DETERMINATION OF ATTRACTANT SEMIO-CHEMICALS OF THE WAX MOTH, *Galleria mellonella* L., IN HONEYBEE COLONIES

BY

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DECLARATION

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I **Charles Atieno Kwadha**, hereby declare that, this thesis is my original work and has not been submitted elsewhere for examination, award of a degree or publication. Where other people's work or my own work has been used, this has properly been acknowledged and referenced in accordance with the University of Nairobi's 'requirements.

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DEDICATION

To my mother Roselyne Anyango and grandfather Mr. Jackton Abonyo

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To the Almighty God, I return all glory and honour.

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ABBREVIATIONS AND ACRONYMS

AFB	American foulbrood
ANOVA	Analysis of variance
APBV	Acute paralysis bee virus
Bt V	*Bacillus thuringiensis* serotype V
DWV	Deformed wing virus
EFB	European foulbrood
FID	Flame ion detection
GC-EAD	Gas Chromatography-Electro-Antennograhic Detection
GC-MS	Gas Chromatography – Mass Spectrometry
GWM	Greater wax moth
HB	Honey bee
icipe	International Centre for Insect Physiology and Ecology
MAs	Modified atmospheres
MST	Male sterile technique
NIST	National Institute of Standards and Technology
ECB	European corn borer
RIFA	Red imported fire ant
SPME	Solid phase micro-extraction
USA	United States of America
UK	United Kingdom
SHB	Small hive beetle

Abstract

The role of honeybees remains crucial in global food security, ecosystem stability and poverty alleviation. However, recent reports indicate a global decline in population of both the feral and domesticated honeybees. The decline is attributed to interaction of myriad drivers including: climatic changes, intensive application of pesticides, habitat alteration, pathogen and diseases, and pest which often act in synergy. Pathogens and pests have been undoubtedly identified as key drivers. Amongst honeybee pests, the greater wax moth, *Galleria mellonella,* has been reported as the most devastating pest. The greater wax moth larvae feed on pollen, honey, wax and occasionally brood. Larvae tunnel through the comb structure, leave masses of web, which result in galleriasis, bald brood and absconding of colonies. Several management strategies including physical, biological and chemical are applied against greater wax moth invasion of honeybee colonies and honeybee product stores, but all have short comings that limit their application. There is scarce information regarding its chemical ecology, thus, determination of semio-chemicals involved in the wax moth behaviour is crucial. In the current study, laboratory raised colony of the wax moth was used to elucidate larva aggregation pheromones and adult female host kairomones. Dual choice assays using Y-tube olfactometer revealed significant attraction of immature instars to only food and frass volatiles, while mature instars were strongly attracted to conspecific cocoon-spinning larva odors only. Analyses and identification in a coupled gas chromatography-mass spectrometry (GC-MS) using NIST libraries revealed presence of three alcohols, alkane and aromatic hydrocarbons in frass and feces volatile extracts, while food extracts in addition to the three classes of compounds in frass, have a ketone, lactone, three monoterpenes and four sesquiterpenes. Furthermore, analysis of cocoon-spinning larva volatile extracts revealed presence of two aldehydes and two alkanes. Wind-tunnel bioassays showed that only mated females significantly respond to honeybee comb odors. Analysis of honeybee comb volatile extracts revealed presence of various classes of organic compounds. However, further analyses in GC with both flame ionization (FID) and electroantennographic detection (EAD) revealed that only 7 compounds viz ethyl propanoate; 2-methyl, ethyl propanoate; ethyl 2-methyl butanoate; 3-methyl butyl acetate, nonanal ,decanal and sylvestrene elicited antennal response in mated female. These results demonstrate that honeybee hive related semiochemicals play crucial role in chemical communication of *G. mellonella* both at larval and adult stages. Further, the results offer a benchmark in developing semio-chemically based management and control system for the pest.

CHAPTER ONE

1.0: GENERAL INTRODUCTION

1.1 Background information

Honeybees, *Apis mellifera* Linnaeus (Hymenoptera: Apidae) (HB), globally plays a central and an inherent role in food security, ecosystem stability and poverty alleviation (Raina *et al.*, 2009, Klein *et al.*, 2007). Moreover, their commercial value attributed to pollination services in forest, agricultural and horticultural crops, and their associated marketable product including honey, brood, propolis and wax enable their utilization as a source of insect-based enterprise (Raina *et al.*, 2009, Klein *et al.*, 2007). Despite the aforementioned essential services, studies show that there is a general global reduction in the HB productivity (Kluser *et al.*, 2010, Meixner, 2010). This is partially attributed to the general decline in HB population due to constant threats posed by several biological and environmental factors (Genersch, 2010, Kluser *et al.*, 2010, Meixner, 2010, Potts *et al.*, 2010). Important among these factors is threat posed by honeybee pests such as the smaller hive beetle, *Aethina tumida* Murray (Coleoptera: Nitidulidae), larger hive beetle, *Oplostomus haroldi* Witte (Coleoptera: Cetoniidae), the greater wax moth (GWM), *Galleria mellonella* Linnaeus (Lepidoptera: Pyralidae) and the invasive mite, *Varroa destructor* Anderson and Trueman (Parasitiformes: Varroidae). Among HB pests, *G. mellonella* has been reported as a key pest species causing significant economic losses (Shimanuki, 1980, Ritter & Akratanakul, 2006).

Galleria mellonella is a devastating pest of honeybee comb across the world (Nielsen & Brister, 1979). The GWM larva feeds on pollen, honey, wax and occasionally brood during which it destroys the comb structure and leaves masses of webs on the frames (Nielsen & Brister, 1979, Shimanuki, 1981, Türker *et al.*, 1993, Williams, 1997). Given its

economic importance, different mitigation strategies such as chemical, physical, biological and semio-chemically based trapping system has been exploited. Despite implementation of these strategies, the GWM pest problem still persist in the beehives (Flint & Merkle, 1983, Hood *et al.*, 2003, Dweck *et al.*, 2010).

1.2 Statement of the problem

Honeybees, *A. mellifera*, as one of the top 5% commercial insects, are significant to the human race both directly and indirectly (Raina *et al.*, 2011). However, productivity of HB is currently threatened by myriad of different interacting biotic and abiotic factors (Shimanuki, 1980, Meixner, 2010, Johnson, 2015). Important among biotic factors is the *G. mellonella*, which causes destruction in the beehive (Nielsen & Brister, 1979). The larva of *G. mellonella* feeds on cast-off honeybee pupal skins, pollen, honey and wax, create tunnels in the comb and leave masses of webs on the frame (Shimanuki, 1980, Williams, 1997, Türker *et al.*, 1993). This results in substantial losses, unsustainability of beekeeping and makes its management a top priority.

1.3 Justification of the study

Management strategies, both chemical and non-chemical techniques, initiated to limit losses associated with pest attack are constrained by a number of challenges due to the delicate nature of the hive environment (Hood *et al.*, 2003, Williams, 1997). Several studies designed to identify sustainable GWM management strategy are underway in different parts of the world (Svensson *et al.*, 2014, Dweck *et al.*, 2010). The recent discovery and incorporation of male pheromones in a pheromone baited trap is a result of such studies (Svensson *et al.*, 2014). Although pheromone baited trap is a promising strategy, it only targets female seeking-males limiting its effectiveness (Flint & Merkle,

1983). This study aimed to close this knowledge gap by identifying chemicals involved in honeybee – GWM interaction and exploring their utility in the pest's control.

1.4 Objectives

1.4.1 General objective

The main objective of the study was to identify and catalogue attractant semio-chemicals of *G. mellonella* in the honeybee colonies.

1.4 2 Specific objectives

a) To elucidate the larval aggregation pheromone of the greater wax moth

b) To identify honeybee colony odors attractive to gravid female wax moth

1.5 Research hypothesis

Volatile cues emanating from honeybee hive attracts the greater wax moth to honey bee colonies.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Background

Pollination services rendered by both feral and commercially managed honey bees, as recently classed in animal pollinator dependency classification system (Klein *et al.*, 2007), remain essential to global food production (Kremen *et al.*, 2007, Meixner, 2010) and ecosystem resilience (Gallai *et al.*, 2009, Bauer & Wing, 2010). Globally, approximately 70% of the principal crops consumed by humans cultivated in mono-and mixed cropping systems largely depend on honey bees for pollination (Klein *et al.*, 2007, Kremen *et al.*, 2007, Meixner, 2010). In 1998, pollination services by *A. mellifera* contributed $3.2 billion to South African and $14.6 billion to the U S economy in 2000 (Muli *et al.*, 2014) with similar services. It is estimated that in the absence of *A. mellifera*, there will be approximately 90% reduction in yield of some vegetables, nuts, fruits and edible oil crops (Klein *et al.*, 2007, Gallai *et al.*, 2009).

As a recognition of their economic potential, the art of beekeeping has been transformed into a tool used to combat poverty, unemployment for rural families, beekeepers and small scale farmers in sub-Saharan Africa, and to promote conservation of natural forests resources (Raina *et al.*, 2011). The reliance of agriculture on honey bees is under threat following the global decline of bee colonies which has resulted in a reduction in the abundance of both managed (National Research Council, 2007) and feral honey bee colonies (Moritz *et al.*, 2007). In effect, many countries in Europe have established rental hives with commercial farmers renting honeybees to supplement pollination services (Carreck & Williams, 1998, Bauer & Wing, 2010).

In the USA, beekeepers lost approximately 36% of their colonies over the winter period of 2007-2008 (Hayes Jr *et al.*, 2008). Previously, about 25% of domesticated colonies were lost between 1985 and 2005 in Europe (Potts *et al.*, 2010). In sub-Saharan Africa, where most economies are agro-based, the disappearance of honey bees is likely to pose a substantial threat to food security and conservation of biodiversity, with scanty data available on such losses. Therefore, if the production of bee pollinated crops that nourish humanity is to be sustained, the recent decline in honey bee population must be reversed (Carreck & Williams, 1998, Carreck *et al.*, 1997).

2.2 Economic value of *Apis mellifera*

Apis mellifera is the most domesticated mobile pollinator organism by man. It's global widespread is attributed to its direct and indirect integral role in the survival of human generations. These gains translate into food security, poverty alleviation, creation of employment and conservation of the ecosystem (Klein *et al.*, 2007, Raina *et al.*, 2011, Bauer & Wing, 2010, Meixner, 2010). Direct benefits linked with honey bee-based enterprises are honey, propolis, pollen, bee venom, royal jelly and bee wax (Krell, 1996). In addition, many local practitioners apply these products in what is termed as apitherapy (Münstedt & Bogdanov, 2009, Krell, 1996).

2.2.1 Honey

Honey is considered; a nutritious food rich in sugars such as fructose and glucose, carotenoids, organic acids, proteins and amino acids (Viuda-Martos *et al.*, 2008). It is consumed either in liquid, crystallized or in combs state by different societies as a food ingredient both at the small-scale and industrialized level in milk, baked, beverage and preserved products; an ingredient in brewing and related industries for the production of

wine, beer and vinegar (Krell, 1996). Even though antibacterial activity of honey remains understudied, it is applied in the treatment of wounds and burns, hepatitis A and in suppressing cardiovascular related diseases (Koç *et al.*, 2011).

2.2.2 Propolis

Propolis has been outlined as one of the hive product with pronounced antifungal activity (Koç *et al.*, 2011). Propolis is widely exploited for dermatological applications, treatment of cardiovascular and respiratory related infections, dentistry, and in suppressing human papilloma virus (HPV) infection (Krell, 1996). Propolis and bee venom are thus considered "drugs from the hive" owing to their pharmacological and biological activities (Koç *et al.*, 2011, Münstedt & Bogdanov, 2009).

2.2.3 Pollen

Pollen normally collected by honey bees during foraging, is valued as a desensitizer in treating allergic patients; dietary supplement for man, domestic animals, laboratory insects and newly established honey bee colonies and as agents of monitoring environmental pollution (Conti & Botrè, 2001). Commercially produced pollen is useful in breeding and pollination programme (Krell, 1996, Crane, 1999).

2.2.4 Bee Venom

Bee venom has been used extensively in some parts of the world, especially in Western Europe and American countries as a desensitizer on persons hypersensitive to honey bee venom and as a treatment for rheumatoid arthritis (Krell, 1996). Despite these observations, the role of bee venom in human medicine warrant further studies to isolate and characterize the active compounds with potential antifungal and antibacterial activities (Koç *et al.*, 2011, Münstedt & Bogdanov, 2009).

2.2.5 Royal jelly

Royal jelly is exclusively the food for the queen larva. It has been widely demonstrated to possess nutritional, pharmacological and biological activities that have attracted its application in pharmaceutical, food and cosmetic industries. Such include anti-inflammatory, anti-bacterial, anti-oxidant and anti-tumor characteristics (Krell, 1996). Biologically, its activities are attributed to its active proteins, fatty acids and phenolic components (Krell, 1996, Crane, 1999).

2.2.6 Bee wax

Natural bee wax has underlying characteristics that make it more lucrative than artificial wax. Local bee keepers use wax for constructing foundations sheets for new colonies, a raw material for candle making, metal casting and modeling. It's unparalled properties (builds stable emulsion, offer a protective layer against ultraviolet rays of sun creams, strengthens actions of detergents and provoke no allergy) has made it an irreplaceable ingredient in cosmetic industry (Münstedt & Bogdanov, 2009). Moreover, bee wax is utilized for production of shoe and furniture polishes; furniture vanishes; anticorrosion for lead accumulators and lubricants; finishes for leather, textile, wood and paper; preparations of waterproof walls and water resistant materials (Crane, 1999).

2.3 Ecosystem value

Ecosystem services are defined as the benefits to human welfare and wellbeing, derived from interactions of mobile organisms inhabiting the diverse ecosystem (Klein *et al.*, 2007, Kremen *et al.*, 2007, Bauer & Wing, 2010). Apiculture and agriculture are interlinked. The prosperity of beekeeping requires succession honey bee forage from both the uncropped and cropped areas, undoubtedly, sustainable production of bee pollinated

crops requires bee pollination (Carreck *et al.*, 1997). Honey bees are considered functional service provider based on the mounting evidence of their involvement in successful pollination of diverse crop species (Gallai *et al.*, 2009, Klein *et al.*, 2008, Kluser *et al.*, 2010, Hoehn *et al.*, 2008). They remain integral pollinators for mono and mixed-cropping systems, in effect, it influences ecosystem interactions, species specialization and adaptation, genetic variation at the floral community level and floral diversity. Pollination as an essential service by honey bees, therefore, directly contributes to ecosystem stability and management (Raina *et al.*, 2011), and indirectly to the well-being of herbivorous species of mammals, birds and their predators (Carreck & Williams, 1998), and ultimately biodiversity.

2.4 Challenges facing honey bees, *A. mellifera*

Interactions of myriad factors ranging from animate to inanimate such as landscape degradation, climate change, application of pesticides, pathogens (bacteria, viruses and fungi), diseases and pests such as kleptoparasites (parasitic mites and beetles) and moths, have been reported to adversely affect honey bees (Johnson, 2015, Oldroyd, 2007, Genersch, 2010, Shimanuki, 1980), with the latter two considered as the most destructive (Genersch, 2010).

2.4.1 Landscape degradation

Intense anthropogenic activities pose the greatest threat to the global ecosystems. Biodiversity is directly endangered by the on-going conversions within the agricultural landscapes (Kluser *et al.*, 2010). In addition, the disruption of the pollinator communities, cast into doubt, the diversity, productivity and sustainability of food output systems. And as Ricketts *et al.* (2008) reported, pollination failure owing to landscape alteration, greatly

impact tropical crops that are primarily pollinated by social bees. Moreover, the declining complexity of native landscape alters temporal and spatial distribution of resource patches, thus, affecting survival and interaction of bees both at individual and community level (Kluser *et al.*, 2010, Brosi *et al.*, 2008, Ricketts *et al.*, 2008, Potts *et al.*, 2010).

