara | | Humid zone | 00°28'2.3"S, 37°43'2.2"E | Jun. 2010 | 4 (1) | None | None |

### 3.3.2 Genetic diversity of *Oplostomus* species

#### 3.3.2.1 Sample preparation and DNA extraction

A subset (n = 5) of each *Oplostomus* species (mixed sexes) collected during field surveys in a previous study in 2009 (Torto *et al.*, 2010a) and 2010 at various sites were killed by freezing at – 20 °C for 30 min. The dead insects were later thawed, cured and the left middle leg pulled off and stored in 85% ethanol at 4 °C prior to shipment. The stored leg samples were then sent to the BOLD (Barcode of Life Data systems) laboratory hosted at the University of Guelph, Canada for DNA barcoding.

Five male insects of each *Oplostomus* species and *Pachnoda gedyei* (which served as an outgroup species) were similarly killed and prepared for subsequent DNA extraction. All samples were stored in 85% ethanol at 4 °C prior to DNA extraction and analysis at *icipe*.

DNA was then extracted from each of these legs, using the CTAB DNA extraction protocol (Powell *et al.*, 2006) with care taken to avoid sample contamination. Ten (10) millilitres of isolation buffer (2X CTAB composed of 100 mM Tris HCL at pH 8.0, 1.4 mM NaCl, 20 mM EDTA and 2% Cetyltrimethyl ammonium bromide-CTAB) containing 80 µl of beta-mercaptoethanol in 50ml falcon tubes was preheated in 65$^0$C water bath for 15 minutes. Insect tissue from each beetle was homogenised in 150 µl of the isolation buffer. Afterwards, 150 µl of SEVAG buffer (comprising chloroform and isoamyalcohol in the ratio 24:1 respectively) was added into the homogenate and mixed gently. The homogenate was centrifuged at 150 rpm for 60 min and later at 4000 rpm for 20 min. The supernatant of the homogenate (~500 µl) was then transfered into a fresh tube and the sediments discarded. Two-third this volume of ice-cold isopropanol was added into the supernatant and the solution mixed gently and incubated at – 20 °C overnight. The solution was then centrifuged at 3000 rpm for 5 min to

precipitate the extracted DNA. The resulting supernatant was decanted carefully to avoid loss of DNA material. The precipitated DNA was washed twice with 70% ethanol, each time centrifuging at 3000 rpm for 5 min and pouring-off the liquid phase. The precipitate was air dried by placing the tubes to lie on their side for approximately 30 min and later resuspended in 40 µl of Tris EDTA (composed of 10mM Tris-HCL at pH 8.0 and 0.25 mM EDTA).

### 3.3.2.2 Polymerase Chain Reaction and DNA sequencing

A 658 bp region of the *mtCOI* gene was amplified by PCR using the primers, LCO-1490 (5' GGTCAACAAATCATAAAGATATTGG 3') forward and HCO-2198 (5' TAAACTTCAGGGTGACCAAAAAATCA 3') reverse, previously described by Folmer *et al.*, (1994) and known to amplify the mtCOI gene in a wide range of invertebrate taxa. Amplification was carried out in 20 µl reaction volumes containing 5U GenScript *Taq* DNA polymerase, 1.25 mM MgCl, 0.5 mM dNTPs, 2 µM of each primer, 1 x GenScript buffer and ~ 10ng of genomic DNA template. The PCR thermocycling was carried out under the following conditions: 2 min at 94 °C; 35 cycles of 1 min at 94 °C, 1 min at 46 °C and 2 min at 72 °C; 10 min at 72 °C; held at 10 °C. The PCR product was separated on a 1.2 % agarose gel with ethidium bromide incorporated. The PCR product of ~ 680 bp (close to the target size) was excised from the gel and purified using GenScript QuickClean 5M *Gel* Extraction Kit (Piscataway, NJ, USA) following the manufacturer's instructions. The purified product was bidirectionally sequenced using the PCR primers on an ABI chain termination sequencing technology at a commercial facility (Macrogen Inc., Korea).

### 3.3.2.3 Damage pattern of *Oplostomus* species

The aim of this experiment was to characterise the pattern of damage caused by *Oplostmus* sp. on honey bee combs. Honey bee combs constructed on standard hive frames (19 in x 9¼ in)

which contained bee brood (larvae and pupae), pollen and honey but were void of adult bees were collected from managed colonies at the *icipe* apiary and brought to the laboratory. Pictures were taken of both sides of each comb after which they were kept in an observation hive (k GS4005 model, Dadant, Hamilton, IL, USA). Adults of both *Oplostomus* species were introduced into the observation hive and kept in the dark for 24 hr at a temperature and relative humidity of 30 ± 1 °C and 50 ± 5%, respectively, to mimic conditions inside a beehive. For *O. haroldi*, three treatments consisting of 20 males only (treatment 1), 20 females only (treatment 2) and a mixture of 10 males and 10 females (treatment 3) were released separately into the observation hive and their damage evaluated. Each treatment was replicated three times. Similarly, two treatments comprising 6 males and 6 females only were tested for *Oplostomus* sp. and also replicated thrice. Treatment sizes were chosen based the highest infestation numbers reported by Torto *et al.* (2010a) for *O. haroldi* and from the first section of this study. After 24 hr, the observation hives were opened and the beetles removed from the frames. The frames were then removed and photographed again. The number of brood, pollen and honey cells damaged by the beetles were counted, recorded and expressed as a percentage of the total number of cells on the frame. The total number of cells on each honey bee frame was estimated using a 10 cm x 10 cm quadrat made of steel wire. The quadrat was gently pushed onto one side of the bee frame and the number of cells within it counted. This procedure was replicated 3 times using both sides of the frames while avoiding areas damaged by the beetle and the 3 replicates used to compute the average cell number per 10 $cm^2$ of the comb. The total cell number on each bee frame was computed using the formula below and assuming perfect symmetry in comb structure:

$$N = 2(n \times q),$$

where N = total cell number on frame, n = average number of cell/ 10 $cm^2$ and q = number quadrats that completely fit one side of the bee comb.

### 3.3.4 Data analysis

#### 3.3.4.1 Distribution of *Oplostomus* species

Logistic regression and pairwise orthogonal comparisons were used to compare beetle counts across apiaries and sites where each beetle species was recovered. Comparisons were carried out only for sites surveyed within the same month of the year. All analyses were done at an $\alpha$ level of 0.05 using the statistical software R version 2.1.3.0. (R, 2010).

#### 3.3.4.2 MtCOI sequence variations and genetic diversity

DNA sequences were edited manually using BioEdit (Hall, 1999) to give consensus sequences. The primer sequences were removed from the consensus sequences resulting in a 658 bp fragment. The sequences were checked for open reading frames using the Transeq program hosted by the European Molecular Biology Open Software Suite (EMBOSS). The 658 bp sequences were then submitted to BOLD.

Multiple sequence alignment was done using ClustalX version 1.81. Phylogenetic relationships between the samples were inferred from trees constructed using the neighbour-joining (NJ) tree method in Mega 4.0 (Tamura *et al.*, 2007), with 1000 bootstrap replicates. Intra-and interspecific sequence divergence distances based on Kimura 2-parameter (Kimura, 1980) between individual species and groups of populations were also determined.

#### 3.3.4.3 Damage pattern of *Oplostomus* species

The proportions of the honey bee frame damaged between treatments for each species were compared using a Kruskal-Wallis ANOVA on ranks and Mann Whitney U. The proportions of brood, honey and pollen cells damaged within each treatment were compared using Kruskal-Wallis ANOVA and Mann Whitney U test on ranks to reveal any food preferences.

Non-parametric tests were used to analyse the data as their variances were unequal. All analysis were done at an α level of 0.05 using the statistical software R version 2.1.3.0. (R, 2010)

### 3.4 Results

#### 3.4.1 Distribution of *Oplostomus* species in Kenya

Only 14.8% out of 108 honey bee colonies inspected in 35 apiaries at 22 locations were infested. Only honey bee colonies in 6 localities showed infestation by both *Oplostomus* species (Figure 3.1). The occurrence of *Oplostomus* sp was limited to the Eastern province while *O. haroldi* was found in the Coast and Eastern provinces (Figure 3.1).

A total of 96 adult *O. haroldi* were recovered from Kitui (11), Mtwapa (2) and Muhaka (83) while 26 *Oplostomus* sp. adults were recovered from Kina (16), Isiolo Central (9) and Il Parua (1) (Figure 3.2). Infestation levels of *O. haroldi* were significantly higher in Kitui compared Mtwapa ($\chi^2$ = 7.21, P = 0.007). Similarly, there were significant differences in counts of *Oplostomus* sp. across sites where it occurred ($\chi^2$ = 3.39, P = 0.009). Nevertheless, pairwise orthogonal comparisons showed that the occurrence of *Oplostomus* sp. did not vary significantly across and within the three locations (P> 0.05). *Oplostomus haroldi* occurred more at higher altitudes than lower altitudes while more *Oplostomus* sp. adults were recovered at lower than higher altitudes (Figure 3.5). The majority of both beetle species were observed on the frames inside the hives (Figure 3.3). The small hive beetle *A. tumida* was also found mostly on the bottom board at all the survey sites co-existing with these scarabs inside the hive (Figure 3.4).

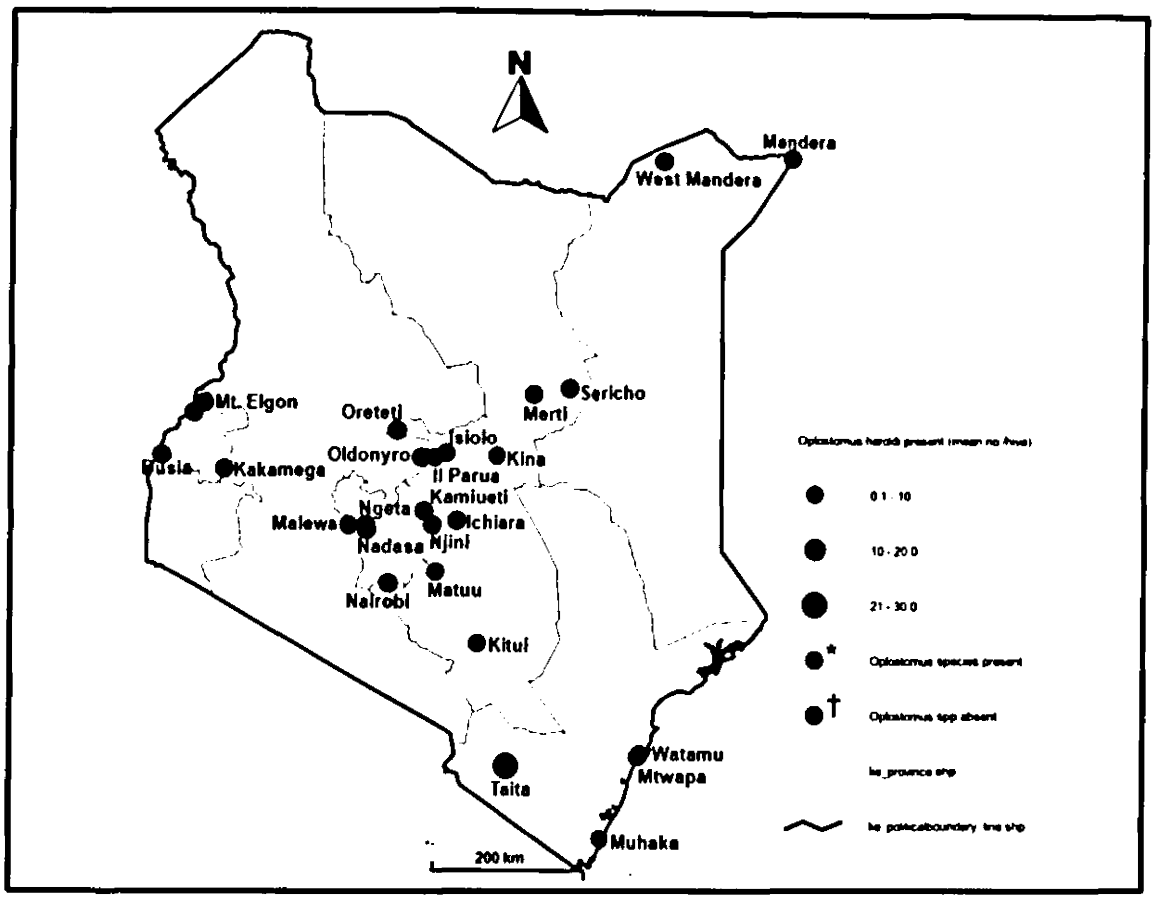



**Figure 3.1** Map of Kenya showing locations surveyed for *Oplostomus* species between June 2009 and May 2011 (modified from Torto *et al.* 2010a). Blue dots represent occurrence of *Oplostomus* sp, red dots presence of *O. haroldi* (and based on beetle counts from Torto *et al.*, 2010a and the current study) and black dots absence of both species. Asterix (*) refers to localities from which statistically similar numbers of *Oplostomus* sp. were recovered and Dagger (†) represents surveyed locations where no Oplostomus beetles were found in honey bee colonies.

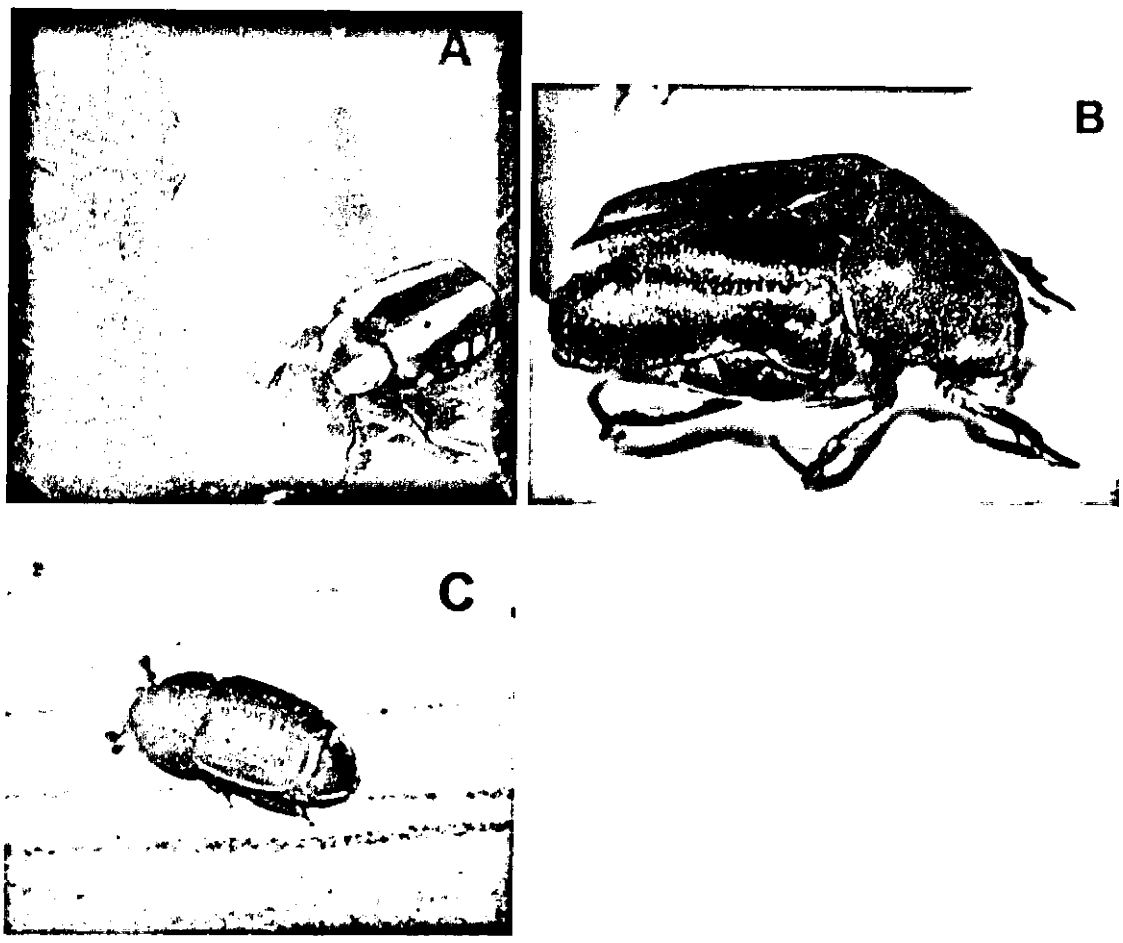



**Figure 3.2** Hive beetle species associated with honey bee colonies in Kenya. (A) *Oplostomus haroldi*, (B) *Oplostomus* sp. and (C) *Aethina tumida* (small hive beetle)

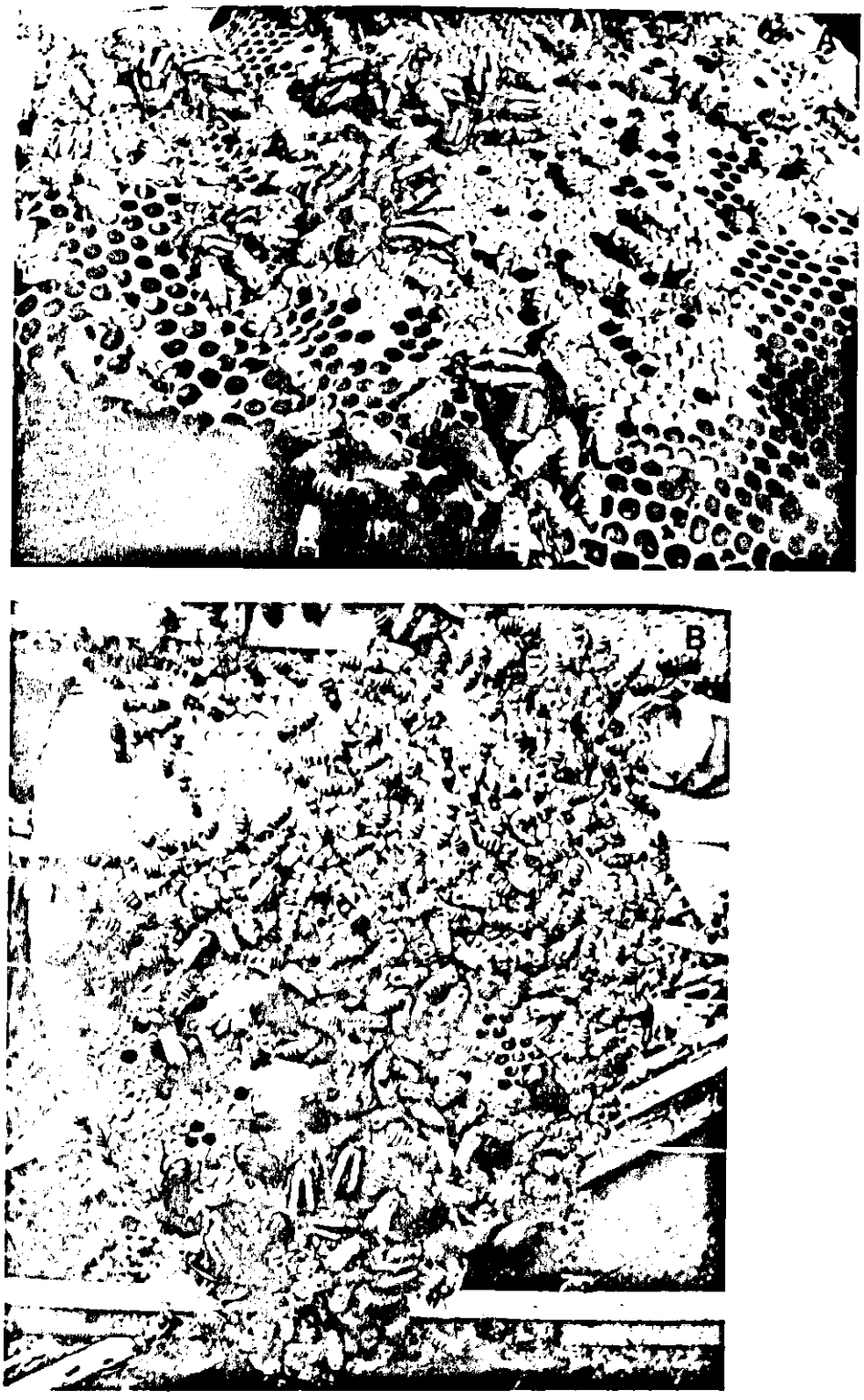



**Figure 3.3** *Oplostomus haroldi* beetles on honey bee frames

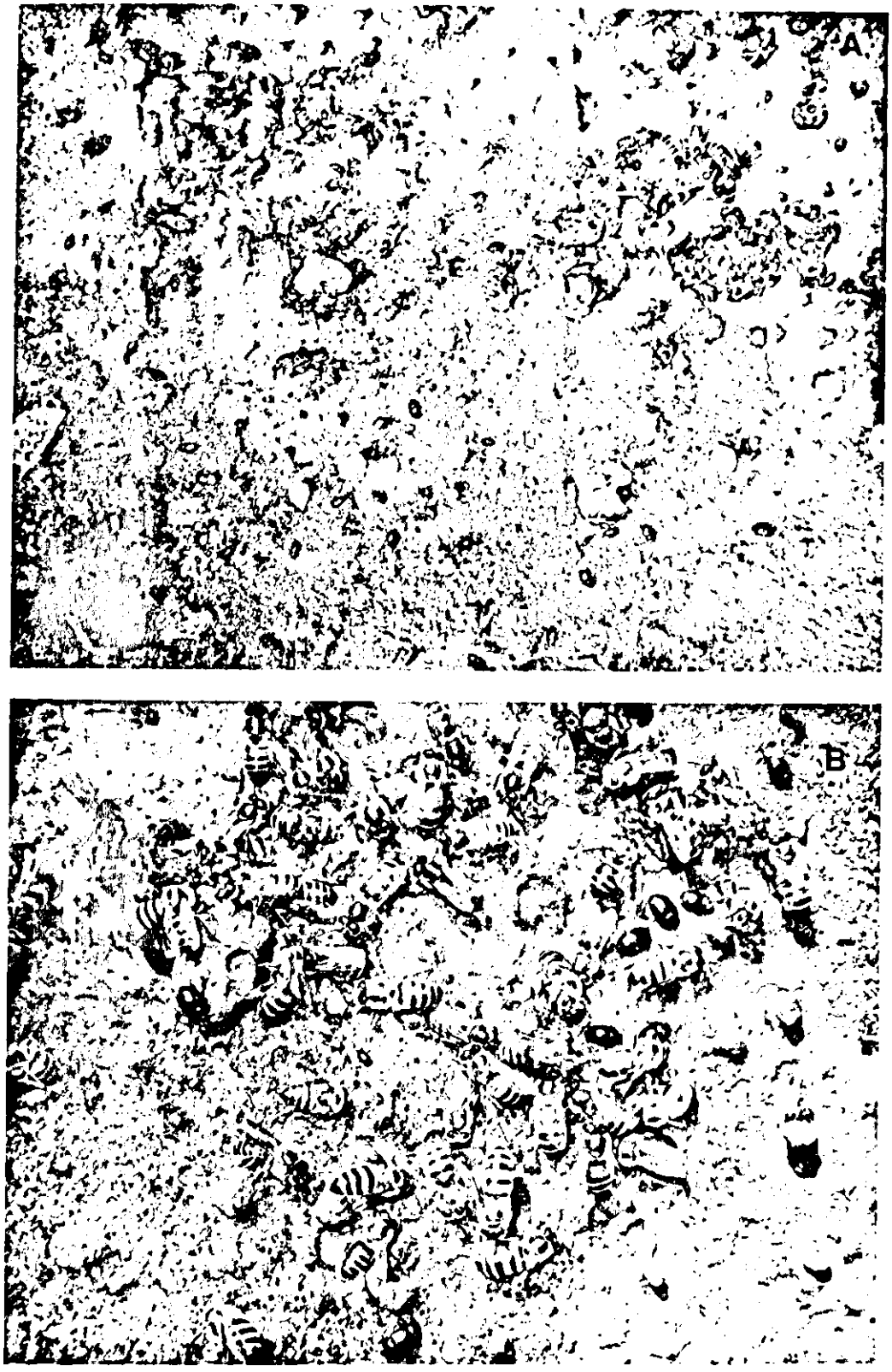



Figure 3.4 Adult *Aethina tumida* on bottom board of a honey bee hive

### 3.4.2 MtCOI sequence variation and genetic diversity

A total of 28 sequences were used for subsequent analyses. All sequences were highly AT biased with an average A+T content of 66%. The average interspecific K2P distance was 18.8%, with the lowest being 17.3%, observed between *O. haroldi* and *P. gedyei*, and the highest 21.3%, observed between *Oplostomus* sp. and *P. gedyei*, (Table 3.2). Intraspecific distances averaged 1.65% and ranged from 1.5% in *O. haroldi* to 1.8% in *Oplostomus* sp.

The evolutionary relationships between both *Oplostomus* sp. and the outgroup *P. gedyei* revealed two clusters, one containing *O. haroldi* and *P. gedyei* and the other *Oplostomus* sp. only (Figure 3.6a). A similar neighbour-joining (NJ) cluster analysis carried out for *O. haroldi* samples from 4 different locations showed that they clustered into 3 groups according to their locations of origin except for the lone sample from Matuu which was part of the Taita group (Figure 3.6b).

