BIOCHEMICAL SIGNALLING IN THE TRI-TROPHIC INTERACTION BETWEEN *Anacardium occidentale*, *Pseudotheraptus wayi* and *Oecophylla longinoda*

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April 2014

DECLARATION

I declare that this thesis is my original work and has not been presented for the award of a degree in any other University or any other award.

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List of Abbreviations and acronyms

ANOVA	Analysis of variance
ARCU	Animal rearing and containment unit
BCED	Behavioural Chemical Ecology Department
EFNs'	Extra Floral Nectaries
DAGs'	Dorsal abdominal glands
GC-MS	Gas chromatography- Mass spectrometry
HIPVs'	Herbivore induced plant volatiles
ICIPE	International Centre for Insect Physiology and Ecology
MTGs'	Metathoracic glands
SPME	Solid Phase Micro extraction
VOCs'	Volatile Organic Compounds
ng/µl	Nanogram per microlitre

Abstract

The relationship between *Pseudotheraptus wayi* (cashew pest), *Anacardium occidentale* (Cashew plant), and *Oecophylla longinoda* (natural enemy) is a tri-trophic interaction that is very important in cashew nut production. However, the chemical ecology of the interaction, specifically between the cashew plant and natural enemy as well as the pest and natural enemy was little investigated. Based on the hypothesis that both interactions were mediated by chemical signals, the objective of this study was to identify these signals and establish their relevance to the species in question. Head space volatiles of the cashew plant, the pest and natural enemy were collected, analyzed in Gas chromatography-mass spectrometry (GC-MS), confirmed and quantified using authentic standards. Behavioral assays were then conducted to establish the relevance of the bio- chemicals. In the pest-natural enemy interaction, the volatiles identified were mainly C_6 compounds (aldehyde, alcohol and the corresponding esters). Hexanal, hexanol, hexyl acetate and hexyl hexanoate were identified in the head space volatiles of the natural enemy as well as the pest. Moreover they were also present in the metathoracic glands of the pest which are primarily devoted to producing chemicals for defense purposes. Previously, these same compounds were shown to constitute the alarm pheromone of the natural enemy as well as defense chemicals of various insect species in the Coreidae family. In another study these four components were also suggested to constitute a candidate sex pheromone of the pest, corroborated in this study by the finding that, the onset and pattern of both mating and production of these chemicals coincide. In the host plant-natural enemy interaction, volatiles from plant parts most vulnerable to herbivory (leaves, fruit and inflorescence) were predominantly monoterpenes and sesquiterpenes. The attractiveness of the crude volatile extracts of the individual plant parts was measured as, total time spent by the natural enemy in either the test or control arm of the Y-tube olfactometer expressed as a percentage of the total time allocated .Crude volatile extracts from leaves and inflorescence were significantly attractive compared to the control (t-test, α=0.05).. However when the extracts were presented together with a food reward (sugar syrup) the responses of the natural enemy were significantly enhanced (ANOVA, α = 0.05). These findings demonstrate that while cashew volatiles are attractive to the natural enemy, they are most relevant when paired with a food reward; a response that is indicative of associative learning. Mirroring, these findings to the natural context suggests that, the natural enemy relies on cashew plant volatiles as predictors for food rewards. The rewards are usually nectar from extra floral nectaries located mainly on plant parts vulnerable to herbivory. Therefore, the rewards not only benefit the natural enemy but also confer an advantage to the plant, by motivating the natural enemy to spend more time around the vulnerable plant parts, effectively deterring herbivory. The wider implication is that the chemical signals identified in the interactions of the natural enemy with both the pest and plant can potentially be exploited for improved crop protection.

CHAPTER ONE

INTRODUCTION

1.1 Background

Pseudotheraptus wayi (Heteroptera: Coreidae), commonly known as the coconut bug is a pest of economic significance in cashew (*Anacardium occidentale*) orchards in the East African coast (Wheatley, 1961). Despite being a low density pest, both adults and the immature forms cause about 60-100% yield losses. As early as 1945, it was observed that trees inhabited by *Oecophylla longinoda* (Hymenoptera: Formicidae) also known as the African weaver ant had reduced pest populations and significantly better yield. This observation was later confirmed by Michael way (1953) where experimental evidence on the ability of the weaver ant in controlling *P. wayi* was first described and more recently by other researchers (Way and Khoo, 1992; Van Mele, 2008; Olutu *et al.*, 2012).

In an ecological context the interaction between *P. wayi* (prey/pest), *A. occidentale* (host plant) and *O. longinoda* (predator/natural enemy) constitutes a tri-trophic relationship. By definition, a trophic level is the space an organism occupies in a food chain, therefore as the name suggests this study was about the association between three organisms belonging to different trophic levels. The three most important characteristics of tri-trophic relationships are; species share feeding links, the alternate trophic levels (plant-predator) share a symbiotic relationship and that organisms in this relationships are linked biochemically (Ahmad *et al.*, 2004)

which formed the framework of this study. The focus was primarily on semiochemicals i.e. communication chemicals produced by animals to trigger a response that impacts the behaviour of the recipient in one or more ways (Torto, 2004; Moraes *et al.*, 2008).

In the context of plant and predator/natural enemy interactions plants have been known to have a long standing association with ants. These associations, co-evolved into mutualistic relationships (Oliveira, 1999; Mithöffer and Boland, 2012; Pringle *et al.*, 2012). The mutualisms are expressed into two contexts; provision of plant rewards (extra floral nectaries (EFNs'), nest sites and accommodation of honey dew producing hemipterans) in exchange for anti- herbivore defense and production of plant Volatile Organic Compounds (VOCs') to signal the natural enemies (Arimura *et al.*, 2005; Heil, 2007) to intervene on behalf of the plant in response to herbivore damage.

The cashew plant expresses EFNs' and is visited by ants all year long and is therefore regarded as a beneficiary of ant protection which is indicative of a mutualistic association (Rickson and Rickson, 1998). Based on these two contexts in which ant-plant mutualisms are expressed, the relevance of rewards and VOCs'on the interaction between the cashew plant and natural enemy/predator was investigated in this study.

With regard to the relationship between the prey/pest and predator/natural enemy, there is no known herbivore that has been documented to rival protector ants such as *O. longinoda* as yet (Arimura *et al.*, 2005). However *P. wayi* belongs to an insect order (Heteroptera) associated with sophisticated chemical defense ability (Aldrich, 1988; Millar, 2005). Likewise, *O. longinoda* is equally endowed with chemical and tactile defense arsenal (Crozier *et al.*, 2010). On that basis it was hypothesized that the interaction between these two species was possibly dominated by interplay of chemical signals.

1.2 Problem statement

For many decades now, *P. wayi* damage has hampered cashew nut farming in the East African region. The current practice in controlling this pest involves indiscriminate pesticide use which is not only cost limiting but also presents a potential threat to the integrity of the eco-system. This makes the use of *O. longinoda* as a bio-control agent favorable, more so because it naturally colonizes cashew trees in this region. it is therefore not only cost efficient but requires little effort if any for use by small holder farmers while at the same time, allowing them to sell their produce in the highly lucrative organic markets that had remained out of reach for long time (Van-Mele, 2008). As with all natural systems, species continually evolve in space and time. Therefore, to leverage on these systems for purposes such as improving agricultural productivity as with the use of bio-control agents such as *O. longinoda*, a holistic understanding of the species interactions is required. Before this study, only the chemical interactions between the pest and

host plant were investigated, while the chemical ecology of the pest- natural enemy and plant-natural enemy relationships remained obscure. This study therefore was an attempt to address the knowledge gap.

1.3 Justification of the study and expected output

Chemical signaling is central to interactions between living organisms. In the advent of integrated pest management (IPM), the exposition of such signals has been invaluable in the quest for highly selective and specific pest control tools. Currently insect and plant derived pest control tools are regarded as more favorable alternatives and are steadily gaining popularity world over. One of such approaches is the use of *O. longinoda*. It has been proven to be an efficacious bio-control agent against *P. wayi* and other pests of economic significance in the fruit and nut industry in East Africa. Similarly, in other parts of the world its counterpart *O. smargdina* ("green ant") has been shown to be equally effective. Tri-trophic relationships involve three organisms belonging to different trophic levels and therefore understanding these systems more so on how to exploit them for agricultural productivity requires a holistic understanding of the individual species interactions. Previously the interaction between pest and the plant had been investigated and some chemical components of the plant odor shown to constitute a candidate *P. wayi* kairomone. However nothing was known about the chemical ecology of the interactions between (a) the pest and its natural enemy, and (b) the host plant and the natural enemy. Therefore, the aim of this study was to address the current knowledge gap and possibly provide insight on ways in which the

chemical ecology of this system can be further exploited for the benefit of crop protection. Similar studies in the past, have led to the discovery of plant compounds such as (*Z*) Jasmone currently in use in wheat plantations and also under investigation on its potential in the manipulation of both pests and natural enemies of other related crops. Such chemical tools are indisputably invaluable to crop protection, emphasizing the need for this study.

1.4 Hypotheses

i. The interaction between *P. wayi* and *O. longinoda* is dominated by chemical signals

ii. The interaction between the cashew plant and *O. longinoda* is mediated by chemical signals

1.5 Objective of the study

1.5.1 General objective

To establish chemical signals mediating the interaction between the *A. occidentale*, *P. wayi* and *O. longinoda*

1.5.2 Specific objectives

i. To identify and determine the role of chemical signals in the interaction between *P. wayi* and *O. longinoda*

ii. To identify and determine the role of chemical signals in the relationship between the cashew plant and *O. longinoda*.

CHAPTER TWO

LITERATURE REVIEW

2.1 Tri-trophic interactions

Tri-trophic interactions involve organisms occupying three trophic levels i.e. plant, herbivore and carnivore (Ahmad *et al.*, 2004). These interactions are core to bio-control and therefore very important in agriculture. Their study is not only important in understanding the species interactions but can also provide useful insights in the manipulation of bio-control agents for improved pest control (Agrawal, 2000). A good example is the manner in which novel chemical components such as (*Z*)-Jasmone, nepetalactone and (*E*)-β-farnesene identified from studies of a similar nature have gained usefulness in pest control (Bruce *et al.*, 2003). (*Z*)-Jasmone for instance, was originally identified in black currant volatiles and was found to repel the lettuce aphid *Nasonovia ribis-nigri* and attract the aphid parasitoid *Aphidius ervi* (Bruce *et al.*, 2003). Currently this chemical compound has found practical application in the control of the grain aphid *Sitobion avenae* in wheat where it is sprayed in low doses to repel the pest as well as attract the parasitoid (Bruce *et al.*, 2003). The compound has been shown to attract *Telenonus podisi* (Hymenoptera: Scelionidae), the egg parasitoids of stink bugs that attack soy beans in Brazil and therefore proposed to have great potential for practical application in that area as well (Moraes *et al.*, 2009).

2.2 *Anacardium occidentale*

Plate 2.1: Ariel parts of *Anacardium occidentale* © C. W. Kung'u

The cashew tree (*Anacardium occidentale* Linnaeus (Sapindales: Anacardiaceae) is native to Brazil, Mexico, the United states of America and exotic to many tropical countries across Latin America, and Africa (Wijit, 1991; Rickson and Rickson, 1998; Aliyu, 2007). The tree is medium sized and grows to a height of about 12m. The leaves are simple, rounded at the ends with short petioles and pale green or reddish when young and dark green when mature (Plate 2.1 and *Agro forestry* database 4.0, 2009). It is an adromonoecious cross pollinated flowering plant with inflorescence occurring in a panicle like cluster as seen in plate 2.1, bearing both haemophroditic and male flowers (Wijit 1991; Aliyu, 2007; *Agro forestry* database 4.0, 2009).

In the east African coast cashew production is predominantly a small holder farmer activity but a very important source of income. In Kenya it is mainly found in

three districts of the Coast province, namely; Kwale, Kilifi, and Lamu. In the past it contributed about 50-90% of the total income of farmers in that area (Waithaka, 2002) .However production has been hampered by a combination of biological, socio-economic and agro-economic factors. Biological factors include pests of economic importance such as the sucking bugs *Helopeltis schoutedeni*, *H. anacardii*, *P. wayi* among others. Diseases such as die back and anthracnose are also common (*Agro forestry* database 4.0, 2009).

2.3 *Pseudotheraptus wayi*

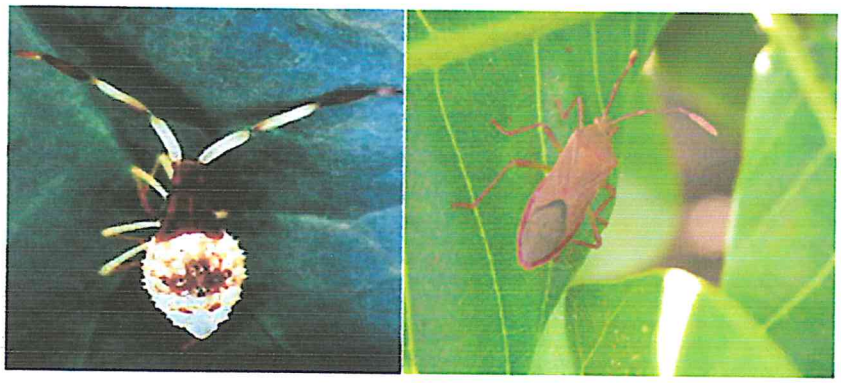

Plate 2.2: Left to right: Nymph and adult *P. wayi*

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Pseudotheraptus wayi (P. wayi) belongs to the suborder Heteroptera, family Coreidae and is commonly known as the coconut bug (Brown, 1955; Way, 1953). Heteroptera are also known as "true bugs" and comprise a group that is both large and widely distributed in the world with some species being important pests of

crops and have been ranked fourth among the most economically important group of insects (Millar, 2005).