2.4.2 Climate change

The changing climatic conditions alter community composition by drifting the spatial range and phenology of plant and pollinator taxons. These changes negatively impact physiology and behaviour of honey bee, flowering, pollen production and availability, pathogen loads, foraging activities and lessen or enhance colony productivity and development (Le Conte & Navajas, 2008). Even though *A. mellifera* has shown plasticity in terms of being the most widely distributed honey bee species, climatic changes might exert unbearable adaptive pressure. Honey bee pathogens and pests such as GWM are confined to the tropical regions, however, the changing climatic conditions has facilitated its spread into new regions (Kluser *et al.*, 2010).

2.4.3 Application of pesticides

Intensive application of pesticides such as coumaphos and fluvinate to manage honey bee pests and diseases, is one of the key factors contributing to excessive honey bee mortality (Kluser *et al.*, 2010, Oldroyd, 2007). In the mid-20th Century, California beekeepers lost approximately 11.5 % honey bee colonies to pesticide poisoning (Oldroyd, 2007, Kluser *et al.*, 2010, Sanchez-Bayo & Goka, 2014). In recent years, colony losses in France have been linked with nicotine-like insecticide Imidacloprid (Oldroyd, 2007). Contrary to direct poisoning, sub-lethal effects of pesticides and acaricides, can consequently affect bee's cell physiology and immunity after prolonged exposure.

Furthermore, a recent study revealed that systemic insecticidal compounds, including: neonicotinoids especially thiamethoxan, impidacloprid and clothianidin, and organophosphates (phosmet and chlorpyrifos) constitute the biggest risk to global apiculture industry (Sanchez-Bayo & Goka, 2014). And as Goulson *et al.* (2015) describes, "When appropriately used, pesticides provide a clear economic benefit, but bring the welfare of bees into direct conflict with industrial agriculture."

2.4.4 Pathogens

2.4.4.1 Bacteria

Bacterial pathogens cause diseases generally termed as brood diseases i.e. American Foulbrood (AFB) and European Foulbrood (EFB) diseases caused by *Paenibacillus larvae* (Bacillales: Paenibacillaceae) and *Melissococcus plutonius* (Lactobacillales: Enterococcaceae) respectively (Genersch, 2010, Bailey, 1983, Oldroyd, 2007). America Foulbrood is considered the most destructive affecting bee brood, especially by beekeepers in temperate and sub-tropical regions (Ritter & Akratanakul, 2006). While EFB is less virulent than AFB, the recently inexplicable economic losses accrued by beekeepers in UK and Switzerland give a contrasting opinion (Genersch, 2010).

2.4.4.2 Viruses

Viral diseases are caused by ribonucleic acid (RNA) viruses affecting both adult bees and brood. The most common honeybee viruses are deformed wing virus (DWV) and acute paralyses bee virus (APBV). Infestation of honey bee colony by DWV and APBV is associated with *Varroa destructor* infestations (Genersch, 2010, Ritter & Akratanakul, 2006).

2.4.4.3 Fungi

Two microsporidia fungal species of the genus *Nosema,* i.e. *Nosema apis* Fries (Dissociodihaplophasid: Nosematidae) and *Nosema ceranae* Fries (Dissociodihaplophasid: Nosematidae), are intracellular parasites which inflict damage to the epithelial cells of the honey bee midgut resulting into death (Fries, 2010). Even though, the two species seem to have a comparable cumulative mortality and virulence rate, inconsistent reports exist on their growth pattern, which is largely attributed to different temperatures and methods used by the researchers (Forsgren & Fries, 2010). The presence of the two *Nosema* sp has been demonstrated across all continents (Genersch, 2010, Fries, 2010, Forsgren & Fries, 2010).

2.4.5 Parasites

2.4.5.1 Parasitic bee mites

Parasitic mites are one of the key factors behind rapid decline in abundance, diversity and survival of honey bees. Three mites pronouncedly linked with the menace are *Varroa destructor, Acarapis awoodi* Rennie (Trombidiformes: Tarsonemidae) and *Tropilaelaps clareae* Delfinado and Baker (Acarina: Laelaeptidae) (Genersch, 2010, Ritter & Akratanakul, 2006). *Varroa destructor*, which is a vector of bee viruses, is considered the most threat to apiculture (Kluser *et al.*, 2010). The mite feed on haemolymph during larval and pupal development, as a result emerging adults have reduced flight and reproduction activities (Forsgren & Fries, 2010, Rosenkranz *et al.*, 2010). *Acarapis awoodi*, the causative agent of Isle of Wight disease, causes significant losses in North America and Europe (Ritter & Akratanakul, 2006). Just like *V. destructor*, *T. clareae* is a haemolymph-sucker causing havoc in tropical Asia.

2.4.5.2 Parasitic beetles

Occurrence of parasitic beetles especially the small hive beetle (SHB) (*Aethin*a *tumida* and *Oplostomus haroldi*) is a source of concern to beekeepers in sub-Saharan Africa. The beetles feed on bee brood, pollen and honey, in the process, they cause damage to the comb and hive resources (Ritter & Akratanakul, 2006, Fombong *et al.*, 2012).

2.4.6 Pests

Amongst the major pests is the GWM, *G. mellonella* , a pyralid whose larva is considered the most destructive pest of stored combs (Nielsen & Brister, 1979, Oldroyd, 2007).

2.4.6.1 The Biology of the Greater Wax Moth, *G. mellonella*

The GWM is a member of the Lepidopteran family Pyralidae. It is distributed ubiquitously throughout the world, in areas where bees are kept (Shimanuki, 1981, Williams, 1997, Svensson *et al.*, 2014). It causes severe damage in the tropics and sub-tropics (Shimanuki, 1980). Most studies on GWM have focused on its role as a model organism for *in vivo* toxicological, physiological and pathological work (Ellis *et al.*, 2013), with little attention for its chemical ecology, especially with regards to its importance to the apiculture industry. The GWM undergoes a complete metamorphosis; egg-larva-pupa-adult (Figure 2.1).

The female of GWM lay approximately 200-300 eggs in crevices or cracks and depressions, though a female can lay up to 1,800 eggs in her lifetime (Williams, 1997, Ellis *et al.*, 2013). Eggs of GWM are spherically shaped with cream to whitish colour (Williams, 1997, Ellis *et al.*, 2013, Smith, 1965). Normally, eggs are laid in clusters for protection against worker bees (Nielsen & Brister, 1979, Smith, 1965). The development of eggs and

ultimate hatching of larvae vary considerably depending on prevailing temperature, but it begins from 3-5 days of oviposition (Smith, 1965, Williams, 1997, Ellis *et al.*, 2013). Upon hatching, larva is approximately 1-3mm in length and whitish in appearance (Ellis *et al.*, 2013, Nielsen & Brister, 1979). The hatched Larvae spontaneously commence feeding and web spinning. Larvae feed on hive components preferably in the order of pollen, wax and honey contained in comb cells (Charles Kwadha, personal observation) as they burrow deep into the midrib of the comb (Nielsen & Brister, 1979, Williams, 1997). During development, GWM larvae undergo 6-8 moulting. Larval development takes between 18-20 days, which depends on food availability and temperature (Williams, 1997, Nielsen & Brister, 1979, Ritter & Akratanakul, 2006). The rapid larval growth rate consequently result in metabolic temperatures higher than that of the surrounding (Williams, 1997). During the last larva instar, mature larvae ceases feeding, spin a hard-webbed cocoon, enters the quiescent period and develop into pupae.

Pupae of the GWM are of the obtect type (Smith, 1965), ranges from 5-7mm in diameter and 12-20mm in length (Ellis *et al.*, 2013). Generally, a newly formed pupa is white to yellow in colour, but gradually transformed into light brown and later to dark brown towards the end of pupation (Ellis *et al.*, 2013). At pupal stage, the sexes exhibit distinct morphological features: In males, there is a pair of small rounded knobs representing the phallomeres on the ventral side of the ninth (9^{th}) abdominal segment, in contrast females lack the phallomeres, but instead, their eighth (8^{th}) abdominal scelerite, have a cloven sternum representing an aperture for the bursa copulatrix (Smith, 1965). The pupal development stage takes between 5-65 days, but just like other GWM life stages, it considerably vary with prevailing conditions (Ellis *et al.*, 2013, Williams, 1997).

The adults of GWM have atrophied mouthparts (Williams, 1997), and as a result, they do not feed. Females are approximately 20mm in length, slightly larger than males, darker in colour, and have a straight apical margins in forewings, unlike their conspecific males that have scalloped wing margins (Williams, 1997, Ellis *et al.*, 2013). The size and color of both sexes vary depending on the larval diet. Dark gray to black adults emerge from larvae fed on honey bee comb, in contrast, those raised on artificial diet become gray to silver-white in colour (Williams, 1997). Adult moths emerge in the hive and fly out regardless of the presence or absence of bees. Female moths always fly back to the hive during the scotophase period after mating. Oviposition takes 2-13 days from the day of emergence. Males have never been observed flying back to the hives (Nielsen & Brister, 1977). The greater wax moth females have considerably lower lifespan than males.

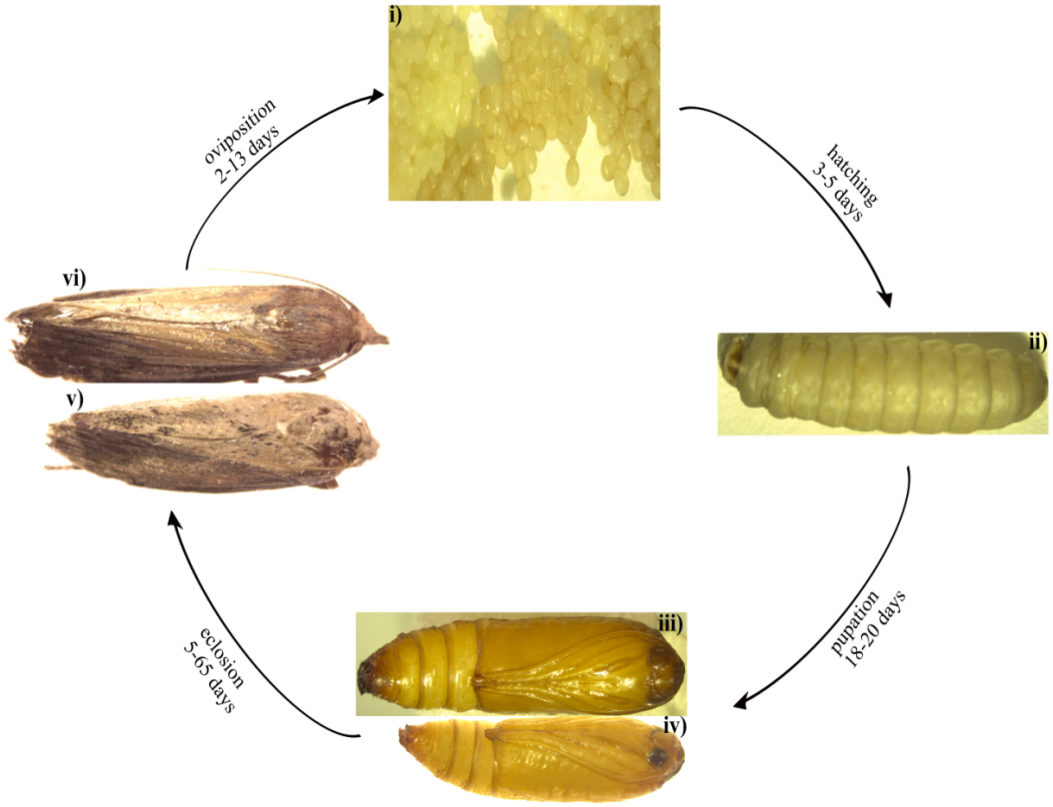

Figure 2.1 Life cycle of the greater wax moth, *Galleria mellonella*. i) eggs, ii) larva, pupae: iii) female and iv) male, adults: v) male and vi) female. Source of literature Williams, 1997; Pictures by Charles Kwadha. Magnification x5625.

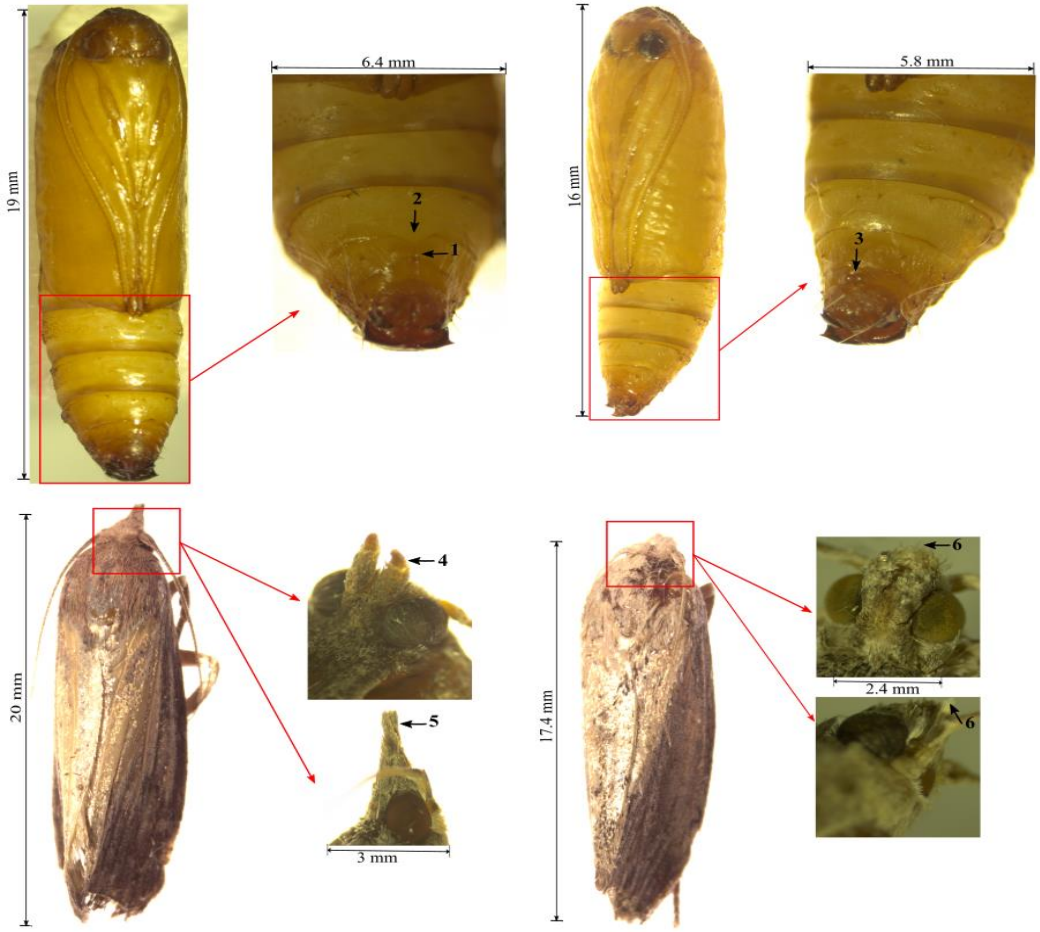

Figure 2.2 Key diagnostic features for male and female of greater wax moth at pupal and adult stages. a) female pupa; 1) and 2), cloven sterna forming copulatrix's aperture, b) male pupa, 3) a pair of small rounded knobs representing the phallomeres, c) wax moth female adult , 4) bifurcated proboscis, 5) labial palps projecting forward (beak-like appearance), D) wax moth male adult, 6) curved and inwardly hooked labial palps (snub-nose appearance). Source (Kwadha *et al.*, 2017).

2.4.6.2 Economic Importance of the greater wax moth

The GWM is considered the most important pest of honey bee products owing to the destructive feeding habit of its larva (Williams, 1997). The larva feeds on pollen, honey, wax, cast-off honey bee pupal skins, and rarely brood, create tunnels in the comb and leave masses of webs on the frame (Türker *et al.*, 1993, Williams, 1997, Nielsen & Brister, 1979, Shimanuki, 1980). Damage occurs as the larvae create silk-lined tunnel through the hexagonal cell walls and over the comb surface. The tunnels and borings created by the larvae on the wax caps makes holes through which honey leak, rendering the sections unmarketable (Shimanuki, 1981). The silken threads entangles emerging bees, which as a result die of starvation, a phenomenon described as galleriasis (Williams, 1997). Moreover, infestation of apiaries by larvae of the greater wax moth, often lead to colony loss, absconding and reduction in size of the migratory bee swarms (Williams, 1997). Even though adult wax moth does not cause any direct damage, they are potential vectors of honeybee pathogens (Charriere & Imdorf, 1999).