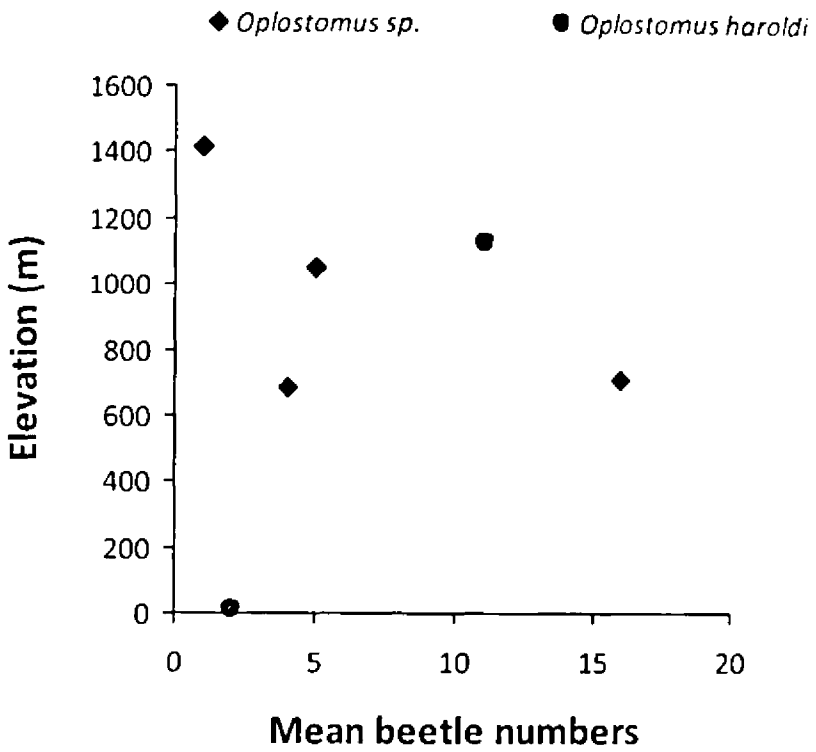



**Figure 3.5** Variation of occurrence of *Oplostomus* sp. and *O. haroldi* with elevation.

**Table 3.2**: Estimates of evolutionary divergence between *Oplostomus* species based on 5' mtCOI gene.

| | *Oplostomus haroldi* | *Oplostomus* sp. | *Pachnoda gedyei* |
|---|---|---|---|
| *Oplostomus haroldi* | | | |
| *Oplostomus* sp. | 0.177 | | |
| *Pachnoda gedyei* | 0.173 | 0.213 | |

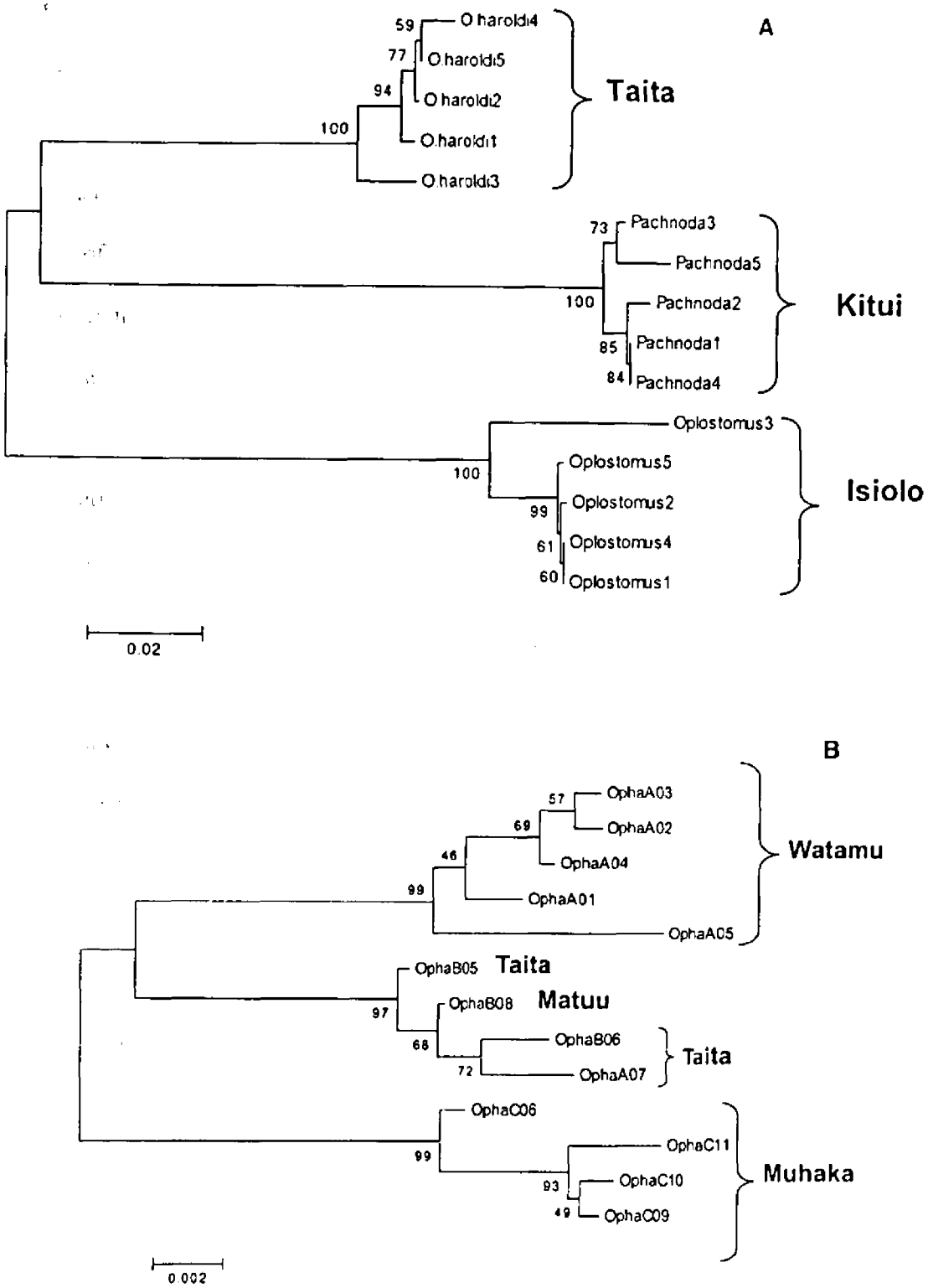



**Figure 3.6** Summary of evolutionary relationship of 5 samples of *O. haroldi* and *Oplostomus* sp. from different localities (A) and 13 samples of *O. haroldi* (B) from 4 different localities analysed using the NJ tree method with *P. gedyei* as an outgroup. This tree was inferred from 1000 bootstraps whose values are shown next to the branches.

### 3.4.3 Damage pattern of *Oplostomus* species

Both species were observed to feed on brood (both capped and uncapped), honey and pollen on the honey combs (Figure 3.7). Their feeding behaviour resulted in a characteristic damage pattern consisting of small (1-3 cells) and large (>5 cells) clusters of damaged cells. The small clusters of cell damage resulted from a beetle feeding on a 4$^{th}$ or 5$^{th}$ instar larva or pupa and sometimes its neighbouring cells as it did not have to burrow into the cell to access food. The large clusters of cell damage resulted from the beetle either burrowing into a single cell containing a 2$^{nd}$ or 3$^{rd}$ instar larva or a capped pupa cell to feed on it and destroying neighbouring cells as it did so due to its large size. This burrowing behaviour was done at an angle to the surface of the comb resulting in a large number of damaged cells than if it were vertically done.

With regards to capped brood, beetles scraped-off their cell cappings and fed on the young developing pupae (white to pink-eyed stages) while avoiding callow unemerged adult bees (purple to black-eyed stages). Beetles also scrapped the caps off capped honey cells and fed on honey. Both species showed a greater preference for brood over honey and pollen (Table 3.3). More damage was recorded on brood ($H=8.011$, $df=2, 9$, $P<0.001$; $U<0.0001$, $P=0.029$ for treatments 1 and 3) in *O. haroldi* as well as in *Oplostomus* sp. The damage recorded for the different treatments in each species did not significantly differ from each other ($H=0.382$, $df=2, 9$, $P=0.826$ for *O. haroldi* and $U=4.0$, $P=1.0$ for *Oplostomus* sp., respectively).

**Table 3.3**: Summary of damage of *Oplostomus haroldi* and *Oplostomus* sp treatments on honey combs under laboratory conditions

| Species | Treatments | Damage on frame | Proportion of brood, honey and pollen | | |
|---|---|---|---|---|---|
| | | | Brood | Honey | Pollen |
| *Oplostomus haroldi* | 1 (10 males & 10 females)* | 1.7 ± 0.74 | 87.4 ± 9.38[a] | 7.6 ± 7.63[b] | 5.0 ± 2.89[b] |
| | 2 (20 males)* | 2.4 ± 1.11 | 54.9 ± 19.5[a] | 40.7 ± 20.64[a] | 4.4 ± 3.53[a] |
| | 3 (20 females)* | 1.7 ± 1.01 | 99.1 ± 0.67[a] | | 0.9 ± 0.67[b] |
| *Oplostomus* sp. | 1 (6 males) | 0.6 ± 0.15 | 100 | 0 | 0 |
| | 2 (6 females) | 0.8 ± 0.38 | 100 | 0 | 0 |

*Treatment row values followed by the same superscripted letter are not significantly different at P=0.05, Kruskal-Wallis ANOVA and Mann-Whitney t-test

### 3.5 Discussion

This study revealed that the large hive beetles *O. haroldi* and *Oplostomus* sp. occupy specific habitat ranges which were apparently non-overlapping and were restricted to the eastern, coastal and highland parts of Kenya. It is not known why they do not overlap although it is possible that Mt. Kenya acts as a physical barrier limiting their spread beyond this landmark feature. These somewhat specific geographic ranges with dissimilar environmental conditions may play an important role in the survival, distribution and possible spread of these species. The observation of significantly more *O. haroldi* adults at a higher altitude (Kitui-1128 m asl) compared to a lower one (Mtwapa-10 m asl) corroborates an earlier study by Torto *et al.*, (2010a). These trends were attributed to the ambient environmental conditions in these areas which permitted the survival of *O. haroldi*. Highlands in Kenya are associated with watersheds and forest, and hence, receive more precipitation which favours the development of these beetles than the lowlands. However, an opposite trend was observed for *Oplostomus* sp. as more adults were recovered at lower altitudes than higher altitudes. Although Isiolo

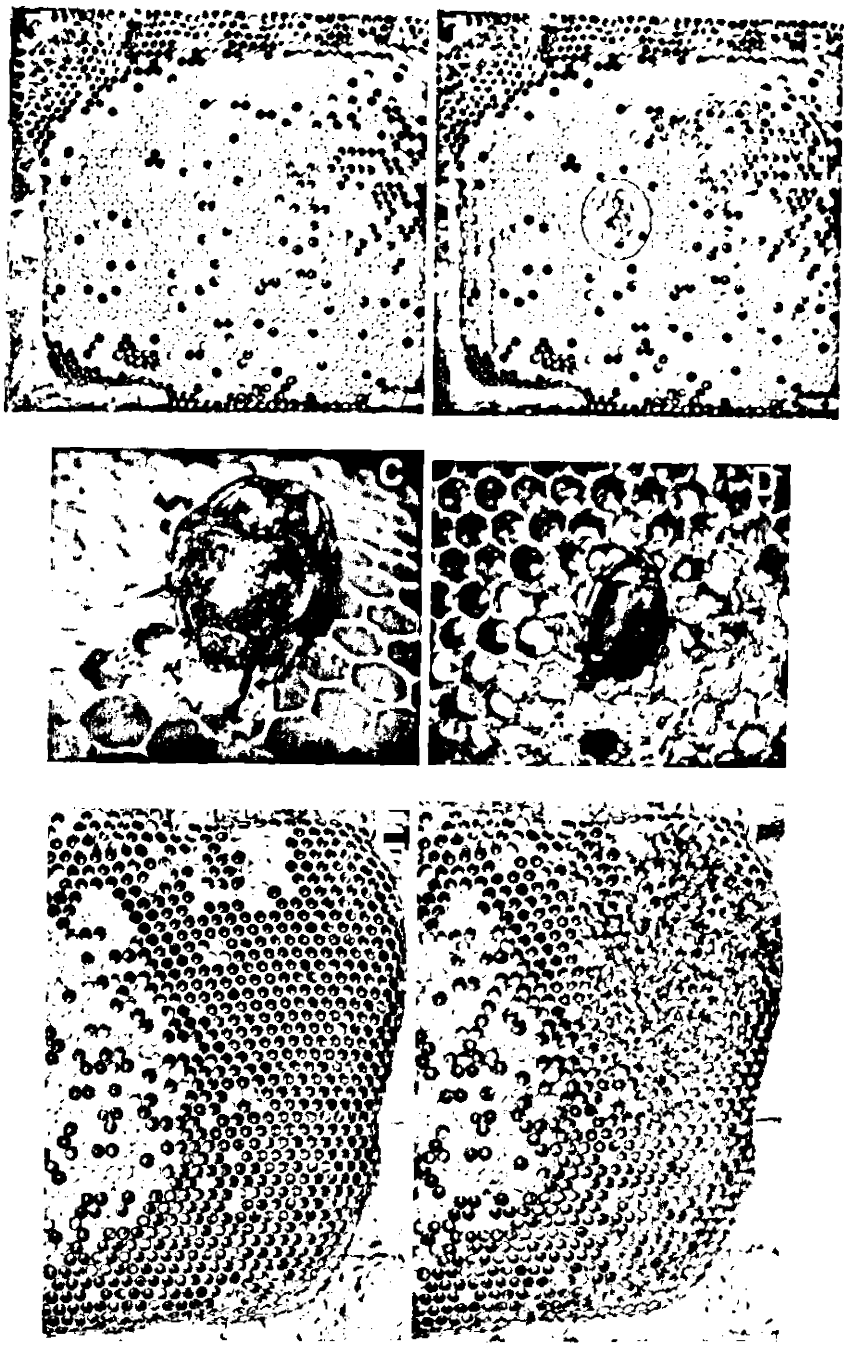


**Figure 3.7** Damage pattern of *Oplostomus species*. (A) section of frame before damage, (B) frame section after damage; white circle shows uncapped and uneaten callowed brood while the red circle shows uncapped and eaten young brood, (C) beetle feeding on pulled-out larva, (D) beetle burrowed deep into comb while feeding on brood, (E) section of frame before damage and (F) section of frame showing damage to uncapped honey cells.

district possesses a hot and semi-arid climate, its sub-locations with lower altitudes (< 1200 m asl) possessed more water bodies, (streams and rivers), a factor which may favour *Oplostomus* sp. occurrence at such elevations compared to higher ones. Infact, apiaries at both Kina and Isiolo Central where 96% *Oplostomus* sp. were recovered were < 50 m from permanent water bodies, which may have increased the beetles' survival chances by providing adequate moisture for the development of their larvae.

More beetles were found on the hive frames for both *Oplostomus* species. These observations tie-up with our earlier findings which showed that *O. haroldi* occurred more on the frames than other sections of the hive. This could be attributed to their large sizes, hard exoskeleton and death feigning behaviour which allows them to escape from defensive tactics of honey bees (Torto *et al.*, 2010a).

The mtCOI gene sequences generated from this study segregated *O. haroldi* from *Oplostomus* sp. and the generated barcodes were submitted to the BOLD database. The A+T content of these sequences were similar to those reported for other beetles (Raupach *et al.*, 2010; Monaghan *et al.*, 2011). Exploratory analyses of mtCOI gene fragment obtained from *O. haroldi* collected at different locations revealed population specific haplotypes. However, the clustering of the outgroup species *P. gedyei* with *O. haroldi* and one *O. haroldi* individual from Matuu with others from Taita suggests the necessity to use additional molecular markers to resolve population related differences. Although, mtCOI was demonstrated as a taxonomic tool, it will be important to incorporate more samples of these beetle species from different locations as well as *Oplostomus fuligineus* so as to gain more insights in the genetic variability of scarab pests of honey bees across the continent. Previous studies by Omondi *et al.*, (2011) and Raupach *et al.*, (2010) showed that mtCOI gene sequences enabled beetle

species discrimination with a higher accuracy compared to other molecular markers such as ITS (internal subscribed spacers) and rDNA (ribosomal DNA). Further studies are required to develop full length primers for the mtCOI gene and test their usefulness alongside RFLP (restriction fragment length polymorphism) in the discrimination of individual beetle species and their populations. The barcodes generated from this study will go a long way to improve the delineation of these beetles from one another.

The damage assays clearly showed that beetles fed on brood, pollen and honey with a greater preference for brood over the others. Their strong preference for brood may come from an innate biological demand for its nutrients which are essential for their survival and reproduction. A previous study by Donaldson (1989) on *O. fuligineus*, a species closely related to *O. haroldi* and *Oplostomus* sp. reported that it fed mainly on brood in the field, and pollen and honey as well in the laboratory but was only capable of reproducing when fed on brood. Besides their damage to hive resources, they also destroy the comb structure. Damage estimates showed that both beetle species can destroy 1.7-2.4% of a honey comb's contents within 24 hr. With these beetles known to be most abundant in the hives during the months of May-June (major wet season) (Torto *et al.*, 2010a) and November to February (minor wet season) (Fombong *et al.*, unpublished) with averages of more than 20 beetles per frame, these laboratory estimates of damage may translate into the loss of 1.02-1.44 honey combs per hive during such periods. This in turn will imply a loss of 1000-4000 potential worker bees assuming a 25-50% brood content of 5000-8000 celled frames, thus making these beetles a serious threat to the survival of honey bees. Besides negatively affecting colony growth, the damage by this beetle will adversely affect food production as more workers will be dedicated to fending-off the beetles and cleaning their mess.

In conclusion, this study provides the first evidence of the detection of *Oplostomus* sp. as a pest of honey bee colonies in addition to the known beetles, *O. haroldi* and *A. tumida* (Torto *et al.*, 2010). It also reports barcodes useful for the discrimination of *Oplostomus* species and, estimates the impact of their damage. This information will be critical to the drawing-up of guidelines to manage the spread of beetles countrywide, particularly the *Oplostomus* species.

## CHAPTER FOUR

## Life history of *Oplostomus haroldi* (Coleoptera: Scarabaeidae) and a description of its third larva instar

### 4.1 Summary

The domestication of the honey bee, *A. mellifera,* within resource-poor rural Africa is constrained by arthropod pests, among which hive beetles are known to cause significant damage. Recently, the scarab beetle *O. haroldi* was reported as a new pest of honey bee colonies in Kenya. With no information available on its biology, this study was carried out to establish its life history under laboratory conditions and to describe its larva for taxonomic purposes. Adult *O. haroldi* collected from honey bee colonies were reared on moist sterilised soil and cowdung. This rearing substrate was sifted daily for eggs which were kept on a similar substrate until the pupal stage, which was marked by the formation of a mud cocoon. Cocoons were incubated in moist sterilsed soil until adults emerged from them. The duration of each developmental stage and the number surviving were recorded. The larva was scarabaeiform, pear-shaped and curved with a well developed head and thoracic legs but without abdominal prolegs. Under laboratory conditions of 25 ± 2°C, 50 ± 5% R.H. and a 10L: 14D photo period, eggs took 11.9 ± 1.3 days to hatch and the 1$^{st}$, 2$^{nd}$ and 3$^{rd}$ larval instars lasted 14.6 ± 2.6, 17.5 ± 2.4, and 34.6 ± 2.4 days respectively. The pupal stage took 31.1 ± 6.7 days with adults capable of living for 2 to 6 months suggesting that *O. haroldi* is multivoltine and can be successfully reared on soil and cow dung.

## 4.2 Introduction

Cetoninae (Coleoptera: Scarabaeidae) is known to contain approximately 3900 beetle species which are familiar to entomologists for their shiny and bright colourful bodies which range in size from a few millimetres to over 110 mm (Micó *et al.*, 2008). Most cetonid adults are diurnal and feed on flowers, plant sap and fruits hence the names fruit and flower chaffers (Donaldson, 1989; Thomas, 1998; Micó *et al.*, 2008). Adults of some cetonids have been reported to be associated with social insects (Donaldson, 1989; Alpert, 1994; Torto *et al.*, 2010a) while their larvae have been reported to feed on decaying plant matter and faeces of herbivores (Donaldson, 1989; Thomas, 1998; Perissinotto *et al.*, 1999; Micó *et al.*, 2008). A number of cetonid beetles have also been ascribed pest status as a result of their diverse feeding habits (Ritcher, 1957; Donaldson, 1989; Thomas, 1998; Johannsmeier, 2001; Torto *et al.*, 2010a).

Identification of insects relies heavily on morphological characteristics with emphasis on life stages which are more commonly encountered in nature. However, among scarab beetles, external larval morphology is increasingly being used to separate species, in addition to adult characters (Ritcher, 1966; Donaldson, 1989; Smith *et al.*, 1998; Perissinotto *et al.*, 1999, 2003; Šípek *et al.*, 2004). Besides identifying insects, an understanding of their life history is imperative to the development of monitoring and management tools for economically important insects (Amarasekare *et al.*, 2008) because it reveals their pattern of development, abundance and their role within their habitats. Behavioural information obtained from the life history can also be useful in separating sibling species as demonstrated by various authors (Alexander & Bigelow, 1960; Craig *et al.*, 1993; Boake *et al.*, 1997).

Unlike most adult cetonids, two members of the genus *Oplostomus* have been reported to be strongly associated with honey bee colonies within their areas of occurrence and are rarely found on nearby flowering plants (Donaldson, 1989; Johannsmeier, 2001; Torto *et al.*, 2010a). Although some information exists on the biology of *O. fuligineus* (Donaldson 1989), similar information is lacking for *O. haroldi*, a closely related species that was recently recorded as a pest of honey bee colonies in Kenya (Torto *et al.*, 2010a). In this chapter, the life history of *O. haroldi* under laboratory conditions is documented and a description of the external larval morphology of its third instar larva provided for the first time.

## 4.3 Materials and Methods

### 4.3.1 Preparation of 3rd larva stage (L3)

Seven third instar larvae (65 – 70 days old) were prepared for dissection by immersing them first in boiling water (to kill them) and then in 50 % and 70% ethanol to dry the specimens. Dead larvae were decapitated and the head heated in 10% sodium hydroxide (NaOH) to macerate muscle tissues present in the head. Descriptions were based on the head capsule, mouthparts and raster pattern. These parts are more conspicuous and easy to visualise at the third instar and they were drawn using an EZ4 D digital stereomicroscope (Leica Microsystems, Heerbrug, Switzerland) connected to a personal computer. The terminology used in morphological descriptions were similar to those previously used by Ritcher (1966) and Smith *et al.* (1998)

### 4.3.2 Life History

Adult *O. haroldi* were collected from honey bee colonies during a survey in June 2009 (Torto *et al.*, 2010a), and brought to the *icipe* insectary (01° 13′ 25.3″ S, 36° 53′ 49.2″ E) where

they were reared on a substrate comprising sterilised moistened soil and crushed cow dung mixed in the ratio 1:1 by volume. The substrate was changed every 3 weeks.

Adult beetles were kept in white rectangular plastic bowls with perforated lids and provided drinking water by placing moist cotton balls (6 – 5 cm in diameter) on the rearing substrate with an equal number of males and females placed together. Temperature, relative humidity and photoperiodicity in the rearing room were maintained at 25 ± 2 °C, 50 ± 5 % and 10L : 14D respectively.

Eggs were recovered by sifting the substrate every 24 hr. Each egg was placed on approximately 5 g of rearing substrate (composed of two parts of sterilised soil and one part sterilised and crushed cow dung) placed on moist filter paper (9 cm in diameter) kept in a Petri dish. One to two millilitres of distilled water was added on to the filter paper every two to three days to keep the eggs moist. Six mated females were kept individually on the rearing substrate and monitored for egg-laying until they stopped. The maximum length and width of each egg was measured every 48 hr from day two up to day eight using a digital stereomicroscope. These measurements were used to compute the change in volume of the egg according to Donaldson (1989) during the incubation period. The incubation period and the survival of a subset of eggs were recorded.

Larvae were kept on a similar rearing substrate but the substrate was changed every two weeks (14 days). Measurements of the maximum head width of each larval instar were made using a digital stereomicroscope. The duration of subsets of insects in each larval instar was recorded.

The formation of a cocoon by third larval instars marked the beginning of the pupal stage. Cocoons were transferred in to rearing bowls containing moist sterilised soil and monitored daily for adult emergence. The pupal period was recorded.

### 4.3.3 Measurements

All observations and measurements were done with EZ4 D digital stereomicroscope (Leica Microsystems, Heerbrug, Switzerland) and a laptop, using the software Leica EZ4 1.7 provided by Leica. Images were taken as basis for drawings.

### 4.3.4 Data analysis

The length, width, and volume of all eggs followed through incubation were compared across the 4 different days of measurement by one way Kruskal-Wallis analysis of variance (ANOVA) and means separated by multiple pairwise Tukey's tests (as this test is highly conservative and only separates means with substantial differences among them). The mean durations of each immature instar were computed from the recorded data for groups of insect followed through each developmental stage. These durations (days) were compared across instars by a one way Kruskal-Wallis analysis of variance (ANOVA) and means separated by multiple pairwise comparisons using Dunn's method (which allows mean separation of treatments with unequal replicates). Larval instar head capsule widths were also subjected to similar ANOVA comparisons. The duration from pupa to adult emergence of males and females was compared using Mann-Whitney t test. The lifespan of both male and female beetles under laboratory conditions were compared using Mann-Whitney t test.

## 4.4 Results

### 4.4.1 Description of the 3rd larval stage (L3)

The body of L3 is yellowish in colour and lightly hirsute with the posterior abdominal segments greatly enlarged to give a pear-shaped body. Spiracles are distinctly dark and present on the prothorax and the first eight abdominal segments and enclosed by a c-shaped bulla.

The cranium is reticulate, shiny and yellow to dark brown. Two dark ocelli are present, one above the base of the each antenna (Figure 4.1a). Epicranial and frontal sutures are present with the former more pronounced than the latter. The epicranial surface has one long seta above each ocellus and two pairs on the lateral edges. The frons has a pair of medialy located posterior setae and a pair of exterior setae. The clypeus is trapezoidal in shape (Figure 4.1a). There is one long seta present on each lateral margin and a pair of more centrally located setae on the postclypeus (if an imaginary line were drawn to divide the clypeus into two symmetrical parts). Pores present on the postclypeus and absent on the preclypeus. The preclypeus is less sclerotised than postclypeus.The labrum is trilobed with rounded lateral margins (Figure 4.1a). A pair of proximal setae and two pairs of distal setae are present on lateral lobes while the apical lobe has a fringe of short setae. The antennae are four-segmented with first segment twice as long as each of the other 3 segments (Figure 4.1a). The antenna has no setae and the apical segment tapers towards the tip with two to three round sensory spots on the dorsal surface and two on the ventral surface.

The mandibles are asymmetrical, similar in size with black and heavily sclerotised scissorial areas (Figures 1b and c). The scissorial area of the right mandible has four teeth and left has three. The outer lateral edge of the mandibles has eight setae, of which four are long and the

other four are short. The molar part of the mandible is dark and heavily sclerotised with the molar part of the right mandible bilobed with the distal lobe larger than the proximal lobe. The molar part of the left mandible is trilobed with the proximal lobe slightly larger than the other two. A brustia of setae is present on basal part of each mandible with two to four dorsomolar setae present at the base of the molar teeth. A single seta is present on the ventral surface of the most distal molar tooth of each mandible. A ventral process is present. Stridulatory areas are present and lighter in colour than the surrounding area.