The adult *P. wayi* is reddish brown in colour, 12 to 14 mm long (Plate 2.2), while nymphs are red brown to green brown in colour and have long antennae (Plate 2.2). It is hemimetabolous (undergoes incomplete metamorphosis) with three life stages; egg, nymph and adult with a mean length of time from egg laying to emergence of adult being about 41 days (Way, 1953; Millar, 2005). The eggs are cream colored, oval in shape and measure about 1.85mm long and 1.26mm wide. The 1st instar measures about 2.7×1.42 and 4.7×2.59mm, 6.3×3.34mm, 8.1×4.35mm and 10.3×5.1mm for the consecutive instar stages (Egonyu, in press).

2.3.1 Economic significance of *Pseudotheraptus wayi*

Plate 2.3: Damaged cashew fruit © www.agriculture.go.tz

P.wayi is a pest of various tree crops that includes; coconut (*Cocos nucifera*), cashew (*Anacardium occidentale*), avocados (*Persea americana*), Macadamia

(*Macadamia integrifolia*), guava (*Psidium guajava*), Mango (*Mangifera indica*) among others (Schoeman *et al.*, 2010; Olotu *et al.*, 2012). The yield losses due to this pest was estimated at 99.8%, 60-100%, 72.6%, 52.2% in coconut, cashew, avocados and guavas respectively (Way 1953; Wheatley, 1961; Meulen, 1992; Meulen, 1994). In cashew it sucks on the sap of young leaves and fruit resulting in lesions that cause premature nut fall and development of secondary infections (Plate 2.3). Although it is a low density pest, as few as 10 insects per hectare are capable of causing extensive damage (Way, 1953; Wheatley, 1961; Way and Khoo, 1992; Van-Mele, 2008; Olotu *et al.*, 2012).

2.3.2 Control strategies for *Pseudotheraptus wayi*

The most common control option is the application of chemical pesticides during the flushing season. Synthetic pyrethroids such as Cypermethrin and Lambda cyhalothrin ("karate") have been in use for decades and are still in use (Nyambo *et al.*, 2003; CABI, 2005; Olotu *et al.*, 2012). However, with the increasing awareness on the potential dangers of pesticides on humans, non target species and the eco-system at large attention is shifting to other safer approaches (Lopez *et al.*, 2005). The use of bio-control agents such as the African weaver ant (*O. longinoda*), have proven efficacious in various parts of Africa and are therefore being popularized (Vander plank, 1960; Way and Khoo, 1992; Van-Mele, 2008; Olotu *et al.*, 2012).

The evaluation of the African weaver ant began from an observation made as far back as 1953 by Michael Way. He observed that trees inhabited by the ant had reduced pest populations. More recently in a study by Olotu *et al.*, (2012) it was found that tree colonized by *O. longinoda* had reduced nut damage (6.2%) compared to uncolonised trees with 21%. These findings validate the African weaver ant to be an efficient control strategy for *P. wayi* in cashew orchards.

2.4 Heteropteran scent gland system

Heteropteran are considered the most successful group of hemimetabolous insects, because of their well-developed scent gland system. It is made up of metathoracic glands (MTGs) exclusive in adults and dorsal abdominal glands (DAGs) in the nymph stage (Millar, 2005; Oduor, 2007; Raska, 2009). At metamorphosis the DAGs are lost (not in all bugs) and are replaced by MTGs. They open laterally between the meso- and metathoracic legs and comprise of a pair of lateral glands (Accessory glands) that empty into a reservoir via a duct (Aldrich, 1988) . There is also another small pair of accessory glands (AG2s) that form outgrowths on the reservoir wall (Aldrich, 1988). There are two types of metathoracic scent glands based on the type of ostiole (region where MTGs; open on the surface of the coreid): diastomien and omphalien type. The diastomien, scent gland opens to the outside through more than one ostiole whereas omphalien opens through one ostiole (Durak and Kalender, 2007b). These glands are primarily developed for

chemical defense although in some species they serve both defense and pheromonal functions (Aldrich, 1988; Millar, 2005).

2.4.1 Heteropteran defence chemistry

Defense compounds produced by "true bugs" *de-novo* tend to be small and simple compounds that are shared across species, genera and even families (Millar, 2005).They are mostly mixtures of saturated or 1,3-unsaturated aldehydes, alcohols, acetate or butyrate esters of the alcohols but the blends are genus specific (Aldrich, 1988). Besides defense they may also have a role as sex pheromones used to attract the opposite sex at lower doses (Millar, 2005; Durak and Kalender, 2007b). In other species such as bugs in the family Pentatomidae, pheromones are entirely unrelated to the defensive chemicals both in chemistry and function (Millar, 2005).

2.4.2 Heteropteran pheromones

Generally pheromones are chemicals produced by animals, able to trigger a behavioral response in either the recipient, the emitter or both within a given species (Torto, 2004; Moraes *et al.*, 2008). The behavioral responses may be immediate and short lived (releaser effect) or long term and irreversible (primer effect) (Torto, 2004). Activities of releaser pheromones are mediated through neural pathways to give rise to motor output while those of primer pheromones are mediated through endocrine pathways (Torto, 2004; Vogt, 2005; Oduor, 2007). Releaser pheromones are classified according to the type of behavior they evoke; sex attraction, alarm, dispersal and aggregation (Torto, 2004; Oduor, 2007).

In Heteroptera several different pheromones have been reported and include sex attractants, aggregation and alarm. Sex attractants are produced by either male or female to mediate successful courtship and mating. Aggregation pheromones can be produced by either or both sexes to attract individuals for mating, feeding and protection. Alarm pheromones warn members of a species of impending danger (Demirel, 2007; Raska, 2009). However, the identification of Heteropteran pheromones is almost always limited by the fact that defensive secretions from the scent glands occur in such large magnitudes, that they tend to overwhelm pheromone components (Millar, 2005).To overcome this limitation, it is important to make a distinction between pheromone and defense compounds or establish their dual function. Techniques such as gas chromatography coupled to a mass spectrometry detector (GC-MS) can be used to analyze chemical contents of the scent glands which can then be compared to volatiles trapped from live adult insects (Durak and Kalender, 2007a).

2.5 Anti-herbivore plant defenses

Plant defenses in response to herbivory may be constitutive or inducible and have either direct or indirect effects on the herbivore (Arimura *et al.*, 2005; Hare, 2011; Mithöffer and Boland, 2012). Direct defense targets the aggressor and manifests in the form of morphological phenotypes such as thorns, prickles, glandular trichomes (may function as both mechanical and chemical barriers), waxes, increased leaf toughness among others (Howe and Schaller, 2008; Mithöffer and Boland, 2012).

Additionally, plants also produce secondary metabolites that may cause unpalatability of the plant or act as anti-digestives and even toxins (Howe and Schaller, 2008). There exists a diverse array of compounds which include cyanogenic glycosides, glucosinolates, terpenoids, alkaloids and latex among others (Mithöffer and Boland, 2012).

On the other hand, indirect plant defenses are important in the recruitment and sustenance of natural enemies (Turlings and Wäckers, 2004). Recruitment is based on the production of Volatile organic compounds (VOCs') that constitute mainly terpenoids, fatty acid derivatives and some aromatic compounds (Hare, 2011; Mithöffer and Boland, 2012). They signal these beneficial organisms to intervene on behalf of the plant during biological challenge by activities such as herbivore feeding, egg deposition and even mechanical damage (Mithöffer and Boland, 2012). Furthermore, these defenses are not only limited to above ground herbivory but have also been demonstrated in below ground herbivory (Bezemer *et al.*, 2003; Van -Dam, 2009; Mithöffer and Boland, 2012).

Sustenance of the natural enemies is facilitated by adaptations such as provision of shelter (domatia) and food sources such as nectar from extra-floral nectaries and food bodies (Turlings and Wäckers, 2004; Arimura *et al.*, 2005). In exchange these natural enemies provide in some cases nutrition but most commonly, they provide anti-herbivore defense (Oliveira *et al.*, 1999; Turlings and Wäckers, 2004).Such

phenomena have been mainly documented with regard to ants where the plants they associate with are regarded as myrmecophilic ("ant loving") (Koptur and Truong, 1998; Cuautle *et al.*, 1998; Heil and Mckey, 2003; Turlings and Wäckers, 2004). These interactions are regarded as ant-plant mutualisms and there are many documented cases (Boucher *et al.*, 1982; Heil and Mckey, 2003; Heil, 2007; Pringle *et al.*, 2012).

EFNs' are nectar rich secretory organs that can be found virtually on any vegetative part of a plant but are not involved in pollination (Arimura *et al.*, 2005). The nectar is mainly constituted of sugars, amino acids and lipids (Gonzalez-Teuber and Heil, 2009). It is produced specifically for the benefit of mutualists such as ants and is therefore a distinctive feature of ant-plant mutualisms (Heil and Mckey, 2003; Turlings and Wäckers, 2004; Gonzalez-Teuber and Heil, 2009). The mechanisms of secretion are not clear since the suggestions that it is both a passive and active process are contradictory. Furthermore the process by which insects locate these organs is also not very clear but it is hypothesized that it could be visually guided since some EFNs' are colored. Additionally herbivore induced plant volatiles (HIPVs') may also serve as attractants and a similarity in the biochemical signaling pathways of both HIPVs' and EFN expression has been suggested (Arimura *et al.*, 2005).

2.6 *Oecophylla longinoda*

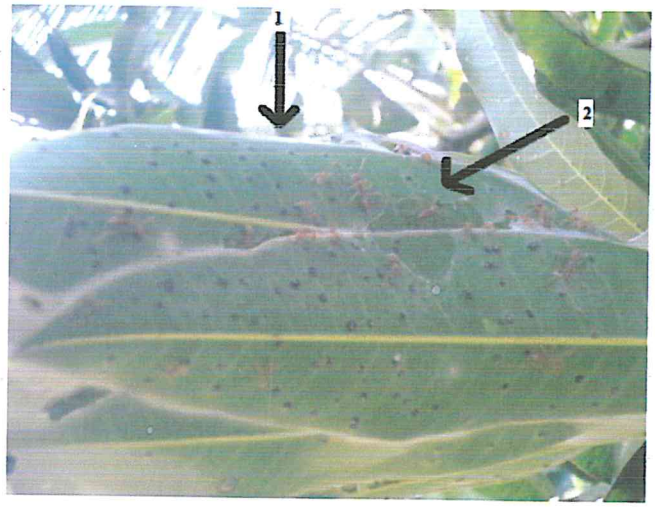


Plate 2.4: (1) *O. longinoda* nest woven by gluing leaves together (2) ants patrolling the nest site © C.W. Kung'u

Oecophylla longinoda (Latrielle) belongs to the insect order Hymenoptera, family Formicidae and is commonly known as the African weaver ant (Bradshaw *et al.*, 1979a; Olotu *et al.*, 2012). It is so called because it lives in nests that are woven by gluing leaves together (Plate 2.4) using silk that is derived from the developing larvae (Morgan, 2008). It is exclusive to the African region while its counterpart *O.smaragdina* (Fabricius) is found in Asia and Australia (Crozier *et al.*, 2010).

Weaver ants are a common feature of bio-control programs in cashew, coconut, mango cocoa and citrus both in Africa and other parts of the world where they are found (Van- Mele and Vayssières, 2007; Van- Mele *et al.*, 2007; Van -Mele, 2008; Olotu *et al.*, 2012). Their success is attributed in part to their highly territorial nature as well as their complex chemical and tactile defensive arsenal (Bradshaw *et*

al., 1979a; Bradshaw *et al.*, 1979 b). It allows them to aggressively protect their habitats from invaders most importantly insect herbivores (Morgan, 2008).

2.6.1 *Oecophylla*- cashew mutualism

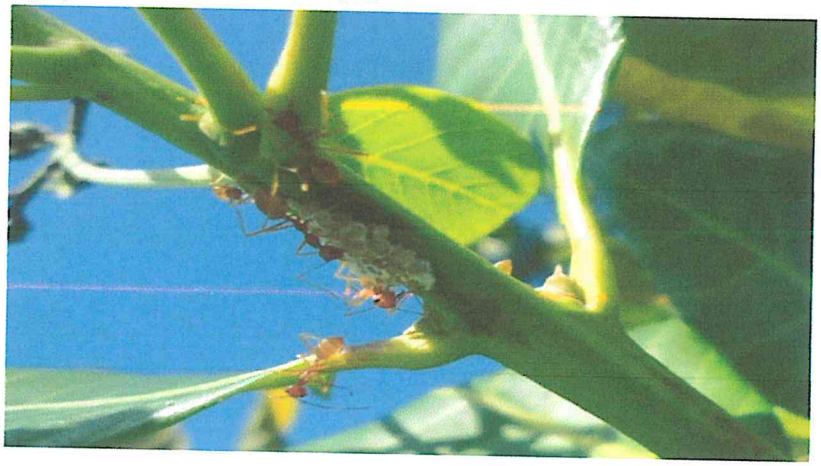


Plate 2.5: *O. longinoda* tending to hemipterans on the cashew plant

© C.W. Kung'u

Mutualisms involve two organisms that associate either directly or indirectly but the relationship is beneficial to both. In direct mutualisms species interact physically and the association is basically built on the exchange of benefits (Boucher *et al.*, 1982). In the context of ant-plant mutualisms, provision of shelter and or food in the form of EFNs', food bodies or hemipterans that are exploited by ants for their sugar and amino acid rich honey dew (Plate 2.5), appears to be the requisite for these associations(Heil and Mckey, 2003; Heil, 2007 ; Mithöffer and Boland, 2012).