Honeybee colonies weakened by pesticide contamination, diseases (Romel *et al.*, 1992), starvation or with low adult populations (Shimanuki, 1980) are most vulnerable to infestation. The damage is significant in warm climatic regions (Shimanuki, 1980). Though no attempt has been made to assess the economic impact of GWM at the global scale, losses attributed to *G. mellonella* infestation in southern United States was approximately to be $3 million and $4 million in 1973 and 1976 respectively (Hood *et al.*, 2003). In Florida and Texas, states with tropical similar climatic conditions, an estimation of $5 and $1.5 losses were experienced per colony in 1997 (Hood *et al.*, 2003). The accumulated economic loss of wax ascribed to *G. mellonella* infestation in Iran, was approximately 38% (Jafari *et al.*,

2010). The destructive nature of the pest is attributed to its high reproductive potential and rapid developmental time (Türker *et al.*, 1993, Warren & Huddleston, 1962, Shimanuki, 1980).

Generally, honey bees have developed hygienic and grooming behaviour against hive intruders, such include; social encapsulation (Ellis *et al.*, 2003, Neumann *et al.*, 2001), thermo-balling (Papachristoforou *et al.*, 2007), active aggression towards adult beetles, removal of the contents of nearby comb cells by workers as they seek out hidden intruders and removal of the immature stages of the intruders (Neumann & Elzen, 2004). In order to escape bee defenses, the GWM employs counter-attack mechanisms, and as such, adults are still able to emerge from pupae encapsulated with a patch of propolis and fly out of the hive whenever aggressively attacked by honeybees (Nielsen & Brister, 1977). Furthermore, during the scotophase when bees are less aggressive, gravid females gain entrance into poorly guarded colonies and lay masses of eggs in crevices and comb cells with loosely packed pollen (Nielsen & Brister, 1977).

2.5 Management of the greater wax moth

Given the economic importance of this insect, it has received considerable attention from the scientific community largely oriented at developing novel ways to manage it. Some of these options include chemical control (using fumigants), physical control, biological control (*Bacillus thuringiensis* and natural enemies), Insect Growth Regulators (IGR), and semiochemical-based control.

2.5.1 Chemical control

Chemical control of the pest using fumigants such as Paradichlorobenzene (PDB), Phlestoxin and Ethyl-bromide, is considered most effective as it is lethal to all its life stages (Shimanuki, 1981, Williams, 1997). Other commonly used fumigants include sulphur dioxide, phosphine and carbon disuphide. However, these fumigants are poisonous to all honey bee stages, sometimes contaminate hive products and render them inconsumable and unmarketable (Hood *et al.*, 2003, Jafari *et al.*, 2010). In addition, the insecticides have detrimental impact both on other beneficial insects and the ecosystem. And as a result, it has generated interest in developing alternative managements for *G. mellonella.*

2.5.2 Physical control

A number of physical methods developed to manage greater wax moth include heating, freezing and modified atmosphere (MAs). Heating sag wax combs, melt honey and reduces its quality (Shimanuki, 1981, Burges, 1978). Freezing and cooling methods are only applied in the absence of honey bee for wax/ empty comb storage, hence their adoption among commercial beekeepers remains questionable (Hood *et al.*, 2003). There are inconsistent results on the use of MAs in controlling GWM, however, there is a consensus that the strategy require use of complicated CO_2 supply vessels which are not easily available to most small scale apiculturists (Donahaye *et al.*, 2000).

2.5.3 Biological control

Biological agents that have been identified include *Bacillus thuringiensis* (H-serotype V) (*B.t*.V), *Bracon hebetor* (Say), *Trichogramma sp*, the red imported fire ant (RIFA) (*Solenopsis invicta*) and the use of male sterile technique (MST).

2.5.3.1 *Bacillus thuringiensis*-H serotype

A case study conducted for three years using foundation wax impregnated with 1% B.t.V, which induce death in the GWM larvae by lysing the midgut, recorded effective control of GWM in the hives during year 1 of study, but mortality trend of the pest reduces in subsequent years even at 2% B.t.V. As the honey bee comb matures in bee hives, impregnated B.t.V is diluted through addition of wax, propolis, cast-off skin and larval cocoons, hence its limited application by commercial bee keepers (Burges, 1977, Williams, 1997, Burges, 1978).

2.5.3.2 *Bracon hebetor*

Bracon hebetor Say (Hymenoptera: Braconidae), is a gregarious larval ectoparasitoid of many Lepidopteran pests of the family Pyralidae, including the greater wax moth. Dweck *et al*. (2010) showed that female *B. hebetor* utilize nonanal, decanal and undecanal, components of the male-produced sex pheromone of GWM, as chemical cues for locating potential oviposition sites of their host. However, no follow-up study on the use of these olfactory cues by the female *B. hebetor* has been carried out in the field. Therefore, more studies are needed to fill this knowledge-gap.

2.5.3.3 *Trichogramma* species

The greater wax moth have been successfully used as a factitious Lepidopteran host for mass rearing of the egg parasitoid *Trichogramma* species (*T. pretiosum, T evanescens* and *T. minutum*) (Boldt & Marston, 1974). Despite these empirical evidence, there is no documented scientific finding showing field application of the parasitoid as a biological control agent of the GWM (Hood *et al.*, 2003).

2.5.3.4 Red imported fire ants (RIFA)

The red imported fire ant feed on immature stages of GWM (Williams, 1997), but when evaluated as a biological control agent of the moth in stored super combs, it could only be effective in combination with promoted light and ventilation conditions (Hood *et al.*, 2003). In addition, RIFA is a predator of ground-nesting bees, a nursery pest which has infested an estimated 106 million ha of land in eastern states of the United States (Hood *et al.*, 2003). In North America, the significant decline in biodiversity of fauna has been linked with the invasion of the natural habitats by RIFA (Wojcik *et al.*, 2001). These facts strongly suggest that it will be inappropriate to use RIFA as the replacement for insecticides, and thus there is need for an effective and environmentally-sound mechanism.

2.5.3.5 Male sterile technology

Male sterile technology (MST) using irradiated pupae were initially developed to control Lepidopteran pests (Bloem *et al.*, 2005). But given the fragility of pupae and high cost of releasing sterile males, irradiated F1 eggs was applied in place of irradiated pupae. However, the emerging irradiated F1 larvae were more destructive raising questions about application of MST (Bloem *et al.*, 2005). Even though exposing male pupae to gamma-

radiation proved effective at 350 Gy (Jafari *et al.*, 2010), the above mentioned shortcomings has limited its application by commercial beekeepers.

2.5.3.6 Natural product-Propolis

Propolis occurs naturally as a honey bee hive component, made from plant's resinous substances collected by foragers, and honey bee salivary gland secretions (Krell, 1996, Garedew *et al.*, 2004). As a beehive component, propolis has only been used by honey bee as a multipurpose cement and varnish, but not, as a control tool against hive-intruders (Garedew *et al.*, 2004, Krell, 1996). Though biological activities of propolis such as bactericidal (against *Bacillus* larvae-causative agent of American Foul Brood) (Krell, 1996), varroacidal (against *Varroa destructor)* (Garedew *et al.*, 2002) and recently IGR have been proven to experimentally reduce infestation by the GWM (Garedew *et al.*, 2004), earlier findings by Johnson *et al.* (1994) suggested that the pest has ability to develop tolerance. Therefore, more studies are needed to ascertain its usefulness as a control tool against *G. mellonella*.

2.5.3.7 Semio-chemicals

Semiochemicals are chemical compounds that are released by living organisms into their environment which elicit either a behavioral or physiological response in insect second organism that perceives the signal. Semio-chemicals are broadly classified into two; pheromones, which mediate intraspecific interactions, and allelochemicals, which mediate interspecific interactions (El-Sayed, 2012). Pheromones are further classified into subcategories including sex pheromone, aggregation pheromone, trail pheromone and alarm pheromone. Allelochemicals are also subdivided into allomones, kairomone and synomones (El-Sayed, 2012). Semio-chemical based control techniques such as use of

pheromone and kairomone attractants offer pest specific and environmentally friendly options (Cook *et al.*, 2007, Witzgall *et al.*, 2008)

The GWM, unlike other lepidopteran species, exhibit a unique pair-forming behaviour characterize by male released acoustic sound and sex-pheromone which attract virgin females (Türker *et al.*, 1993). Some components of GWM sex pheromone have been identified as undecanal identified by Roller *et al.* (1968); nonanal (Leyrer & Monroe, 1973); decanal, hexanal, heptanal, undecanal and 6,10,14 trimethylpentacanol-2 (Lebedeva *et al.*, 2002) and 5,11-dimethylpentacosane (Svensson *et al.*, 2014). Trials with a mixture of nonanal and undecanal (ratio of 7:3) in baited traps intercepted only male-seeking virgin females (Flint & Merkle, 1983). Although a blend of 2/7/54 ng of undecanal/nonanal/5, 11-dimethylpentacosane corresponding to 0.1 male equivalent was more effective, the females preferred whole male-body extract (Svensson *et al.*, 2014). Therefore, some components of GWM sex pheromone are still unknown. These shortcomings necessitated the need to identify GWM host-kairomones and larva aggregation pheromones and to develop a more effective semio-chemical based attractant targeting both gravid females and larvae.

The hive environment is known to host a variety of resources attractive to various arthropods and exploited by them as food for adults and immatures, and as oviposition sites (Fombong *et al.*, 2012). Some of these arthropods such as the small hive beetle have been shown to exploit the unique smells associated with these resources to locate their hosts (Torto *et al.*, 2005, Suazo *et al.*, 2003). Despite the obvious role of colony odours as olfactory cues exploited by their arthropod associates, their involvement in the GWM host location has received minimal attention. It is a well-known fact that the GWM infest stored

honey combs with a preference for older ones (Nielsen & Brister, 1977) and their larvae aggregate inside the hive (Nielsen & Brister, 1979). These observations suggest the involvement of kairomones and pheromones in the GWM behaviour respectively. However, follow-up studies to identify the active components have not been pursued. This study aim to close this knowledge gap by identifying the active components of the above mentioned semio-chemicals and explore their utility as novel control options for within and without colony control of the GWM.

CHAPTER THREE

3.0: MATERIALS AND METHODS

3.1 Test insects

The greater wax moth colonies were raised on artificial diet. Different developmental stages were selected to help in understanding the role of chemical signals in their behavior. Honeybee colonies and products, provided odour sources

3.1.1 Honey bee colonies

Honeybee colonies maintained by the International Centre of Insect Physiology and Ecology (*Icipe*) in standard Langstroth hives in three apiaries within Karura forest, Nairobi, were used as the source of bee colony odor.

3.1.2 Wax moth rearing and maintenance

The greater wax moth larvae were manually collected by a pair of forceps from wax moth infested apiaries in Karura forest (36.8347°E and 1.23442°S), Nairobi. Field collected larvae were used to establish a laboratory colony at the insect rearing facility set at 26±2ºC and 28±3% temperature and relative humidity (RH) respectively at *icipe.* Larvae were maintained on an artificial diet made of Wax (45 g), honey (225g), maize flour (301g) and yeast-brewers (90g), a modified diet from that previously developed by Ramarao *et al.* (2012). The diet was prepared in three steps. In the first step, yeast and maize flour were mixed in a plastic bowl (the mixture hereafter referred to as mix 1). In step two, wax and honey were melted in a metallic pot on a hot plate maintained at 60-70 °C (hereafter referred to as mix 2). In the last step, mixes 1 and 2 were combined into a homogenous

paste to form the larval food. The colony was raised on this diet on a rectangular plastic bowls (8.6″ × 6.1″ × 3.2″) with perforated lids (Figure 3.1).

Figure 3.1 Wax moth rearing containers: larval container, side (A) and dorsal (B) view; adult container, side (C) and dorsal (D) view.

Sheets of paper towel (6 cm x 12 cm) pleated into a fan-like shape were placed on top of the diet to serve as pupation sites for mature larvae. Cocoons formed on the papers were removed and incubated into adults in similar rearing bowls under room temperature and R.H of 26 ± 2^0C and 28 ± 3% respectively. Pupae of 5-10 days old were separated into males and females by carefully opening up their cocoons and sexed based on the following morphological features; 1) males identified by a pair of external phallomeres on the ventral

side of the ninth abdominal, and 2) females identified by the bursa copulatrix aperture present on the eight abdominal sternum (Smith, 1965, Ellis *et al.*, 2013). Eclosed adults were further sexed morphologically using labial palps (Williams, 1997).

Five (5) couples were placed in rearing bowls provided with pleated sheets of paper towel (each was 1 cm x 1 cm x 6 cm) to serve as the oviposition substrates. The sheets of paper were inspected on a daily basis for egg clutches and any egg found was harvested by cutting the portion of the paper on which they lay. The cut portions were transferred into bowls containing rearing diet for nourishment. All the wax moth stages used in various behavioural experiments were conditioned by maintaining them in the experimental room at least three hours prior to each experiment. The colonies were maintained at 12L: 12D photoperiod and only insects showing no signs of physical injury (missing leg, antenna and body appendages) were used in the subsequent experiments.

3.2 Determination of the greater wax moth larva aggregation pheromone

Previously, wax moth larvae were observed aggregating under natural conditions (Nielsen & Brister, 1979). However, factors that induce the behaviour have never been elucidated. In the current study, larval aggregation was assessed under laboratory conditions. Based on the observations, it was hypothesized that the behaviour is induced and maintained partly by pheromone signals. To prove the hypothesis, behavioural and analytical chemistry techniques were employed.

3.2.1 Assessment of larval aggregation under laboratory conditions

To test the assumption that the difference between *G. mellonella* larvae that pupate in solitude and clusters is zero, ten (10) immature (3-5th instar) and mature (8th instar) larvae were placed at the center of sterile Petri-dishes (Pyrex® glass) (9 cm ID) and allowed to pupate. Larval instars were distinguished based on their color and size (Smith, 1965). The set-up was placed in a dark cupboard to mimic the hive environment. Observation was made after 24 and 48 h and the number of larvae were recorded as either solitary or aggregates. This step was replicated ten times using fresh immature and mature larvae.

3.2.2 Larval orientation towards different numbers of larval cocoons

Larval orientation to 1, 2 and 4 larval cocoon(s) was separately studied by sticking cocoon(s) at one end of a Pyrex® glass Petri-dish (9 cm ID) on a piece of masking tape. Folded piece(s) of masking tape stuck at the opposite end served as control. Ten (10) 8th instar larvae were released at the center of a Petri-dish and after 24 h, data was recorded as previously described (Section 3.2.1). The set-up was replicated eight times for each number of larval cocoon(s).

3.2.3 Assessment of the role of semio-chemicals in larva aggregation behaviour

The following experiment was undertaken as a follow-up to observations made in aggregation experiment (Section 3.2.1) to determine the response of immature (3-5th instar) and mature (8th instar) larvae to odour sources:

1) cocoon-spinning larvae versus clean air (hereafter referred to as control)

2) mature larvae versus control

3) newly spun cocoon case versus control

4) frass + feces versus control

5) food (honey comb with honey + pollen) versus control

All these experiments were conducted in a custom built Y-tube olfactometer (18.5 cm L; 12.0 cm ID × 9.4 cm L; 7.6 cm ID × 9.4 cm L; 7.6 cm ID) (Sigma Sci LLC, Gainesville, Florida, USA) (Figure 3.2), as previously described by (Hern & Dorn, 2004) but with modifications. In brief, humidified and charcoal filtered air from a compressed air tank was split into two streams using a four-arm air olfactometer air delivery system. The air stream delivered into either arm of the olfactometer flowed at a rate of 0.3 L/min. An odour holding glass jars were attached to each arm of the Y-tube olfactometer. A vacuum pump was attached to the base of the Y-tube olfactometer and extracted air at a rate of 0.6 L/m. The odour holding glass jars separately contained twenty (20) cocoon-spinning larvae, twenty (20) mature larvae, newly spun cocoon case (0.114 g), frass + feces (10.0g) and food (10.0g) which served as the odour sources in their respective set-up. The jars were fit with aeration ports and glass fritz barriers to push and pull air through them respectively. Similar clean empty glass jars served as controls.