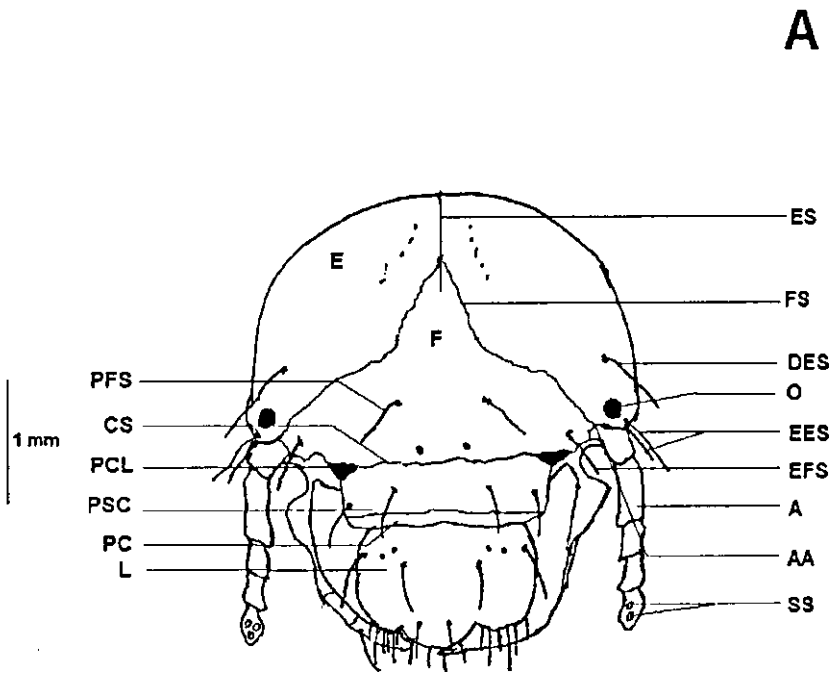
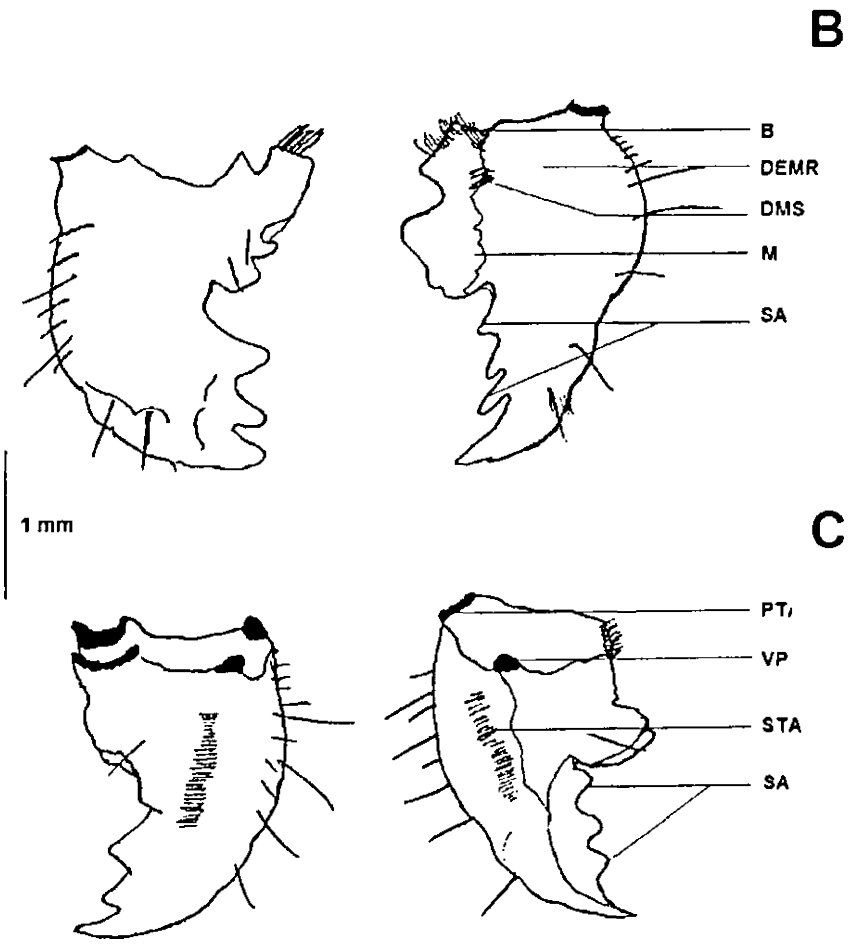


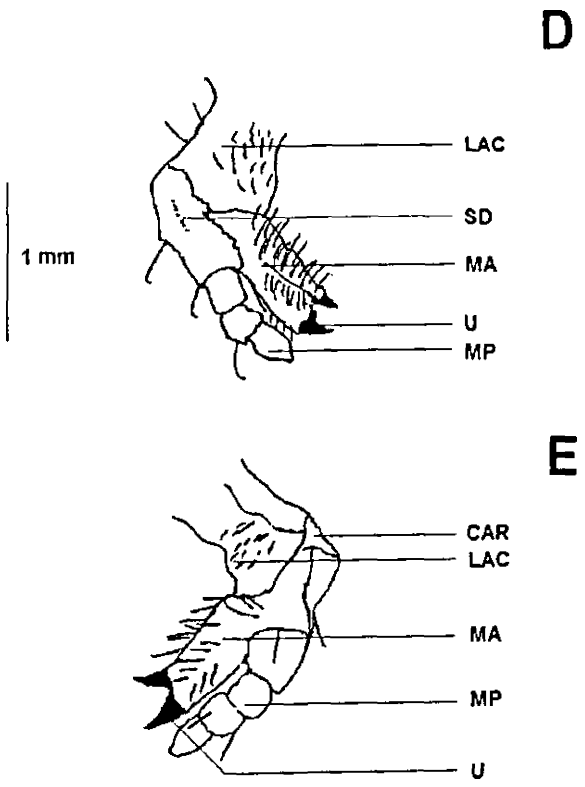

**Figure 4.1A** – External morphological features of *Oplostomus haroldi* larva head capsule. A - antenna; AA - anterofrontal angle; CS - clypeofrontal suture; DES - dorsoepicranial seta; E - epicranium; EES - exterior epicranial setae; EFS - exterior frontal seta; ES - epicranial suture; F - frons; FS - frontal suture; L - labrum; O - ocellus; PC - preclypeus; PCL - precoilia; PFS - posterior frontal seta; PSC – postclypeus and SS - sensory spots.

A maxillary cardo with fine setae is present on the interior dorsolateral surface (Figures 4.1d and e). The exterior dorsolateral surface bears a row of five to seven small denticulate stridulatory teeth. Galea and lacinia are fused to form a mala with two similarly sized unci, one terminal and the other below it. The mala has dorsoventral rows of short sharp setae. The maxillary palp has three segments with a pair of setae present on middle segment, one dorsolaterally located and the other ventrally.

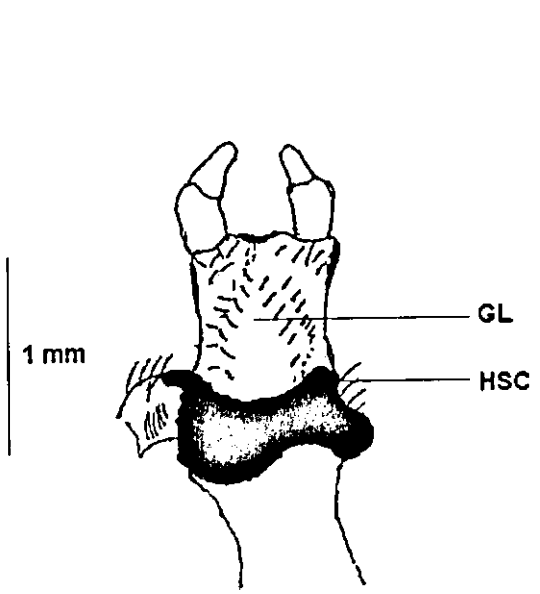



**Figures 4.1 B and C** - Dorsal (**B**) and ventral (**C**) surfaces of mandible. B - brustia; DEMR - dorsoexterior mandibular region; DMS - dorsomolar setae; M - molar lobe; SA - scissorial area; PTA - postartis; STA - stridulatory area and VP - ventral process.

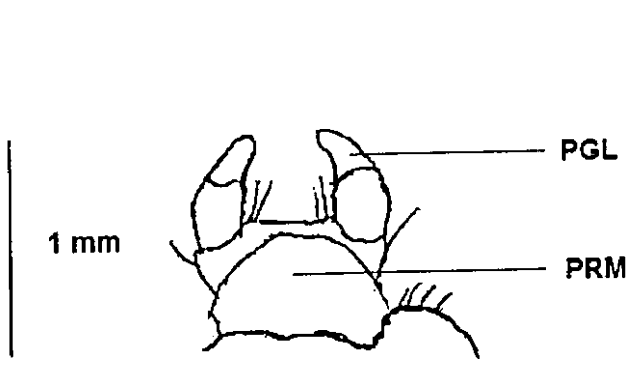
The labium possesses a pair of short setae on the prementum and the glossa is covered with an ordered pattern of short setae and sensilla (Figures 4.1f and g). The hypopharyngeal sclerome is asymmetrical and bears a truncate process on the left side which is absent on the right. The lateral lobe has a group of short setae.




**Figures 4.1 D and E -** Dorsal (**D**) and ventral (**E**) surfaces of the maxilla. LAC - labacoria; MA - mala; MP - maxillary palp; U – uncus and CAR – cardo.



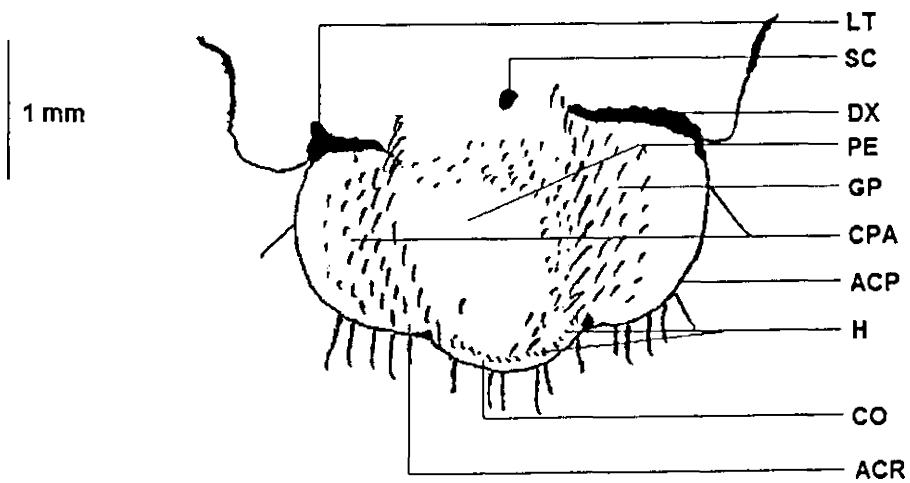

**Figures 4.1F** - Dorsal surface of labium. GL – glossa and HSC - hypopharyngeal sclerome.




**Figures 4.1G** - Ventral side of labium. PGL – paraglossa and PRM - prementum.

The epipharynx has round margins with the apical margin in corypha region protuberant (Figure 4.1h). Acroparia is prominent and bears long thin setae. The zygum and epizygum are absent. A clithra is also absent. Plegmatium is absent on each lateral margin. The acanthopariae are asetaceous. The gymnopariae have short setae. The chaetopariae have long setae surrounding a slightly indented pedium. The haptomerum has a single row of heli. A sclerotised triangular laeotorma is present on the left side of epipharynx and an elongated dexitorma on the right side.

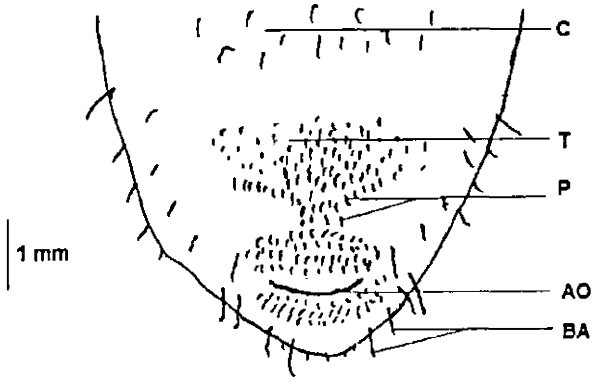
H




**Figures 4.1H** - Epipharynx. ACP - acanthoparia; ACR - acroparia; CO - corypha; CPA - chaetoparia; DX - dexitorma; GP - gymnoparia; H - heli; LT - laeotorma; PE – pedium and SC - sense cone.

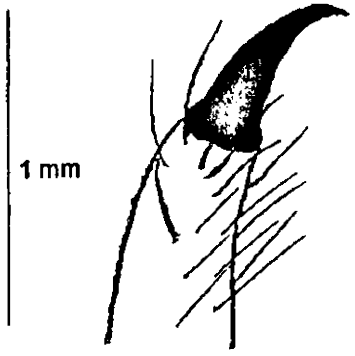
The raster lacks a septula (Figure 4.1i). The tegilla extends from the barbulae to palidia and is covered with many sharp-pointed short setae. Presepular hamate setae are absent. Barbulae have long slender setae and the campus has sparse short setae. The anal opening is transverse and surrounded by several rows of short setae. The mesothoracic claw is sharp, slighly curved and without setae (Figure 4.1j).

I




**Figures 4.1I –** Raster Pattern of *O. haroldi*. AO - anal opening; BA - barbulae; C - campus; P – palidia and T - tegilla.

J




**Figures 4.1J -** Lateral side of mesothoracic claw showing its asetaceous form.

### 4.4.2 Life history

Mated females of *O. haroldi* started egg laying 8-10 weeks after their collections from the field. Females were observed to lay eggs singly, in pairs or triplets and enclose them within moulded lumps of substrate. Females laid an average of 72.7 ± 22.4 eggs (N = 6) throughout the egg-laying period which lasted 4-8 weeks. Halting of egg-laying was observed when the rearing substrate became dry. Newly laid eggs were white and oval. As eggs developed, they became spheroid as indicated by their length: width ratio (Table 1). The egg size increased from the day it was deposited up to about day 6 after which it decreased slightly with significant differences in the average length ($H$ = 103.2, $df$ = 3, $P$ <0.001), width ($H$ = 101.7, $df$ = 3, $P$ <0.001) and volume ($H$ = 104.5, $df$ = 3, $P$ <0.001) during development (Table 1) (Figure 4.2). The incubation period was the shortest and lasted 7-13 days (Table 3). A few hours before hatching, the abdominal setae and sclerotized mandibular teeth darkened and became visible under the chorion. Egg survival under the described laboratory conditions was 98.4% (N = 129).

Newly hatched larvae were generally white with a white head capsule which turned brown after a few hours, matching the colour of the setae and spiracles. The width of the head capsule was significantly different between the larval instars ($H$ = 46.03, $df$ = 2, $P$ <0.001) (Table 2). The mean width of the head capsule was 1.66 ± 0.05 for the first instar, 2.53 ± 0.08 mm for the second instar and 3.98 ± 0.13 mm for the third instar (Table 2). The developmental time for the third larval and pupal instars were significantly longer than those for the other instars but not significantly different between the 3$^{rd}$ instar larva and pupa ($H$ = 248.1, $df$ = 4, $P$ <0.001 ) (Table 3). The survival rate of the first, second and third instars were 73.7%, 93.3% and 96.3%, respectively. Fungal growths observed on some dead larvae were

later identified as *Metarhizium anisopliae* (identification carried out by Ouna E., APU- *icipe*, Nairobi) strains and may have been the cause for the larval death.

The pupal stage was recognised by the oval mud cocoon casing. Cocoons were formed within the rearing substrate without any anchoring to the wall of the container. The duration of the pupal stage was shorter compared to total larval developmental time (Table 3). Pupal survival was moderate (54.2%, N = 48), with adults emerging from the surviving pupae with a sex ratio of 1:1 (N = 26 males and females). The developmental time of male and female pupae did not differ significantly ($P = 0.979$). An examination of cocoons from which adults failed to emerge revealed that all dead insects were in their pupal stage indicating that the larva to pupa transformation was successful for all beetle cocoons monitored for adult emergence. However, no entomopathogens were observed to have infected the pupae.

The total duration of the life cycle from the egg to the newly emerged adult stage took 86–145 days (about 3-5 months) (Figure 4.3). Adult beetles (both laboratory-bred and field collected) were observed to live for up to 24 weeks under laboratory conditions, with females living longer than males. Adult males were observed to survive for 29–112 days (N = 55) whereas females survived for 31–168 (N = 40), these differences being significant under laboratory conditions ($U = 767$, $df = 1$, $P = 0.012$).

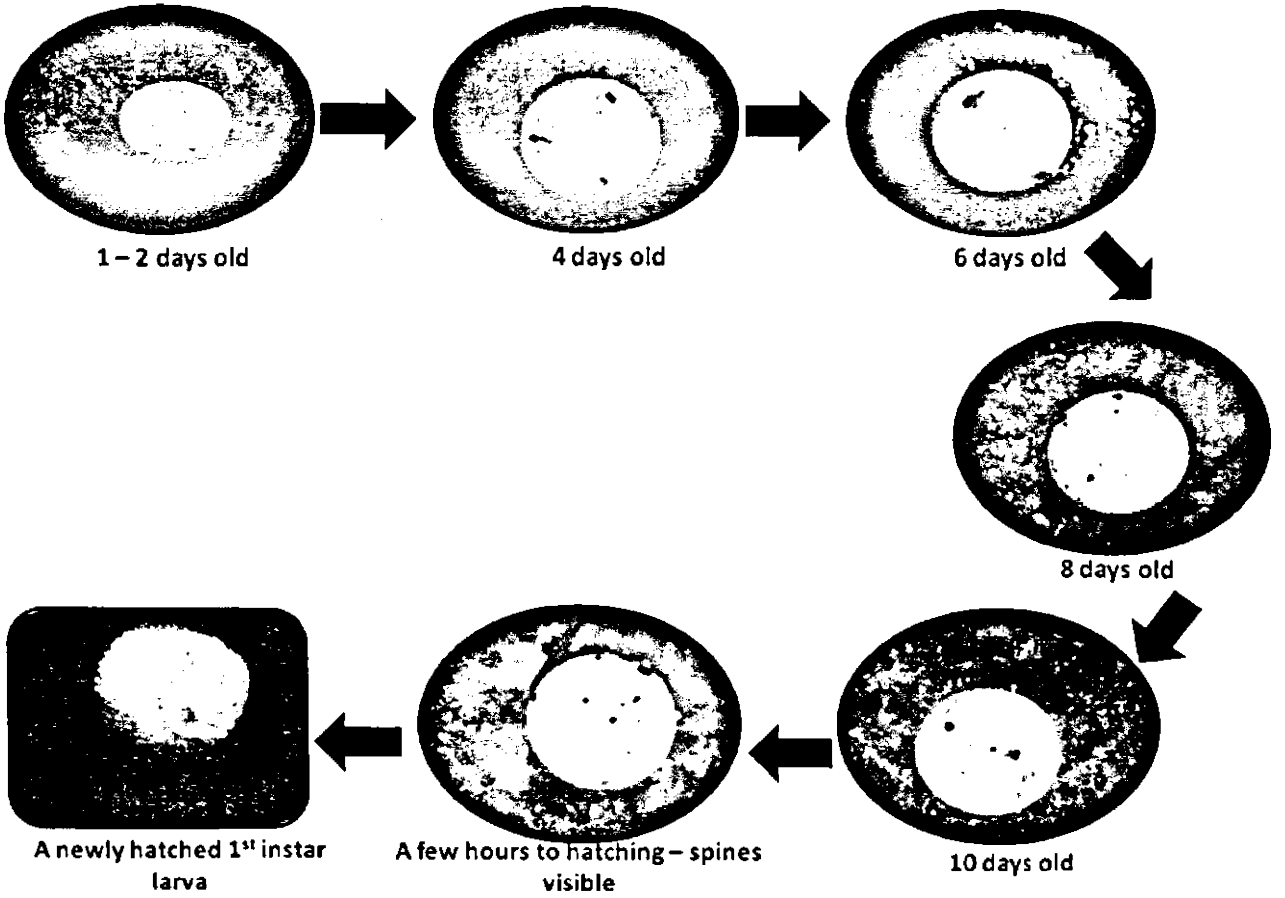



**Figure 4.2** Development of *Oplostomus haroldi* from egg to first instar larva.

**Table 4.1**: Summary of changes in shape and volume of *Oplostomus haroldi* egg during incubation period under laboratory conditions

| Days after egg deposition | N | Length (mm)† | | Width (mm)† | | Volume ($mm^3$)† | | Length:width | |
|---|---|---|---|---|---|---|---|---|---|
| | | Mean ± S.D. | Range | Mean ± S.D. | Range | Mean ± S.D. | Range | Mean ± S.D. | Range |
| 2 | 44 | 2.02 ± 0.06c | 1.90 – 2.16 | 1. 46 ± 0.04c | 1.35 – 1.54 | 2.59 ± 0.16c | 2.33 – 3.03 | 1.37 ± 0.05 | 1. 29 – 1.54 |
| 4 | 44 | 2.48 ± 0.12b | 2.03 – 2.70 | 2.26 ± 0.14b | 1.44 – 2.44 | 7.00 ± 1.00b | 2.53 – 8.73 | 1.10 ± 0.05 | 1.03 – 1.41 |
| 6 | 44 | 2.54 ± 0.10ab | 2.01 – 2.70 | 2.31 ± 0.14ab | 1.49 – 2.45 | 7.37± 0.87ab | 2.65– 8.94 | 1.10 ± 0.04 | 1.05 – 1.36 |
| 8 | 44 | 2.54 ± 0.09a | 2.01 – 2.70 | 2.31 ± 0.12a | 1.49 – 2.45 | 7.50 ± 0.89a | 2.64 – 8.73 | 1.10 ± 0.04 | 1.05 – 1.35 |

All rearing experiment were carried out under temperature, relative humidity and lighting conditions of 25 ± 2 °C, 50 ± 5 % and 10L : 14D, respectively. †Mean values followed by the same letter per column are not statistically different (one-way Kruskal-Wallis ANOVA)

**Table 4.2**: Mean (± S. D) of head capsule width of the larval instars of *Oplostomus haroldi*

| Instar | N | Width (mm)† | Range (mm) |
|---|---|---|---|
| First instar | 18 | 1.66 ± 0.05c | 1.56 – 1.72 |
| Second instar | 15 | 2.53 ± 0.08b | 2.41 – 2.66 |
| Third instar | 20 | 3.98 ± 0.13a | 3.66 – 4.11 |

†Values followed by the same letter per column are not statistically different (based on a one-way Kruskal-Wallis ANOVA)

**Table 4.3**: Mean (± S. D.) of developmental time (days) of the immature life stages of *Oplostomus haroldi*

| Instar | N | Duration (days)† | Range (days) |
|---|---|---|---|
| Egg | 127 | 11.9 ± 1.3d | 7 – 13 |
| First larval instar | 70 | 14.6 ± 2.6c | 11 – 20 |
| Second larval instar | 42 | 17.5 ± 2.4b | 13 – 23 |
| Third larval instar | 54 | 34.6 ± 2.4a | 29 – 40 |
| Pupal stage | 26 | 31.1 ± 6.7a | 26 – 49 |

All rearing experiment were carried out under temperature, relative humidity and lighting conditions of 25 ± 2 °C, 50 ± 5 % and 10L : 14D, respectively. †Values followed by the same letter per column are not statistically different (one-way Kruskal-Wallis ANOVA)

## 4.5 Discussion

This study provides for the first time the life history of *O. haroldi* under laboratory conditions and a description of the third instar larva with emphasis on taxonomically important characters. The third larval stage of *O. fuligineus* has been described to possess up to 11 maxillary (stridulatory) teeth (Donaldson, 1989), while this study showed that *O. haroldi* has up to 7 maxillary teeth with similar function, clearly depicting a difference in the number of this structural character. However, lack of pictorial descriptions of the larva of *O. fuligineus* poses a challenge to the compilation of the morphological differences which exist between larvae of the two species, which could be relevant for taxonomy.

It was evident from our results that the eggs of *O. haroldi* are smaller than those of *O. fuligineus* (Donaldson, 1989). Egg size was used to show changes during incubation because our attempts to weigh them failed due to their highly hygroscopic nature, and, as a consequence, the recorded weight was highly variable. The cessation of egg deposition by females during drier periods implies that females possess the ability to determine moisture

levels suitable for egg development and survival of newly hatched larvae. This phenomenon has also been observed in other scarabs (Soltani *et al.*, 2008). The differences in head capsule width of various instars of the larval stage of scarabs have been employed in identifying larval instars (Donaldson, 1989; Soltani *et al.*, 2008), as well as in taxa other than Scarabaeidae. Data obtained from this study show that differences in head capsule width can be used to delineate the various larval instars.

The results showed that *O. haroldi* eggs apparently survived better than the other life stages while the pupal stage showed the least survival under laboratory conditions. The enclosure of eggs within lumps of rearing substrate might serve to provide the necessary moisture level for their development and thus explain the relatively high survival rate. Egg enclosure may also offer protection from predators in the natural setting. The high mortality (26.3%) recorded in the first larval instar during the larval stage of the life cycle is an indication of their vulnerability. Although the beetle seemed to survive up to six months in the laboratory, it is not known how long beetles can survive inside honey bee colonies. The suitability of the substrate used in this study can only be confirmed by carrying out comparative studies where beetles are kept on different substrates. Initials efforts to rear them on banana in our laboratory which were reported to be attractive to another member of the *Oplostomus* genus (Donaldson, 1989), were not successful, suggesting that this beetle may not be highly polyphagous.

Cocoon formation in *O. haroldi* was similar to that described by Donaldson (1989) for *O. fuligineus* as they were not attached to the walls of the rearing container. This observation is another behavioural similarity shared by both species in addition to they being pests of honey bee colonies. The cause of the very high pupal mortality observed during the laboratory

rearing still remains a mystery and suggests that other factors (both biotic and abiotic) other than faulty larval-pupal transformations and entomopathogens might have been the probable cause of death.

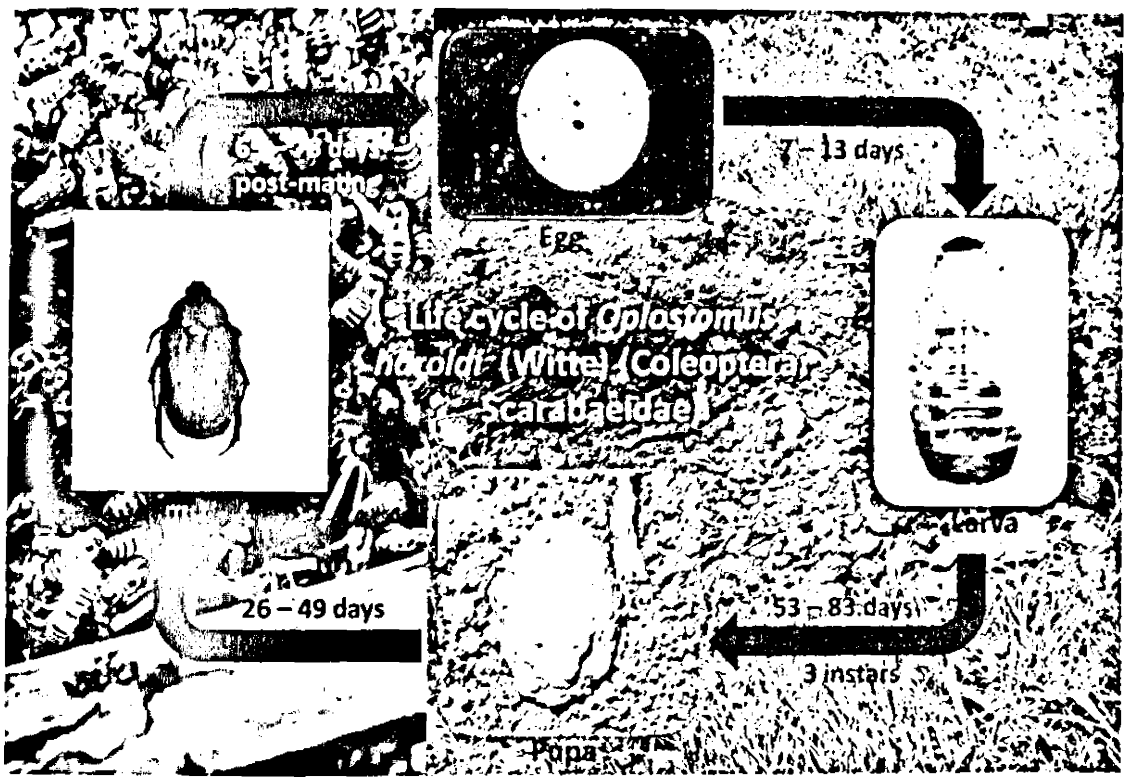



**Figure 4.3** Diagram showing the life cycle of *Oplostomus haroldi*

With *O. haroldi* capable of completing its life cycle within three to five months, this species can therefore produce between two to four generations per year depending on the prevailing climatic conditions. Local climatic conditions can have a direct bearing on the level of honey bee colonies infestations by shortening or prolonging the life cycle as suggested by Ritcher (1957) and Torto *et al.* (2010a). Previous studies showed that beetle populations infesting honey bee colonies were male biased with female numbers varying significantly between seasons (Torto *et al.*, 2010a). Upon mating inside the hive, females are bound to leave the hives in search of suitable egg-laying sites, and this might explain the difference in sex ratios

of beetles inside the hives. No previously reported sex ratio disparities were observed during the laboratory rearing of this beetle.