This seems to be the case with the interaction between the cashew tree (*Anacardium occidentale*) and *Oecophylla* ants. The tree is ant visited all year round and *Oecophylla* is the most pre-dominant species (Way, 1953; Rickson and Rickson, 1998). Furthermore the tree expresses extra floral nectaries on young leaves, inflorescence and fruit (plant parts are considered most vulnerable to herbivory) (Wijit, 1991; Rickson and Rickson, 1998). These EFNs' are exploited by the ant visitors and in the process they counter any herbivores that may be feeding on the plant (Wijit, 1991; Way and Khoo, 1992). Additionally the plant also accommodates hemipterans that produce sugar and amino acid rich honey dew also for the benefit of the ants (Rickson and Rickson, 1998).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Research site

The study was carried out at the Behavioral and Chemical Ecology Department (BCED) of *icipe*- Duduville campus, Nairobi Kenya (1°13'16"S 36°53'46"E). The insect populations under study were reared in both indoor and outdoor insectaries at the Animal Rearing and Containment Unit (ARCU-*icipe*) according to the in-house standard operating procedures.

3.2 Insects

3.2.1 *Pseudotheraptus wayi*

Insects were obtained from an existing colony established from field collected insects in 2010.They were reared in 46×46×46 cm perspex cages housed in an indoor insectary at 27-28°C, 60-85% relative humidity, lighting regime of 12:12 hours light: dark and fed on French beans and water.

3.2.3 *Oecophylla longinoda*

The colony was established from weaver ant nests collected in 2012 from the Mtwapa area (3.9500° S, 39.7444° E) in Kikambala divison of Kilifi district in the coast province of Kenya. The insects were then transported to an outdoor insectary in the Animal Rearing and Containment Unit at *icipe*, Duduville campus. They were inoculated on mango seedlings (≈ 3½ ft high) and allowed to habituate and weave fresh nests. The temperature of the insectary was in the range of 24-28°C with relative humidity between 55-65%. They were maintained on a diet of

6% sugar water and powdered fish meal (silver cyprinid- "omena") as a protein source.

3.3 *P. wayi -O. longinoda* interaction

Six adult *P.wayi* males, six adult females and six 5th instar nymphs were divided equally into test and control groups. Each group was introduced into a glass vial (2.5cm × 7.5cm) and allowed to settle. Three ants were then carefully introduced into each of the vials which were then secured using a piece of perforated aluminum foil (Chandaria Ind. Ltd, Nairobi, Kenya) and a rubber band to allow for air circulation. A Supelco SPME fiber, (Sigma-Aldrich, Supelco Park, Bellefonte, PA, 16823 USA) was then inserted into the aluminum cover to adsorb head space volatiles for 15 minutes. After the 15 minutes the SPME fibers were withdrawn from the vials and chemical analysis of the volatiles carried out using Gas Chromatography-Mass Spectrometry (GC-MS).

3.4 Collection and preparation of scent gland secretions

3.4.1 Adult metathoracic glands (MTGs')

Insects were anesthetized in CO_2 (BOC Kenya ltd) for 2 minutes and killed by freezing at -20°C. The appendages were then removed and the body pinned ventral side up on a paraffin wax dissecting platform. A sharpened micro capillary tube (75mm/75µl D.A 1.5-1.6mm, Hirschmann, West Germany) slightly broken at the tip was used to pierce between the 3rd and 4th thoracic segment. An aliquot (2µl) of a clear fluid was drawn and diluted in 1ml hexane (Analytical grade -Sigma Aldrich, 3050 Spruce street St. Louis, MO). The extract was then passed through a

Pasteur pipette (Sigma-Aldrich) loaded with magnesium sulphate (Roth GmbH) and a glass wool plug. The eluate was collected in a glass vial and stored at - 20°C for chemical analysis in GC-MS.

3.4.2 Nymphal dorsal abdominal glands (DAGs')

P wayi nymphs were anaesthetized in CO_2 for 2 minutes and killed by freezing at -20°C. The insects were pinned dorsal side up on a paraffin wax dissecting platform. A sharpened micro capillary tube (75mm/75µl D.A 1.5-1.6mm, Hirschmann, West Germany) was used to pierce the specialized cuticular area that appears like two nodules. An aliquot (1-2µl) of a clear fluid was withdrawn and diluted in 1ml analytical grade hexane (Analytical grade -Sigma Aldrich, 3050 Spruce street St. Louis, MO). The extract was then passed through a Pasteur pipette (Sigma-Aldrich) loaded with magnesium sulphate (Roth GmbH) and a glass wool plug. The eluate was collected in a glass vial and stored at - 20°C for GC-MS analysis.

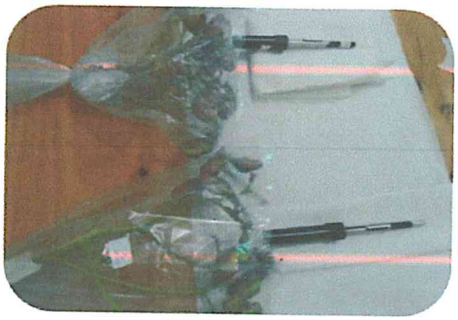
3.5 Collection and preparation of *P. wayi* head space volatiles

The head space volatiles of male and female *P.wayi* obtained from a mixed colony were collected consistently for 40 days. Collection began at 4 days after adult emergence and was done consistently at an interval of 4 days throughout this period. Volatiles were collected on Super Q from 6pm-6am (Scotophase) which is the peak period for *P.wayi* mating (Egonyu, 2013). Super Q was then eluted with 200µl hexane (Analytical grade -Sigma Aldrich, 3050 Spruce street St. Louis, MO) and sample stored at -20°C for GC-MS analysis.

3.6 Collection and preparation of cashew head space volatiles

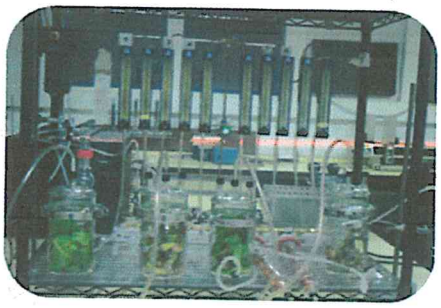
Outdoor volatile collection pump

(a)



Solid Phase Microextraction (SPME)

(b)



Indoor volatile collection equipment

(c)

Gas chromatography - mass spectrometry

(d)

Plate 3.1: Volatile collection and chemical analysis instruments © C. W. Kung'u

Headspace volatiles were collected from cashew plants (Plate 3.1 (a)) growing at the Kenya Agricultural Research Institute (KARI) orchards in the Mtwapa area of Kilifi district in the coastal region of Kenya (3.9500°S and 39.7444°E). Volatiles were collected from leaves, fruit and inflorescence on Super Q and Supelco SPME fibers (Sigma-Aldrich, Supelco Park, Bellefonte, PA, 16823 USA) for comparative purposes (Plate 3.1 (b) and (c)). About 12-15 leaves, 9-12 fruits and 10-15

inflorescences were bagged (large size oven bags 355mm×508mm- classic consumer products INC.266 South Dean street, Engle wood, NJ,USA) separately and volatiles collected for 12hours (Super Q) and 8 hours (SPME) . Each plant part was sampled four times and in every instance a different plant was used. Volatiles trapped on Super Q adsorbent were eluted using 500 µl dichloromethane (Analytical grade -Sigma Aldrich, 3050 Spruce street St. Louis, MO) and stored at -80°C (New Brunswick refrigerator, U725-86G model) for use in GC-MS analysis (Plate 3.1 (d)) and bioassays. Each individual sample contained 12 plant hour equivalents (PHE) where 1PHE= volatiles emitted by the part sampled on each individual plant.

3.7 GC-MS analysis

Volatiles collected in all the experiments were analyzed by coupled gas chromatography-mass spectrometry on an Agilent technologies series A 7890 GC coupled to a 5975C (inert XL/EI/CI MSD) triple axis mass detector, equipped with a HP-5MS column 30 m× 250 µm×0.25 µm in the electron impact mode at 70 eV. The GC oven temperature was 35°C for 5min with a rise of 10°C/min to 280° C for 10.5min then 50°C/min to 285°C and held at this temperature for 9 min. Identification of compounds was done by comparison of mass spectral data with library data (Adams2 and NIST05) .Confirmation was done by using authentic standards under the same GC-MS analytical conditions employed for analysis of the crude volatiles.

3.8 Cashew- *O. longinoda* interaction

3.8.1 Y-tube olfactometer assay

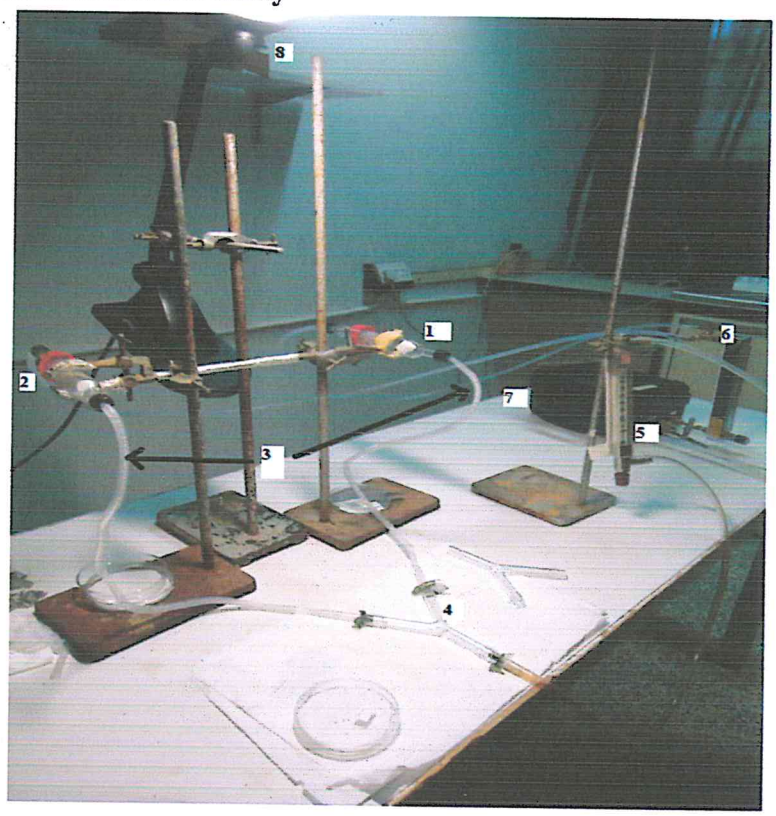


Plate 3.2: (1) Test arm (2) Control arm (3) Teflon tubes (4) Y-tube (5) Vacuum flow meter (6) Air supply flow meter (7) Battery powered pump (8) Light source© C.W. Kung'u

A Y-tube olfactometer set up in plate 3.2 (arm length: 7×7cm, internal diameter: 1cm) was used with one arm connected to the test odor (0.6 PHE of the crude extract of cashew volatiles dissolved in 25µl DCM and loaded on 3×3cm Whatman filter paper N^{o} 1) and the other connected to a control (a similar volume of solvent also loaded on a similar size and type of filter paper) via Teflon tubes. An energy-saving bulb (Philips stick -11W) emitting white light was suspended 55cm above

the bioassay arena to provide illumination. Charcoal-filtered clean air was passed through the Teflon tubes into each arm of the olfactometer at a flow rate of 348 ml/min and pulled out of the main arm of the olfactometer at 348 ml/min by a battery powered portable vacuum pump. Individual worker ants were released in the main arm of the Y-tube and allowed to settle for 5 min. The pump system was turned on to test ant preferences to the test odor and the control. Preference was determined when the insect traversed the entire stem of the olfactometer and another additional 2cm into either arm which was determined using a line marked on both arms. Each ant was allocated a total of 5 minutes in the olfactometer and the cumulative amount of time spent at each arm recorded. Odor preference for each plant part was tested against 10 individual ants and the experiment replicated five times in each case.

3.8.2 Olfactory - reward assay

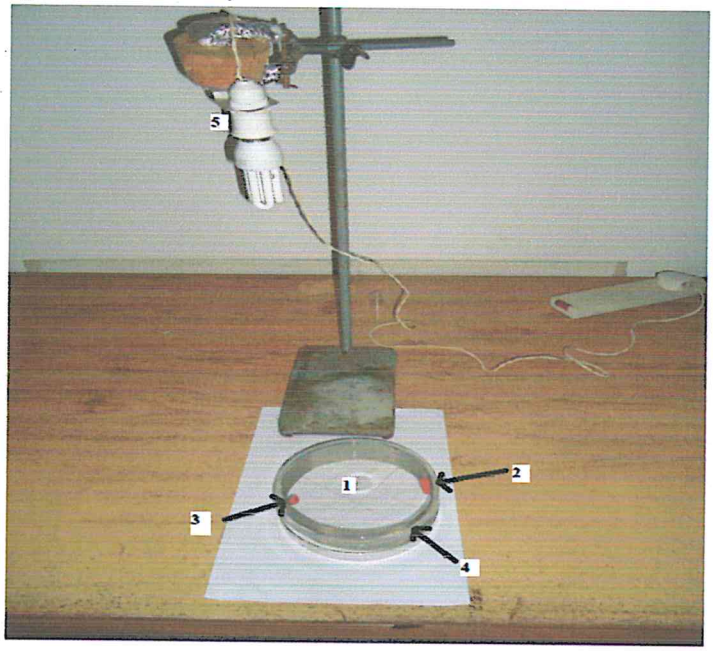


Plate 3.3: Olfactory-reward assay; (1) Petri dish arena (2) Rubber septum with test odor (3) Rubber septum with control odor (4) Wire gauze (5) light source

The bioassay was carried out in a glass petri dish, 15 cm wide x 3.5 cm high lined with a circular wire mesh screen (mesh size ≈ 1.19mm) and divided into four equal quadrants (Plate 3.3). Two quadrants were designated as test and control whereas the remaining two were left as free zones. 0.6 PHE of the crude extracts of volatiles dissolved in 25µl DCM was dispensed on a rubber septum and 50µl dichloromethane on another rubber septum. Both septa were left to stand for about 5minutes to allow the solvent to vaporize. The septum loaded with crude extract of cashew volatiles, was suspended on the wire gauze in the test quadrant of the bioassay arena using a pin and the other in the control quadrant. Ten droplets of

glucose syrup (2 parts glucose: 1 part water w/w (200% glucose syrup) were applied on the wire mesh in both test and control quadrants using a fine camel hair brush. At this concentration each droplet of the sugar syrup was held on the mesh screen without flow. An ant held in a small glass vial (21mm× 34mm) was then placed at the centre of the bioassay arena and allowed to crawl out of the vial into the arena after which the vial was then removed and the arena covered. Each ant was allocated 10 mın to walk around the arena. The time spent and the frequency of sugar feeding in the test and control quadrant was then recorded. Each odor was presented together with sugar syrup droplets and tested against 10 individual ants and the experiment replicated five times for each odor. The numbers of feeding attempts by each individual ant in either or both the test and control quadrant were also recorded.