Mature and immature larvae (Figure 3.3), were individually introduced into the arena at the base of the Y-tube olfactometer. Larvae that crossed the score-line (5cm from the introduction chamber) within 2 min were considered responders or otherwise, non-responders. The time spent by each respondent larva at each arm of the olfactometer was recorded during an observation period which lasted for 5 minutes. For each odour source, the experiment was replicated using 25 mature and immature larvae. Each individual was used only once. The position of test and control odour sources was reversed after every 3 replicates to avoid positional bias. The entire experiment was performed during the scotophase period from 18:30 – 00:00 h under red light illumination provided by a red

fluorescent tube (4″ and 36W) placed 1.5 m above the olfactometer and room conditions of 25±2 ^{0}C and 28±2 % relative humidity.

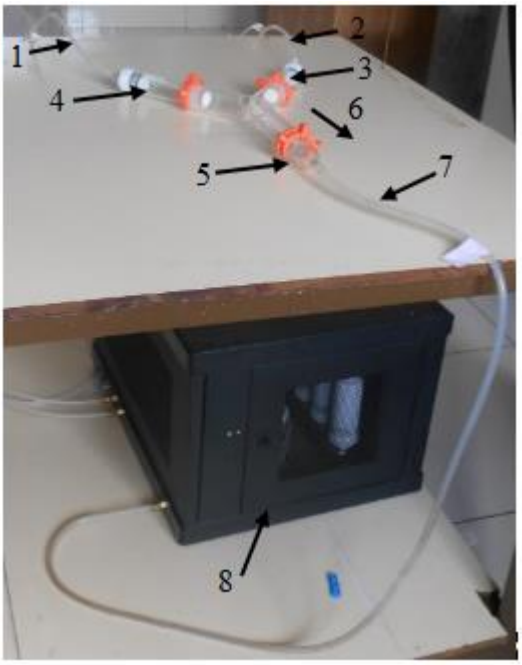

Figure 3.2 A diagrammatic over-view of the Y-tube set-up for larval assays: 1) and 2) inlet tubings, 3) treatment odour adapter, 4) empty odour adapter (control), 5) specimen adapter, 6) direction of air flow, 7) vacuum tubing and 8) air deliver system.

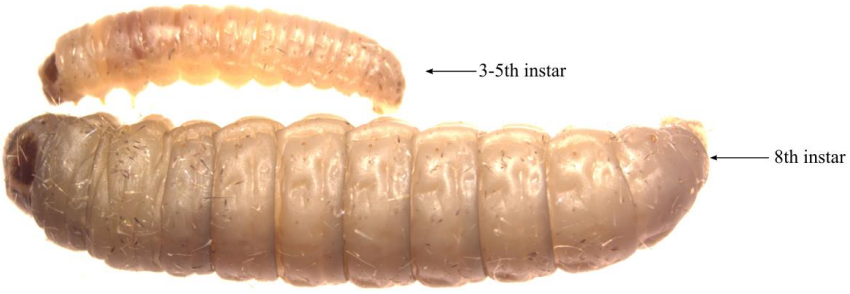

Figure 3.3 *Galleria mellonella* larval instars used as respondents in the Y-tube olfactometer assays: Magnification 5X.

3.2.4 Odour collection

Volatile odours from 1) cocoon spinning larvae, 2) food (honey bee comb with honey and pollen) and 3) frass + feces were separately trapped for 48 h by aeration and adsorption using a pre-cleaned (dichloromethane and hexane) Super Q, in a customized volatile trapping system. Two hundred cocoon spinning larvae were placed in a Pyrex quick-fit glass. An empty Pyrex quick-fit glass served as control. Similar quick-fit glasses were used for food (10.02 g) and Frass + Feces (10.02 g). The volatiles were trapped by pulling air at 50 mL/min over the odour sources in Pyrex-quick fit glasses through Super Q. All the Super Q traps were eluted using 300 µL hexane and stored at – 80 °C before analysis. Hexane was chosen as the extraction solvent based on the polarity of the target compounds (Torto *et al.*, 2013). Headspace volatile from 200 pupae were also collected but on a solid phase micro-extraction fibres (SPME) for 45 minutes. Prior to use, spindle micro-extraction (SPME) fibres were conditioned in a GC-injector at 280 °C for 30 minutes. The pre-conditioned SPME was subsequently wrapped with aluminium foil to prevent contamination of the fibre. To collect odours, the pre-conditioned SPME was then inserted in a volumetric flask (internal volume 100mL) containing 200 pupae, and covered with aluminium foil.

3.2.5 Assessing response of larvae to the extracted volatiles

Based on the Y-tube olfactometer assays, solvent extracts of odour sources (cocoon spinning larva, food and Frass + Feces) attractive to the larvae, were further evaluated at different doses. Volatile extracts were expressed as cocoon-spinning larval hour equivalent (CSLHE), honey bee comb hour equivalent (HBCHE) and frass feces hour equivalent (FFHE). Three (3) different doses (1µL 10 µL and 100 µL) of CSLHE, HBCHE and FFHE

were used in testing response of mature and immature larvae trapped volatiles. For each dose, equal volumes of treatment (extracts) and solvent control (hexane) were loaded onto Whatman No.1 filter paper (2 cm^2) and placed in the Y-tube olfactometer, near the orifice of each arm. Impregnated filter papers were allowed to air dry for approximately 2 min. A total of 25 replicates were used per dosage during which the treatment and control filter paper strips were replaced at an interval of 3 replicates to minimize variability of odor strength. In addition, the Y-tube arms were reversed at 180º after every 3 replicates to minimize positional bias. All tests were performed under similar conditions as previously highlighted (Section 3.2.3).

3.2.6 Analysis and identification of volatiles

To identify components in the Super Q extracts, analysis of the volatile extracts were conducted using an Agilent Technologies on an HP-7890 gas chromatograph equipped with an HP-1 capillary Column (30m × 0.25 mm I.D., 0.25 μm film thickness; Agilent), and coupled to HP 5975 mass spectrometer (EI, 70 eV, Agilent, Palo Alto, California, USA). Hydrogen was used as a carrier gas at a flow rate of 1.0 mL/min. The oven temperature was held at 35 ºC for 5 min, then programmed to increase at 10ºC /min to 280ºC and maintained at the temperature for 10 min. For each Super Q extract, an aliquot 50μL of the extract was analyzed in a splitless mode using helium as a carrier gas and its components separated based on the programmed temperatures. However, SPME fibres were analyzed immediately by injecting collected volatile into the GC-system, though with the same column and settings described in the preceding sentences. The compounds in the extracts were identified by comparing the mass spectra with those in the NIST library (Torto *et al.*, 2013). Components of the volatiles were identified using only GC-MS since

the larval antennae were so minute to be used for coupled gas chromatography-electroantennographic detection (GC-EAD). The structures of the identified compounds were drawn using a JavaScript toolkit for chemical graphics, interfaces and informatics (Burger, 2015).

3.3 Determination of the hive odours attractive to the greater wax moth adult

Previously, it was reported that only female moths would fly back to honeybee colonies. Moreover, the females were having spermatophore full of eggs, which might be an indication that they were gravid. It is on this basis that the current experiment was formulated to determine the role of host volatiles in attracting adult wax moth.

3.3.1 Assessing behavioural kairomone mediated host attraction (Wind-tunnel behavioural assays)

To ascertain the role of volatile cues from honey bee comb (with honey, pollen and brood) in mated female wax moths host-attraction, behavioural responses with modification of a previously described protocol (Torto *et al.*, 2005), were performed by a dual choice bioassay in Plexiglass wind-tunnel (30 cm×30 cm×121 cm) (Sigma Sci LLC, Gainesville, Florida, USA) (Figure 3.4). Both ends of the wind tunnel were impregnated with charcoal air filters which cleaned the air drawn through the tunnel by a centrifugal fan and an exhaust blower, both of which worked simultaneously. The activated charcoal-impregnated fibers also served as an air diffuser. These assays were conducted under red illumination provided by a 34-Watt fluorescent tube placed 2 m above the wind tunnel. All bioassays were carried out between 1830 – 0000 h, which coincide with the peak activity period of the moth. A honeybee frame (45 cm x 2 cm x 24 cm) containing honeybee comb with honey, pollen and brood, placed in an experimental cage (50 cm × 10 cm × 25 cm),

was connected to one inlet pipe of the wind tunnel (test odours) and a similar empty cage (control) connected to the second inlet pipe. The experimental cages were connected to the wind-tunnel using Teflon tubings.

The rearing cages containing the moths were placed in the behavioural laboratory, 3 h for acclimatization prior to tests. Twenty five (25) naïve, mated males and females of 1-3 days old were used during the test. Each moth was placed in a Petri-dish on top of the release cage. The release cage was placed at 105 cm away from the odour source and 15 cm above the wind-tunnel floor. Only moths that engaged in flight activities oriented towards odour source, and finally landed on either traps were recorded and considered for analysis. Each test moth was allowed 5 min to choose the preference odour source. An individual was used only once. The position of the test and control odour was switched after every 3 replicates to minimize error due to positional bias.

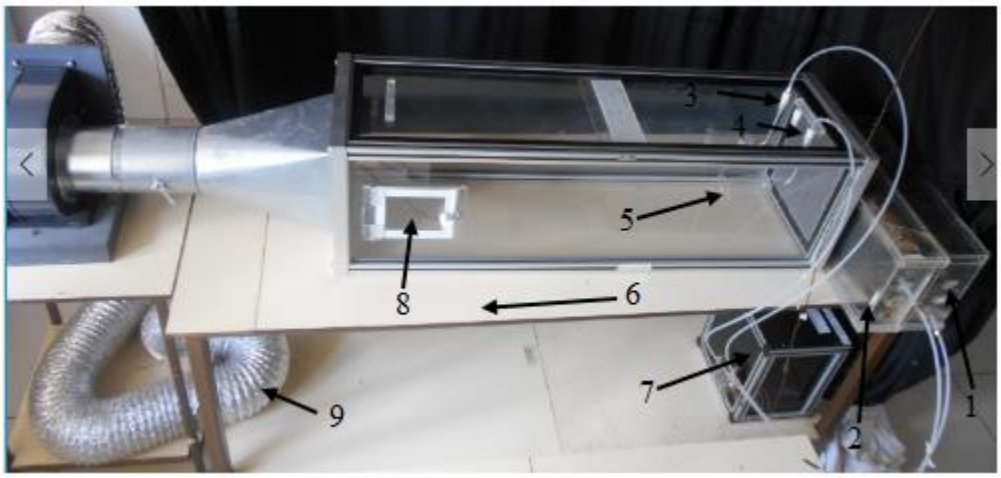

Figure 3.4 A diagrammatic presentation of the wind-tunnel set-up for adult wax moth assays: 1) empty (control) cage, 2) treatment cage containing honeybee comb, 3) inlet port connected to control cage, 4) inlet port connected to treatment cage, 5) traps, 6) direction of airflow, 7) air delivery system, 8) side door and 9) vacuum tubing.

3.3.2 Odour collection

Volatile odour from honeybee comb with honey, pollen and brood was collected by a dynamic flow system, similar to that described in section 3.2.4. In brief, a honey bee comb with honey, pollen and brood in a rectangular transparent experimental cage and a similar empty cage (control), was connected to the trapping system. Volatiles were collected by pulling humidified-charcoal-filtered air through pre-cleaned Super Q for 48 h. Entrained volatiles were eluted using 300µL of GC/GC-MS grade hexane. The eluents were stored at -80°C prior to bioassays and analysis.

3.3.3 Assessing response of adults to honey bee comb extracted volatiles

The Super Q entrained volatiles were used as the odour sources to determine the behavioral activity of the honey bee comb (with honey, pollen and brood) extracts on mated female moths. Doses were expressed as honey bee comb hour equivalent (HBCHE= volatiles released from the honey bee comb within 1h). Three different doses of the extract were used (6HBCHE, 12HBCHE and 24HBCHE). A dual choice wind-tunnel set-up was used as the bioassay arena. The odour stimuli were prepared by loading equal amounts of trapped honey bee comb volatile cues (treatment) and solvent control (hexane) onto Whatman No.1 filter paper (3 x 3 cm) and randomly assigned to each of experimental cages. Impregnated filter papers were allowed to air dry for approximately 2min. The treatment and control filter paper strips were replaced at interval of 3 replicates to minimize variability of odour strength. In order to eliminate positional bias, the experimental cages containing the treated filter paper strips were rotated after every 3 replicates. All tests were performed under similar conditions as elaborated in section 3.3.1.

3.3.4 Coupled Gas Chromatography-Electroantennography (GC-EAG)

The identification of physiologically active components in headspace extract of honey bee comb was carried out in GC-EAD using 1-3 days old mated female *G. mellonella*. The analysis was conducted on a Hewlett-Packard (HP) 5890 Series II GC equipped with an HP-1 column (30m × 0.25 mm I.D., 0.25 μm film thickness) (Agilent, Palo Alto, California, USA). Hydrogen was used as the carrier gas. Volatile extracts were analyzed in a splitless mode at an injector temperature of 280°C. The split valve was delayed for 5 min. The oven temperature was programmed from 35°C and held for 3 min, then at 10°C/min to 280°C, and held for 10 min. The effluent from the column was split 1:1, for simultaneous detection by flame ionization detector (FID) and electroantennographic detection (EAD). For the EAD detection, silver-coated wires in drawn-out glass capillaries (1.5 mm I.D) pulled to a fine end with an electrode puller and filled with Ringer saline solution (Torto, 2005) served as reference and recording microelectrodes. Mated females' antennae were prepared by first cutting the base of the head and distal end of antenna with a scalpel. The head was connected to the reference electrode, and the antennal tip was connected to the recording electrode mounted on a micromanipulator. Charcoal filtered and humidified stream of air was passed over the antennal preparation at 1mL/sec. The microelectrodes were connected through an antennal holder to an AC/DC amplifier in DC probe (Syntech, Hilversum, the Netherlands). Amplified EAD and FID signals were captured and analyzed simultaneously using a data acquisition controller (IDAC-4, Syntech, the Netherlands) and a GC-EAD program (EAD 2000, Syntech) on a PC. An aliquot (5 μl) of the honey bee comb volatile extract was analyzed using fresh antennae of mated female moths for repeated sample analysis. Linked

GC-EAG analyses of extracted honeybee comb volatiles were replicated three times using different female antennal preparations.

3.3.5 Analysis and identification of honeybee comb volatiles

Analyses of honeybee comb extracts trapped in Super Q were conducted using an Agilent Technologies on an HP-7890 GC coupled to HP 5975 mass spectrometer (EI, 70 eV, Agilent, Palo Alto, California, USA) equipped with an HP-1 capillary Column (30m × 0.25 mm I.D., 0.25 μm film thickness). The oven was set at similar conditions as previously described in section 3.2.6.

3.3.6 Data Analysis

Chi-square (χ^2) goodness-of-fit with Yate's correction for continuity was used to test the hypothesis that the difference between solitude and aggregating larvae is zero. Data on Y-tube response of larval stages were subjected to circular transformation and subsequently analysed using two sample t-tests (SigmaPlot version 11.0 and Coda Pack version 2.01.15). To test the hypothesis that volatiles emanating from honeybee comb do not play a role in gravid female attraction, percentages of moths captured in test and control traps were arcsine transformed ($\sqrt{p}$) in order to determine deviation from normality before analysis by (χ^2) goodness-of-fit with Yate's correction for continuity. All data were subjected to Shapiro-Wilk (W) test to confirm normality prior to transformations and analysis. Unless stated, statistical analyses were performed in R software Team (2015) at significance level of 5%.