Initial observations of *M. anisopliae* infecting larvae of *O. haroldi* suggest that the entomopathogen could be used to control the pest. However, detailed field studies are required to locate the beetle breeding sites and to assess their ecological importance in nutrient recycling prior to consider using *M. anisopliae* as a control strategy. This study also demonstrates that it is possible to breed *O. haroldi* in the laboratory on a diet composed of cow dung and soil.

# CHAPTER FIVE

## Behavioural and electrophysiological responses of *Oplostomus haroldi* (Coleoptera: Scarabaeidae) to honey bee odours

### 5.1 Summary

*Oplostomus haroldi* (Coleoptera: Scarabaeidae) is a pest of honey bees that threatens the beekeeping industry in East Africa. The adults feed on brood, honey and pollen. Current cultural management tools for this beetle have failed to reduce its population thereby prompting a search for more effective ways of managing it. The response of this beetle to honey bee volatiles was studied using laboratory olfactometric bioassays, coupled gas chromatography electroantennographic detection (GC-EAD) and gas chromatography mass spectrometric (GC-MS) analyses. Olfactometer bioassays showed that both sexes of the beetle were significantly more attracted to honey bee colony odours than blank controls. Coupled GC-EAD analysis showed that antennae of the beetle detected 12 components of colony odours. GC-MS analysis identified seven of these components, six of which were esters. These results show that *O. haroldi* detects candidate kairomones from honey bee volatiles. Behavioural studies of these candidate kairomones will provide baseline data for formulation of a lure for the management of this beetle.

### 5.2 Introduction

Apiculture is a fast growing economic activity and enjoys a continuously expanding popularity within Kenya where it provides both a livelihood to subsistence small scale rural farmers and crucial ecosystem services (Raina, 2006). However, its expansion is greatly challenged by the arthropods pests *Aethina tumida* (Coleoptera: Nitidulidae), *Oplostomus haroldi* (Coleoptera: Scarabaeidae) and *Varroa destructor* (Parasitiformes: Varroidae) found

recently as pests of honey bee colonies in various parts of Kenya (Torto *et al.*, 2010a; Frazier *et al.*, 2011). With *A. tumida* and *V. destructor* known to be invasive pests of honey bee colonies across continents, there has been a continuous effort by various researchers to understand different aspects of their biology. These efforts have resulted in the development of fairly successful management methods against *A. tumida* (Arbogast *et al.*, 2007; Torto *et al.*, 2007b) and *V. destructor* (USDA, 2009) based on knowledge about their chemical ecology.

*Oplostomus haroldi* unlike the other two pests has been reported only in East Africa (Njau *et al.*, 2009; Torto *et al.*, 2010a) and has the potential to cause serious havoc to honey bee colonies (see chapter 3), thus, the need to develop management tools which will go a long way to minimise such damage. Pest management tools based on the chemoecological interactions between honey bees and their pests have given promising results (Arbogast *et al.*, 2007; Torto *et al.*, 2007b; USDA, 2009). Furthermore, *O. haroldi* does not produce any airborne pheromones but relies on a contact pheromone for mate recognition (see chapter six). Based on this finding, it was hypothesised that *O. haroldi* locates honey bee colonies using hive odours. The results presented herein, report the identification of candidate kairomonal components from honey bee odours as a first step towards the identification of behaviourally-active components and subsequently, development of a lure for management of the beetle.

## 5.3 Materials and methods

### 5.3.1 Insects

Beetles used for the bioassays were collected from honey bee colonies at the coast of Kenya (Watamu and Taita localities) between January 2010 and February 2011 and were maintained in the laboratory as previously reported by Torto *et al.* (2010a). The individual sexes were

kept separately in rearing bowls with perforated lids (19 cm x 14 cm x 10 cm) and maintained on a diet of sterilised cow dung and soil mixed in the ratio 1:1 by volume. Prior to bioassays, beetles were starved for 24 hr. Insects used for bioassays were checked for injuries and missing body parts and only ones with no signs of injury were used.

### 5.3.2 Bioassays with hive odours

The response of adult beetles to honey bee odours was recorded using a dual choice olfactometer (84 cm x 7 cm x 7 cm) made from glass and aluminum (Figure 5.1). Medical air (which is equivalent to purified atmospheric air) from a compressed air tank was further purified by passing it through an activated charcoal filter and then split into two streams at a T-connection, immediately followed by two flow meters, which supplied air into each arm of the olfactometer at a rate of 0.25 liter/min. An electrically powered vacuum fan was used to pull out air from the center of the olfactometer at 0.5 liter/min. The test area was evenly illuminated by two 40W fluorescent light bulbs placed 1.5 m directly above the bioassay setup as the beetle is diurnal.

The odour sources consisted of honey bee odours (comprising ~300 worker honey bees and a cut section of a honey bee frame containing live brood, pollen and honey stores) and a blank control which comprised medical air.

Each insect was used only once during the bioassays which were conduction between 0900 – 1600 h. Test beetles were introduced into the test arena singly via an entrance underneath the vacuum fan by briefly lifting it up and replacing it afterwards to reconnect the fan to the setup. The beetles were then allowed 10 minutes to respond to the odour sources. A test beetle was considered to have responded to a particular odour source if it either stayed most

or spent more time in the arm of the olfactometer bearing that odour. After 10 min, the fan was again disconnected from the bioassay setup and the insect removed. The position of the odour sources were reversed in between replicates to minimise positional bias. The response of 20 male and 20 female beetles were recorded using this bioassay setup. All bioassays were conducted at a temperature and relative humidity of 29 ± 2 °C and 50 ± 5 % respectively.

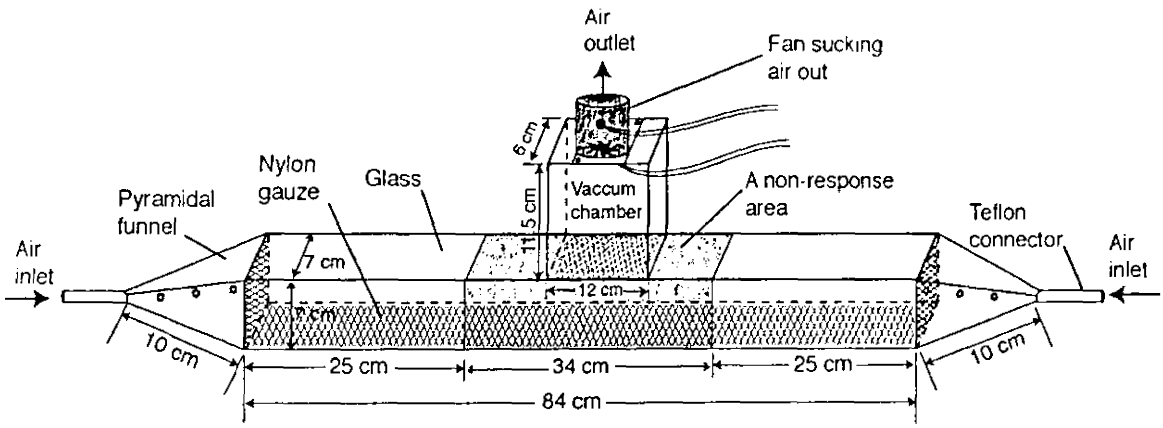



**Figure 5.1** Dual choice olfactometer used for bioassays

### 5.3.3 Volatile collection from honey bee colonies

Volatiles were collected from intact honey bee colonies using a mobile vacuum pump system (Analytical Research Systems Inc., Gainesville, FL USA) on cleaned Super Q traps (30 mg, Alltech, Nicholasville, KY). An air flow meter was connected via its air outlet to the vacuum terminal of the mobile pump and through its inlet to a flexible Teflon tube. A Super Q trap was placed at the open end of the Teflon tube. A hive was briefly opened and the Teflon tube end with the Super Q trap placed gently in between two frames inside the hive and its lid returned (Figure 5.2). Hive odours were collected on the trap by pulling air through it at a rate of 500 ml/min for 3 hrs. The Super Q trap was washed with 150µl of dichloromethane (DCM) under ice and the eluent pushed through the trap using a gentle stream of charcoal-filtered nitrogen ($N_2$). The eluate was stored at -20 °C prior to analysis. This procedure was repeated 4 times using the same honey bee colony to obtain enough samples as needed for gas chromatographic analyses.

**Figure 5.2** Volatile trapping system used to collect odours from honey bee colonies.

### 5.3.4 Analysis of volatiles

Coupled gas chromatography-electroantennographic detection (GC-EAD) analysis was carried out on a Hewlett-Packard (HP) 5890 Series II gas chromatograph equipped with a capillary column HP-5 MS (30 m × 0.25mm ×0.25µm, ID and film thickness) with nitrogen as the gas carrier. Extracts were analysed in split less mode at an injector temperature of 250°C and a split valve delay of 1 min. The oven temperature was held at 35°C for 5 min, increased to 280°C at 10°C/min, and then held at this temperature for 20.5 min. The column effluent was split 1:1 for simultaneous detection by FID and EAD. For EAD detection, silver wires in drawn out glass capillaries filled with ringer solution served as the reference and recording electrodes. The distal and proximal segments of the antenna of *O. haroldi* were

placed in contact with the microelectrodes and humidified air delivered at 1 ml/s over the mounted antenna. The microelectrodes were connected via an antennal holder to an AC/DC amplifier in DC mode (Syntech, Hilversum, The Netherlands). A GC program (Syntech GC-EAD 2000, Hilversum, The Netherlands) was used to simultaneously record and analyse the amplified EAD and FID signals on a PC. Three micro litres of the extract were analysed with either fresh male or female antenna for repeated sample analysis.

Coupled gas chromatography-mass spectrometry were carried out on an Agilent Technologies 7890A gas chromatograph equipped with a capillary column HP-5 MS (30 m × 0.25mm ×0.25µm, ID and film thickness) and coupled to 5795C mass spectrometer. One micro litre of each sample was injected in the split less mode (Inlet temperature = 250°C, Pressure = 6.83 psi), and helium was used as the carrier gas at 1.0 ml/min. The oven temperature was held at 35°C for 5 min, increased to 280°C at 10°C/min, and then held at this temperature for 20.5 min. EAD-active compounds were identified by comparing their mass spectral data with those from the NIST 08 library of the mass spectrometer and by retention times with those of authentic commercial samples. The amounts of identified components were quantified by comparison of their relative peak areas to that of ethyl nonanoate (200 ng) as an external standard.

### 5.3.5 Statistical analysis

The percentage of insects positively responding to both hive and blank odours were compared using a one sample chi square analysis. Different concentrations of the external standard ethyl nonanoate were used to construct a calibration curve used in estimating the amount of each biologically active hive odour component being produced by the colony.

## 5.4 Results

### 5.4.1 Bioassays with honey bee odours

Of the beetles tested, 90% males and 85% females were attracted to honey bee odours while 10% of males and 15% of females positively responded to the blank controls (Figure 5.3). There were no non-responders. The responses of both sexes to honey bee odors was significantly higher compared to their controls (One sample chi square test, $\chi^2$=64, P< 0.001 for males; $\chi^2$=49, P< 0.001 for female).

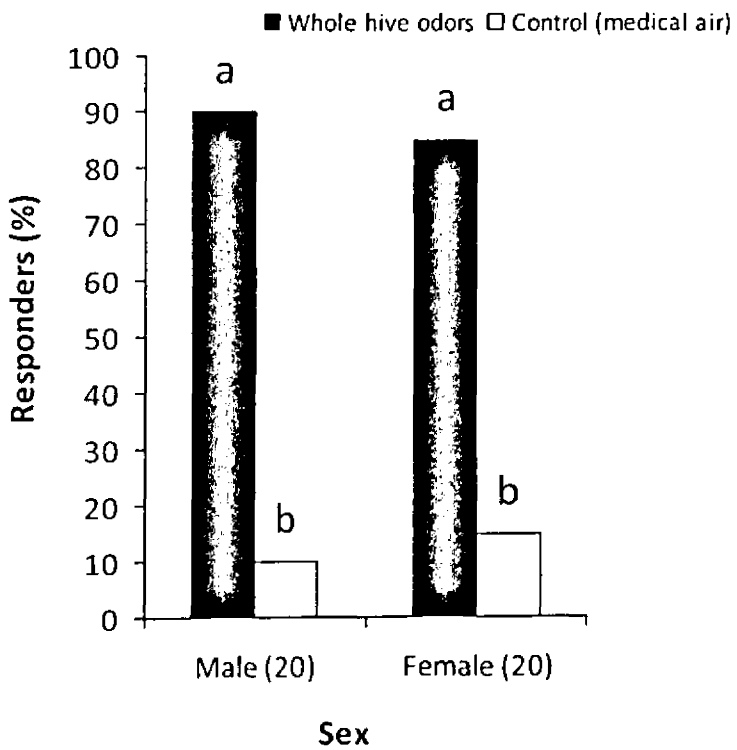



**Figure 5.3** Behavioural responses of *Oplostomus haroldi* to honey bee odours. Pairs of open and closed bars followed by the same letter are not significantly different at P= 0.05

### 5.4.2 Analysis of volatiles

The GC-EAD profiles of volatiles collected from the hive showed that both male and female beetles consistently detected 12 components using their antennae (Figure 5.4a). The EAG-

active components of honey bee volatiles were tentatively identified by GC-MS analysis, which compared mass spectra data from the samples with library data (NIST 08). Six of these components were esters and, one alcohol, (Figure 5.5b) all of which had their identities confirmed by GC-EAD analysis with a mixture of authentic standards of these compounds (Figure 5.4b). These seven compounds were 2, 3-butanediol, butyl acetate, isopentyl acetate, butyl acetate, hexyl acetate, pentyl isobutanoate and methyl benzoate. The relative amounts of EAG-active compounds produced by the honey bee colony varied from one another (Figure 5.5a).

**5.5 Discussion**

It is clearly evident from this study that both sexes of *O. haroldi* rely on honey bee odours to locate host colonies which may account for both sexes infesting colonies. A similar response to honey bee odours has been reported for *Aethina tumida*, another beetle pest of honey bees (Suazo *et al.*, 2003; Torto *et al.*, 2005).

The coupled GC-EAD analyses showed that *O. haroldi* detects honey bee odours. Hive odours consist of a mixture of volatiles originating from adult bees, bee larva, pollen, honey and possibly waste material. The detection of adult bee volatiles (Torto *et al.*, 2005) by the antenna of the beetle is not surprising as they form the majority of individuals in the hive at all times. The somewhat strong antennal response to methyl benzoate compared to other hive odour components in both analyses involving the crude and mixed standard samples may imply that this compound is central to the orientation of a host-seeking beetle towards a host colony. However, bioassays using individual and mixed preparations of pure synthetic versions of these compounds are needed to confirm the role of methyl benzoate and the other biologically-active components.

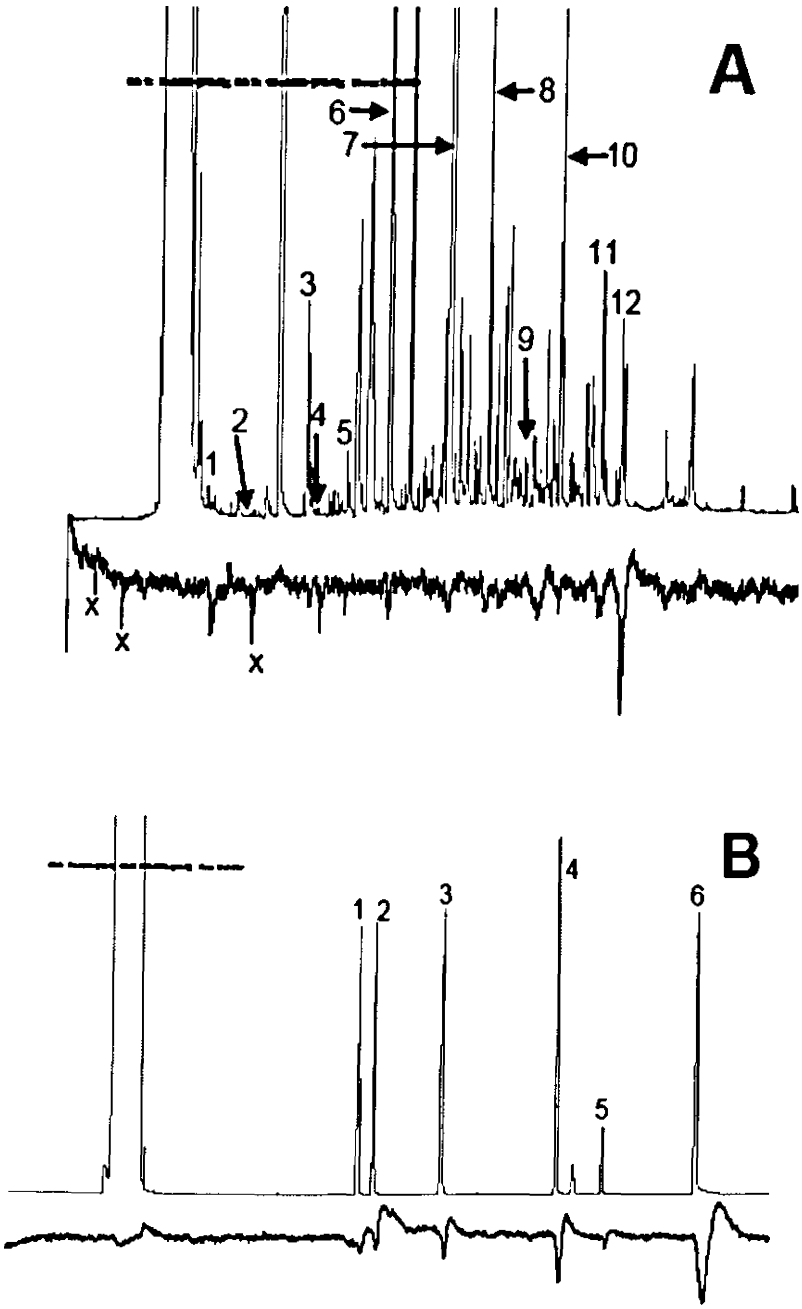
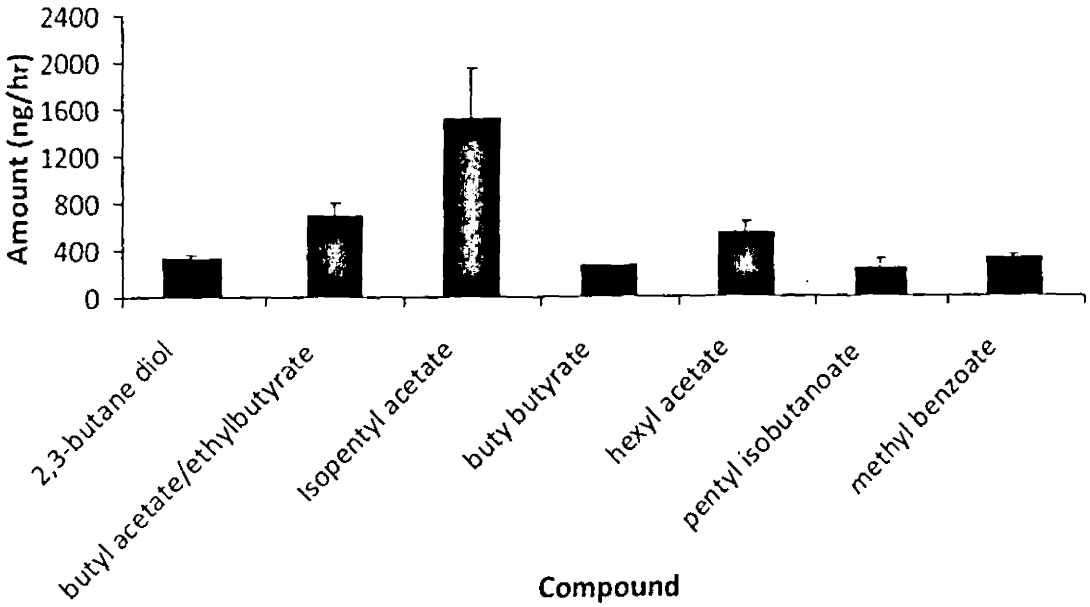



**Figure 5.4** Antennal responses of *Oplostomus haroldi* to (A) hive odours, peaks (1-4) unidentified, (5) - 2,3-butane diol, (6) - butyl acetate, (7) - isopentyl acetate, (8) - unidentified, (9) - butyl butyrate, (10) - hexyl acetate, (11) - pentyl isobutanoate, and (12) - methyl benzoate and (B) a mixture of ethyl butyrate (1), butyl acetate (2), iospentyl acetate (3), butyl butyrate (4), hexyl acetate (5), pentyl isobutanoate (6) and methyl benzoate (7). Peaks marked 'x' represent electric spikes which are false biological responses.

A



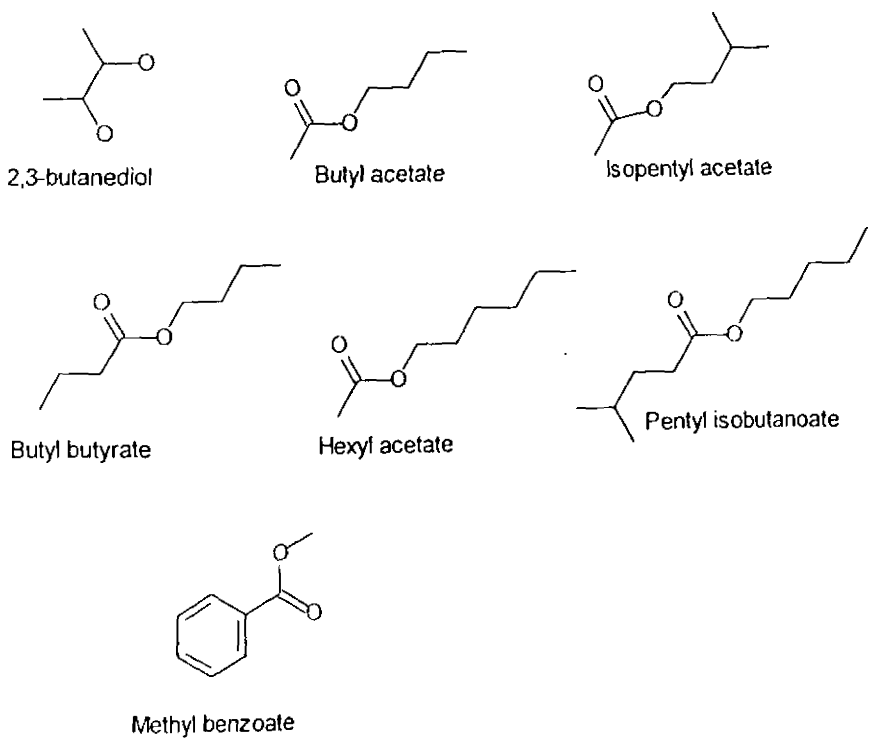




**Figure 5.5** (A) Relative amounts (ng/hr) of the EAG-active compounds produced by the honey bee colony under study and (B) chemical structures of compounds listed above.

With the compounds detected by *O. haroldi* also known to be detected by *A. tumida*, it may be possible to formulate a lure which can be used to manage both beetle species.

The response of *O. haroldi*'s antenna to ethyl butyrate (a fermentation product) in the standard mixture raises questions about the 'real' range of hive odour components and esters detectable by this beetle. Studies by Khbaish (2010) showed that *Pachnoda marginata* and *P. interrupta* both cetonid beetles respond to acids, alcohols, terpines, aldehydes, ketones and esters, some of which are also known to be produced by worker honey bees (Suazo *et al.*, 2003; Torto *et al.*, 2005). It will be interesting to find out if *O. haroldi*, another cetonid and pest of honey bees can detect and respond to these compounds.

In summary, this study presents evidence of olfactory detection of honey bees by *O. haroldi*, knowledge which is imperative towards the formulation of a lure for its management.

# CHAPTER SIX

## Mating Behaviour and Evidence of a Contact Sex Pheromone in *Oplostomus haroldi* (Coleoptera: Scarabaeidae)

### 6.1 Summary

Mating and pheromonal communication was investigated in the scarab *Oplostomus haroldi*, a pest of honey bees in East Africa. Mating bioassays in a Petri dish arena revealed that males showed specific behaviours which resulted in mating. A male got arrested upon making mouth contact with the elytra or pronotum of the female, aligned its body to that of the female, mounted and copulated with her. The last stage was followed by a post-copulatory stage. The duration of each stage was significantly lower than the following stage, with the last stage, a post-copulatory, stage being the longest. In choice bioassays, twice as many males responded to dead unwashed females (control) than to dead solvent-washed females. These results were confirmed when female extracts were applied to dead solvent-washed females. Furthermore, in palpectomy assays, males detected females using their maxillary palps and not with labial palps. Coupled GC-MS analysis of cuticular extracts showed identical profiles for males and females, comprising hydrocarbons of chain length ranging from $C_{23}$ to $C_{29}$, which differed quantitatively between the two sexes. When the most dominant compound, *(Z)*-9-pentacosene present in the cuticular extract of females was applied on to dead pentane-washed males, and presented to living males, it elicited arrestment, alignment and mounting behaviours in the males. These results confirm the presence of a contact sex pheromone in the scarab beetle, with *(Z)*-9-pentacosene as a component of this pheromone.

## 6.2 Introduction

The cetonid *Oplostomus haroldi*, is a newly recorded pest of honey bee colonies in Tanzania (Njau *et al.*, 2009) and Kenya (Torto *et al.*, 2010a). In Kenya, although beekeeping is widely practiced all over the country, this beetle occurs mainly in honey bee colonies in two major beekeeping areas of the country - the coastal lowlands which are sub-humid and, the humid to semi-arid eastern highlands which have two wet and dry seasons (Torto *et al.*, 2010a). Larval and adult stages of the beetle develop in different environments, with larvae in the soil and adults in the honey bee colony. In the colony, adults feed on brood, pollen and honey, destroying the comb structure as they feed (see chapter three). Current methods of managing the beetle in Kenya are mainly cultural involving reduction of the hive entrance to keep the adult beetle out and manual removal of beetles during hive inspection (Johannsmeier, 2001).

Semiochemical studies on the beetle and other cetonids is largely unknown although recent work showed that both adult males and females of the beetle are attracted to honey bee volatiles (Torto *et al.*, 2010a). This study suggested that the beetle has the sensory system to detect odours. To understand sex attraction in the beetle, the behaviour of both males and females was monitored in the honey bee hive where it was observed that mating was initiated by males. When the male approached a female, it made mouth contact first with the female's pronotum and elytra before mounting and copulation. It was therefore hypothesised that sex attraction in *O. haroldi* involves contact semiochemicals. To test this hypothesis, experiments were conducted to determine the specific behaviours involved in mating, mode of mate recognition and chemical nature of the signal mediating the mating process.