3.9 Data management and analysis

GC-MS data was recorded in Microsoft Excel 2007 in form of Instrument responses (peak areas) of compounds identified in the analysis of crude head space volatiles. A representative authentic standard for each chemical group identified in the extracts was selected. Five samples of known concentrations of the standard were prepared and analyzed in GC-MS using the same conditions employed for the crude extracts. The peak areas of the standards were then plotted against their respective concentrations. Linear correlations were generated for the plots and only those with a correlation co-efficient (R^2) of $\geq$ 0.9 were acceptable. The concentrations of the compounds identified in the crude volatile extracts were then

determined by extrapolating their peak areas in the calibration curves generated using the authentic standards. In the olfactometer assays the responses of *O. longinoda* to cashew volatiles (presented singly and with to a reward) and control odors were also recorded in Microsoft Excel 2007. The data was then imported to the R version 2.15.1 statistical software (Core team, 2012) where pair wise comparisons of the responses of *O. longinoda* to the individual cashew odors and the control odor was assessed using students *t*-test (α=0.05). One-way ANOVA (α=0.05) and post-hoc analysis (Tukey's test α=0.05) were then used to compare the difference in responses to the leaves, fruit and inflorescence odor when presented alone and together with sugar rewards. Similarly student-*t* test (α=0.05) as well as a one-way ANOVA (α=0.05) was used to assess the influence of cashew volatiles on the frequency of sugar feeding in the rewards assay

CHAPTER FOUR

RESULTS

4.1 *Pseudotheraptus wayi*

4.1.1 Life cycle

The first stage in the life cycle is the egg. These are pale yellow in colour and are oval shaped measuring approximately 1.07 mm (Plate 4.1). The average development time from egg to adult was 35 days at 27-28°C and 67-80% relative humidity and progressed through five successive instar stages (Plate 4.2). In the egg stage, fertilized eggs were distinguished from sterile ones by the presence of a red eye spot visible to the naked eye. Hatching took place within 3-8 days after oviposition. The transition time for the subsequent instar stages was; 1st instars (2-4 days), 2nd instars (4-10 days), 3rd instars (2-9 days), 4th instars (2-7 days) and 5th instar (2-6 days).

Plate 4.1: *P. wayi* eggs © C. W. Kung'u

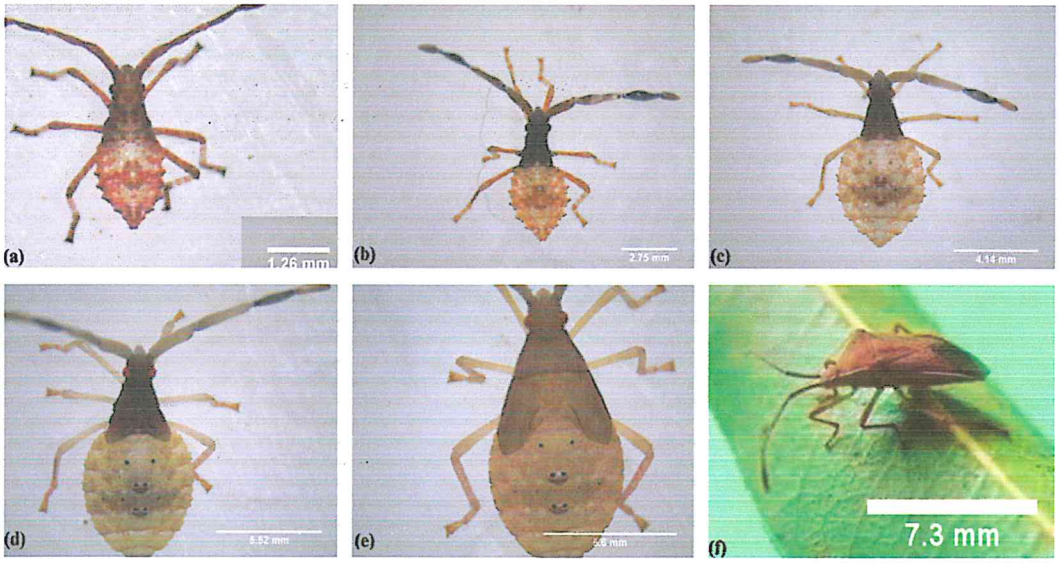


Plate 4.2: a, b and c (1st, 2nd and 3rd instar), d, e and f (4th, 5th instar and adult insect) © C. W. Kung'u

4.1.2 Survival of immature *P. wayi* in laboratory conditions

The progression of immature *P. wayi* through the various instar stages was monitored and recorded over a period of 7 months as shown in Figure 4.1 (n=100). Mortality rates of 1st and 3rd instar stages were found to be consistently higher (60 and 80% respectively) compared to the other stages in all the months except May where 100% survival of 3rd instars was recorded.

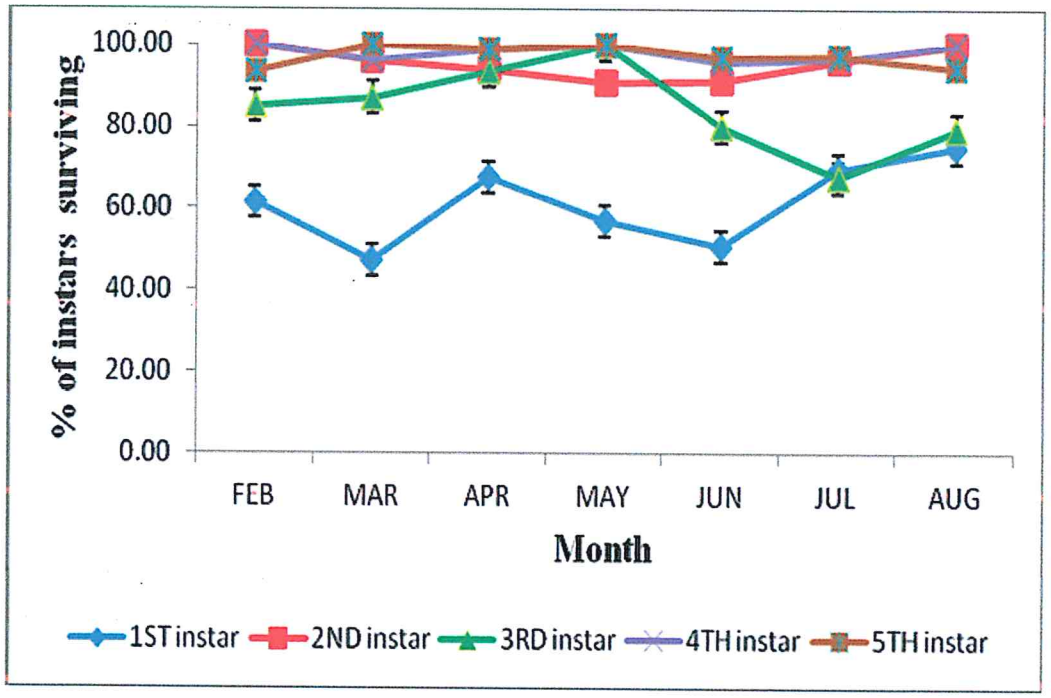


Figure 4.1: Survival of 1st to 5th instar *P. wayi* stages in laboratory conditions

4.2 *P. wayi – O. longinoda* interaction

4.2.1 Chemical analysis of head space volatiles

The interaction between both adult sexes of *Pseudotheraptus wayi* (prey) and *O. longinoda* (predator) were observed to occur in two distinct forms ; (a) compromise where both prey and predator co-existed and (b) combat where the predator viciously attacked the prey. The chemical compounds identified in the head space volatiles during combat were (1) hexanal (2) hexanol (3) hexyl acetate (4) hexanoic acid (5) octanol (6) undecane (7) hexyl butanoate (8) 2-butyl, octen-2-enal (9) octyl acetate (10) hexyl hexanoate (11) heptyl hexanoate (exclusively in females) and octyl hexanoate (12) (exclusively in males) (Figure 4.2 and 4.3). During compromise, hexanal (1), hexanol (2), hexyl acetate (3), octanol (5), undecane (6), 2-butyl, oct-2-enal (8) and octyl acetate (9) were detected in females whereas only hexyl acetate (3) and undecane (6) were detected in males (Figure 4.2 and 4.3). The GC-MS output, retention times (RT) and structural identities of these compounds are shown in Figures 4.2, 4.3 and Table 4.1.

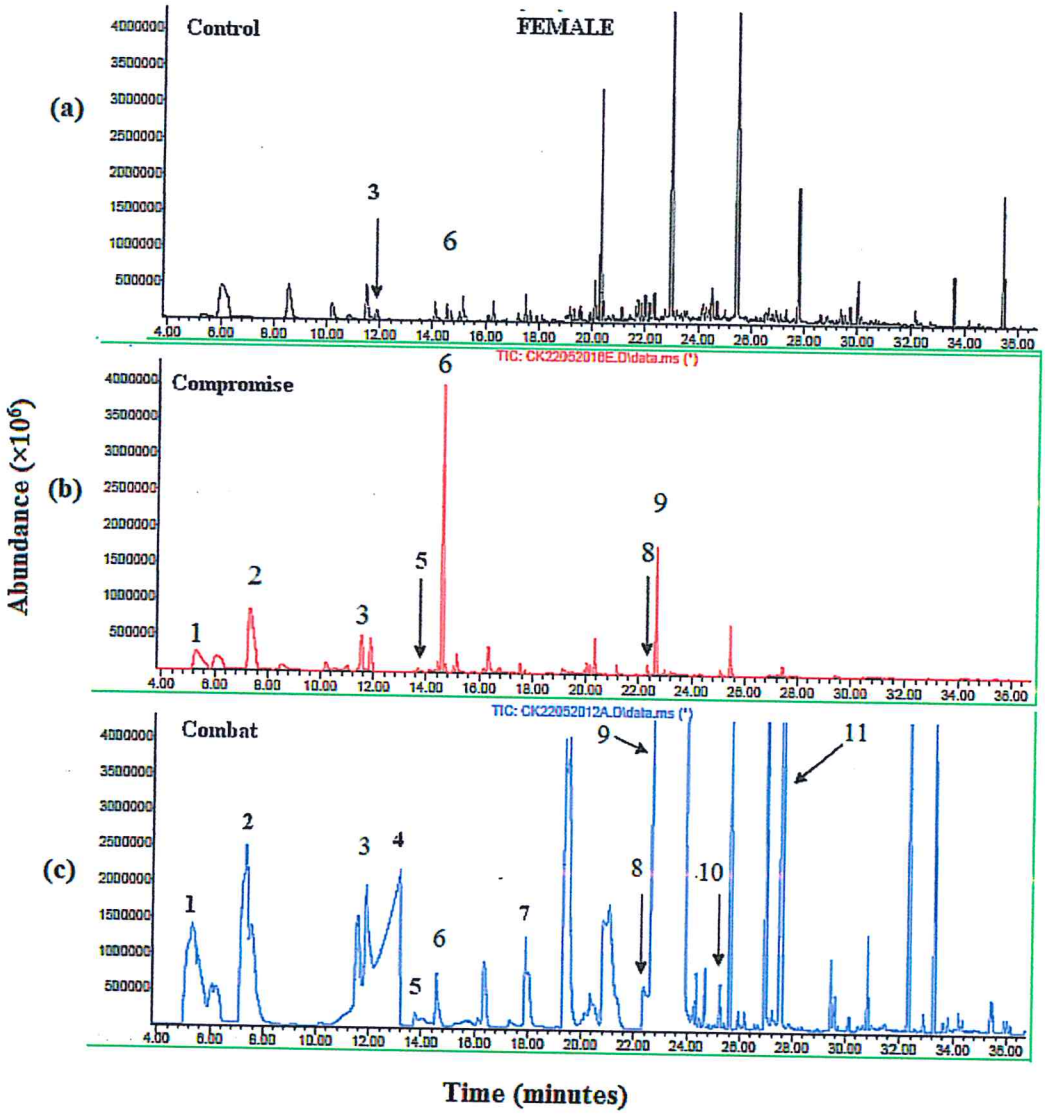


Figure 4.2: GC-MS chromatograms comparing the head space volatiles in the compromise and combat interactions between female adult *P. wayi* and *O. longinoda* ; (1) hexanal (2) hexanol (3) hexyl acetate (4) hexanoic acid (5) octanol (6) undecane (7) hexyl butanoate (8) 2-butyl, octen-2-enal (9) octyl acetate (10) hexyl hexanoate (11) heptyl hexanoate