CHAPTER FOUR

4.0 RESULTS

4.1 Determination of the greater wax moth larva aggregation pheromone

The data showed that the greater wax moth larvae exhibit aggregation behaviour. The behaviour is induced partly by an aggregation pheromone emanating from conspecific cocoon-spinning mature larvae. Mass spectrometric analysis revealed that the major components of the pheromone are nonanal, decanal, tridecane and tetradecane.

4.1.1 Evidence of larval aggregation in the laboratory

Larval aggregation bioassays carried out in the Petri-dish arena, showed that more larvae grouped in clusters of 2-5 and >5 (Figure 4.1 and Figure 4.2) individuals.

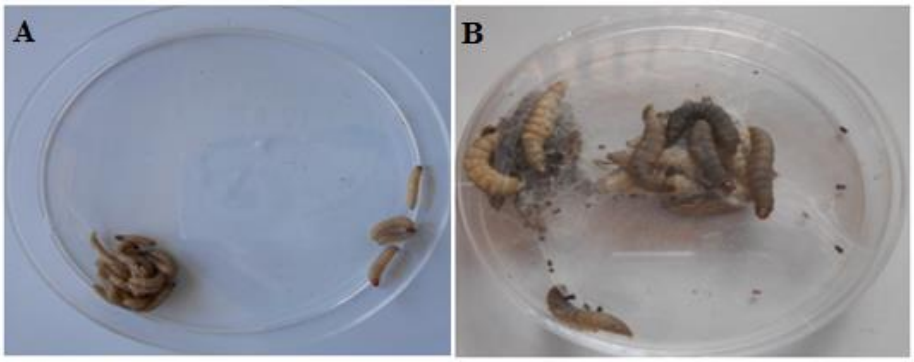

Figure 4.1 Larval aggregation behaviour in the Petri-dish arena: (A) 3-5th instars after 24 h and (B) 8th instars after 24 h

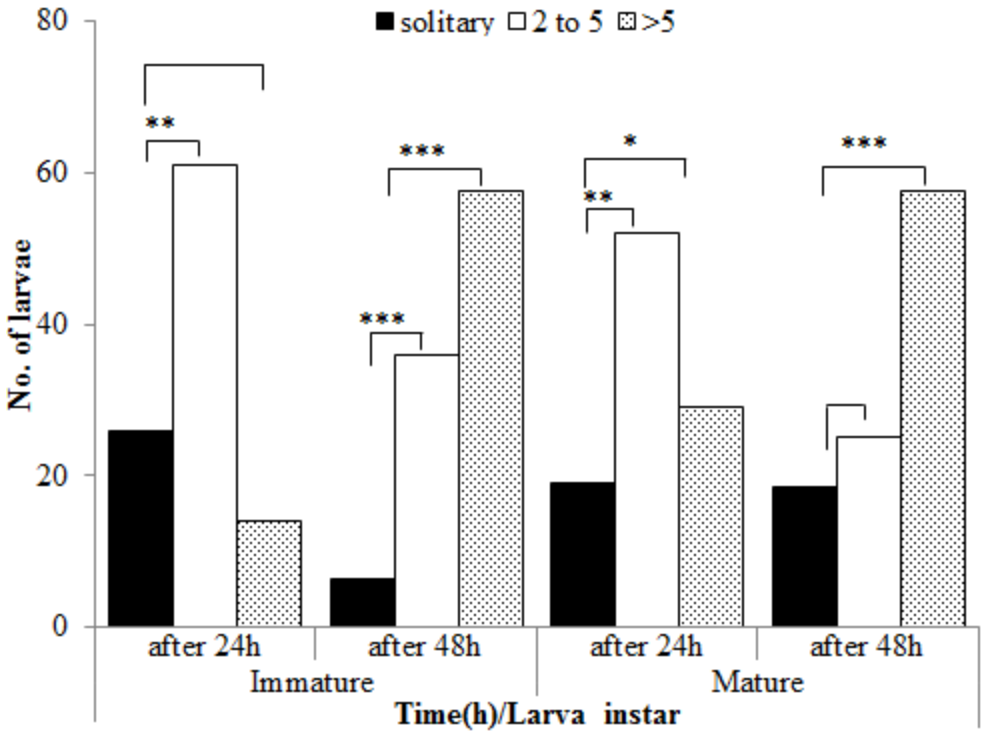

Figure 4.2 Aggregation behaviour of *G. mellonella* larva in the Petri-dish. An asterisk indicate significant difference by χ^2; α=0.05, n=100 (*p≤0.05, **p≤0.01 and *** p≤0.001).

4.1.2 Larval orientation towards different numbers of larval cocoons

A mild dose effect was observed as the number of clustering larvae increased with increasing number of larval cocoons (Figure 4.3). However, this increase was not linear as the 2-5 cluster size records reduced with increasing test odour concentration whereas a continual increase was observed for clusters with over 5 individuals.

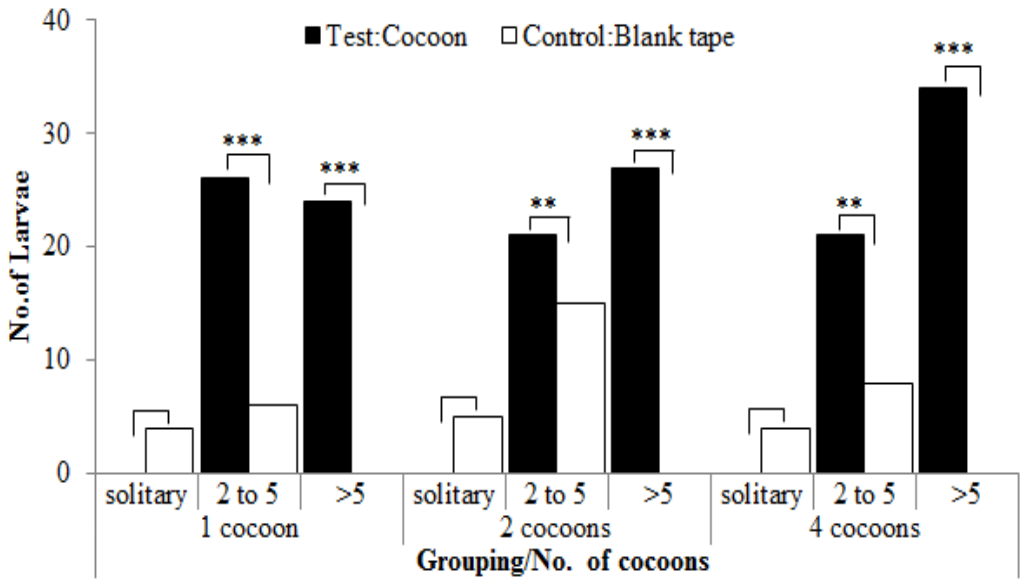

Figure 4.3 Aggregation behaviour of the mature larvae in the presence of cocoons. An asterisk indicate significant difference by χ^2; α=0.05, n=80 (*p<0.05, **p<0.01 and *** p<0.001).

4.1.3 Behavioural evidence for the role of semio-chemicals in larva aggregation

The Y-tube olfactometer assays (Table 4.1), confirmed the earlier results of mature larva attraction to cocoon-spinning larva (t_{48} = 2.27; P = 0.028) which was not observed in immature larvae (t_{48} = 1.9; P = 0.064). A similar response pattern was observed in both mature and immature larvae for mature larva odours (mature larva - t_{48} = 2.71; P = 0.009; immature larva - t_{48} = 0.851; P = 0.339). Contrary to attractive cocoon-spinning and mature larva odours, both larva stages did not show any preference for newly spun cocoon cases over their controls (mature larva - t_{48} = 0.567; P = 0.574; immature larva - t_{48} = 0.156; P = 0.876). Immature larvae were significantly more attracted to odours from frass and feces compared to the control (t_{48} = 2.84; P = 0.007) while mature larvae showed no preference

for either of these odours ($t_{48} = 1.47$; $P = 0.149$). A significant preference was displayed by immature larvae to food (honey bee comb with honey and pollen) odours over the control ($t_{48} = 3.22$; $P = 0.002$) while no significant preference was observed in mature larvae ($t_{48} = 0.356$; $P = 0.723$)

Table 4.1: Olfactometric response of larva to odour sources.

Respondent	Stimuli		t-values	p-values
	Odour source	Control		
8th instar larvae	Cocoon-spinning larva [1.826]	Clean air [0.138]	2.270	0.028*
	Mature larva [0.589]	Clean air [1.842]	2.710	0.009**
	Newly spun cocoon cases [0.765]	Clean air [1.180]	-0.567	0.574
	Frass + Feces [1.459]	Clean air [0.372]	1.470	0.149
	Newly spun cocoon cases [0.335]	Frass + Feces [-0.415]	1.080	0.285
	Honey bee comb (honey + pollen)[0.008]	Clean air [-0.225]	0.356	0.723
	Honey bee comb (honey + pollen) [0.854]	Frass + Feces [-1.239]	2.400	0.021*
3-5th instar larvae	Cocoon-spinning larva [0.126]	Clean air [1.204]	1.900	0.064
	Mature larva [-1.913]	Clean air [-2.089]	0.851	0.399
	Newly spun cocoon cases [-0.056]	Clean air [0.056]	-0.156	0.876
	Frass + Feces [2.256]	Clean air [-2.317]	2.840	0.007**
	Newly spun cocoon cases [-1.007]	Frass + Feces [1.087]	-3.540	0.001***
	Honey bee comb (honey + pollen) [1.142]	Clean air [-0.925]	3.220	0.002*
	Honey bee comb (honey + pollen) [0.827]	Frass + Feces [-0.442]	4.230	0.001***

Values in square brackets are mean time (after circular and log transformation) spent by larvae in the respective Y-tube arm.
An asterisk indicate significant difference by two sample t-test α=0.05, n=25 (*P<0.05, **P<0.01 and *** P<0.001).

4.1.4 Assessing response of larvae to the extracted volatiles

Mature larvae exhibited significant preference for cocoon-spinning larval extracts only at the intermediate hour equivalent (t_{48} =2.21; P = 0.032), with no bias in mature larva's attraction to either of the odours at 1CSLHE (t_{48} =0.0209; P = 0.983) and 100CSLHE (t_{48} =1.06; P = 0.296). All tested doses of cocoon-spinning larval extracts (1CSLHE, 10 CSLHE and 100 CSLHE) (Figure 4.4), did not elicit any significant attraction of immature larvae (t_{48} =0.947; P = 0.348, t_{48} =0.920; P = 0.362 and t_{48} =1.29; P = 0.205 respectively). Though not significant, mature larvae showed preference for control over frass and feces extracts at all doses tested (1FFHE - t_{48} =0.149; P = 0.883, 10FFHE - t_{48} =0.885; P = 0.381 and 100FFHE - t_{48} =0.106; P = 0.916). In contrast, frass and feces extracts attracted immature larvae at all the hour equivalents, but the attraction was significant only at 100FFHE (t_{48} =3.02; P = 0.004), unlike at 1FFHE and 10FFHE (t_{48} =1.87; P = 0.068 t_{48} =1.91; P = 0.062 respectively) (Figure 4.5). Response of immature larvae to food extracts (Figure 4.6), was significantly higher at 1HCHE (t_{48} =5.30; P = 0.001) than at 10HCHE (t_{48} =2.24; P = 0.03). However, a significantly higher preference for control was displayed at 100HCHE (t_{48} =2.22; P = 0.031). In mature larvae, attraction to food extracts was apparent only at the 10HCHE dose (t_{48} =2.88; P = 0.006), at the lowest level tested there was a significant preference for control over test (t_{48} =2.75; P = 0.008) while at the highest dose tested there was no preference displayed (t_{48} =0.849; P = 0.40).

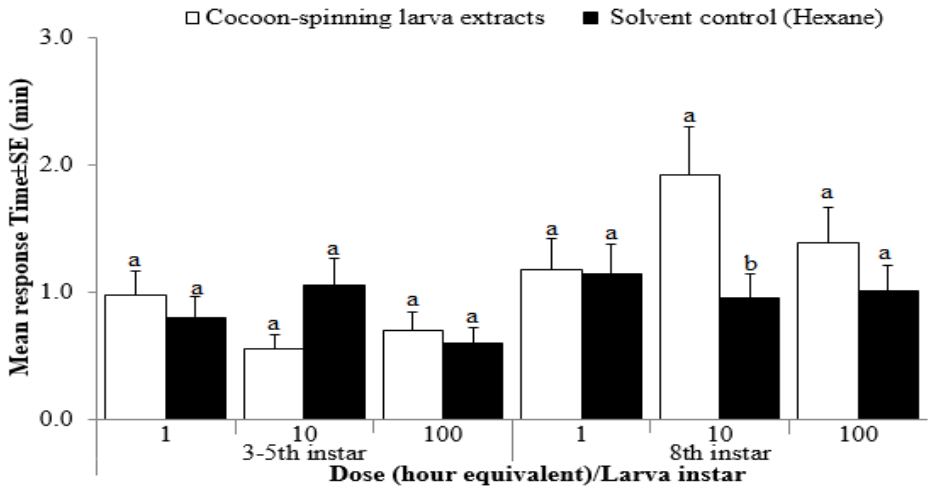

Figure 4.4: Behavioural response of *G. mellonella* larvae to cocoon-spinning larva extracts against control in the Y-tube.; two sample-t-test (α=0.05, n=25). Pairs of white and black bars with different letters represent statistically different behavioural responses at α=0.05. Error bars indicate standard errors.

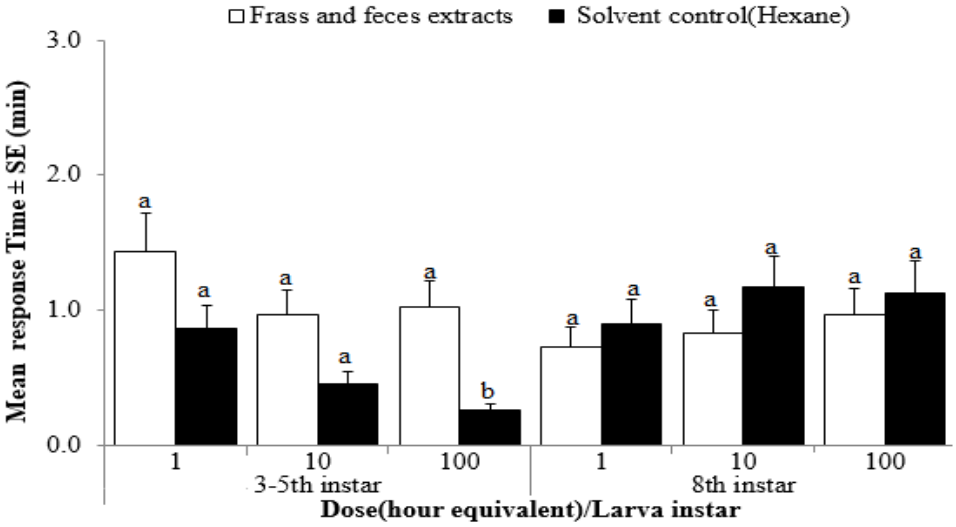

Figure 4.5: Behavioural response of *G. mellonella* larvae to frass + feces extract against solvent control in the Y-tube; two sample-t-test (α=0.05, n=25) Pairs of white and black bars with different letters represent statistically different behavioural responses at α=0.05. Error bars indicate standard errors.