## 6.3 Materials and methods

### 6.3.1 Insects

Beetles used for the bioassays were collected from honey bee colonies at the coast of Kenya (Watamu and Taita localities) between January 2010 and February 2011 and were maintained in the laboratory as previously reported by Torto *et al.* (2010a). The individual sexes were kept separately in plastic rearing bowls with perforated lids (19 cm x 14 cm x 10 cm) in groups of 20 individuals and maintained on a diet of sterilised cow dung and soil mixed in the ratio 1:1 by volume. Prior to bioassays, the separate sexes were removed from the rearing container and kept on moist cotton wool for about 3 hrs to allow them to rub off any food material and debris stuck on their body surface.

### 6.3.2 Mating behaviour of *Oplostomus haroldi*

Individual males and females were placed in glass Petri dishes (9 cm in diameter) lined with filter papers and the mating sequences displayed by the different sexes were videotaped using a SONY™ cybershot digital camera. The recordings for 10 couples were played back and analysed to reveal the specific behaviours involved in the mating process.

### 6.3.3 Mating bioassays

Two groups of males and females (20 each), precleaned as described above in section 6.3.1 on insects, were transferred into empty plastic rearing bowls to allow them to copulate. Beetles that copulated were immediately decoupled and then transferred into separate bowls to be used for further assays. Two types of bioassays were carried out as follows:

First, pairs of dead females of similar size and body color were freeze-killed at – 20 °C for 30 min and then allowed to thaw to room temperature. The females were then placed at opposite

ends of a filter paper in a Petri-dish where they were held by masking tape (Figure 6.1). One of the dead females had its cuticular components stripped-off by washing it in 3 ml of analytical grade pentane (Sigma-Aldrich, St. Louis, Missouri, USA) for 10 min. This was repeated three times using fresh solvent and then air-drying the beetle in the hood for 30 minutes. A pair of females was presented to five living males individually in the Petri dish arena. This procedure was repeated four times using fresh pairs of dead females and fresh males. Each bioassay run was stopped when a male made mouth contact with both females and attempted to mate with either of them. The percentage of males making mouth contact, mounting and attempting to copulate with the dead females was recorded.

Using a similar procedure, a second bioassay was carried out to confirm the role of cuticular components in the mating. Cuticular components of the females were stripped-off by washing the beetles three times in 3 ml each of analytical-grade n-pentane as in the previous bioassay. Different doses of the female extract (0.1, 0.2, 0.4, 0.8, 1 and 2 female equivalents [FE]) were re-applied topically on to the body of one female and an equivalent volume of solvent applied on the other. For each female equivalent (FE), 20 males were individually presented to females and their behaviour in the test arena videotaped using a SONY™ cybershot digital camera. The percentage of males making mouth contact, mounting and attempting to copulate with the dead females was recorded. Test females were changed after 5 replicates. Once a mounted male attempted to copulate the dead female by extrusion of its aedeagus, it was disrupted from doing so and the bioassay considered complete if the male had previously made mouth contact with the other female. All bioassays were carried out under laboratory lighting conditions with a room temperature and relative humidity of 25 ± 2°C and 47 ± 5%, respectively.

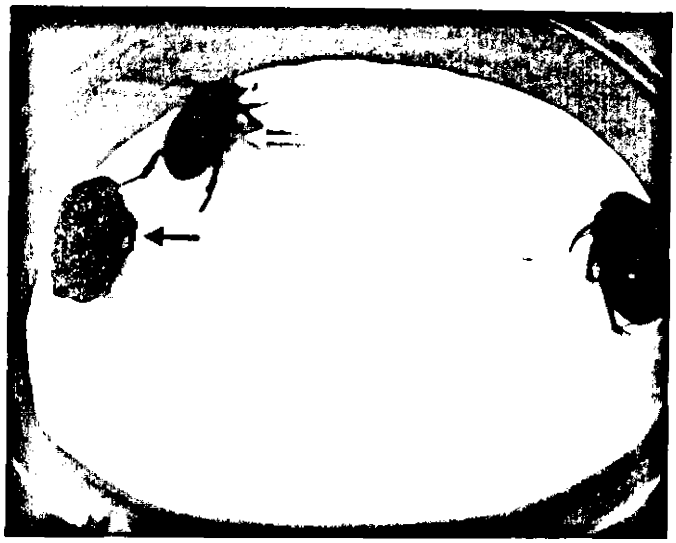


**Figure 6.1** Mating bioassay setup showing dead female beetles (with black and red arrow indicating control and test females respectively) stuck at opposite sides in a Petri dish with a live male (indicated by white arrow) walking in between them.

### 6.3.4 Role of mouth palps in mating

To establish the role of the palpi in mating, labial palps of one group of 5 males and the maxillary palps of another group of 5 males were removed using a pair of sterilised fine scissors and a stereomicroscope. Prior to each assay, 10 males were placed individually in a glass Petri dish (9 cm in diameter and lined with filter paper) containing 3 female beetles and the male allowed to interact with them. Once the male was observed attempting to copulate with a mounted female it was separated from her and she was kept aside in another Petri dish while the male was returned into the bioassay arena. A fresh female from the laboratory colony was then placed in the Petri dish to replace the mounted one which had been set aside. This procedure was repeated again until three females which the male had attempted copulating with were obtained. This bioassay was repeated for each of the 10 males until a total of 30 females (3 mounted by each male) selected by the 10 males were set aside in groups of 3 according to the males which mounted them for subsequent bioassays.

A first group of five males had their labial palps removed and a second group their maxillary palps. Each male was then placed in a glass Petri dish lined with filter paper together with the 3 females it had previously attempted to copulate with and allowed to mate with any one of them. The number of males in each group mounting and copulating with females was recorded. All bioassays were videotaped with a digital camera and conducted under temperature and relative humidity conditions similar to that used in the mating bioassays.

### 6.3.5 Ultra structure analysis of mouth palps

Adult male and female beetles were killed by freezing and preserved in ethanol (70%). The anterior portion of the head, including the mouthparts, was excised using micro dissection scissors and cleaned by sonication in distilled water with a small quantity of dishwasher detergent. The cleaned parts were air dried and mounted on SEM stubs using double-sided adhesive discs and graphite paste (Electron Microscope Sciences (EMS), Hatfield, PA). The mounted specimens were held for 2-3 days in a desiccator over silica gel for final drying, then sputter coated with gold-palladium in a Denton Vacuum Desk V (Denton Vacuum, Inc., Moorestoen, PA). Specimens were viewed and micrograhs taken at 10.0 kV using a Hitachi H4000 scanning electron microscope (Hitachi High Technologies America, Schaumberg, IL.)

### 6.3.6 Extraction of Cuticular Hydrocarbons (CHCs)

Cuticular components of frozen-killed individual adult males (11) and females (9) were extracted in 3ml of pentane (GC-grade, Sigma-Aldrich, St. Louis, Missouri, USA) for 5 min. All the extracts were concentrated under nitrogen to 500 µl. The extracts were then stored at -20 °C prior to GC-MS analysis.

### 6.3.7 Identification of Cuticular Hydrocarbons (CHCs)

Coupled gas chromatography-mass spectrometry (GC-MS) analysis was carried on an Agilent Technologies 7890A GC equipped with an HP-1 capillary column (30×0.25 mm ID×0.25 μm film thickness) coupled to a 5795C MS. One micro liter of each sample containing 50 ng of an internal standard (1-tridecene) was injected in split less mode with helium as carrier gas at a flow rate of 1.0 ml min−1. The oven temperature was initially held at 35°C for 5 min, increased to 280°C at 10°C/min, and then held at this temperature for 15 min. Spectra were recorded at 70 eV in the electron impact (EI) ionization mode. Tentative identification of the peaks was made by comparing spectral data with NIST 05 library data. The identities of 11 out of 20 components were confirmed by co-injection with the authentic hydrocarbon standards; *n*-Tricosane, *n*-Tetracosane (Alltech Associates Inc. Illinois, USA), *n*-Pentacosane (Kok lab. Inc., New York, USA), *n*-Hexacosane, *n*-Heptacosane, *n*-Octacosane, *n*-Nonacosane (Analabs Inc., Connecticut, USA), *(Z)*-9-Pentacosene, *(Z)*-9-Hexacosene, *(Z)*-9-Heptacosene and *(Z)*-9-Nonacosene (provided by Prof. Jocelyn Millar, University of California-Davis, USA).

### 6.3.8 Bioassays with authentic *(Z)*-9-pentacosene

A stock solution of *(Z)*-9-pentacosene (1μg/μl) was prepared. One, two and four female equivalents of this solution corresponding to 46.5 μg, 93 μg and 186 μg of the compound were tested for mating activity in males. Two frozen-killed males similar in size and body colour were stripped-off of their cuticular hydrocarbons as done for females in the mating bioassays and stuck at opposite ends of a filter paper using masking stick tape and placed in a glass petridish with a lid (males were used as attempts to use pieces of glass rods and plastic beetle dummies were unsuccessful). For controls, an identical volume of solvent only was applied on the other male. Each treatment and control were presented to 15 males and their

responses recorded by videotaping their behaviour in the Petri dish arena. The responses of males to the treatment and control including arrestment, alignment, mounting and copulation were analysed from playing back the video recording.

### 6.3.9 Statistical Analyses

The duration of each stage of the mating process was computed from the replayed videos and expressed as a percentage of the total duration for 10 mating couples. The durations were then subjected to a one way Kruskal-Wallis analysis of variance on ranks as the data did not possess equal variances.

A one sample chi square test was used to compare male beetle responses to test and control treatments in all the different mating bioassays. The relative amounts (ng) of each CHC component for each insect were pooled together for each sex and compared for both sexes using Mann-Whitney U test which does not require that the data sets be normal and their variances homogenous. All statistical analyses were carried out using SAS 9.1.2. statistical software (SAS Institute, 2003).

## 6.4 Results

### 6.4.1 Mating Behaviour of *Oplostomus haroldi*

After a male made contact with a living female using its mouth palps it exhibited a series of progressive behaviours which culminated in copulation. Upon contact, the male continuously palpated the female along its body (= arrestment stage), aligned its body with that of the female as it continued palpating it (= alignment stage), mounted the female and gripped her in between the prothorax and mesothorax with its forelegs (= mounting stage) and attempted to connect its genitalia with the abdominal tip of the female (=copulation stage) (Figure 6.2).

Following successful mounting and copulation, each male did not immediately dismount but cleaned its aedeagus and continued to palpate the pronotum of the female in a post-copulatory stage. Due to the brief nature of the first stages, their duration was pooled together for statistical analysis. Mating lasted 11 to 59.3 min with the post copulatory stage being 40-70% of the total mating duration (one way kruskal-wallis analysis of variance H = 3553, df = 3, P = <0.001) (Figure 6.3).

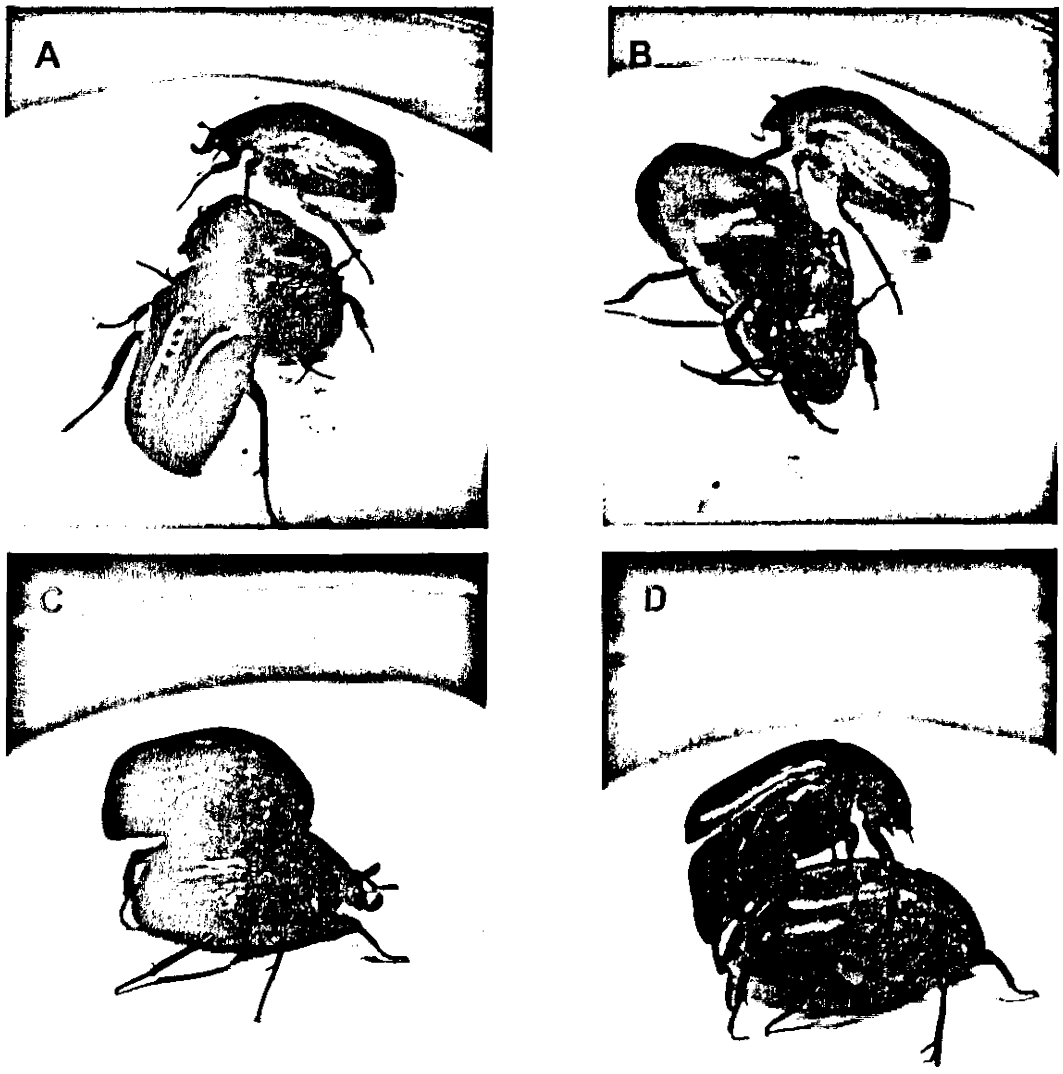



**Figure 6.2** An illustration of the sequence of behaviours that lead to copulation in *Oplostomus haroldi*. A- Arrestment of male beetle following mouth contact with female elytra, B- Alignment of male to female's body, C- Mounting of male on female's back and D- Copulation.

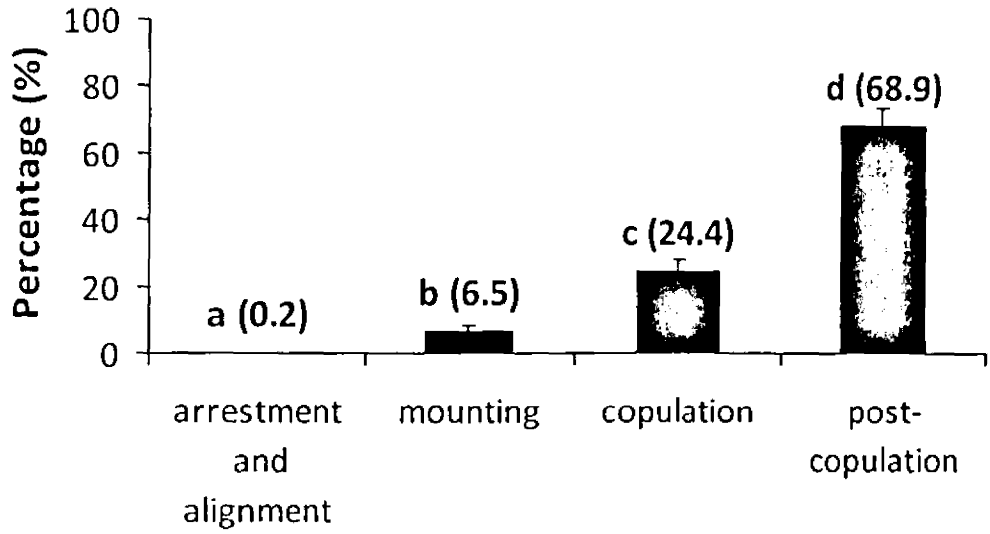
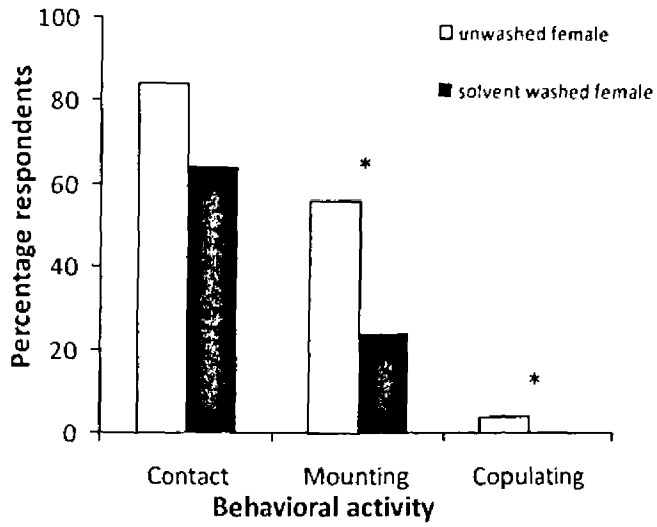



**Figure 6.3** Mean duration of arrestment and alignment, mounting, copulation and post-copulatory stages of the mating process expressed as percentages of the total mating duration for 10 mating couple. Mean separation based on a one way Kruskal-Wallis ANOVA.

### 6.4.2 Mating bioassays

Living males showed significantly stronger preferences for unwashed dead females to solvent washed dead females with more beetles mounting and attempting to copulate with the former than the latter (one sample $\chi^2$=2.7, df=1, P=0.1 for mouth contact; $\chi^2$=12.8, df=1, P<0.001 for mounting; $\chi^2$=4, df=1, P=0.046 for copulation) (Figure 6.4a). Similarly, males showed higher preferences for solvent washed females treated with female extracts than solvent washed females. These responses were concentration-dependent and were significant for mounting at 0.2 FE ($\chi^2$=11, df=1, P<0.001), and 2.0 FE ($\chi^2$=5, df=1, P<0.025) and copulation at 0.2 FE only ($\chi^2$=11, df=1, P<0.001) (Figure 6.4b).

A




B

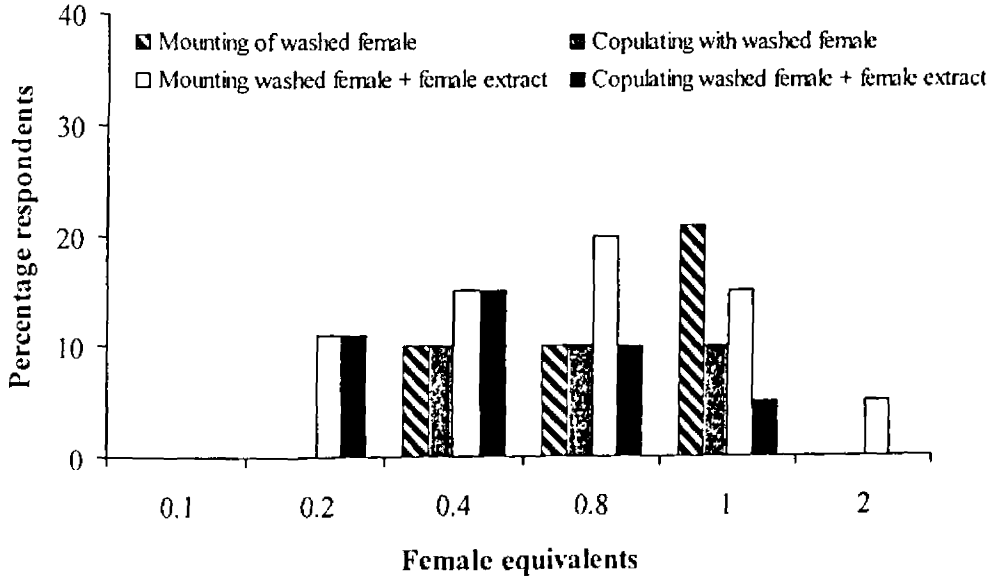



**Figure 6.4** Male responses to female treatments in mating bioassays. (A) The percentage of male beetles making mouth contact with female bodies, mounting females and attempting to copulate in first set of mating experiments. (B) The percentage of male beetles mounting female treatments and attempting to copulate with them in second set of bioassays. *Pair of bars representing statistically different behavioural responses.

### 6.4.3 Role of mouth palpi

All the labial palpectomised males successfully mounted females. Three out of 5 attempted copulation with females. On the other hand, maxillary palpectomised males showed no mounting and copulation attempts with females.

### 6.4.4 Fine structure of labial and maxillary palps

Scan electron microscopy revealed that both maxillary and labials palps possessed the same types of sensory sensilla typical of insects (Slifer, 1970; Zacharuk, 1980). These sensilla were long and short thin-walled trichoid sensilla distributed along the sides of both palps, thick walled basiconic sensilla with pores at their tips, and sensilla surrounded by collars (enclosed sensilla) (Figure 6.5). The basiconic and enclosed sensilla occurred only at the tip of the palps with 4-6 fold more of the former than the later sensillum on both palps. The maxillary palp tip has 4-5 times more sensilla than the labial palp tip in total (Figure 6.5).

### 6.4.5 Cuticular hydrocarbon profile of male and female beetle

Coupled gas chromatography-mass spectrometric (GC-MS) analysis of cuticular extracts of both sexes revealed the presence of several hydrocarbons ranging in chain length between $C_{23}$ and $C_{29}$ (Figure 6.6). Of these components, the identities of 11 were confirmed by comparison of their spectral data with those of authentic samples. These included *n*-tricosane (1), *n*-tetracosane (4), *(Z)*-9-pentacosene (6), *n*-pentacosane (7), *(Z)*-9-hexacosene (9), *n*-hexacosane (10), *(Z)*-9-heptacosene (12), *n*-heptacosane (13), *n*-octacosane (16), *(Z)*-9-nonacosene (18) and *n*-nonacosane (19) (Figure 6.7). The mass spectra of peaks 2, 5, 8, 11, 14, 15, 17 and 20 corresponded to unidentified methyl-branched saturated hydrocarbons.

Quantitative analysis showed that *(Z)*-9-pentacosene was present in significantly higher amounts in the extracts of females than males (Mann-Whitney U= 23, P=0.048) (Figure 6.8). In males, 12 of their dominant compounds were present in significantly higher amounts compared to their female counterparts (Mann-Whitney U< 24, P<0.05) (Figure 6.8) and comprised 1 unsaturated, 5 saturated and 6 methyl-branched saturated hydrocarbons.

### 6.4.6 Mating bioassays with *(Z)*-9-pentacosene

At 1, 2 and 4 FE of *(Z)*-9-pentacosene, the proportion of males arrested did not differ significantly between the test and control (one sample $\chi^2 < 3.841$ df = 1, P> 0.05) (Table 1). However, at 1 and 2 FE the proportion of males that aligned and mounted the test and control subjects differed significantly (Table 6.1). At 4 FE, the arrestment behviour of males was not significantly different for the test and control beetles. Overall, *(Z)*-9-pentacosene elicited an arrestment, alignment and mounting response but no copulation from males which varied with the dose of the compound.

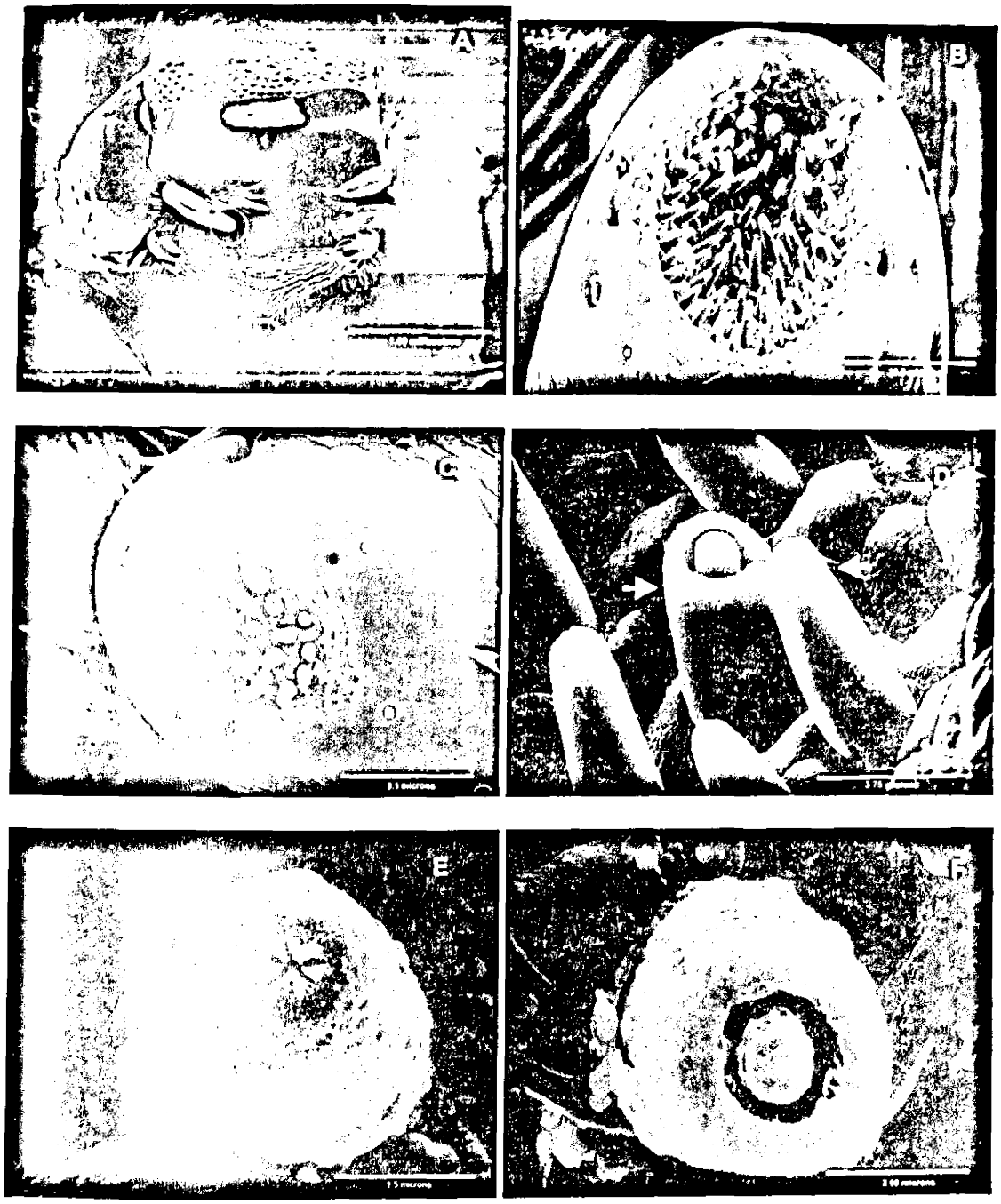



**Figure 6.5** Scan electron micrographs. A- frontal view of mouth showing labial palps (circled in red) and maxillary palp (circled in blue), B- maxillary palp tip, C- labial palp tip, D- sensilla types present on both palps (white arrow indicates enclosed sensillum and red arrow basiconic sensillum), E- basiconic sensillum with apical opening and F- enclosed sensillum.