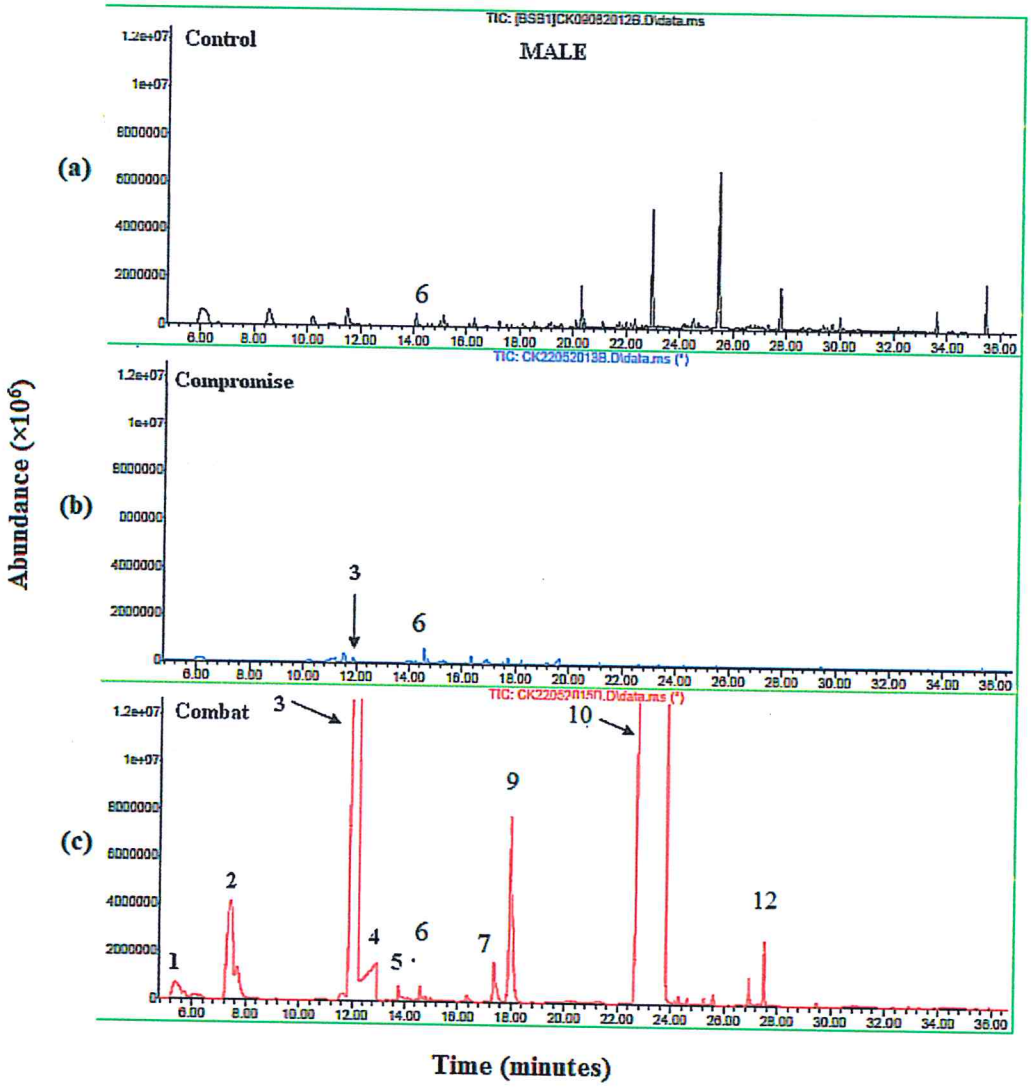
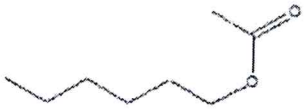
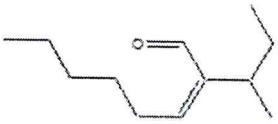
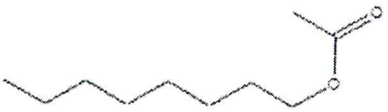
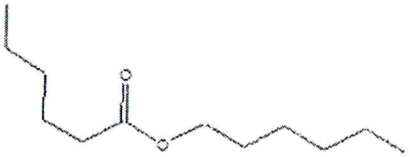
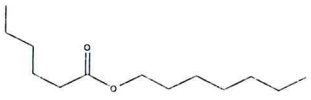
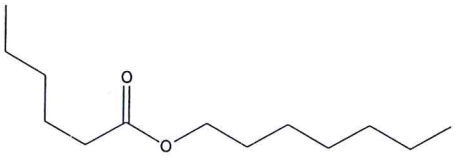


Figure 4.3: GC-MS chromatograms comparing the head space volatiles in the compromise and combat interaction between adult male *P. wayi* and *O. longinoda* ; (1) hexanal (2) hexanol (3) hexyl acetate (4) hexanoic acid (5) octanol (6) undecane (7) hexyl butanoate (9) octyl acetate (10) hexyl hexanoate (12) octyl hexanoate

Table 4.1: Summary table on the chemical and structural identities of the compounds detected in the head space volatiles during both compromise and combat interactions between both sexes of adult prey and predator

No	Chemical ID	RT(min)	Structure
1	Hexanal	5.308	
2	Hexanol	7.329	
3	Hexyl acetate	11.874	
4	Hexanoic acid	13.148	
5	Octanol	13.757	
6	Undecane	14.551	
7	Hexyl butanoate	17.326	
8	2-butyl,oct-2-enal	22.361	
9	Octyl acetate	17.896	
10	Hexyl hexanoate	23.245	
11	Heptyl hexanoate	25.261	
12	Octyl hexanoate	27.574	

The interaction between immature prey (instars) and predator was exclusively combative. The chemical compounds detected in the head space were; (*E*)-hex-2-enal (1), (*E*,*E*)-hex-2,4-dienal (2), (*E*)-oct-2-enal (3) and 2-cyclohexen-1,4-dione (4) as illustrated in the GC-MS chromatogram in figure 4.4. The retention times of the compounds as well as their structural identities are summarized in table 4.2.

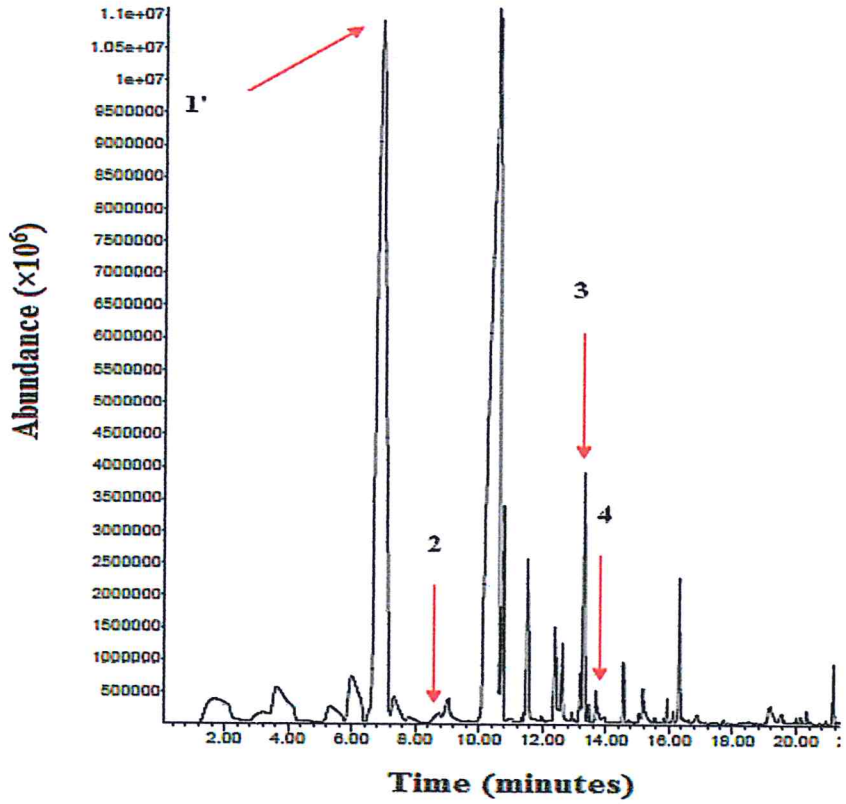


Figure 4.4: GC-MS chromatogram of the head space volatiles in the interaction between immature *P. wayi* and *O. longinoda*; (1) (*E*)-hex-2-enal (2) (*E*, *E*)-hex-2, 4-dienal (3) (*E*)-oct-2-enal (4) 2-cyclohexen-1,4-dione

Table 4.2: Chemical and structural identities of compounds identified in the combat interaction between immature prey and predator

No.	Chemical ID	RT(min)	Structure
1	(*E*)-hex-2-enal	7.194	
2	(*E*, *E*)-hex-2,4-dienal	8.781	
3	(*E*)-oct-2-enal	13.357	
4	2-cyclohexen-1,4-dione	13.740	

Chemical analysis of the head space volatiles in whole insect extracts of the predator (*O. longinoda*) identified, hexanal (1), hexanol (2), hexanoic acid (3) hexyl acetate (4) octanol (5) undecane (6) octanoic acid (7) and hexyl hexanoate (8) as shown on the GC-MS chromatogram in Figure 4.5. The retention times and structural identities of the compounds are summarized in table 4.3.

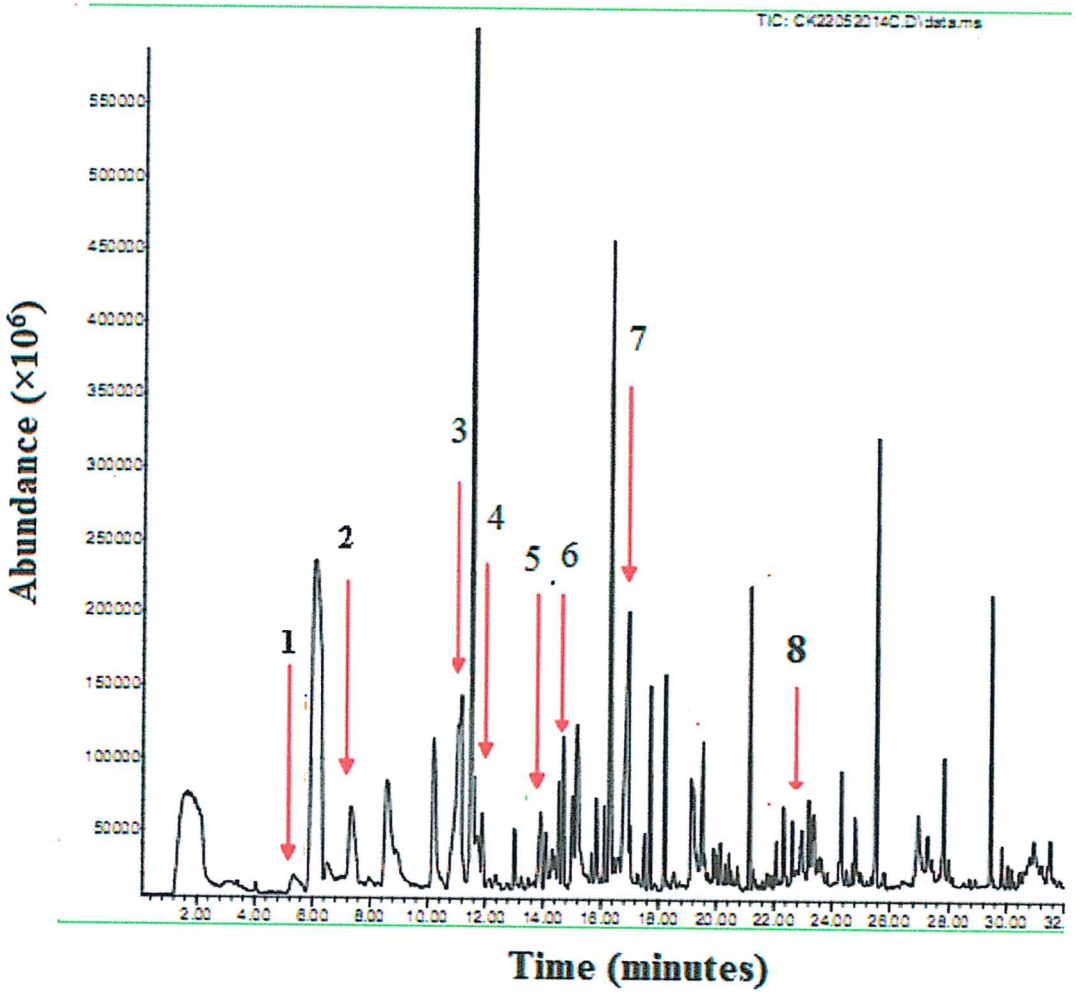
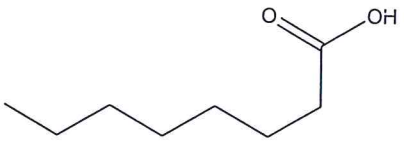
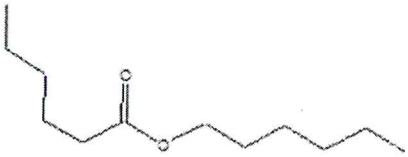


Figure 4.5: GC-MS chromatogram of the head space volatiles of *O. longinoda* whole insect extracts; (1) hexanal (2) hexanol (3) hexanoic acid (4) hexyl acetate (5) Octanol (6) undecane (7) octanoic acid (8) hexyl hexanoate

Table 4.3: Chemical and structural identities of compounds identified in GC-MS analysis of head space volatiles of *O. longinoda* whole insect extracts

No.	Chemical ID	RT(min)	Structure
1	Hexanal	5.371	
2	Hexanol	7.384	
3	Hexanoic acid	13.148	
4	Hexyl acetate	11.874	
5	Octanol	13.757	
6	Undecane	14.551	
7	Octanoic acid		
8	Hexyl hexanoate	23.245	

4.2.2 Behavioral observations in the prey-predator interaction

Besides the collection of head space volatiles during the prey-predator interaction, the behaviour of both species (*P. wayi* and *O. longinoda*) was also observed and recorded. The findings are summarized in table 4.4 below.