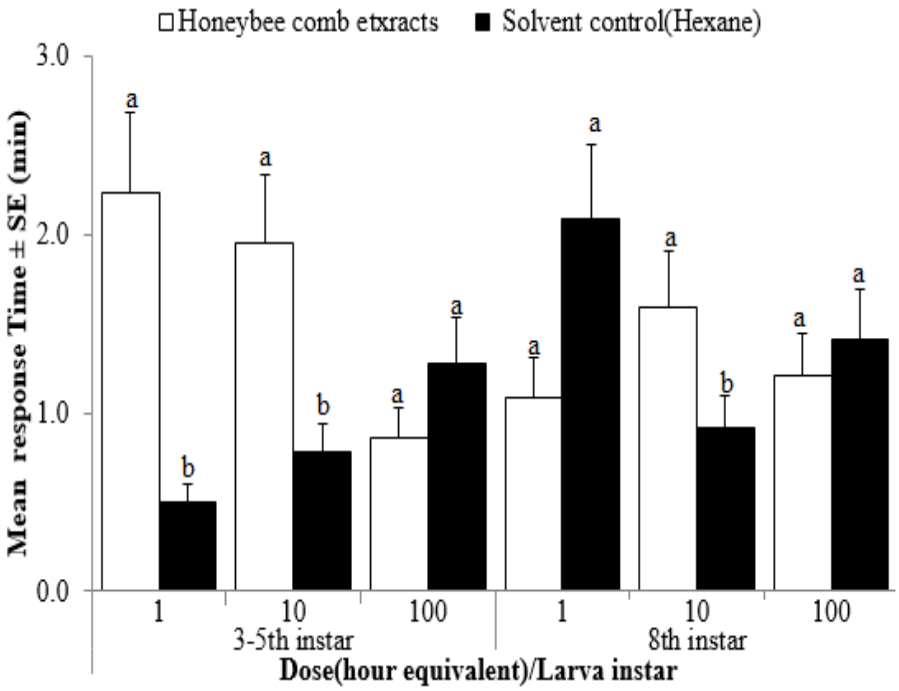

Figure 4.6: Behavioural response of *G. mellonella* larvae to larval food extract against solvent control in the Y-tube; two sample-t-test (α=0.05, n=25) Pairs of white and black bars with different letters represent statistically different behavioural responses at α=0.05. Error bars indicate standard errors.

4.1.5 Analysis and identification of volatiles

GC-MS analysis of SPME and Super Q extracts of pupae and cocoon-spinning larva (Figure 4.7), revealed the presence of various compounds, four of which were identified by GC-MS library (NIST05a.L, Adams2.L and Chemecol.L) as nonanal, decanal (only in pupae derived volatiles), tridecane and tetradecane. The proportion of each compound was determined using relative ratios which showed that decanal was the most abundant in the extract (Table 4.2).

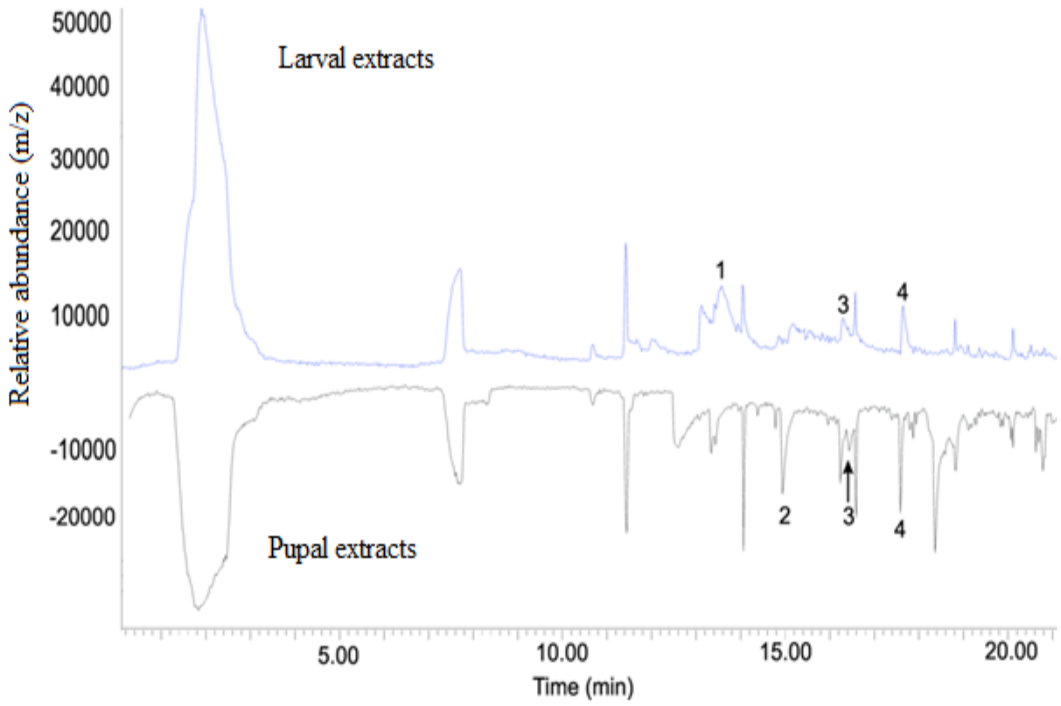

Figure 4.7: Gas chromatography-mass spectrometry profiles for cocoon-spinning larva and pupal volatile extract

Table 4.2: Mass spectral data and ratio of compounds identified in cocoon-spinning larva and pupa volatile extract.

Peak no.	Retention Time(min)	ID	Mean ratio $(\bar{x} \pm S_{\bar{x}})$
1	13.66	Nonanal	0.5193 ± 0.177
2	15.154	Decanal	1.000 ± 0.000
3	16.318	Tridecane	0.4177± 0.033
4	17.664	Tetradecane	0.4460 ± 0.132

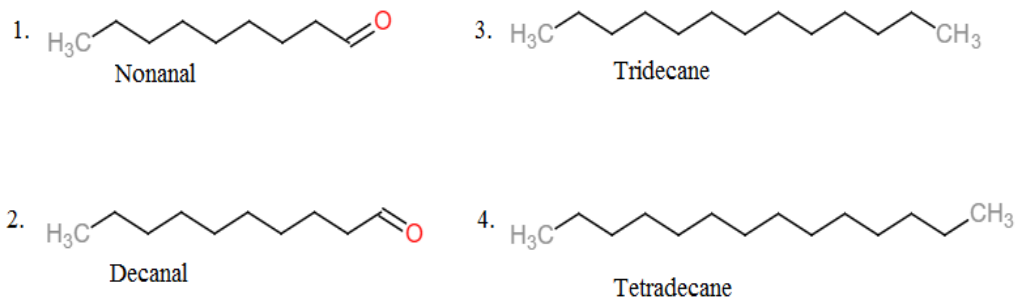

Figure 4.8: Chemical structures of compounds identified in cocoon-spinning larva and pupa volatile extract.

Analysis of larval frass and feces volatile cues trapped in Super Q established the presence of 2-methyl-2-Pentanol, Octane, 2-methyl-2-Pentanethiol, 2,2-Bis (chloromethyl)-1-propanol, 3,3,6-Trimethyl-1,5-hepatadien-4-0l and 3,3,6-Trimethyl-1,5-hepatadien-4-0l. (Figure 4.8). Their GC-MS peaks were used to quantify the amounts present in the extracts, which showed that octane and 2-Pentanethiol, 2-methyl- are the least and most abundant components respectively (Table 4.3).

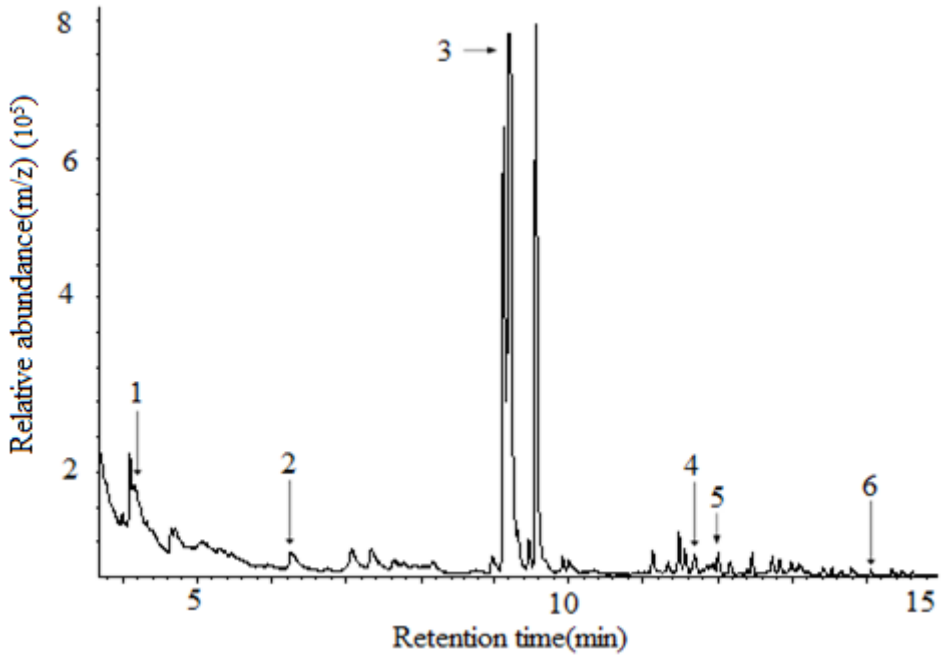

Figure 4.9: Gas chromatography-mass spectrometry trace profile for *G. mellonella* larval frass and feces volatile extract.

Table 4.3: Mass spectral data and ratio of compounds identified in frass and feces volatile.

Peak no.	Retention Time(min)	ID	Mean ratio $(\bar{x} \pm S_{\bar{x}})$
1	4.2	2-Methyl-2-pentanol	0.1040±0.027
2	6.25	Octane	0.0198±0.010
3	9.219	2-Methyl-2-pentanethiol	1.0000±0.190
4	11.694	2,2-Bis (chloromethyl)-1-propanol	0.0459±0.005
5	12.008	3,3,6-Trimethyl-1,5-hepatadien-4-ol	0.4631±0.263
6	14.335	Naphthalene	0.0339±0.016

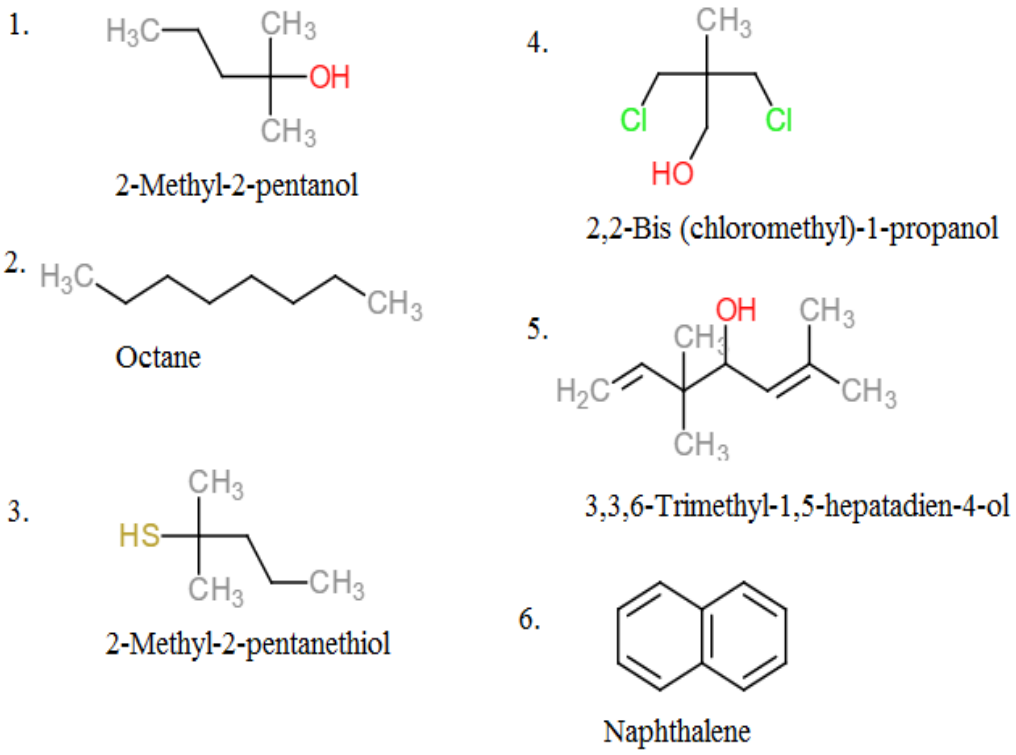

Figure 4.10: Chemical structures of compounds identified in frass and feces volatile.

A total of eighteen compounds were identified in coupled GC-MS analysis of food extracts including octane, styrene, nonane, 2(3H)-furanone-dihydro-5-methyl, benzyl

alcohol, 3,7-dimethyl-1,3,7-octatriene, linalool oxide <cis-> (furanoid), 2-nonanone, phenyl ethyl alcohol, 2H-pyran-3-ol, 6-ethenyltetrahydro, linalool oxide<cis->(pyanoid), naphthalene, 2-methoxy-4-methyl- phenol, exo-2-hydroxycineole, alpha.-cubebene, caryophyllene, germacrene D and alpha.-farnesene (Figure 4.9). Based on quantification analysis, caryophyllene was the most dominant (Table 4.4).

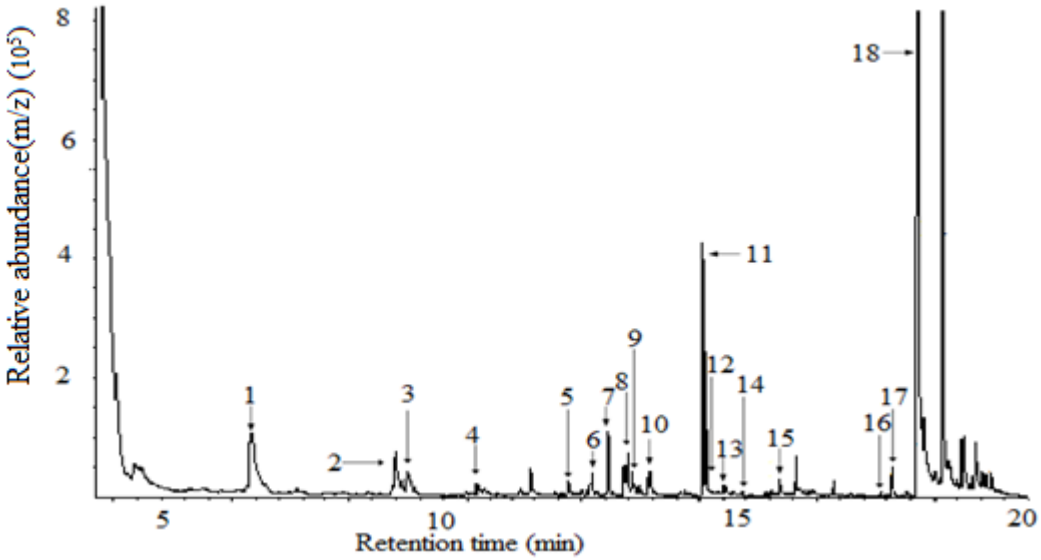

Figure 4.11: Gas chromatography-mass spectrometry profile for *G. mellonella* food (honeybee comb with pollen + honey) volatile extract.

Table 4.4: Mass spectral data and ratio of compounds identified in larval food volatile extract.