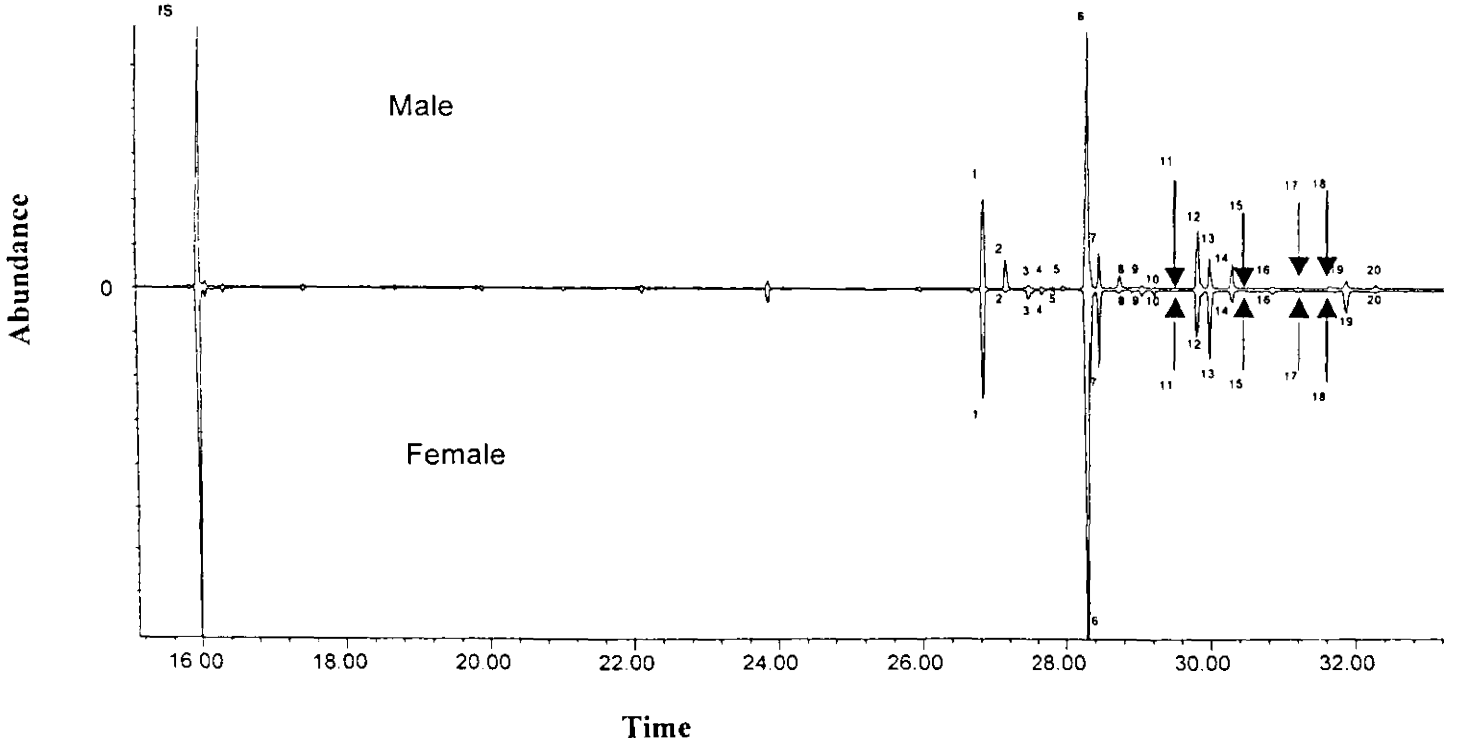



**Figure 6.6** Qualitative comparison of whole male and female cuticular hydrocarbon profiles. Peaks 1 to 20 represent n-tricosane (1), Unidentified methyl-tricosane (2), Unidentified tetracosene(3), n-tetracosane (4), Unidentified methyl-tetracosane (5), *(Z)*-9-pentacosene (6), n-pentacosane (7), Unidentified methyl-pentacosane (8), *(Z)*-9-hexacosene (9), n-hexacosane (10), Unidentified methyl-hexacosane (11), *(Z)*-9-heptacosene (12), n-heptacosane (13), Unidentified methyl-heptacosane (14), Unidentified methyl-heptacosane (15), n-Octacosane (16), Unidentified methyl-octacosane (17), *(Z)*-9-nonacosene (18), n-nonacosane (19), Unidentified methyl-nonacosane (20) and Internal standard (IS)

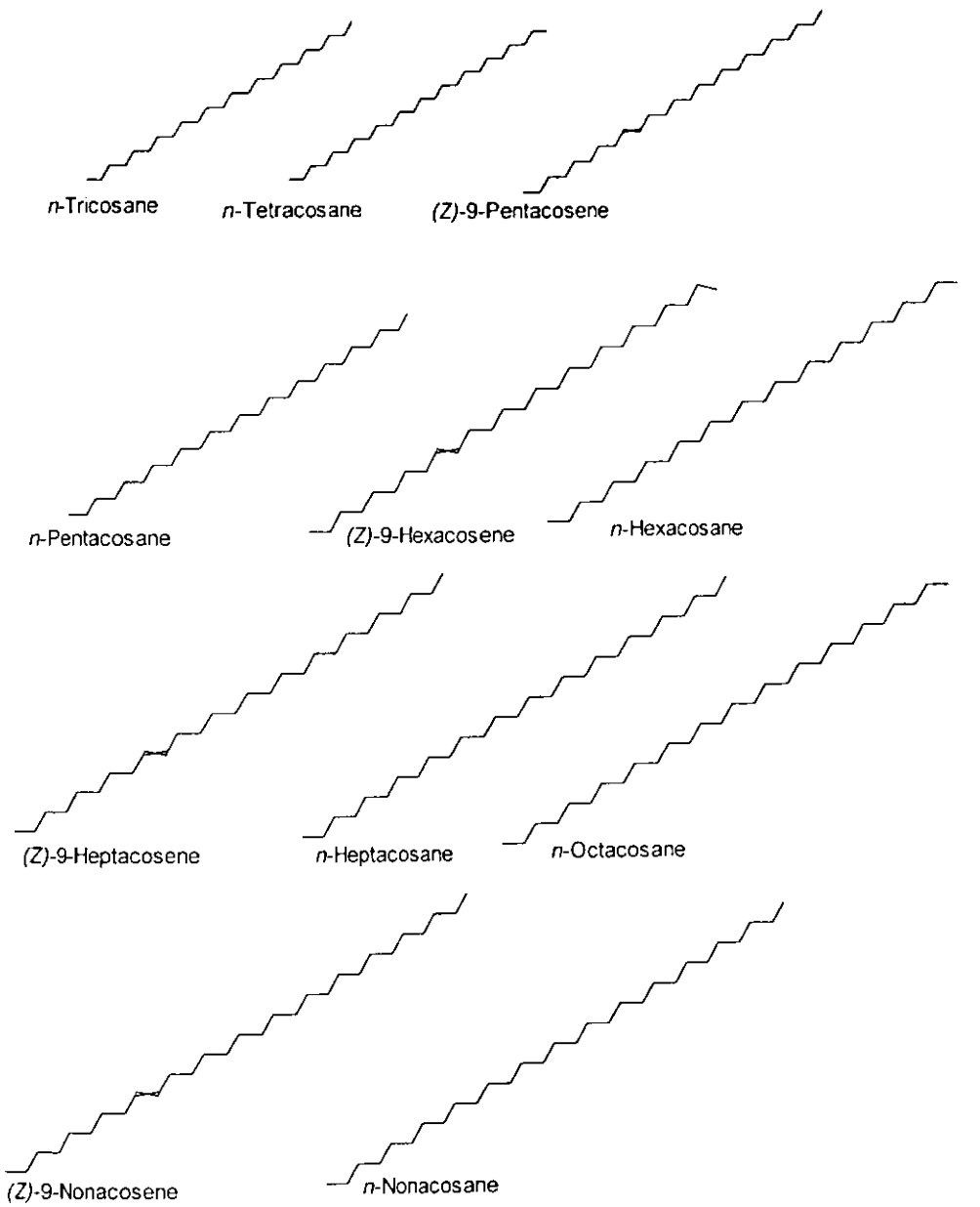



**Figure 6.7** Chemical structures of cuticular hydrocarbons identified from both male and female beetles.

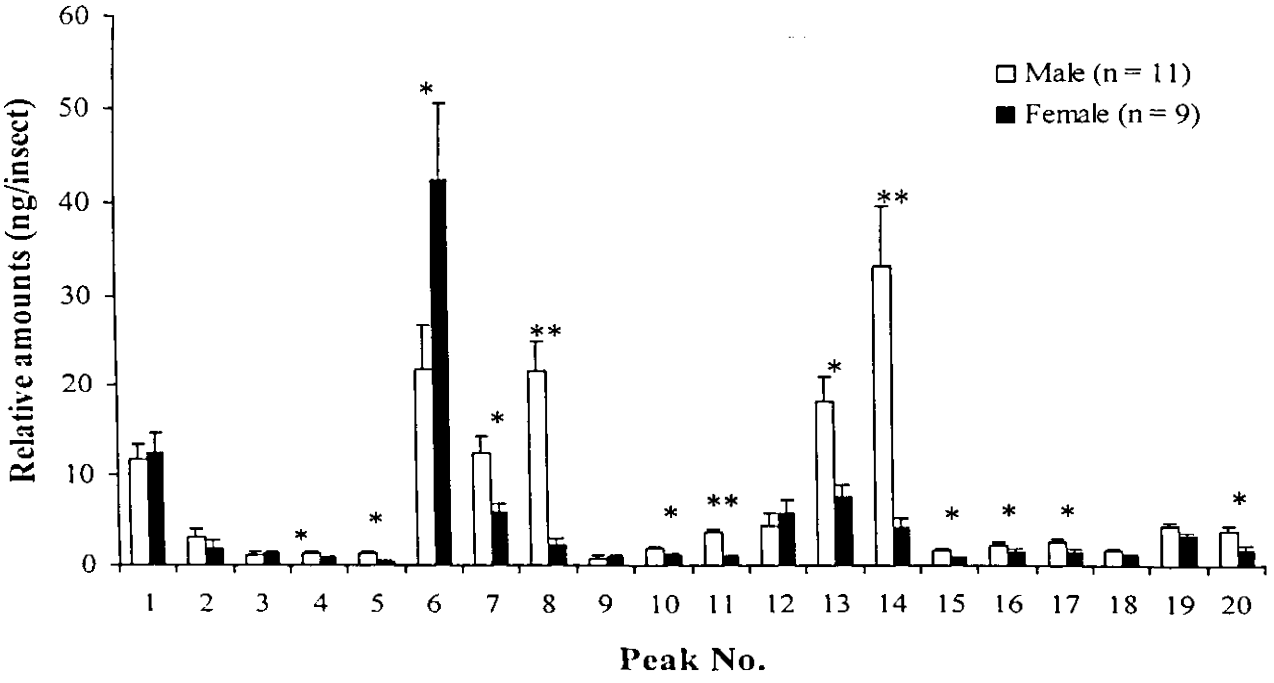



**Figure 6.8** Quantitative comparison of male and female cuticular hydrocarbon components. Pairs of bars with an asterix (*) above them indicate that their amounts are significantly different. * $p < 0.05$, ** $p < 0.001$ based on Mann-Whitney U tests. Peak identities are identical to those given in figure 6.5

**Table 6.1**: Response of male *Oplostomus haroldi* to solvent-washed dead males to *(Z)*-9-pentacosene was applied at different doses.

| Compounds and dosages | No. of responding males Treatment (%) | Control(%) | No. of males responding to each step in the behavioural sequence to the treatments and control (in parentheses) Step 1 | Step 2 | Step 3 | Step 4 |
|---|---|---|---|---|---|---|
| Z-(9)-pentacosene (1 FE = 46.5 µg) | 13 (86.7%) | 12 (80%) | 12 (13) | 7 (1)** | 1 (0)* | 0 (0) |
| Z-(9)-pentacosene (2 FE = 93 µg) | 15 (100%) | 100 (100%) | 15 (15) | 5 (2)* | 2 (0)** | 0 (0) |
| Z-(9)-pentacosene (4 FE = 186 µg) | 13 (86.7%) | 14 (93.3%) | 13 (14) | 4 (3) | 0 (0) | 0 (0) |

**$p < 0.001$ and *$p < 0.05$ based on a one sample chi square analysis comparing percentages of males behavioural responses to both treatments. Steps 1, 2, 3 and 4 refer to arrestment, alignment, mounting and copulation stages of the mating process of *O. haroldi*

### 6.5 Discussion

This study has shown that four specific behaviours viz arrestment, alignment, mounting and copulation mediate the mating process of *O. haroldi*. Very similar behaviours have been reported mediating the mating process of some Coleoptera (Geiselhardt *et al.*, 2009; Lelito *et al.*, 2009, Luo *et al.*, 2011). The relatively short duration of the first two stages, i.e., arrestment and alignment, is not surprising as it implies that *O. haroldi* can sample rapidly the cuticular profile of conspecifics in order to identify a suitable mate from a large group of conspecifics. It also implies that the receptors males rely upon for contact discrimination between males and females are highly specialised. The post-copulatory phase in *O. haroldi* was the longest, possible for a number of reasons. Firstly, it protected their mates from other male competitors (a direct benefit) and secondly, ensured the continuity on their genes as has been reported in some other beetles (Alcock, 1991; Shivashankar and Pearson, 1994; Facundo *et al.*, 1999; Harari *et al.*, 2003; Flay *et al.*, 2009; Lou *et al.*, 2011).

Behavioural assays with cuticular extracts of females of the scarab *O. haroldi* elicited sex attraction from males that are typical of related Coleopterans using contact sex pheromones for communication. Examples of this mode of communication have been reported for some species of the families Staphylinidae, Coccinellidae, Curculionidae, Chrysomelidae, Cerambycidae (Geiselhardt *et al.*, 2009) and Buprestidae (Lelito *et al.*, 2009). An earlier study demonstrated the involvement of body semiochemicals as mating cues in the scarab *Oryctes rhinoceros* L but this study failed to ascertain its nature (Mini, 2000). Previous studies on chemical communication in scarabs only revealed the existence of airborne aggregation and sex pheromones (Leal, 1998; Larsson *et al.*, 2003). This study therefore constitutes the first evidence of a contact sex pheromone in the family Scarabaeidae implying that the utilisation

of contact semiochemicals for communication in Coleopterans and other insects at large may be more widespread than presently known.

During hive inspection, mating pairs of the beetle were found indicating that despite the darkness of the hive environment the different sexes of the beetle are able to locate each other. In such an environment and with hostile honey bee hosts, exploiting a sex communication mechanism involving less volatile compounds and their detection with sensory structures other than the antennae would favor the beetle. In palpectomic assays we found that only the maxillary palps were involved in mate recognition. A closer examination of the palps using scan electron microscopy revealed the presence of identical sensory receptors in both sexes and palp types. This may imply that these sensilla serve multiple roles in *O. haroldi*. The maxillary palp possessed 4-5 times more sensory receptors at its tip compared to that of the labial palps, indicating that the maxillary palp may be used for various purposes including sex discrimination, as discovered in this study.

Once inside the hive the beetle often evades aggression from its host by 'playing dead or thanatosis', a behaviour which involves the tucking of the antennae beneath the eyes, the bending of the head downwards and staying still. In such a protective position, the antennae become unavailable for tactile chemoreception and thus necessitate the use of other sensory receptor bearing body parts. The use of the maxillary palp over the labial palp is advantageous in that it shows greater flexibility in its movement (since it point of origin is the cardo of the maxillary palp which allows it to be moved independently or along with the maxillae) than that of the labial palps which are fixed to side of the labium. The maxillary palp therefore appears to be more sensitive for the detection of conspecifics and potential mating partners and is more protected as it originates in the buccal cavity. This mode of contact

chemoreception represents an important ecological adaptation of *O. haroldi* to its environment.

GC-MS analyses showed that cuticular components of the two sexes were qualitatively similar, but varied quantitatively comprising hydrocarbons of chain length, ranging from $C_{23}$ to $C_{29}$. These quantitative differences may constitute the baseline for sex discrimination in *O. haroldi* as has been demonstrated in the cerambycid *Megacyllene robiniae* (Ginzel *et al.*, 2003) and other Coleoptera (Geiselhardt *et al.*, 2009; Lelito *et al.*, 2009) and other insects (Howard and Blomquist, 1982, 2005; Eliyahu *et al.*, 2008; Woodbury and Gries, 2007; Raina *et al.*, 2003) which rely on contact semiochemicals for mating.

Our behavioural assays showed that *(Z)*-9-pentacosene, the most abundant component of the female CHC profile elicited a partial sequence of the full mating behaviour. This clearly demonstrates that it is a component of the female contact sex pheromone. Interestingly, this compound has been reported to play a pheromonal role in contact recognition in the cerambycid *Megacyllene robiniae* (Ginzel *et al.*, 2003) and the mirid *Lygocoris pabulinus* (Drijfhout and Groot, 2001). The fact that *(Z)*-9-pentacosene is a pheromone in two different orders, Coleoptera and Heteroptera, suggests that different insects from different orders may utilize similar compounds for similar or different purposes. However, it is known that pheromonal activity in insects usually involves blends of compounds. As such further studies will be required to identify the full blend of compounds involved sex attraction in *O. haroldi*.

# CHAPTER SEVEN

## *Musa acuminata* x *M. balbisiana* (Zingiberales: Musaceae) and *Mangifera indica* L. (Sapindales: Anancardiaceae) fruits as potential alternate hosts of *Aethina tumida* (Coleoptera: Nitidulidae)

### 7.1 Summary

The small hive beetle *Aethina tumida* (Coleoptera: Scarabaeidae) is a parasitic pest of honey bees of African origin and European ones in North America and Australia following its recent introduction in these areas. In European honey bee colonies, both adult and larva stages of the beetle wreck havoc on honey bee colonies by feeding on pollen, bee brood and honey. Despite its pest status, little is known about the ecology in its native habitat. However, the beetle has been observed feeding on fruits in areas devoid of social bees suggesting that it utilises alternate hosts for survival. The attractiveness of banana, *Musa sp.* and mango, *Mangifera indica* to *A. tumida* were studied using laboratory bioassays and field traps baited with these fruits at two beekeeping sites as they were the most widely grown fruits in major beekeeping areas. Olfactometric experiments showed a significant attraction of *A. tumida* to banana and mango odours compared to blank controls. Coupled GC-EAD analysis revealed that *A. tumida* consistently detected 14 and 25 components of the banana and mango odours, respectively. Coupled GC-MS analysis showed that both fruit odours consisted mostly of esters which partially mimicked honeybee volatiles. Baited trap captures confirmed the attractiveness and host potential of these fruits for the beetle as both adults and larvae were recorded in traps at both locations; Gede, a lowland forest area and Nairobi, a high altitude semi-arid area. Significantly more beetles were captured at Gede than in Nairobi and corroborated earlier findings of studies conducted in Kenya. These findings constitute the first evidence of *A. tumida* reproduction on fruits in nature and demonstrate its ability to exploit alternate hosts for survival.

## 7.2 Introduction

The existence of alternate hosts for a good number of insect pests for reproduction and survival has been well documented (Christenson and Foote, 1960; Prokopy and Boller, 1976; Fitt, 1989; Ananthakrishnan, 1993; Talekar and Shelton, 1993; Turgeon *et al.*, 1994). For invasive insect pests, finding and exploiting such hosts has greatly contributed to their successful spread (Christenson and Foote, 1960; Prokopy and Boller, 1976). Whether the exploitation of closely related or unrelated host species by a single insect is controlled via a predefined genetic mechanism or is the result of an adaptive survival strategy, there is no doubt that the ability to switch between highly similar or dissimilar hosts has greatly contributed to the successful establishment and continued spread of some invasive species (Moeser and Vidal, 2004). No matter which of the aforementioned mechanisms best describes the survival pattern of a particular invasive insect pest, different host species used by the same insect must share a unique similarity which the insect pest is capable of recognising and associating it to its original host. Such uniquely shared characters can therefore serve as a benchmark for the detection and assessment of potential hosts by an insect.

The small hive beetle *Aethina tumida* (Coleoptera: Nitidulidae) is an African native which is scarcely considered an economically important pests of honey bees within its native range in sub-saharan Africa (Lundie, 1940; Schmolke, 1974). However, it has become an economically important pest of European honey bees in the United States and Australia following its recent introduction into these areas (Sanford, 1998; Neumann and Elzen, 2004). Efforts aimed at reducing populations of the beetle in managed honey bee colonies have only been partially successful. For example, although flight traps and bottom-board traps baited with yeast-inoculated pollen dough work effectively for the beetle, these trapping systems are only effective as monitoring tools (Teal *et al.*, 2006; Arbogast *et al.*, 2007; Torto *et al.*,

2007b). An alternative lure for the pollen dough developed from fruit odours to mass trap this beetle has shown promise only under laboratory conditions (Duehl *et al.*, Unpublished data). Monitoring of honey bee colonies for *A. tumida* showed that they occurred all year round (Torto *et al.*, 2010b). Nationwide surveys in Kenya showed that small hive beetles are widely distributed in honey bee colonies across the country (see chapter 3), yet their successful reproduction in these colonies is rare. Recent trappings of larvae using a larva trap confirmed this observation (Arbogast *et al.*, Unpublished). Only 41 larvae were trapped from 3 colonies after 12 months, suggesting the use of alternate hosts for survival.

It was hypothesised that *A. tumida* survives in its areas of occurrence by either taking over weak honey bees colonies and successfully reproducing in them, or on ripe fruits as alternate hosts. Unlike in the USA where the beetle is able to successively reproduce in such weakened honey bee colonies, its successful reproduction in the African honey bee colonies is rare (Fombong and Torto, Unpublished). The rarity of African honey bee colonies overtaken by *A. tumida*, high abundance of this beetle in major fruit growing areas (Torto *et al.*, 2010a) and the observation of adults on ripe sweet banana spurred the interest to investigate existence of alternate hosts of *A. tumida*. Electrophysiological experiments and olfactometric bioassays were carried out to determine the attractiveness of sweet banana, *Musa acuminate* x *balbisiana* var. apple and mango, *Mangifera indica* var. peach sabre, to *A. tumida* under laboratory and field conditions.

## 7.3 Materials and Methods

### 7.3.1 Insects

Virgin *A. tumida* adults 7-14 days old were used for the bioassays and obtained from a laboratory colony maintained at *icipe* using the method described by Torto *et al.* (2010a).

Prior to bioassays, insects were starved for 24 hr with males kept separately from females. Only insects showing no sign of physical injury or missing any body parts were used as test subjects in the bioassay.

### 7.3.2 Fruits

Mature ripe sweet banana (var. apple) and mango (var. peach sabre) fruits (Figure 7.1a and b) were obtained from supermarket outlets in Nairobi and Mombasa. These fruits were used in this study because they are widely grown across major beekeeping areas in Kenya.

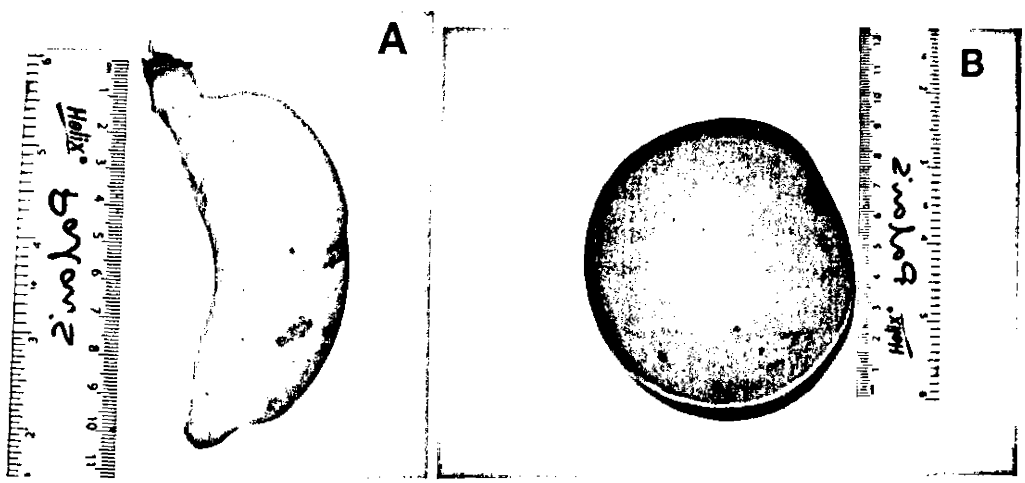



**Figure 7.1** Mature ripe fruits used for this study. Sweet banana (A) and Mango (B).

### 7.3.3 Bioassays with ripe banana and mango fruits

All bioassays were conducted using a glass Y-tube olfactometer consisting of a glass Y-tube (base, 7.5-cm-long; Y-arms, each 7.5-cm long; internal tube, 10 mm in diameter) designed by Carroll *et al.* (2006) and connected at its two arms and base to similar-sized flexible teflon tubings which were in turn attached to the odour and vacuum sources. A screen mesh was placed at each end of the olfactometer to prevent test insects from getting out of the test arena into the Teflon tubing. Odour sources were placed in 2 L glass chambers with lids containing inlets to push in air and outlets through which the odours within the chambers were pulled out into the Y-tube test arena. Charcoal-purified air originating from a compressed air tank was passed into the odour chambers at a flow rate of 250 ml/min. One of the Y-arms was

connected to an odour source (test odour) and the other to an empty jar with only clean air (control odour) passing through. All bioassays were carried out under red light illumination provided by two 40W red bulbs placed 1.5 m above the test arena. Temperature and relative humidity conditions were maintained at 25 ± 1°C and 50 ± 5 %, respectively.

The olfactory responses of male and female *A. tumida* to test odours were studied using the Y-tube olfactometer. Test odour sources included 100 g of ripe mangos and bananas while blank controls were clean air. The test insects were released individually into the test arena through the base of the Y-tube and allowed to settle down for two minutes. The insect was then monitored for eight minutes more and the bioassay stopped after 10 min. The insect was considered to have made a choice if it visited a particular arm of the Y-tube more or stayed in it more than in the other parts of the Y-tube. A no choice response was recorded if the insect stayed in the base arm of the Y-tube for more than 5 mins. The odour sources were switched in between replicates to avoid positional bias. For each sex, 25 replicates were carried out for each test odour.

### 7.3.4 Field trapping of *A. tumida* using mango and banana baited traps at two beekeeping sites

Small hive beetle flight trap baited with ~100g of ripe bananas or mangos (Figure 7.2) were setup at two apiary sites within Kenya to determine whether beetles were attracted to these fruits under field conditions.