Table 4.4: Summary of the behavioral observations recorded during the prey predator interaction

Interaction	**Behaviour**	
	Oecophylla longinoda	*Pseudotheraptus wayi*
Compromise	1. Circling the prey with mandibles wide open 2. Rubbing of forelegs using mandibles then rubbing each other's mandibles 3. Rapid antennal movement 4. Touching the prey using the antennae	1. Circling potential predator 2. Rapid antennal movements 3. Directing the posterior end towards the predator when touched by the antennae
Combat	1. Rapid snapping of the mandibles 2. Vicious biting and pulling of the limbs of the prey until paralysis occurs 3. Rapid attenation, curling positions and intense grooming.	1. Production of an odorous irritant emission detectable by the human nose 2. Paralysis and death

4.3 *Pseudotheraptus wayi* scent gland system

4.3.1 Adult metathoracic gland (MTGs')

In both sexes, the gland appears as a pale orange colored structure weighing ≈ 0.25mg and located on the ventral side between the 3rd and 4th segment in the thoracic area (Plate 4.3).

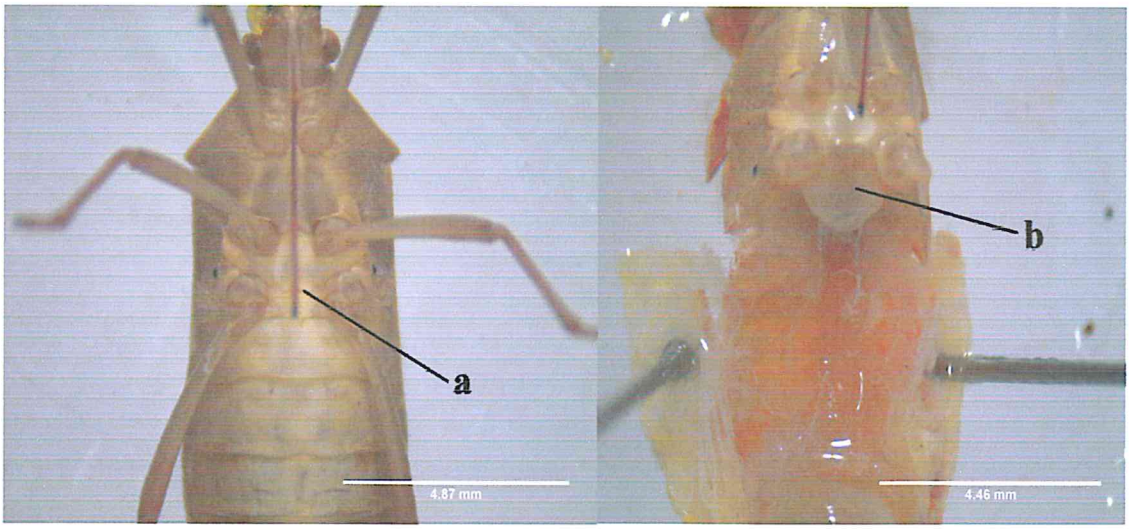


Plate 4.3: (a) 3rd and 4th thoracic segments (b) metathoracic gland attached to thoracic tissues © C. W. Kung'u

4.3.2 Nymphal dorsal abdominal glands (DAGs')

These glands were bright orange, paired, sac like structures weighing ≈ 0.1mg each. They are located underneath the fatty tissue of the abdominal cavity of all instar stages and open through a specialized area of the cuticle on the dorsal surface as illustrated in plate 4.4 below.

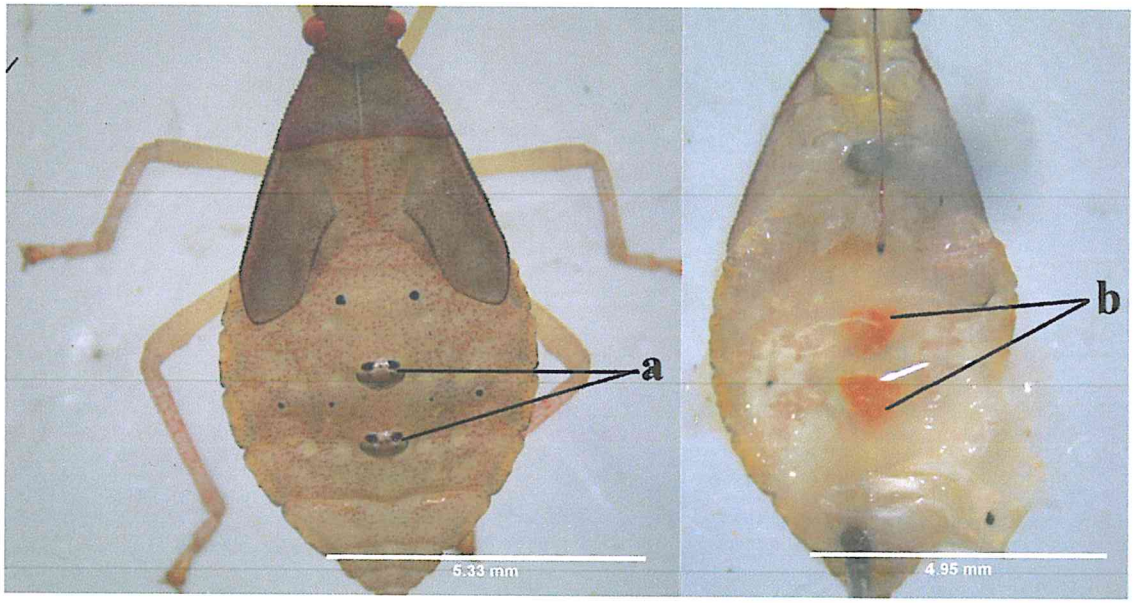


Plate 4.4: (a) dorsal abdominal gland openings (b) a pair of dorsal abdominal glands embedded on the abdominal wall underneath fatty tissues ©C. W. Kung'u

4.3.3 Chemical analysis of metathoracic gland secretions

In both adult sexes the chemical components detected were; hexanal (1), hexanol (2), hexyl acetate (3) and hexyl hexanoate (4) as illustrated in the GC-MS chromatogram in Figure 4.6. Hexyl acetate and hexyl hexanoate were the most abundant compounds in females and hexanal and hexyl hexanoate in males. Three of the four compounds detected (hexanal, hexanol and hexyl acetate) were ten-fold more in the female gland compared to the male. The retention times and structural identities of these compounds are shown in Table 4.5.

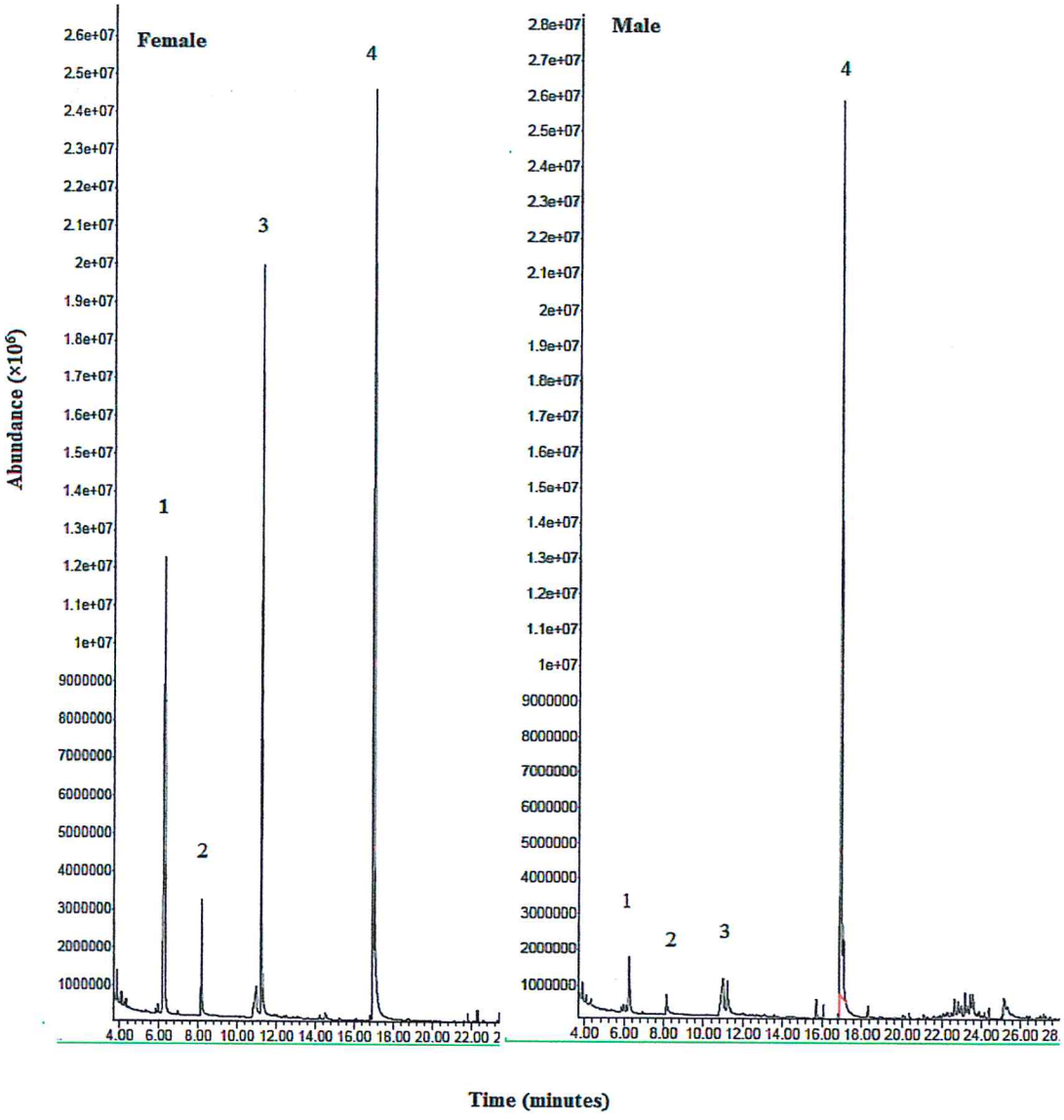
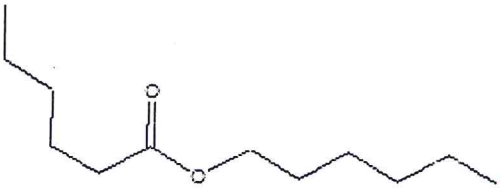


Figure 4.6: GC-MS chromatograms of adult female and male MTG secretion; (1) hexanal (2) hexanol (3) hexyl acetate (4) hexyl hexanoate

Table 4.5: Chemical and structural identities of chemical components identified in GC-MS analysis of adult female and male metathoracic gland secretions

No.	Chemical ID	RT(min)	Structure
1	Hexanal	6.3	
2	Hexanol	7.384	
3	Hexyl acetate	11.2	
4	Hexyl hexanoate	17.5	

4.3.4 Chemical analysis of dorsal abdominal gland secretions

In the DAG secretions only one compound (*E*)-hex-2-enal was identified as illustrated in the GC-MS chromatogram in figure 4.7. The retention time and the structural identity are shown in table 4.6.