Peak no.	Retention time(min)	ID	Mean ratio $(\bar{x} \pm S_{\bar{x}})$
1	6.338	Octane	0.0684±0.024
2	8.73	Styrene	0.0082±0.001
3	8.994	Nonane	0.0206±0.010
4	10.178	Dihydro-5-methyl 2 (3H)-furanone	0.0085±0.020
5	11.768	Benzyl alcohol	0.0091±0.020
6	12.014	3,7-Dimethyl-1,3,7-octatriene	0.0040±0.000
7	12.457	Linalool oxide <cis-> (furanoid)	0.0352±0.002
8	12.774	2-Nonanone	0.0177±0.004
9	13.153	Phenylethyl alcohol	0.0123±0.002
10	14.057	6-Ethenyltetrahydro-2,2,6-trimethyl2H-pyran-3-ol,	0.1052±0.003
11	14.203	14.52 Linalool oxide<cis>(pyanoid)	0.0066±0.001
12	14.316	14.84 Naphthalene	0.0053±0.001
13	14.424	2-Methoxy-4-methylphenol,	0.0089±0.003
14	14.73	Exo-2-hydroxycineole	0.0049±0.003
15	17.107	Alpha-cubebene	0.0032±0.001
16	17.732	Caryophyllene	1.0000±0.000
17	18.495	28.15 Germacrene D	0.0265±0.001
18	18.702	Alpha.-farnesene	0.0440±0.003

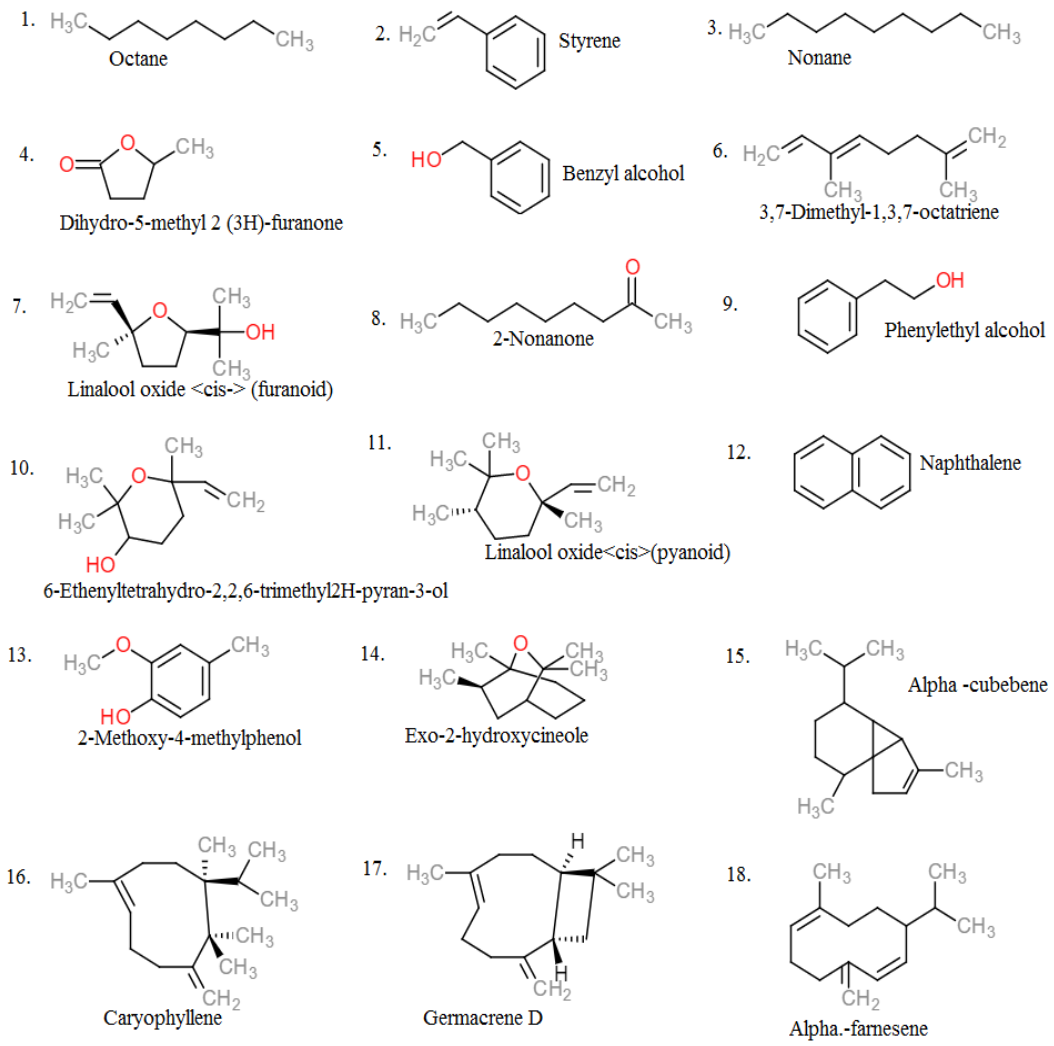

Figure 4.12: Chemical structures of compounds identified in larval food volatile extract.

4.2 Determination of the hive odours attractive to the greater wax moth adult

In the wind-tunnel assays, only mated female moths were strongly attracted to odour plume emanating from honeybee colonies. GC-EAD analysis revealed the presence of seven antennally active compounds that consistently evoked antennal response in mated female moths.

4.2.1 Behavioural evidence for kairomone–mediated host attraction

The Wind-Tunnel assays (Figure 4.10), confirmed the involvement of honey bee comb released volatile compounds in GWM attraction. There was no significant difference in the number of both the virgin and mated male moths that landed on the traps (virgin males - χ^2 = 0.1922, df =1, p= 0.661 and mated males - χ^2 = 0.26224, df =1, p= 0.609), similar trend was displayed by virgin females (χ^2 = 2.386, df =1, p= 0.122). However, odours from honeybee comb elicited more behavioral activity in mated females resulting in landing of more moths compared to the control. Moreover, the landed females displayed intense probing of the surface (χ^2 = 7.9341, p= 0.005).

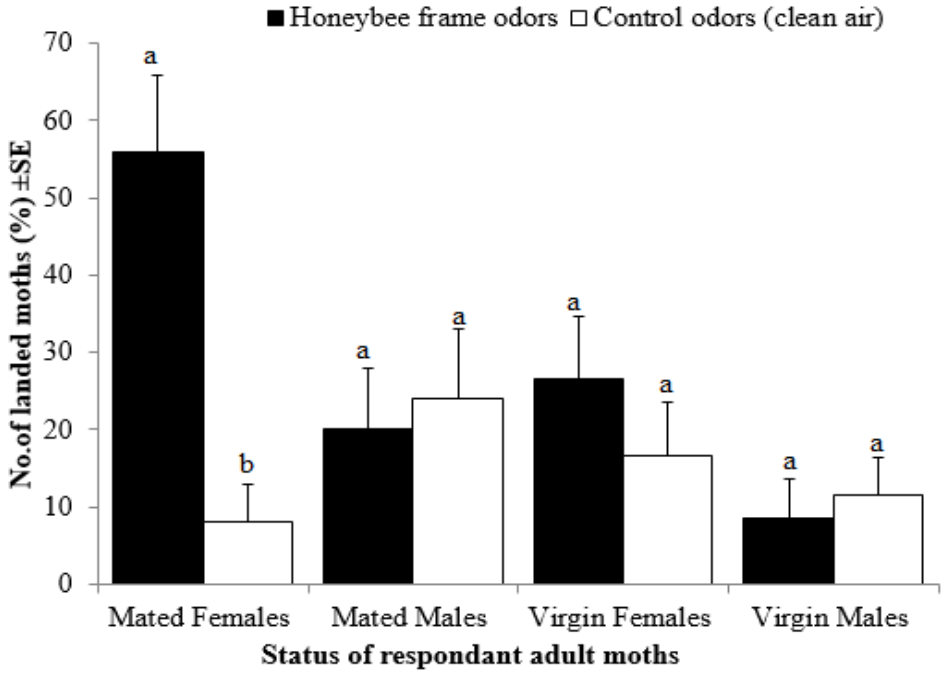

Figure 4.13: Behavioural responses of *G. mellonella* adult moths to honeybee comb volatiles against control (clean air). Pairs of white and black bars with different letters represent statistically different behavioural responses by χ^2 at α=0.05, n=25. Error bars indicate standard error of the mean.

4.2.2 Response of adult GWM to honey bee comb odour extracts

Mated female moths further demonstrated a dose-dependent significant difference between the honeybee comb entrainment and control (Figure 4.11). All the doses tested aroused significant upwind attraction with subsequent landing. A significantly greater number of moths landed on the test trap at 24 hour equivalent of honeybee comb volatile trapping (χ^2 = 18.484; df =1; p= 0.00001714), while the least attraction exhibited at 6 hour equivalent (χ^2 = 4.7586; df =1; p= 0.02915). The intermediate hour equivalent (12HCHE), displayed more significant difference (χ^2 = 10.091; df =1; p= 0.00149) than the minimal dosage tested.

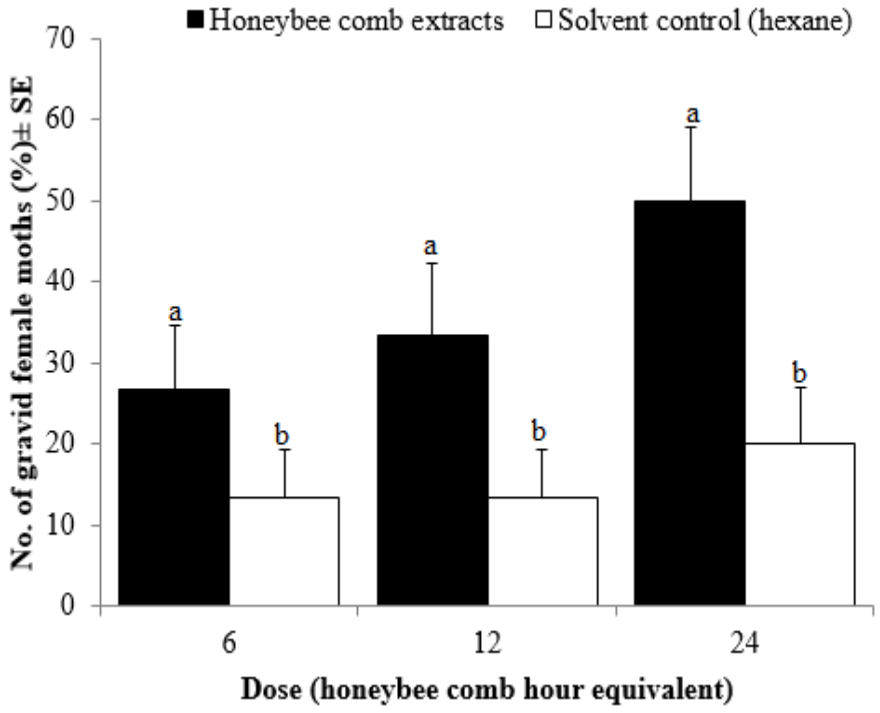

Figure 4.14: Behavioural responses of G. mellonella female moths to honeybee comb volatiles against solvent control (hexane). Pairs of white and black bars with different letters represent statistically different behavioural responses by χ^2 at α=0.05, n=25. Error bar indicate standard error of the mean.

4.2.3 Identification of honeybee comb odour components

Gas chromatographic (GC) analyses of Super Q trapped honeybee comb volatile with both flame ionization (FID) and electroantennographic detection (EAD) revealed presence of seven (7) EAD active compounds (Figure 4.12), that consistently elicited antennal response in mated females. All the EAD active peaks were identified by their GC retention time and comparison with GC-MS mass spectral data. The compounds were identified as esters (ethyl propanoate; 2-methyl, ethyl propanoate; 2-methyl ethyl butanoate; 3-methyl butyl acetate), aldehydes (nonanal and decanal) and a terpene (sylvestrene). Unlike immature stages, the adult female attractant blend seems to be predominated by organic esters (Table 4.5).

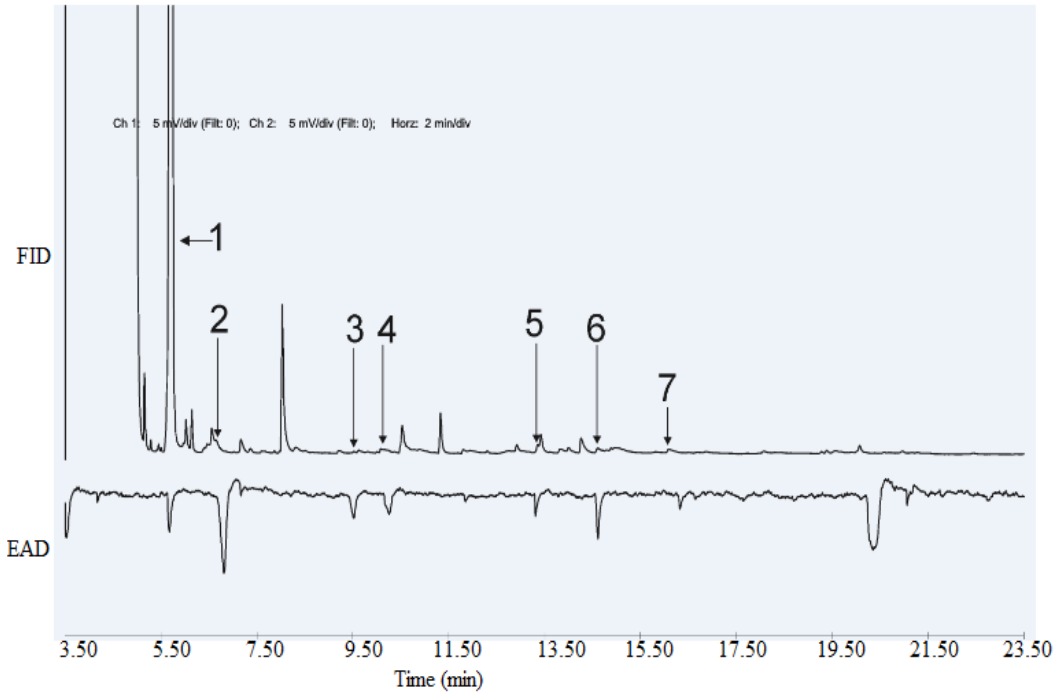

Figure 4.15: Gas chromatography–flame ionization detector coupled with gas chromatography-mass spectrometry –electroantennographic detection recording from GWM gravid female antenna in response to 5µl of honeybee comb extract**.**

Table 4.5: Mass spectral data and ratio of EAD active compounds identified in honeybee comb volatile extract.

Peak No.	Retention time(min)	ID	Mean $(\bar{x} \pm S_{\bar{x}})$
1	3.9	Ethyl propanoate	1.0000±0.000
2	5.04	Ethyl 2-methylpropanoate	0.4266±0.033
3	7.78	Ethyl 2-methylbutanoate	0.0944±0.008
4	8.49	3-Methyl butyl acetate	0.0569±0.007
5	11.55	Sylvestrene	0.2030±0.000
6	12.86	Nonanal	0.8065±0.104
7	14.55	Decanal	0.1338±0.030

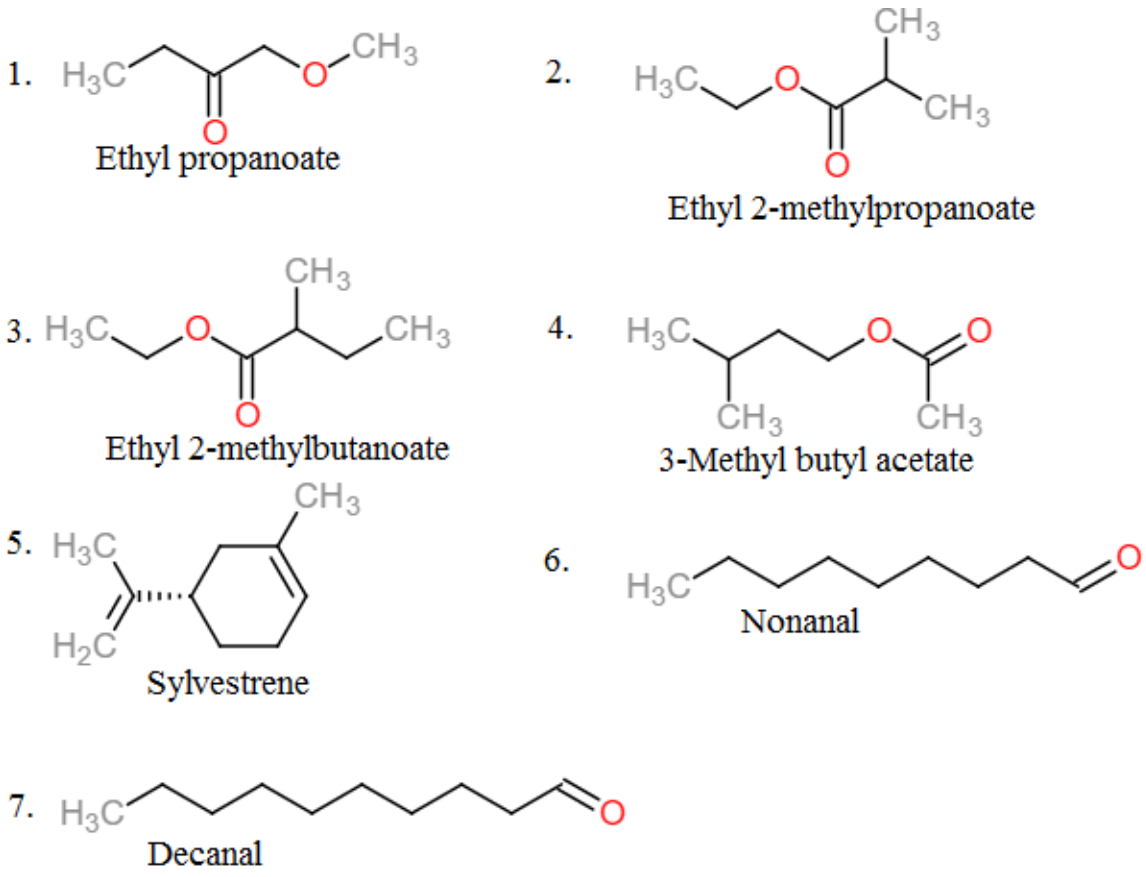

Figure 4.16: Chemical structures of compounds detected by antennae of gravid female wax moth in honeybee comb volatile extract.