Site 1 (*icipe* campus apiary)

This site was located on the *icipe* main campus (01° 13' 25.3"S, 036° 53' 49.2" E) in Nairobi. Five traps baited with bananas only were placed at 100-500 m away from the managed honey

bee colonies as mango trees at this site do not bear fruits due to its high altitude (Figure 7.3a). Each trap was suspended at 1.5-2.0 m above the ground by hanging it on a tree branch which also shielded it from direct sunlight. Grease was applied around the midsection of the straps used to suspend each trap to prevent tree dwelling ants from gaining access to the bait. Fresh whole ripe bananas (100g) were cut into two pieces with their peels on and placed into each trap along with two 15 ml plastic vials filled with water and containing a dental wick sticking out through a hole bored in their lids to keep the baits moist and humid. A kill strip (Hercon® Vaportape II, Gempler's, WI, USA) was placed inside the trap to kill trapped beetles. The traps were removed after 6-7 days and their contents checked for *A. tumida* adults which were then sexed. Thereafter, traps were cleaned, recharged with fresh bait and redeployed. Trapping at this site was carried out for 9 months from 1st September 2010 – 30th May 2011.

Site 2 (Gede Ruins museum apiary)

This apiary was located within the compound of the Gede ruins museum (03° 18' 24.3" S, 040° 1' 4.4" E) at the Coast province. At this site, six traps (three baited with bananas and three baited with mangoes) were placed at 25 m, 50 m and 100 m away from the centre of the apiary (Figure 7.3b). The traps were setup in a manner identical to those at the *icipe* apiary. This experiment ran for 15 weeks from 20th February – 4th June 2011.

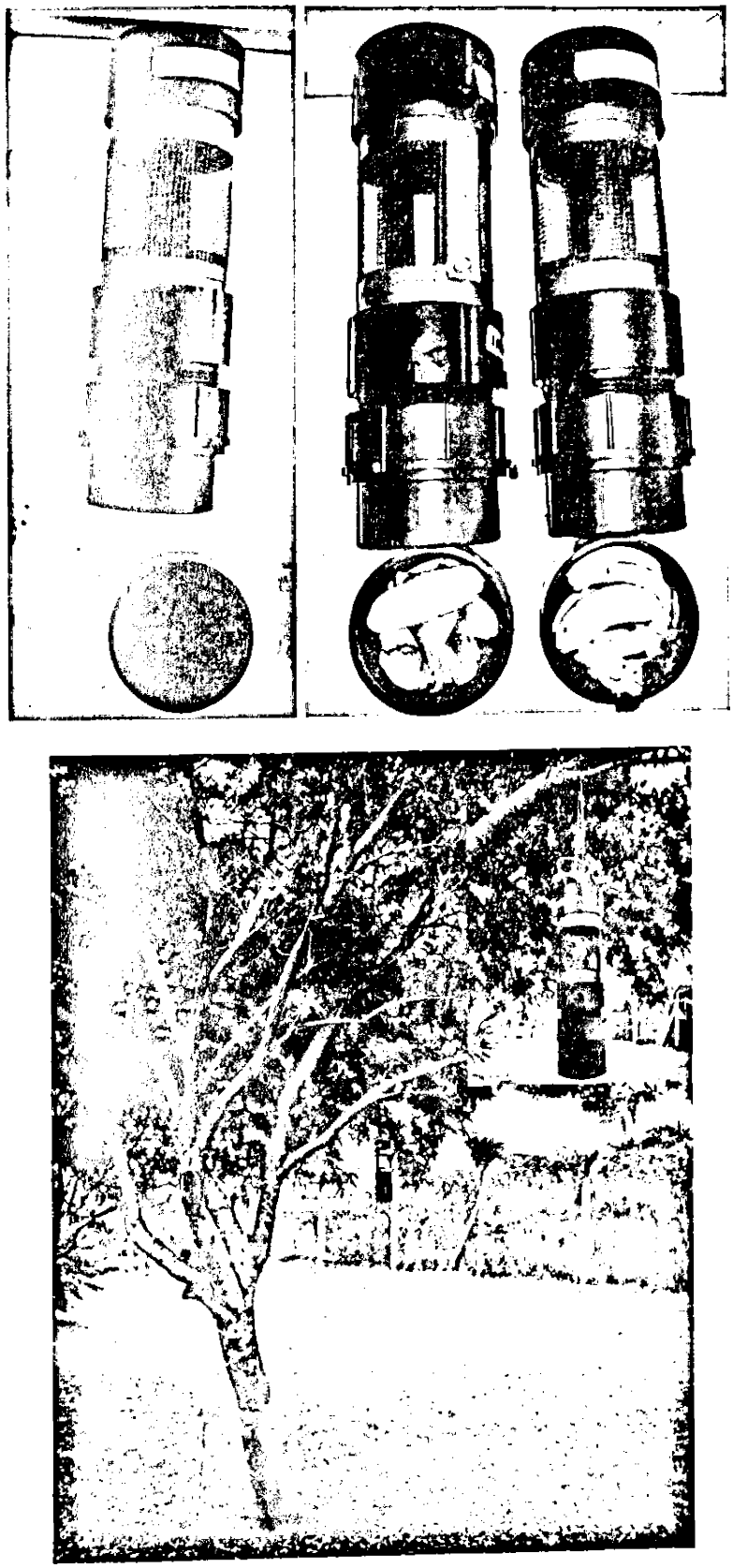


**Figure 7.2** *Aethina tumida* flight trap showing two banana-baited traps and trap placement in the field.

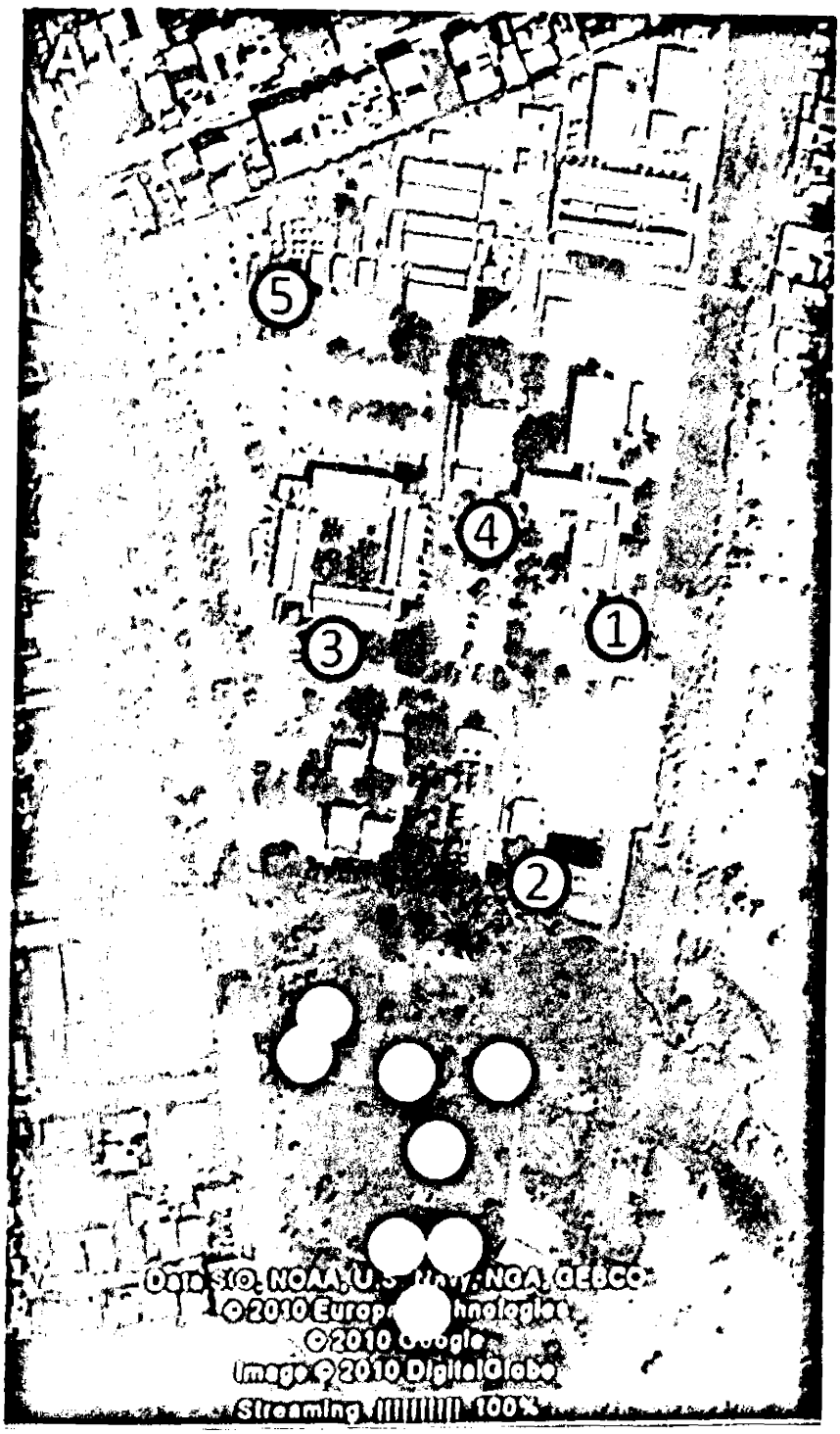



**Figure 7.3A** Layout of baited *A. tumida* flight traps at site 1. Yellow and white circles represent honey bee colonies and trap positions respectively.

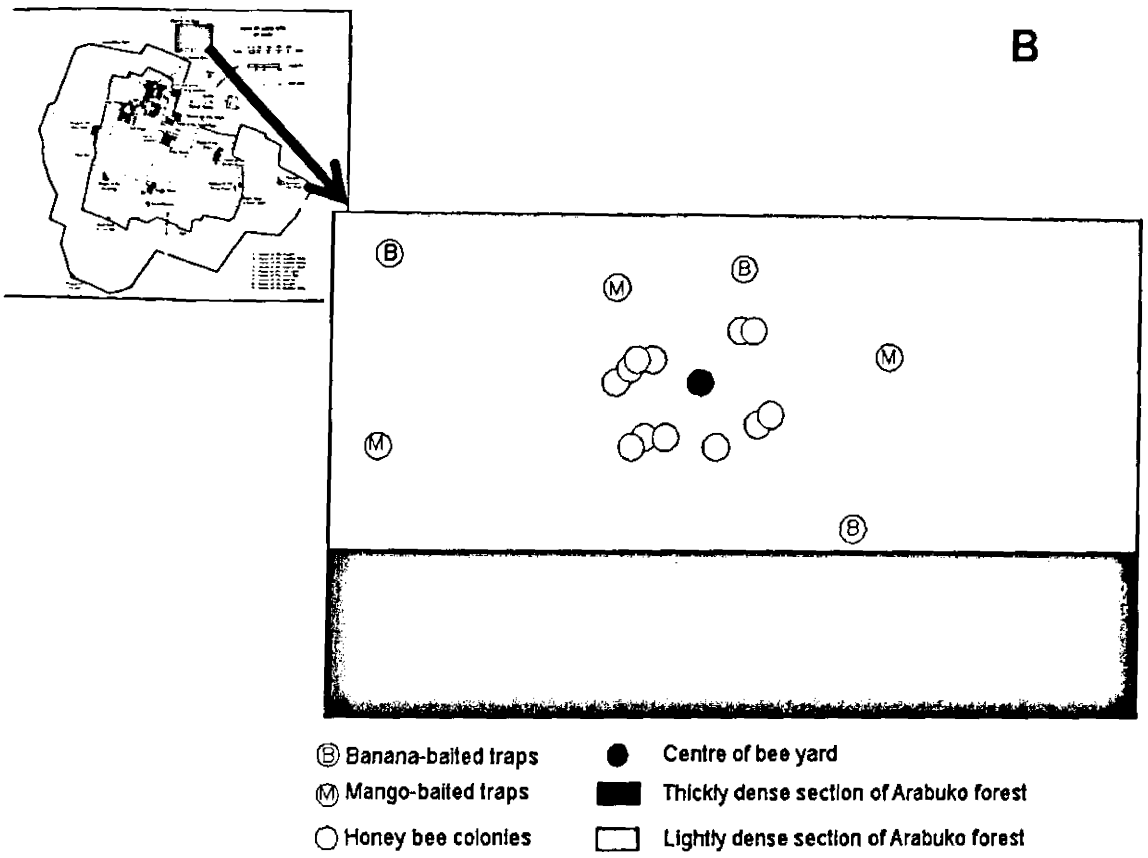



**Figure 7.3B** Layout of baited *A. tumida* flight traps at site 2 (Gede Ruins Museum).

### 7.3.5 Volatile collection

Volatiles were collected from 100g of ripe mango and banana fruits using a push and pull volatile entrainment system (Analytical Research Systems Inc., Gainesville, FL USA) on cleaned Super Q trap (30 mg, Alltech, Nicholasville, KY). Fruits were placed in a 2 L glass volatile collection chamber. Charcoal-filtered and humidified air was continuously pushed into the collection chamber from a compressed air tank and pulled through the Super Q trap by a vacuum pump at a rate of 500 ml/min for 2 hrs. Odours emanating from the fruits were then adsorbed on the trap. The Super-Q traps were eluted with 150µl of dichloromethane (DCM) under ice and the eluent was pushed through the trap using a gentle stream of charcoal-filtered nitrogen ($N_2$). The eluate was stored at -20 °C prior to analysis.

### 7.3.6 Analysis of volatiles

Coupled gas chromatography-electroantennographic detection (GC-EAD) analysis was carried out on a Hewlett-Packard (HP) 5890 Series II gas chromatograph equipped with a capillary column HP-5 MS (30 m × 0.25mm ×0.25µm, ID and film thickness) with nitrogen as the gas carrier. Extracts were analysed in the splitless mode at an injector temperature of 250°C and a split valve delay of 1 minute. The oven temperature was held at 35°C for 5 min, increased to 280°C at 10°C/min, and then held at this temperature for 20.5 min. The column effluent was split 1:1 for simultaneous detection by FID and EAD. For EAD detection, silver wires in drawn out glass capillaries filled with Ringer solution served as the reference and recording electrodes. The distal and proximal segments of the beetle antenna were placed in contact with the microelectrodes and humidified air delivered at 1 ml/s over the mounted antenna. The microelectrodes were connected via an antennal holder to an AC/DC amplifier in DC mode (Syntech, Hilversum, The Netherlands). A GC program (Syntech GC-EAD 2000, Hilversum, The Netherlands) was used to simultaneously record and analyse the amplified EAD and FID signals on a PC. Four micro litres of the both banana and mango extracts were analysed with either fresh male or female antenna in repeated sample analysis.

Coupled gas chromatography-mass spectrometric (GC-MS) analysis of volatile extracts eluted with DCM were carried out on an Agilent Technologies 7890A gas chromatograph equipped with a capillary column HP-5 MS (30 m × 0.25mm ×0.25µm, ID and film thickness) and coupled to 5795C mass spectrometer. One micro litre of each sample was injected in the split less mode (Inlet temperature = 250°C, Pressure = 6.83 psi), and helium was used as the carrier gas at 1.0 ml/min. The oven temperature was held at 35°C for 5 min, increased to 280°C at 10°C/min, and then held at this temperature for 20.5 min. EAD-active compounds were identified by comparing their mass spectral data with those from the NIST 08 library of the

mass spectrometer and by retention times with those of authentic commercial samples. The amounts of EAG-active components in both mango and banana volatiles were quantified using the external standard, ethyl nonanoate. Only components also known to be produced by worker honeybees were quantified.

### 7.3.7 Statistical analysis

The response of each beetle sex to odour pairs was compared using a one sample chi square ($\chi^2$) analysis. Similarly, the total male and female beetles caught by traps at site 1 and site 2 according to host fruit type were compared using one sample chi square ($\chi^2$) analysis. The 9-months weekly trap catches per baited trap at site 1 were subjected to a logistic regression using R statistical software (R, 2010). The effect of rainfall on total monthly trap catches at site 1 was determined by linear regression analysis in R statistical package with the exemption of December 2010 as rainfall data for this month were not collected (due equipment breakdown). Trap catches for the mango and banana baited traps at site 2 were compared among trap distances per fruit and across fruits, across fruit regardless of trap distance and, across fruit types and trap distances. Weekly trap catches per bait type at site 1 were also subjected to linear regression analyses with their corresponding rainfall values to determine the effect of rain on beetle captures. All analyses were carried out at an $\alpha$-level of 0.05.

## 7. 4 Results

### 7.4.1 Bioassays with ripe banana and mango fruits

A total of 72% male and 60% female beetles were attracted to mango volatiles compared to 16% males and 24% females which were attracted to the blank control. The remainder 12% males and 16% females did not show any attraction to either odour sources and were considered non-respondents. Both beetle sexes significantly responded to mango odours

compared to the blank controls ($\chi^2$ = 35.64, df = 1, P < 0.001 for males; $\chi^2$ = 15.94, df = 1, P < 0.001 for females) (Figure 7.4a). Although males showed a stronger response compared to females, both responses were not statistically different from each other ($\chi^2$ = 1.09, df = 1, P = 0.296).

Similarly, 88% males and 60% females were attracted to banana odours as against 4% males and 40% females attracted to the blank control. Only 8% of males did not show any attraction for either odour sources. Again, beetles showed a significant preference for banana odours over the control ($\chi^2$ = 94.63, df = 1, P < 0.001 for males; $\chi^2$ = 4.00, df = 1, P = 0.046 for females) (Figure 7.4b). However, the responses of males were significantly higher compared to that of females ($\chi^2$ = 5.30, df = 1, P = 0.021)

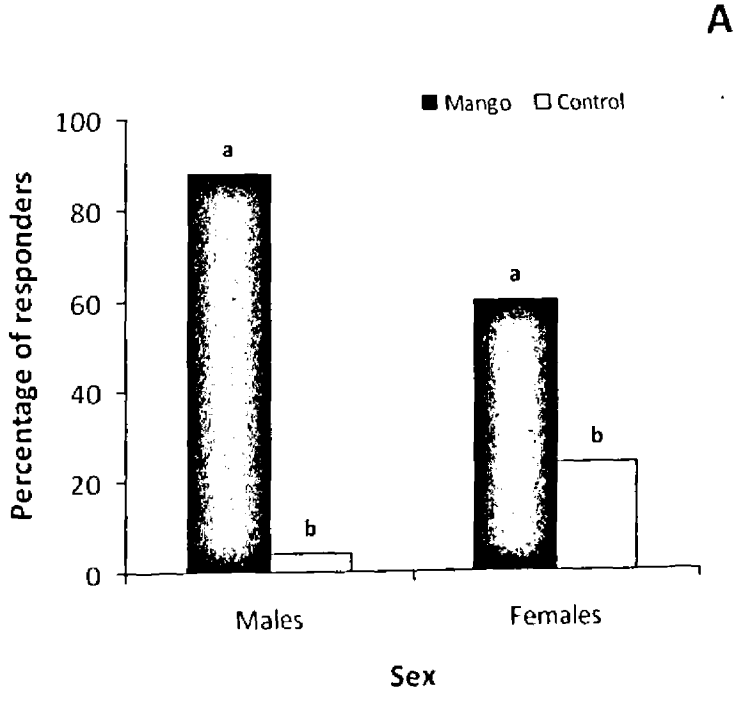



**Figure 7.4A** Responses of *Aethina tumida* to mango odours

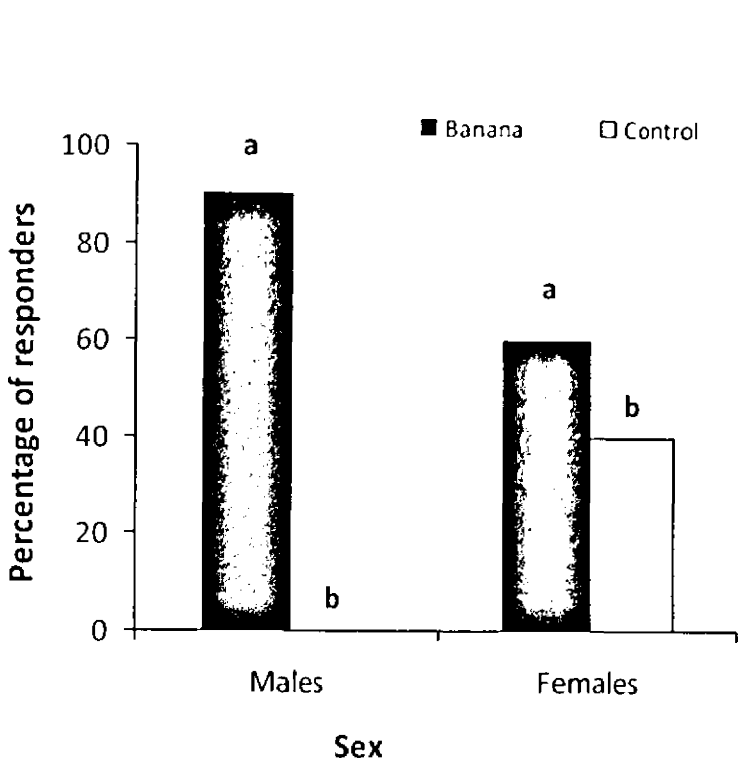



**Figure 7.4B** Responses of *Aethina tumida* to banana odours.

### 7.4.2 Field trapping of *A. tumida* using mango and banana baited traps at two beekeeping sites

**Site 1 (*icipe* campus apiary)**

A total of 26 beetles were captured by the banana-baited traps. Of these, 14 were males and 12 were females with no significant difference in the sex ratio ($\chi^2 = 0.15$, df = 1, P = 0.695). Starting with the trap closest to the apiary and moving further way, traps 2, 3, 1, 4 and 5 caught 8, 8, 6, 2 and 2 beetles, respectively. Trap captures varied with their distance from the apiary. These traps varied significantly in their beetle capture throughout the entire nine months period ($\chi^2 = 3.93$, df = 4, 195, P = 0.004) and with rainfall (Figure 7.5a). Linear regression analysis of rainfall and trap catches were negative and non-significant (F= 2.59, df= 1,6, P= 0.158) (Figure 7.5b).

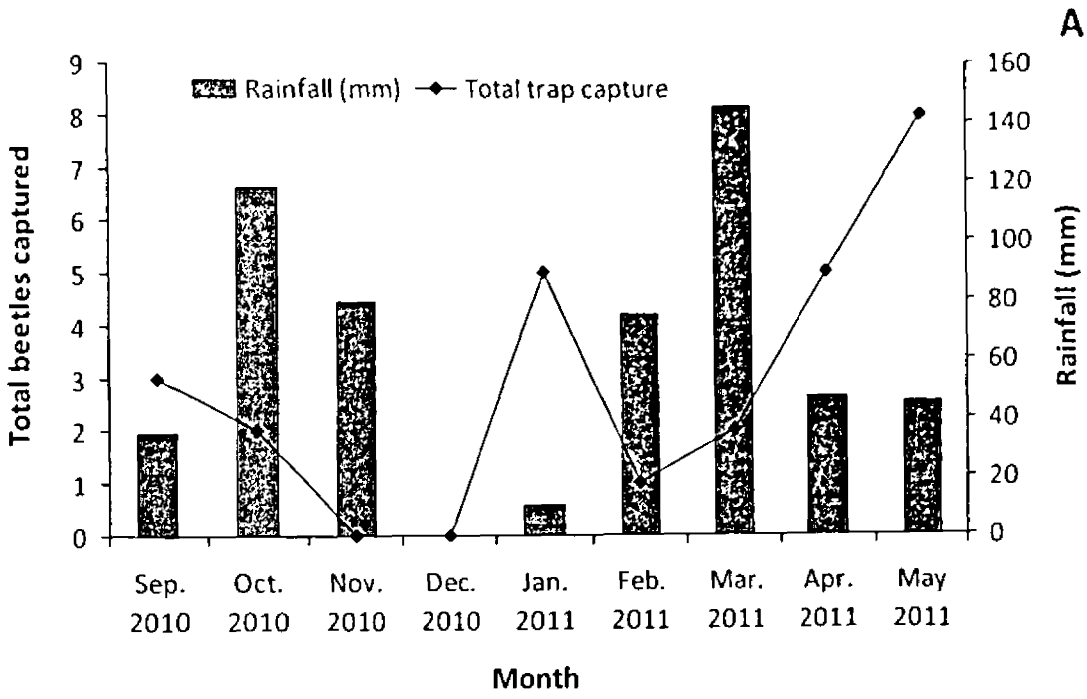



**Figure 7.5A** Total beetle capture/month throughout the 9-month trapping period.

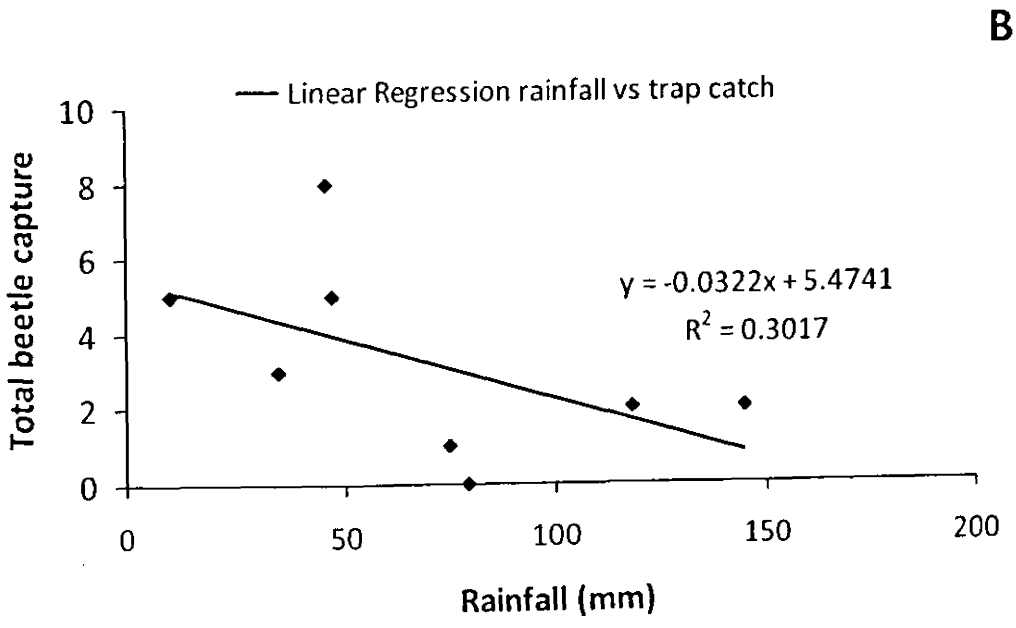



**Figure 7.5B** Linear relationship between trap captures and rainfall at the *icipe* main campus site.

**Site 2 (Gede Ruins Museum apiary)**

A total of 73 and 140 beetles were captured in the traps baited with banana and mango, respectively. Of the beetles caught in the banana-baited trap, 31 were males and 42 were females whereas 64 males and 76 females were captured in the mango-baited trap. The mango-baited traps captured significantly more beetles than those baited with banana ($\chi^2$ = 21.08, df = 1, P < 0.001). There was no significant difference between the proportion of male and female beetles captured by either the banana-baited traps ($\chi^2$ = 1.03, df = 1, P = 0.31) or the mango-baited traps ($\chi^2$ = 1.66, df = 1, P = 0.197). Banana-baited traps deployed at distances of 25, 50 and 100 m away from the apiary captured 15, 27, and 31 beetles, respectively. A similar but reverse trend was observed for mango-baited traps at 25 m, 50 m and 100 m which caught 60, 50, and 30 beetles, respectively. Besides adult beetles, a total of 31 third and fourth instar *A. tumida* larvae were recovered from both trap types (26 from mango and 5 from banana-baited traps).