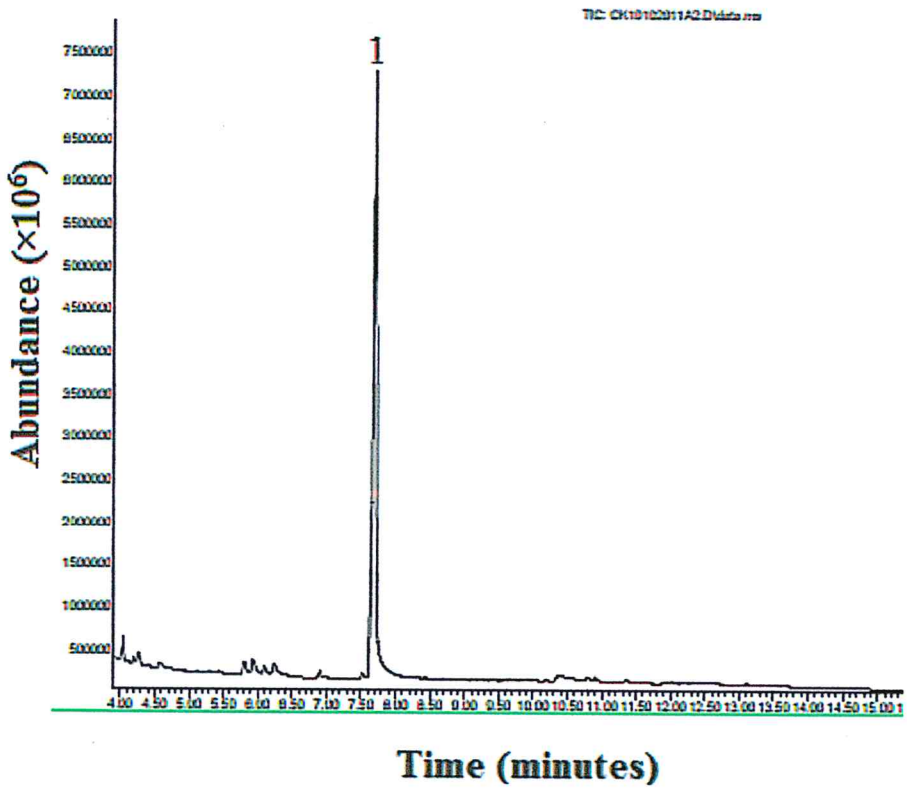
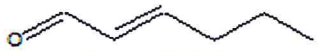


Figure 4.7: GC-MS chromatogram of the chemical components identified in the DAG secretions of immature *P. wayi* ; (1) (*E*)-hex-2-enal

Table 4.6 : Chemical and structural identity of components in the DAG secretion

No.	Chemical ID	RT(min)	Structure
1.	(*E*) –hex-2-enal	7.8	

4.4 Sexual signaling in *Pseudotheraptus wayi*

4.4.1 Age related volatile production

Over a 40 day period only four chemical components (hexanal, hexanol, hexyl acetate and hexyl hexanoate) dominated the head space volatiles collected from live adult male and female insects (Figure 4.8 and 4.9). The quantities of these

compounds were determined using calibration curves with authentic standards (Appendices 1, 2, 3 and 4). In females, the onset of volatile production was 4days, peaking at 8 days then gradually reducing and ceasing by the 16th day. After the 16th day volatile production did not occur until the 24 day after which production increased gradually before peaking again at 32 days then gradually reducing and stopping at 36 days (Figure 4.9). In males onset of volatile production was at 12 days then peaking at 16 days and finally stopping by the 20th day. At 24 days another volatile production phase began, peaking at 28 days and gradually declining through to 36 days (Figure 4.8). The most abundant compound in the first phase of volatile production in males were hexanal and hexyl hexanoate (Figure 4.8) while females were dominated by hexyl acetate and hexyl hexanoate (Figure 4.9).

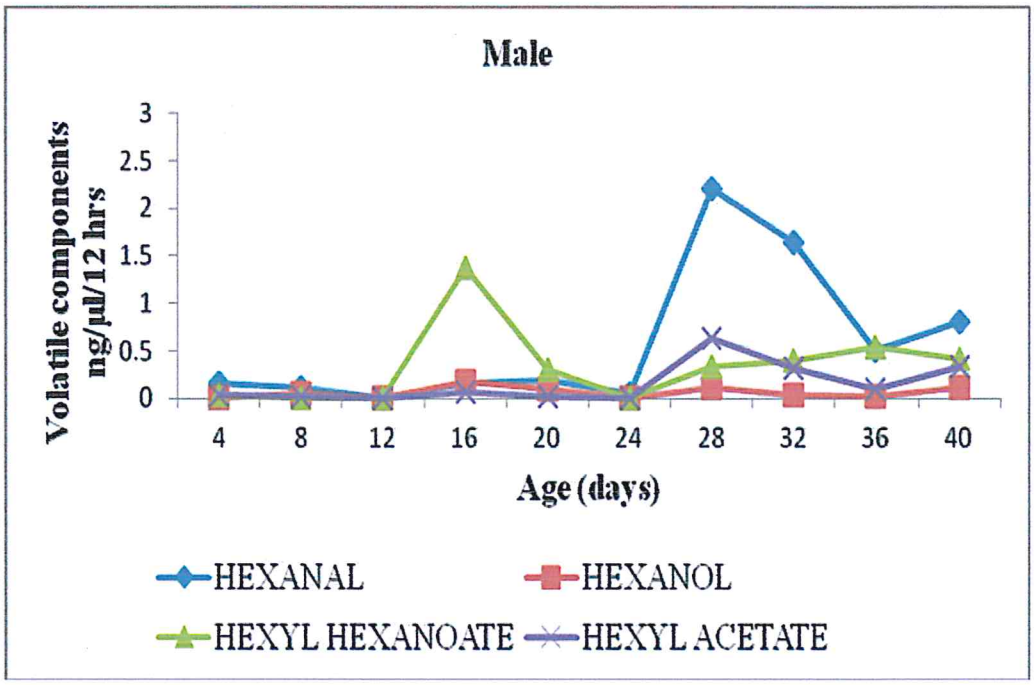


Figure 4.8: Volatile emissions of male *P. wayi* between 4 and 40 days

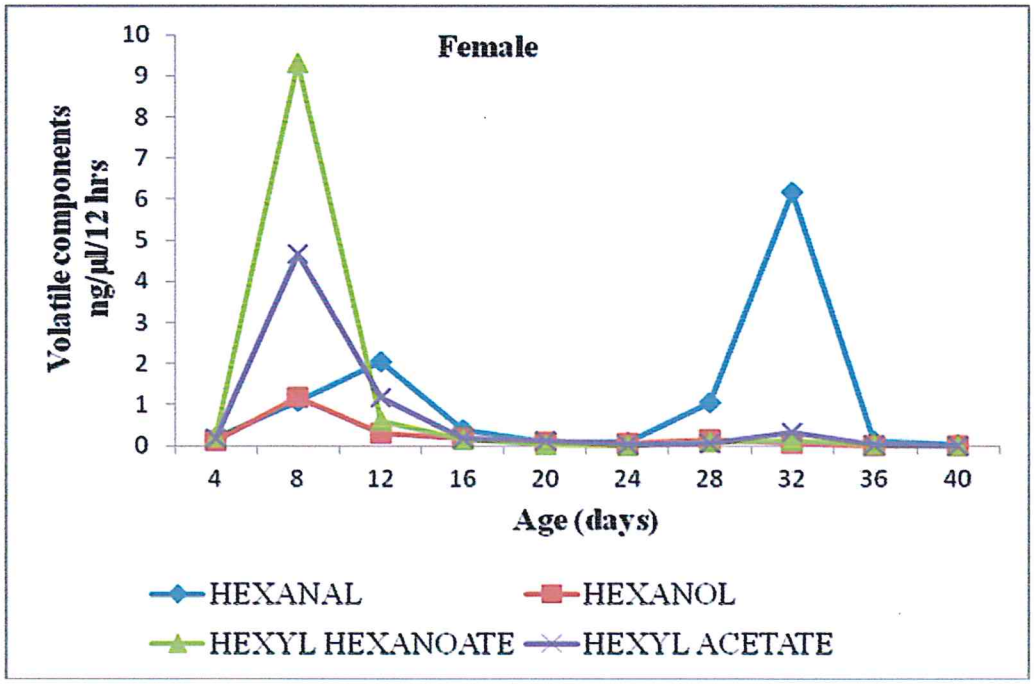


Figure 4.9: Volatile emissions of female *P.wayi* between 4 and 40 days

4.4.2 Mating pattern

The first mating pairs were observed at 8 days after emergence of adult insects. After this period the numbers gradually increased up to 16 days. After this period, there were no significant increments in the number of mating pairs until 28 days later which was then followed by a decline all through to 40 days (Figure 4.10).

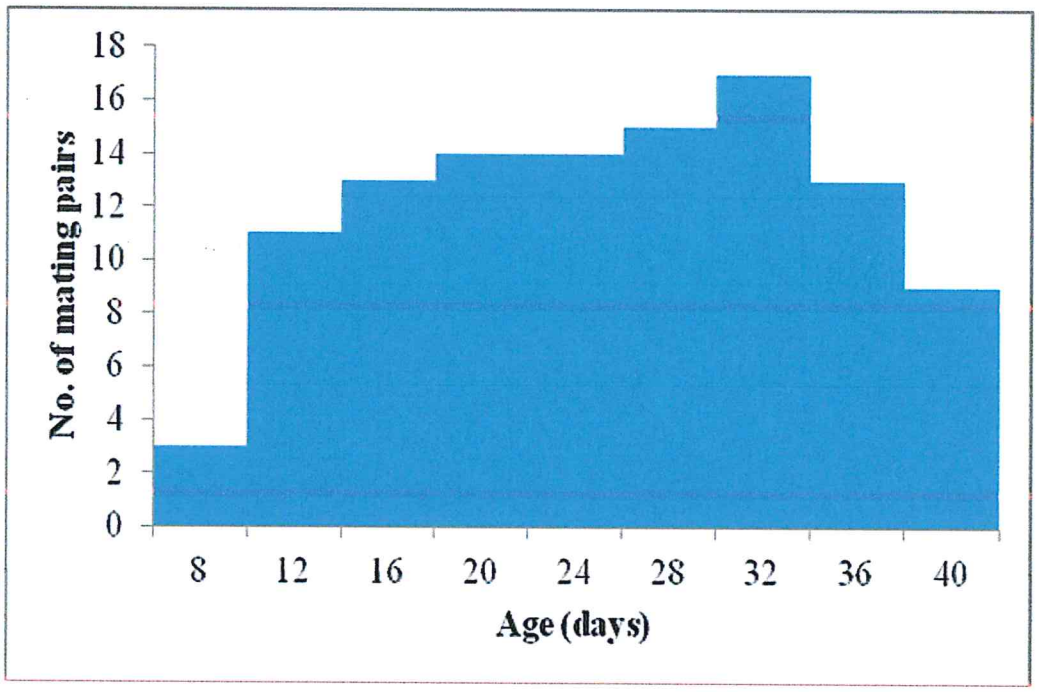


Figure 4.10: *P. wayi* mating pattern

4.5 Cashew -*O. longinoda* interaction

4.5.1 Y-tube olfactometer assay

The olfactometer responses of African weaver ant to cashew volatiles from leaves, fruit and inflorescence are shown in Figure 4.11. Using paired T test at $\alpha = 0.05$, it

was found that, the time spent in the test arm of the olfactometer by the ants was significantly higher compared to the control arm for the leaves and inflorescence (leaves/control p = 0.005159, Inflorescence/control p = 0.006289). For the fruit volatiles, despite there being no statistical significance (p = 0.2727) at the specified alpha level, ants spent a slightly higher amount of time in the test arm compared to the control. Overall the amount of time spent in the test arms for all the three test odors was not statistically different (F (2,147) = 0.123, p = 0.884).

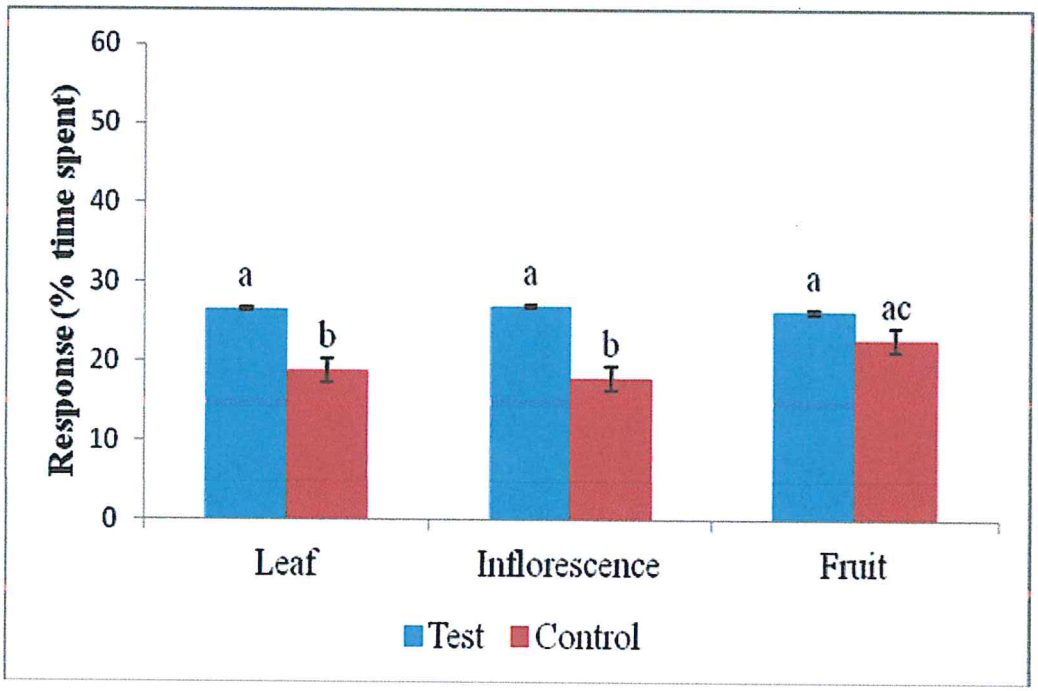


Figure 4.11: Bars represent ant responses (time spent as a percentage of the total time each individual ant is allocated in the olfactometer) to different cashew volatiles. Means with similar letters are not significantly different (p>0.05). Means with different letters are significantly different (p<0.05)

4.5.2 Olfactory- reward assay

In this assay (Figure 4.12) the time spent within the test and control quadrant was significantly different at α 0.05 (Leaves/Control p = 2.81e-06, Inflorescence/Control p = 1.019e-09, Fruit/Control p = 1.787e-11). However when responses to the three test odors were compared there was no significant difference at α 0.05 (F (2, 147) = 1.916, p = 0.151) (Figure 4.12). Interestingly the responses in the presence of cashew volatiles alone (Figure 4.11) compared with cashew volatiles paired to sugar reward (Figure 4.12) differed significantly α 0.05 (F (5, 294) = 16.54, p = 2.17e-14). Post-hoc analyses at α 0.05 indicated significant differences in all the test odors ($p < 0.001$)