CHAPTER FIVE

5.0: DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 DISCUSSION

5.1.1 Determination of the greater wax moth larval aggregation pheromone

While aggregation of hatched larvae from eggs oviposited in clusters might appear logical, it is not always the case, at least in the first larval instars (Clark & Faeth, 1998). The Petri-dish results, albeit under laboratory conditions, augment previous field observations of *G. mellonella* larva behaviour (Nielsen & Brister, 1979, Williams, 1997). Further, it demonstrates that GWM larva aggregation is exhibited across larval instars, therefore, based on the statistical analyses, the null hypothesis that larva of GWM spin cocoon in solitary was rejected.

Olfactometric assays showed that immature instars were strongly attracted to frass and food derived volatile, but indifferent to volatiles emanating from the mature counterparts, on the contrary, mature instars were only responsive to cocoon-spinning larva cues. These observations suggest that there are two larva aggregation patterns in GWM larvae with respect to cocoon-spinning, frass and food volatiles. The interpretation could further be reiterated by the differentiation of extract components revealed by GC-MS analysis. GC-MS analysis and identification using NIST05a, Adams2 and Chemecol libraries detected presence of three alcohols (2-pentanol 2-methyl-, 2-pentanethiol 2-methyl-, 3, 3, 6-trimethyl-1 5-hepatadien-4-0l), an alkane (octane) and aromatic hydrocarbon (naphthalene) in frass volatile extracts. In addition, four alcohols (benzyl alcohol, exo-hydroxycineole, phenol 2-methoxy-4,methyl and phenylethyl alcohol), two

alkanes (octane and nonane), one aromatic hydrocarbon (styrene), one ketone (2-nonanone), one lactone (2(3H)-furanone, dihydro-5-methyl), three monoterpenes (1,3,7-octatriene, 3,7-dimethyl; cis-linalool oxide (furanoid) and cis linalool oxide (pyanoid) and four sesquiterpenes (α cubebene, α-farnesene, caryophyllene and germacrene D) were identified in food volatile extracts.

Recently, octane was reported to act as synergist to sex pheromone of two moths, *Lobesia botrana* Den. & Schiff and *Cydia pomonella* Linnaeus (Gurba & Guerin, 2015). Naphthalene has been identified in lychee stink bug, *Tessaratoma papillosa* Drury (Hemiptera: Pentatomidae), as a component of nymphal released volatiles (Wang *et al.*, 2015) and as a component of dissected wing extracts from swallowtail butterfly, *Papilio protenor* Cramer (Lepidoptera: Papilionidae) (Ômura *et al.*, 2012) but whether it is an active component of a semiochemical in these species remains to be elucidated. To date, there exists dearth literature regarding the biological activities of the identified alcohols.

The most abundant component in food extracts, caryophyllene, alongside α-farnesene, α-cubebene and germacrene D, have previously been reported in grape shoot volatiles, *Vitis riparia*, as components of a lure that significantly attract females of grape berry moth, *Paralobesia viteana* (Cha *et al.*, 2008). Yusuf *et al.* (2014), found that, octane, which was part of a 17-component identified from the cues of termite gallery soil and termites, enhanced attraction of the termite raiding ants, *Pachycondyla analis* Latreille (Hymenoptera: Formicidae). Benzyl alcohol has only been reported as a component of an airborne aggregation pheromone for bed bugs, *Cimex lectularius* Linnaeus (Hemiptera: Cimicidae) (Siljander *et al.*, 2008). Some of the monoterpenes identified in food extracts, have been demonstrated to induce a chemotaxis response of two-spotted oak buprestid

adults, *Agrilus biguttatu* (Coleoptera: Buprestidae) towards host tree , *Quercus robur* (L) (Fagaceae) foliage volatiles (Vuts *et al.*, 2015) and as active components of floral scent extracts of fairy fans, *Clarkia breweri* (Onagraceae) that elicited strong GC-EAD response in the white-lined sphinx moths, *Hyles lineata* (Lepidoptera: Spingidae) (Raguso *et al.*, 1996*)*, however, its noteworthy that the latter did not perform any behavioural experiment to verify whether the compounds are attractant, arrestant or repellants.

Mature instars were attracted to conspecific cocoon-spinning larva extracts consisting of an aldehyde (nonanal) and two alkanes (tridecane and tetradecane). Further, mature larva aggregation was significant in the presence of pupae. Nonanal and decanal have been previously reported as a component of sex pheromone of GWM produced by wing glands (Leyrer & Monroe, 1973, Flint & Merkle, 1983, Lebedeva *et al.*, 2002, Romel *et al.*, 1992). However, according Svensson *et al.* (2014), decanal though elicited female antennal response, it was " a typical contaminant." Similarly, the two aldehydes viz. nonanal and decanal, were part of a n 8-component blend emitted from fifth instars that evoked aggregation response from fifth instars of *S. gregaria* Forskal (Torto *et al.*, 1996). Jumean *et al.* (2005), identified nonanal and decanal as part of the 11 component larva aggregation pheromone of *C. pomonella*. In addition, nonanal and decanal were part of European honeybee hive-produced components that were highly attractive to SHB, *A tumida*, in a wind tunnel bioassays (Torto *et al.*, 2007). Furthermore, the role of nonanal and decanal as components of kairomone has also been reported in *B. hebetor*, a parasitoid which utilize components of GWM sex pheromone to locate its host.

In the European corn borer (ECB), *Ostrinia nubilalis* Hubner (Lepidoptera: Crambidae), nonanal and decanal which were parts of maize plant derived volatile, elicited

strong electroantennographic response of gravid ECB (Molnár *et al.*, 2015). The two alkanes (tridecane and tetradecane) have recently been reported as components of a six-compound spittle mass blend, that induce and regulate aggregation behaviour in spittlebug, *Callitettix versicolo* Fabr (Hemiptera: Cercopidae) (Chen & Liang, 2015). The previous findings suggest the involvement of the aldehydes, alkanes, alcohols and monoterpenes as components of semiochemicals that play augmentative role in different behavioural aspects of an array of insect families, however, the role of these compounds has not previously been established in *G. mellonella* larvae.

Lack of strong attraction of 3-5th instars to cocoon-spinning larva extracts could perhaps imply that: 1) immature larval instars can discriminate between potential food sources and competitors on the basis of chemical cues emanating from them. Such an ability to assess of their immediate surrounding would maximize their chances of survival. This argument might be supported by earlier observation by Williams (1997) who reported incidences of cannibalism by mature larvae towards their disadvantaged immature larvae and pupae. It is also possible that pieces of larval body observed after isolating a mixed group of mature and immature larvae (personal observation) could be as a result of cannibalism, 2) larva aggregation behaviour in *G. mellonella* first instars, is induced and modulated in part, by a complex set of organic compounds viz hydrocarbons, terpenes, lactones, and alcohols produced by frass and food. It is therefore possible that volatiles emanating from cocoon-spinning larvae facilitate different benefits and costs for first and later instars.

In addition, strong attraction of mature larvae to only conspecific cocoon-spinning larva extracts could suggest that; 1) feeding behaviour differs between the immature and

mature larval instars. Nielsen and Brister (1979), reported that the late instars larvae would move away from food once they finish feeding, an observation that would corroborate the argument. Moreover, different feeding behaviour and physiology have been reported for Lepidopteran families (Zalucki *et al.*, 2002, Denno & Benrey, 1997, Inouye & Johnson, 2005), and therefore, it should not be assumed that first instar larvae are simply small versions of later instars (Zalucki *et al.*, 2002); 2) mature instars could be using odors released by conspecific cocoon-spinning larvae as a signal of suitable pupation sites. Previously, Duthie *et al.* (2003) concluded from their studies that the presence and developmental stage of conspecifics is crucial in pupation site selection by fifth-larval instars of *C. pomonella*, an observation that could support the latter argument.

The differential responses to cocoon-spinning larva, frass and food volatiles by mature and immature larval instars presumably indicate that the perceived *G. mellonella* larva aggregation behaviour induced by olfactory cues could be more intricate than previously imagined. Previous authors have suggested several hypotheses to explain evolution and maintenance of Lepidopteran larva grouping behaviour including i) enhancement of feeding, growth and development in *C. janais.* Drury (Lepidoptera: Nymphalidae) (Denno & Benrey, 1997), ii) procurement of future mates and enhancement of fitness amongst *C. pomonella* larvae (Duthie *et al.*, 2003), and iii) protection against predators (Clark & Faeth, 1997). But as Clark and Faeth (1998) suggested, these hypotheses are not mutually exclusive. Although Williams (1997) reported production of substantial quantities of metabolic heat at the centre of aggregating GWM larvae, which he suggested was as a result of rapid larval growth rate, he did not allude it to any of the above hypotheses. Further, high temperatures have been observed in aggregating larvae

(Charles Kwadha, personal observation), which might support enhancement of feeding, growth, and development hypothesis, but, adaptive significance of larva aggregation in *G. mellonella* warrants further investigations.

The present study indicates a great diversity of organic compounds emanating from cocoon-spinning larvae, frass and larval food, and therefore, it could be possible that these compounds either play a primary role or contribute to different aspects of the behaviour. Further, the observations suggest that these compounds might work synergistically. For instance, synergism has been reported earlier between plant volatiles and sex or aggregation pheromones (Honda, 1995), and thus, cannot be ruled out as a possibility in the current study. Majority of the identified compounds can be traced to plants' origin as highlighted in a previous study which identified a vast number of organic compounds in floral scents of insect pollinated flowers (Knudsen *et al.*, 1993). Moreover, larvae of *G. mellonella* feed on honey bee products which are inherently of plants origin and therefore, identification of floral scent compounds in these extracts is not a surprise.

Previously, it was not known how GWM larvae dispersed or removed from honeybee combs would move in the direction of the combs and locate it (Nielsen & Brister, 1979). The results presented in this study has for the first time, revealed involvement of mature larva, food and frass volatiles in *G. mellonella* larva aggregation and a more complex chemical communication system of modulating grouping behaviour in the instars. It is not known whether all these compounds would evoke electrophysiological response, thus it would be worthy to pursue such questions.

5.1.2 Determination of the greater wax moth adult attractant (kairomones)

The wind-tunnel assays showed that honeybee comb releases olfactory cues that evoke strong response from gravid females and neither from naïve males and females nor mated males. Previously, Nielsen and Brister (1977) noted that eclosed females would fly back to the hives immediately after mating process. Although no explanation was put forward by the authors to support their observation, it is likely from the current study that host-volatile could be the inducer of orientation towards honeybee hives.

Analysis of volatile extracts, revealed the presence of four esters, two aldehydes and a terpene, with esters consistently dominant. Ethyl 2-methyl butanoate and ethyl 2-methyl propanoate have been previously reported as components of a 7-compound defense secretion employed by fifth larval instars of swallowtail butterflies (Lepidoptera: Papilionidae) against larger invertebrates (Ômura *et al.*, 2006). Gries *et al.* (1994) found that ethyl propanoate was a constituent of a four–ester volatile blend from African palm oil that act as synergistic kairomone for both male and female African palm weevil, *Rhynchophorus phoenicis* L (Coleoptera: Curculiniodae). In addition, ethyl propanoate was part of sugarcane derived volatile components that exhibited kairomonal synergism in field studies to elucidate attractiveness of host kairomone baited traps for the West Indian sugarcane weevil, *Metamasius hemipterus* Oliv (Coleoptera: Curculiniodae) (Perez *et al.*, 1997).

3-methyl butyl acetate is a well-known defensive compound in Coleopterans, occurring in abdominal glands of thirteen rove beetle species (Huth & Dettner, 1990), while in honeybees, *A. mellifera* (Hymenoptera: Apidae), the ester has been explicitly studied as a component of worker bee alarm pheromone (Collins *et al.*, 1989, Torto *et al.*, 2007).

However, despite the fact that alarm pheromone mediate the first step in honeybee colony defense (Collins *et al.,* 1989), hive intruders such as SHB are sufficiently attracted by alarm pheromone (Torto *et al.,* 2005), and more interestingly at lower doses than the threshold perceived by worker bees (Torto *et al.,* 2007). And as Suazo *et al.* (2003), Torto *et al.* (2005) and Fombong *et al.* (2012) highlighted, each honeybee hive components inherently poses unique chemical signals that bridge association between honeybees and arthropods such as beetles and moths. From the current study, it is therefore likely that gravid female wax moth utilize components of worker bee alarm pheromone as an attractant.

Nonanal and decanal have been previously identified as major components of GWM sex pheromone (Leyrer & Monroe, 1973, Flint & Merkle, 1983, Romel *et al.,* 1992) and aggregation pheromone (Jumean *et al.,* 2005). Similarly, the two aldehydes were part of an 8-compound blend that attracted SHB (Torto *et al.,* 2005) and as constituents of a kairomone for ECB (Molnár *et al.,* 2015). It is not surprising that these EAD active compounds were components of honeybee derived volatile because just like the preceding organic compounds identified in cocoon spinning larva and frass volatiles, they too can be traced to plants origins (Knudsen *et al.,* 1993), an argument supported by the fact that honeybees forage on plants. Even though previous studies showed the involvement of the four esters and two aldehydes identified in Super Q trapped honeybee comb volatile as pheromone and kairomone components, the current work, for the first time report the involvement of these organic compounds in attraction of gravid female wax moth. It is worth noting that no previous work has ever reported any semio-chemical role of sylvestrene and therefore the current study is the first to implicate the compound with a role in an insect's induced behaviour.

5.2 CONCLUSIONS

In this study, the presented results show that;

1. Larvae of *Galleria mellonella* exhibit aggregation behaviour both at first and late instars, and that the behaviour is mediated by cocoon-spinning larva and frass, and host volatiles
2. Frass and food odours partly induce strong response in first instar larvae.
3. Cocoon-spinning larva and pupa produce aldehydes and alkanes that elicit strong response in late instar larvae.
4. Organic volatile compounds emanating from honeybee comb only attracts mated females. Esters are the dominant constituent of attractant compounds of *G. mellonella* mated females.

5.3 RECOMMENDATIONS

Many research questions have risen during the research work;

1. Previous authors have suggested various hypotheses to explain significance of aggregation in Lepidopteran species (Denno & Benrey, 1997, Duthie *et al.*, 2003, Clark & Faeth, 1997), but in *G. mellonella* larva, the significance of aggregation remains unknown, this knowledge gap warrants further studies.
2. Even though GC-MS analysis has revealed the components of larval frass and food volatiles, the minute nature of larval antennae could not permit determination of the active compounds through GC-EAD. Therefore, there is need to elucidate the activity of the identified compounds in larval behaviour.
3. The efficacy of the identified components of aggregation pheromone and attractants either as single compounds or as blends requires evaluation.

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