Beetle captures in banana- and mango-baited traps varied with rainfall throughout the trapping period (Figure 7.6a). Logistic regression analysis showed that no significant difference existed in trap captures at different distances for the mango bait ($\chi^2$ = 0.63, df = 2, 42, P = 0.535) or banana bait ($\chi^2$ = 0.65, df = 2, 42, P = 0.527). A similar analysis did not show any significant difference in trap captures for the bait type regardless of trap distance ($\chi^2$ = 3.29, df = 1, 28, P = 0.073) or bait type in combination with trap distance ($\chi^2$ = 1.18, df = 1, 28 P = 0.322). Scatter plots of beetle captures with rainfall revealed a non-linear association between the two variables for both banana- (Figure 7.6b) and mango- baited traps (Figure 7.6c) as trap captures increased initially with rainfall but decreased as rainfall intensified.

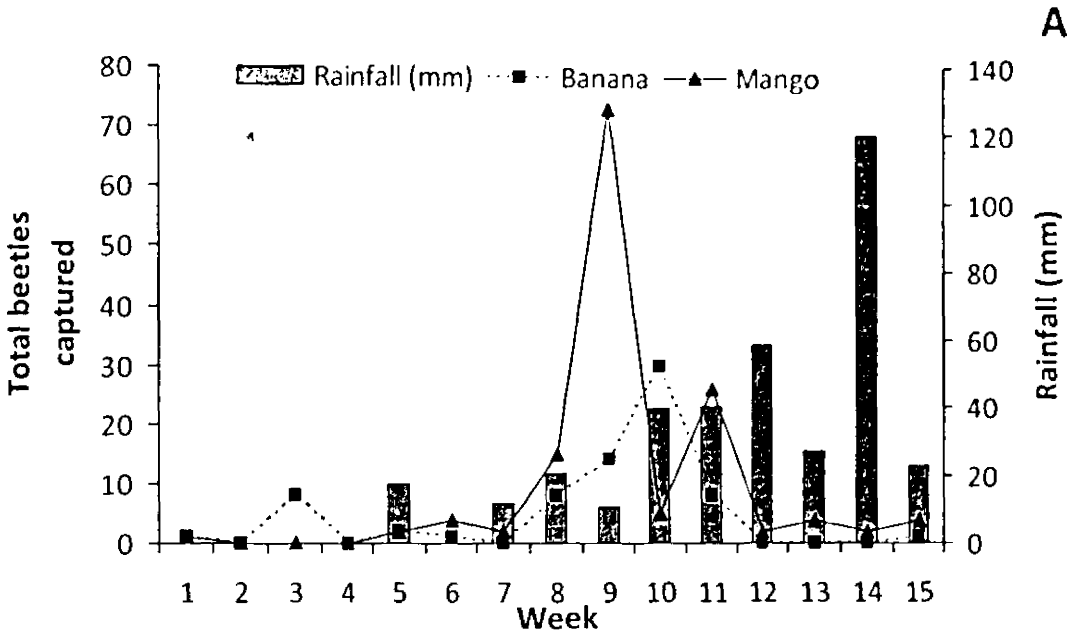



**Figure 7.6A** Total beetle capture/month throughout the 15 weeks trapping period

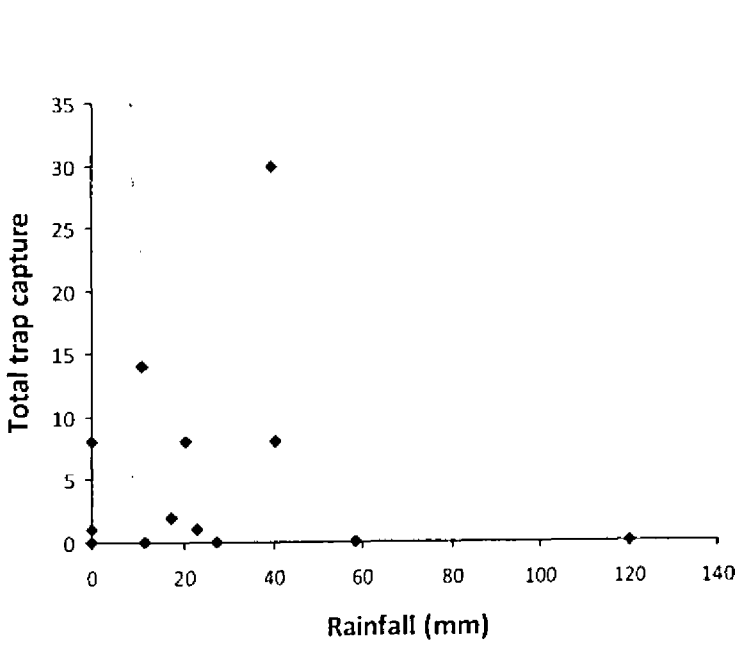



**Figure 7.6B** Scatter plot showing relationship between banana-baited trap captures and rainfall at the Gede ruins museum site.

C

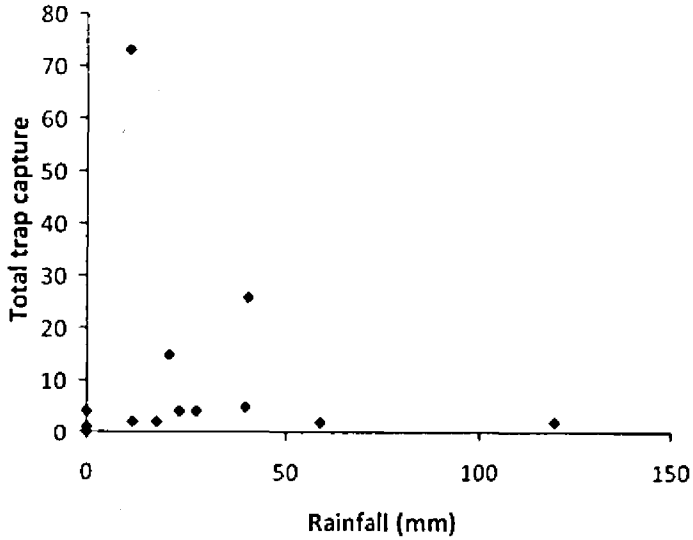


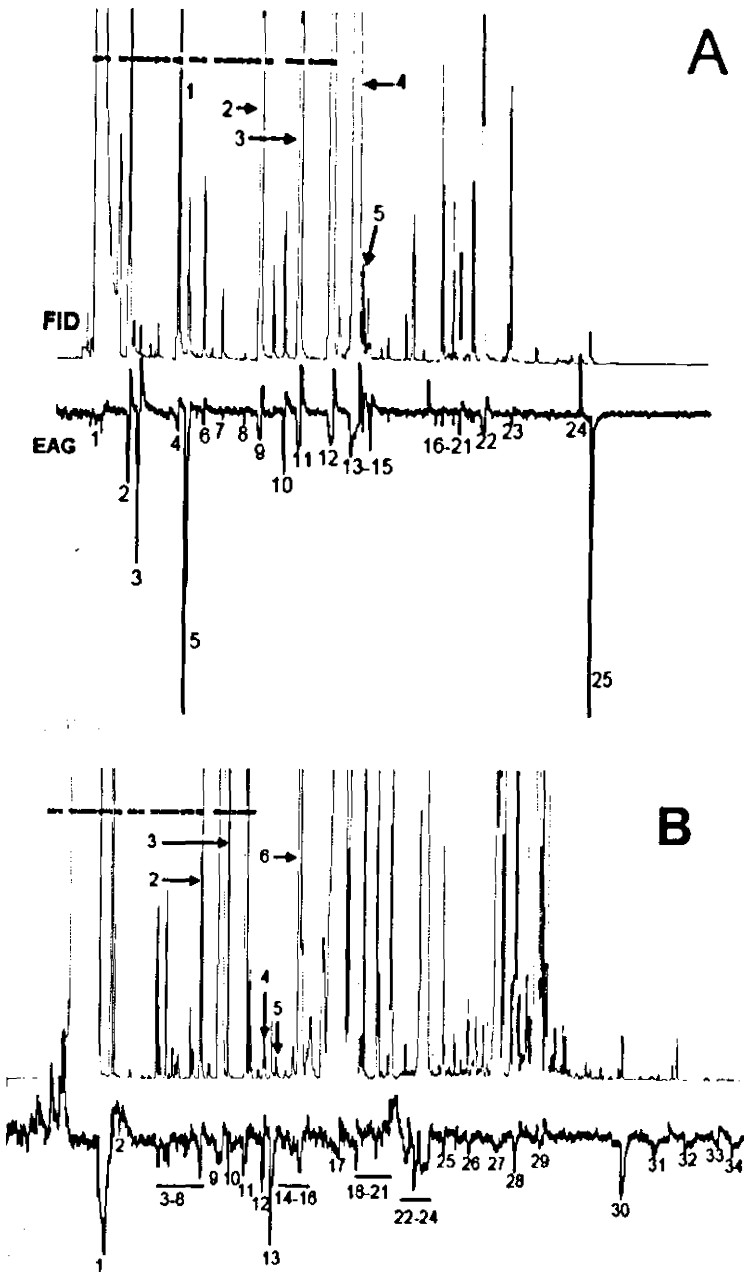

**Figure 7.6C** Scatter plot showing relationship between mango-baited trap captures and rainfall at the Gede ruins museum site.

### 7.4.3 Analysis of volatiles

Coupled gas chromatography-electroantennographic detection analyses gave qualitatively different volatile profiles for the ripe mangoes and banana. The mango volatiles possessed more EAG-active components compared to that of the banana (Figure 7.7). Male and female beetles consistently detected 14 and 25 components in the banana and mango volatiles, respectively. The maximum number of EAG-active components detected for banana and mango volatiles were 25 and 37, respectively.

Identification of several of the EAG-active components by GC-MS analysis revealed that they were mainly esters. A number of these compounds have been reported as components of worker honey bee odours (Torto *et al.*, 2005). These compounds included 2-pentanone, isobutyl acetate, butyl acetate, 2-heptanone and beta-pinene shown in Figures 7.8 and 7.9. A

comparison of the relative amounts of these compounds in both banana and mango volatiles is summarised in Figure 7.8




**igure 7.7** Representative chromatographs showing EAG-active components in banana (A) nd mango (B) odours Peaks labelled on the FID trace represent volatiles components known occur in honey bees while those on the EAG trace represent the maximum number of ological active components. Peaks 1, 2, 3, 4, 5 and 6 represent 2-pentanone, isobutyl etate, butyl acetate, 2-heptanone and beta-pinene, respectively.

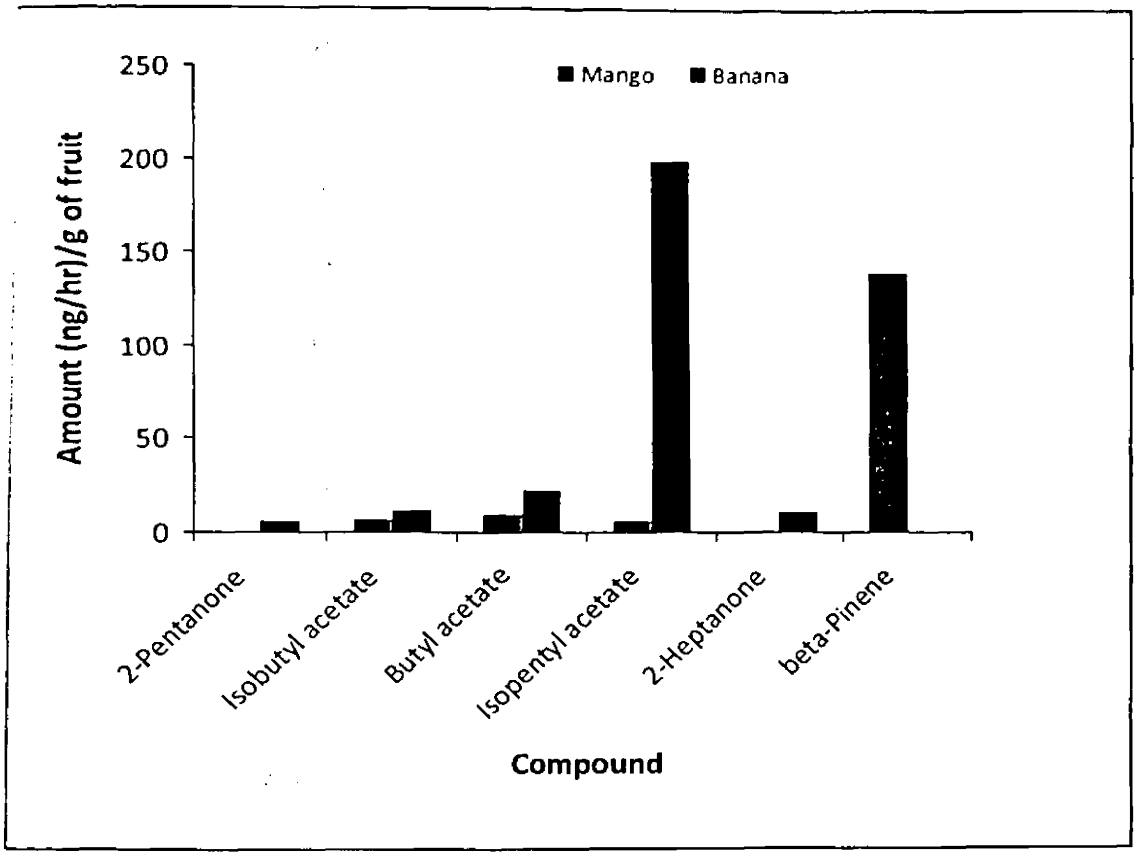



**Figure 7.8** Relative amounts of honey bee components found in both mango and banana volatiles.

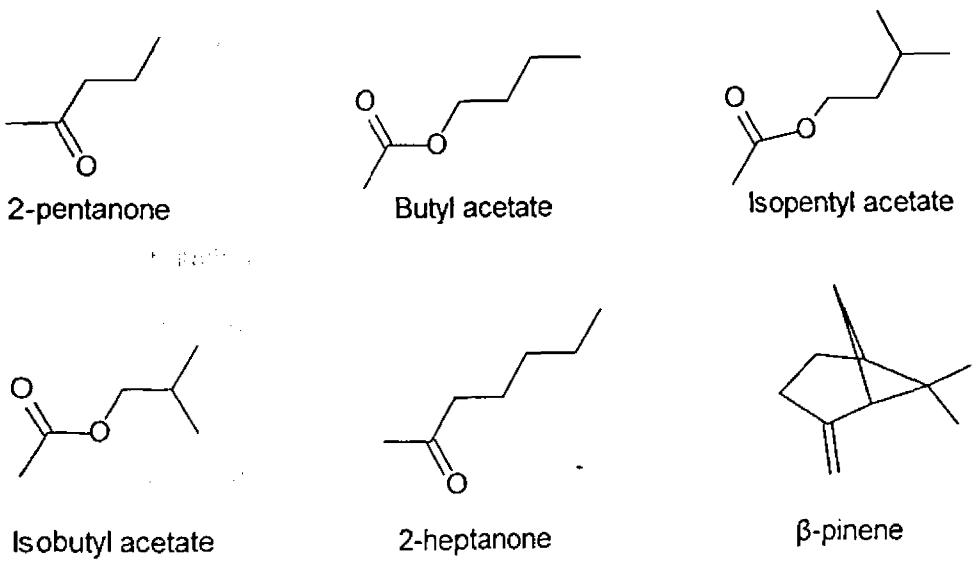



**Figure 7.9** Chemical structures of identified honey bee odour constituents mimicked by both fruits

## 7.5 Discussion

Behavioural assays clearly demonstrated the attraction of small hive beetles to volatiles from ripe fruit bananas and mangos. However, females showed less attraction to fruit odours compared to males. This observation may have been due to their unmated status as mated females are known to show strong attraction to honey bee and fruit odours (Suazo *et al.*, 2003, Torto *et al.*, 2005, Duehl *et al.*, unpublished) and oviposit almost anywhere once disturbed as a survival mechanism (Torto, pers. comm.). These results corroborate earlier studies by Buchholz *et al,* (2007) and Arbogast *et al.* (2009b) which demonstrated the ability of these beetles to reproduce on fruits as alternate host under laboratory conditions.

The field trapping experiments at both sites demonstrated the attractiveness of banana and mango fruit types to *A. tumida* adults. The capture of more beetles by banana-baited traps at Gede compared to Nairobi accords credence to previous findings which showed that more beetles occurred in Gede than in Nairobi (Torto *et al.*, 2010a). Mango-baited traps caught twice the number of beetles caught in the banana-baited traps, suggesting that the former is more attractive than the later, and which confirms the GC-EAD and GC-MS results. These findings imply that in the formulation of a lure for this beetle, the inclusion of biologically active compounds which are not produced by honey bees may greatly improve the performance of such a tool. These results show that fruit-baited traps can serve as a simple, effective and affordable way of detecting the presence of *A. tumida* presence in an apiary.

The results of coupled GC-EAD and GC-MS analyses showed that the volatiles of these fruits contained five antennally-active components identical to some of the constituent compounds of honey bee odours. However, these compounds represent only 18.5% of these fruit volatiles compared to the 27 antennally-active components identified in honey bee volatiles by Torto *et*

*al.* (2005). Although banana volatiles contained more of these five honey bee volatile components compared to mango, the latter had 1.7 times more EAG-active components than the former. Behavioural studies with blends of these compounds would provide more information on the importance of these compounds in beetle attraction.

Trap captures for mango-baited traps decreased with increasing distance from the apiary in Nairobi and Gede. Although these finding agree with that of Arbogast *et al.*, (2009a) the banana-baited traps performed differently at the coast for unclear reasons. Apart from adult beetles, larvae were also recovered in the traps at Gede and this is a clear proof of beetle reproduction on these fruits in nature. This demonstrates the potential of these fruits to serve as alternate host for *A. tumida.* The large number of both adult and larva stage in mango-baited traps may also be attributed to its high water content (82 % of total fruit weight) compared to 76% of banana (NEVO, 2011). *Aethina tumida* is a sap beetle and the large larva numbers from traps with mango are indication of a greater amount of sap oozing-out of this fruit in the traps.

The relationships observed between trap captures and rainfall at the various sites is interesting. Normally, one would expect more beetle captures with rainfall as reported by previous studies (Torto *et al.*, 2007b, 2010b). These observed relationships need to be interpreted with caution. Firstly, the pollen dough substrate used in previous studies relationships was of a particular consistency and inoculated the yeast *Kodamaea ohmeri* (Arbogast *et al.*, 2007; Torto *et al.*, 2007b, 2010b; Benda *et al.*, 2008), a symbiont of the beetle which may have prevented the colonisation of the same substrate by other microorganisms. In this study, cut slices of fruits were used as bait and not inoculated with yeast but left to ferment under natural conditions. It is likely that the trends observed in this

study were affected by the varying climatic conditions, agents of fermentation available in the natural setting and the consistency of fruit pulps. The decrease in trap catches during wetter than drier periods may have been due to rapid fermentation of fruits resulting in the production of odour bouquets unattractive to beetles. However, more studies are needed in this regard to elucidate how environmental factors can affect fruit bait performance.

Overall, this study has demonstrated the host potential of banana and mango for *A. tumida*, and their possible use as affordable baits for detecting the presence of this beetle.

# CHAPTER EIGHT

## General Discussion, Conclusions and Recommendations

### 8.1 General Discussion

This study was carried out with overall aim of documenting beetle pests of honey bees in Kenya and understanding several basic aspects of their biology which could provide information on how best their infestations and damage can be managed. In this regard, the nationwide occurrence of hive beetles, life history of *Oplostomus haroldi* and its inter-specific and intra-specific interaction with its conspecifics and host respectively, and potential alternate hosts of *Aethina tumida* were studied.

Country-wide suveys covering the major beekeeping and honey production areas of the country revealed the presence of three beetle species as pests of honey bee hives; *A. tumida* (Coleoptera: Nitidulidae), *O. haroldi* and *Oplostomus* sp. (Coleoptera: Scarabaeidae). The closeness of *O. haroldi* to *O.* sp. was confirmed by molecular analysis of their cytochrome oxidase subunit I (mtCOI) gene following their identification by expert taxonomists. Similar molecular analyses have been carried out for *A. tumida* and demonstrated mtCOI gene as a useful tool for tracking its global spread (Evans *et al.*, 2003; Lounsberry *et al.*, 2010). The occurrence of *Aethina tumida* was pan-national while the scarabs were found only in the eastern and coastal parts of the country. All three beetles showed an uneven distribution on the different sections of the hive, a pattern probably resulting from their manner of interaction with honey bees.

Only the adult stage of the scarabs caused damage by feeding on bee brood, pollen and honey with a greater preference for brood while both adult and larva stages of *A. tumida* caused

similar damage inside the hive (Lundie, 1940). The damage pattern of these two *Oplostomus* species tied with what had been reported for another species of the same genus *Oplostomus fuligineus* (Donaldson, 1989).

With *O. haroldi* and *A. tumida* recorded as the most abundant beetle pests of honey bees at various beekeeping areas nationwide (Torto *et al.*, 2010a), a profound understanding of their biology became imperative. With the basic life history of *A. tumida* already documented by Lundie (1940), this study focused on that of *O. haroldi*, for which information was non-existent. Laboratory studies clearly revealed that *O. haroldi* was a multivoltine species, results which further support its occurrence pattern reported by Torto *et al.* (2010a). However, several efforts to locate the breeding sites of this beetle for their possible control were not successful, prompting the search for alternate environmentally benign management options. This led to the examination of the interspecific and intraspecific interaction between this beetle and its honey bee host.

Behavioural assays, coupled GC-EAD and GC-MS analyses showed that *O. haroldi* utilises a female contact sex pheromone for mate recognition while honey bee odours provide the cue for host location by both sexes. These findings imply that honey bee odours serve as kairomones for this beetle and a blend of the bio-active components of bee odours can potentially be used as a lure to manage this beetle outside the honeybee colony. Similar studies carried out for the small hive beetle led to the formulation of a lure which was partially successful. Instead, a natural formulation comprising *Kodamaea*-inoculated pollen dough releasing a blend of these odours has been successfully used to monitor beetle populations. The observation of large number of small hive beetles adults but rarely larvae in

honey bee colonies strongly suggested the presence of alternate host for this beetle within its native host range.

Following this observation, behavioural assays, coupled GC-EAD and GC-MS analyses, and field trapping using apple banana and peach sabre mango varieties clearly showed that they can serve as alternate host for the small hive beetle.

### 8.2 Conclusions

The present study targeted several aspects of the ecology of beetle pests of honey bee colonies in Kenya with the overall aim of generating knowledge that can be used to develop effective management tools and strategies in the near future. These hive beetles are *A. tumida*, a nitidulid and two scarabs *O. haroldi* and *Oplostomus* sp. The ecological aspects investigated included the occurrence and geographical distribution in Kenya and inside the hive, their life histories under laboratory conditions and their mode of host and conspecific location (interspecific and intraspecific interactions). This study also investigated the potential of mango and banana as alternate hosts of *A. tumida* and their possible role in the survival of this beetle.

Nationwide surveys for the occurrence and infestation levels of beetle pests in honey bee colonies were carried out to understand their distribution and roles in honey bee colonies. The findings showed that:

1. All three beetle species (*A. tumida*, *O. haroldi* and *Oplostomus* sp.) occur in honey bee colonies in Kenya, with *A. tumida* having a cosmopolitan distribution whereas *O. haroldi* and *Oplostomus* sp. are restricted to the eastern and coastal parts of the country.

2. Beetle infestation levels in honey bee colonies vary spatially and seasonally strongly suggesting that climatic factors influence beetle development and distribution.
3. Inside the hive, *A. tumida* is found more on the bottom boarrd while *O. haroldi* and *Oplostomus* sp. occur mostly on the frames, a distribution pattern which may be attributed to their different sizes and their counter strategies to avoid defences of their host.
4. Molecular analysis of a section of the mitochondrial genome of *O. haroldi* and *Oplostomus* sp. confirmed that although they are very close taxonomically they are distinct species.
5. *Oplostomus haroldi* and *Oplostomus* sp. cause damage by feeding on bee brood, pollen and honey inside the honey bee hive, with a greater preference for bee brood.

The life cycle of *O. haroldi* was studied under laboratory conditions to obtain estimates of its duration. This knowledge is essential in developing models to predict outbreaks of this beetle pest and ensure better ways of managing it. The findings of this study support the conclusion that *O. haroldi* takes 3 - 5 months to develop from egg to adult, is moltivoltine and its abundance in managed honey bee colonies varies across seasons.

From the bioassay findings and GC-EAD analyses of this study, it can be concluded that:

1. Olfactory detection of honey bee odours serves as a long-range cue of host location by *O. haroldi*. Although similar studies were not carried out for *Oplostomus sp.*, it is most probable that it also depends on similar long range olfactory cues to detect its host.
2. Since both sexes of *O. haroldi* infest honey bee colonies and respond to their host odours, it is unlikely that they rely on air-borne pheromones for conspecific communication.

3. *Oplostomus haroldi* relies on a female contact pheromone to distinguish males from females and, engage females in a mating process. Sensory receptors present on the tip of the maxillary palps are central to perception of the female contact pheromone. The palp tips possess two receptor types, any of which may be responsible for sensing the contact pheromone components. Cuticular components of both sexes are composed of hydrocarbons with *(Z)*-9-pentacosene, the most dominant female component having a pheromonal role.

Similar bioassays, coupled GC-EAD, and GC-MS analyses and trapping experiments aimed at evaluating the potential of ripe mango and banana fruits as alternate host of the *A. tumida* resulted in the following findings:

1. Olfactory detection of odours from both mango and banana fruits was responsible for their attractiveness to *A. tumida*. Some of these components mimicked honey bee volatiles.
2. These fruits attract *A. tumida* adults and serve as a substrate for breeding, thereby demonstrating their potential as alternate hosts of this beetle.

In conclusion, the results of this study represent a significant contribution to the biology and chemical ecology of hive beetles. These results are considered a foundation towards the deeper understanding of the biology of hive beetles and the development of effective and efficient tools for their management.

### 8.3 Recommendations

Despite the results of this study, there are still knowledge gaps that need to be addressed. These include:

1. Determination of the seasonal abundance of *O. haroldi* and *Oplostomus* sp towards the development of models to predict the outbreak of these pests.
2. Elucidation of the breeding sites of of *O. haroldi* and *Oplostomus* sp. Such studies could unveil cues exploited by the female to assess and select suitable oviposition sites. Such cues can be exploited in future for the interception of gravid females and suppress the population of this beetle.
3. Evaluation of the effect of soil type, temperature, water content and dietary nutrients present in decaying plant materials ingested by larva stages and hive products consumed by adults on the growth, fertility, fecundity and development of these scarabs.
4. Identification of behaviourally-active compounds from honey bee volatiles for *Oplostomus* species
5. Determination of the full blend of the female contact pheromone, mechanisms used by females to recognise conspecifics and to prevent interbreeding with similar-looking-scarabs.
6. Documentation of the full range of fruits which can serve as alternate hosts to the small hive beetle, *A. tumida* and a screening of their volatile profile for new biologically active compounds towards the development of more effective lures.

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