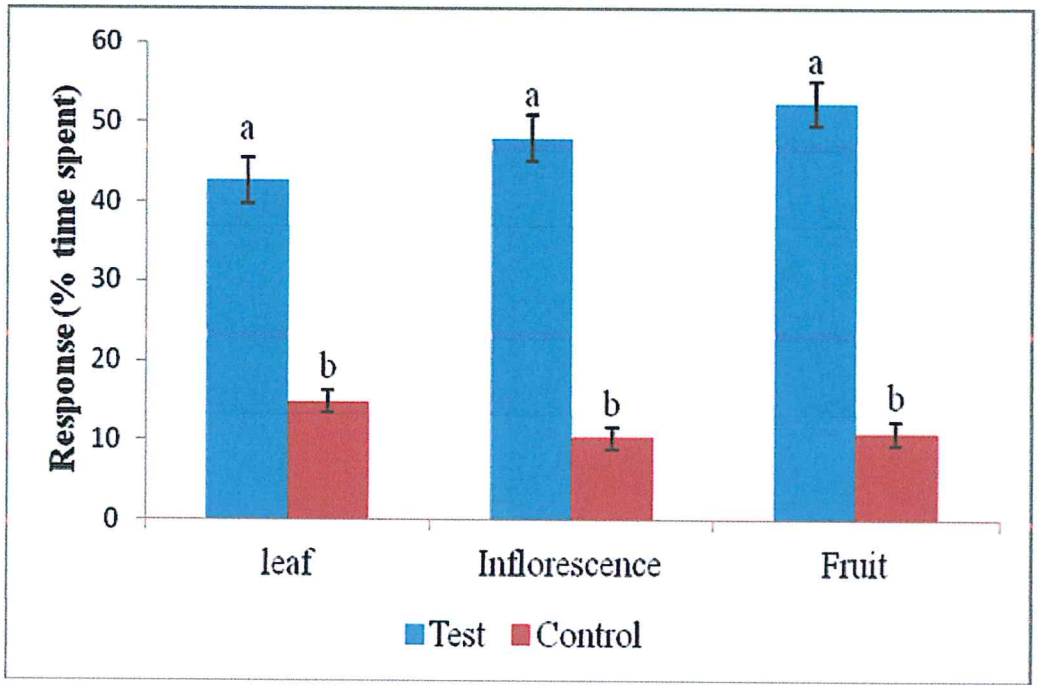


Figure 4.12: Bars represent ant responses (time spent as a percentage of the total time each individual ant is allocated in the olfactometer) to cashew volatiles paired

to a sugar reward. Means with similar letters are not significantly different (p>0.05).Means with different letters are significantly different (p<0.05).

4.5.3 Influence of cashew volatiles on reward exploitation

The frequency of sugar feeding within the test quadrant was significantly higher than in the control quadrant in all the three odors tested ($p = 0.0004$, p<0.0001 and p <0.0001 for the leaves, fruit and inflorescence odor respectively) as shown in Figure 4.13 . However, the frequency of feeding in the presence of the three test odors did not differ significantly (F=1.6581, df=2; p=0.194) across treatments.

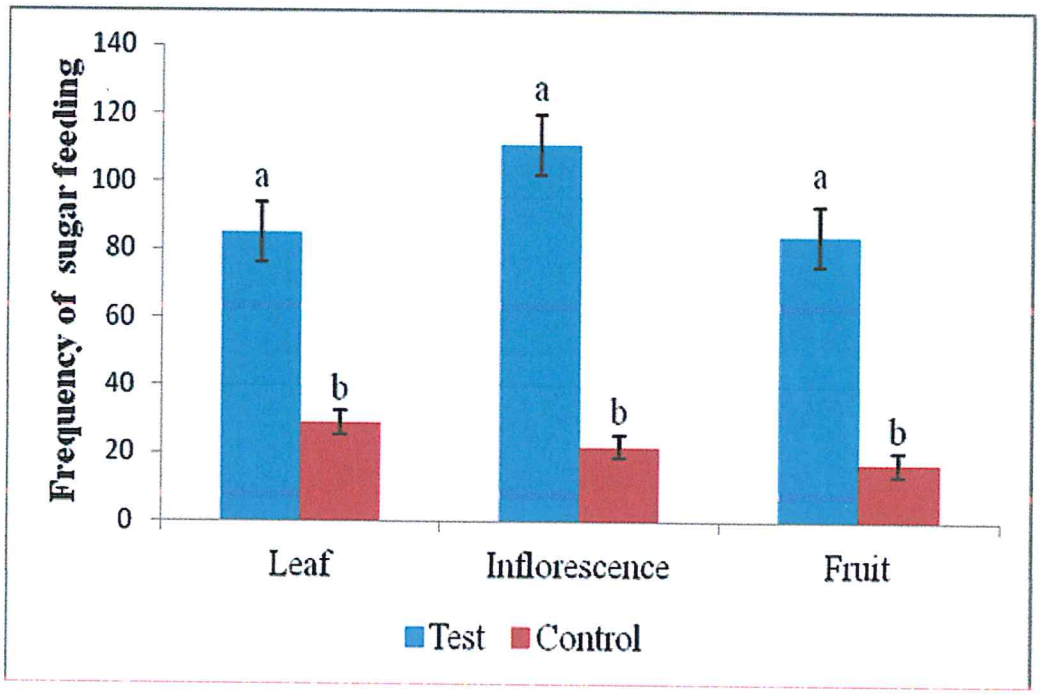


Figure 4.13: Bars represent the total number of feeding attempts observed and recorded. Means with similar letters are not significantly different (p>0.05). Means with different letters are significantly different (p<0.05).

4.5.4 Chemical composition of cashew plant volatiles

GC-MS analysis (Figures 4.14, 4.15 and 4.16) showed that head space volatiles of fruits, leaves and inflorescence were predominantly terpene compounds with monoterpenes comprising ≈60% of all the terpenes detected whereas sesquiterpenes comprised the remainder. α- pinene and (*Z*)-β-ocimene were the most abundant monoterpenes whereas copaene and α- farnesene were the most abundant sesquiterpenes.

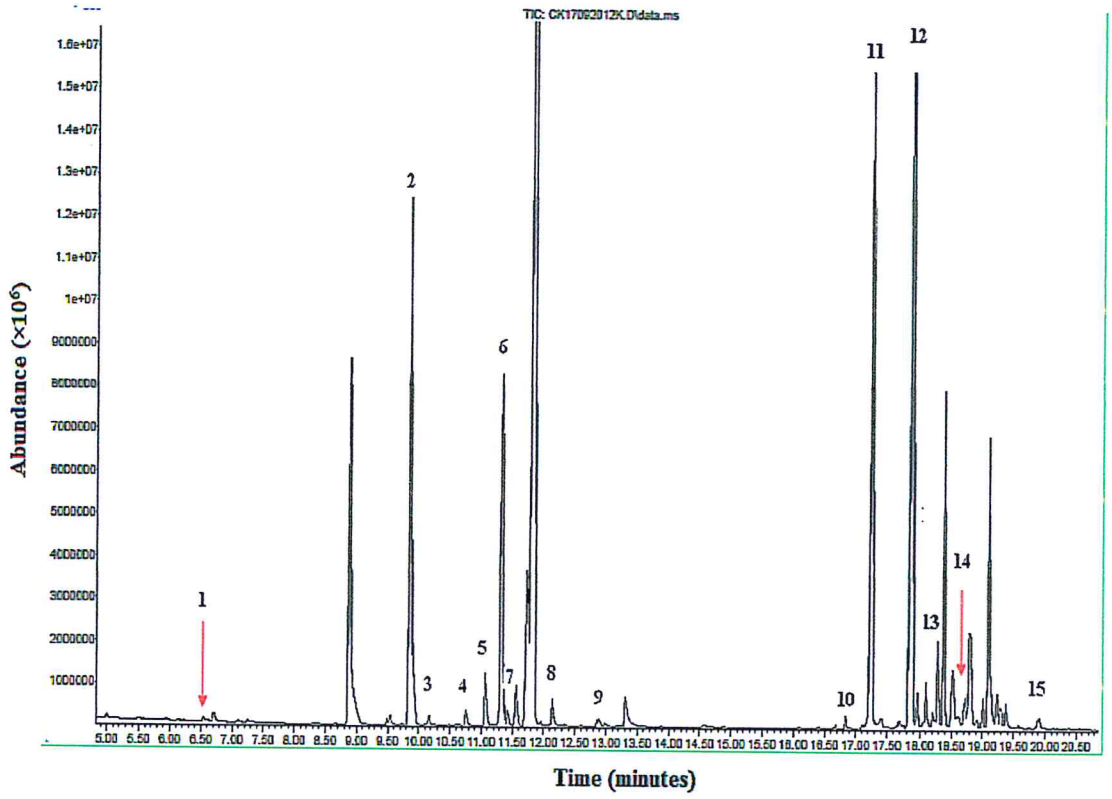


Figure 4.14: GC-MS chromatogram of cashew fruit head space volatiles; (1) hexanal (2) α-pinene (3) camphene (4) β-pinene (5) β-myrcene (6) α-phellandrene (7) 3-carene (8) (*Z*)-β-ocimene (9) 4-carene (10) α-cubebene (11) copaene (12) caryophyllene (13) α-caryophyllene (14) δ-selinene (15) caryophyllene oxide

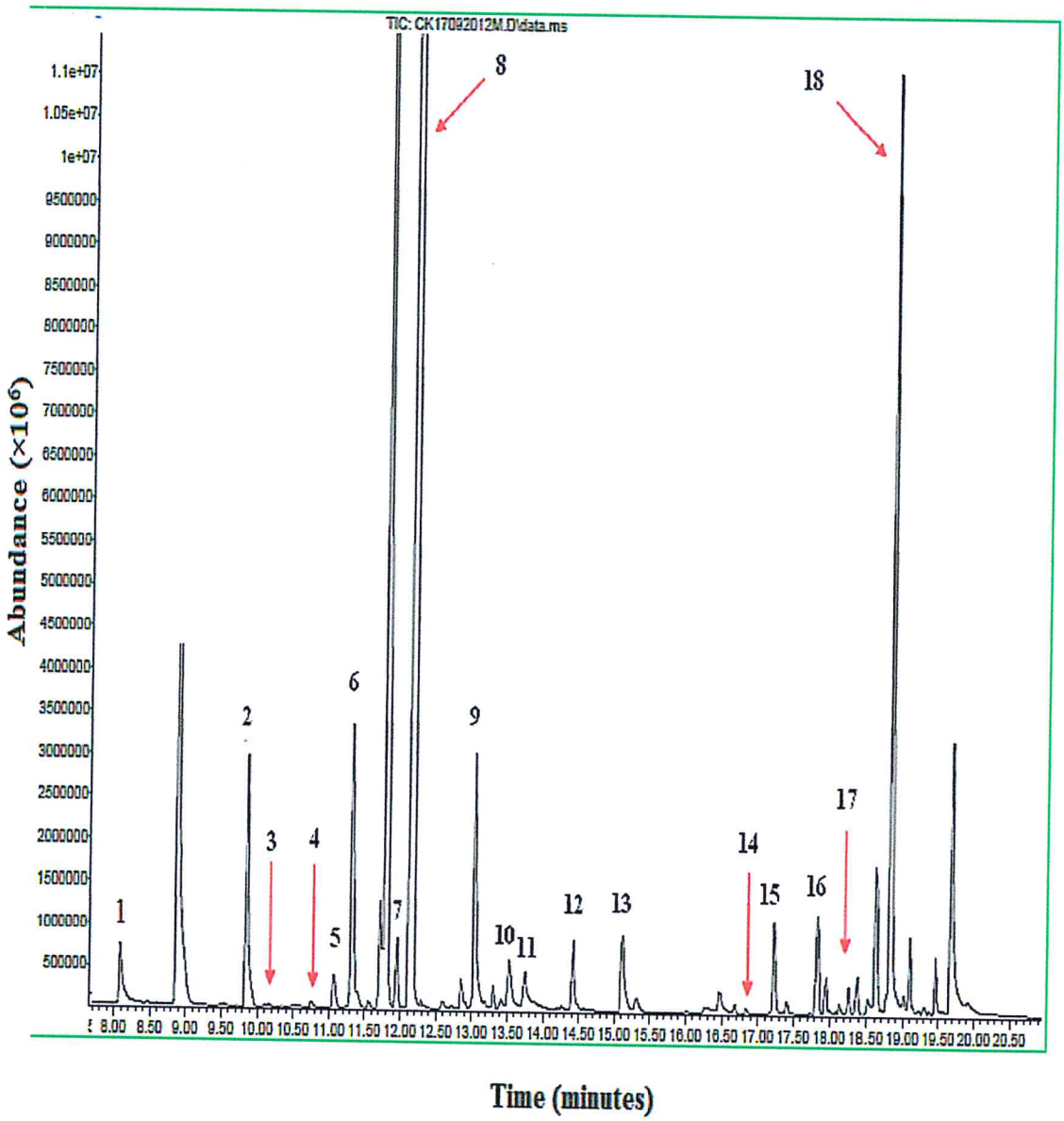


Figure 4.15: GC-MS chromatogram of the head space volatiles of cashew inflorescence; (1) 3-hexen-1-ol (2) α-pinene (3) camphene (4) β-pinene (5) β-phellandrene (6) α-phellandrene (7) (*E*)-β-ocimene (8) (*Z*) β-ocimene (9) linalool (10) allo-ocimene (11) (*Z*) 3-hexenyl-iso-butyrate (12) 3-hexenyl butanoate (13) (*Z*)-3-hexnyl valerate (14) α-cubebene (15) copaene (16) caryophyllene (17) α-caryophyllene (18) α-farnesene

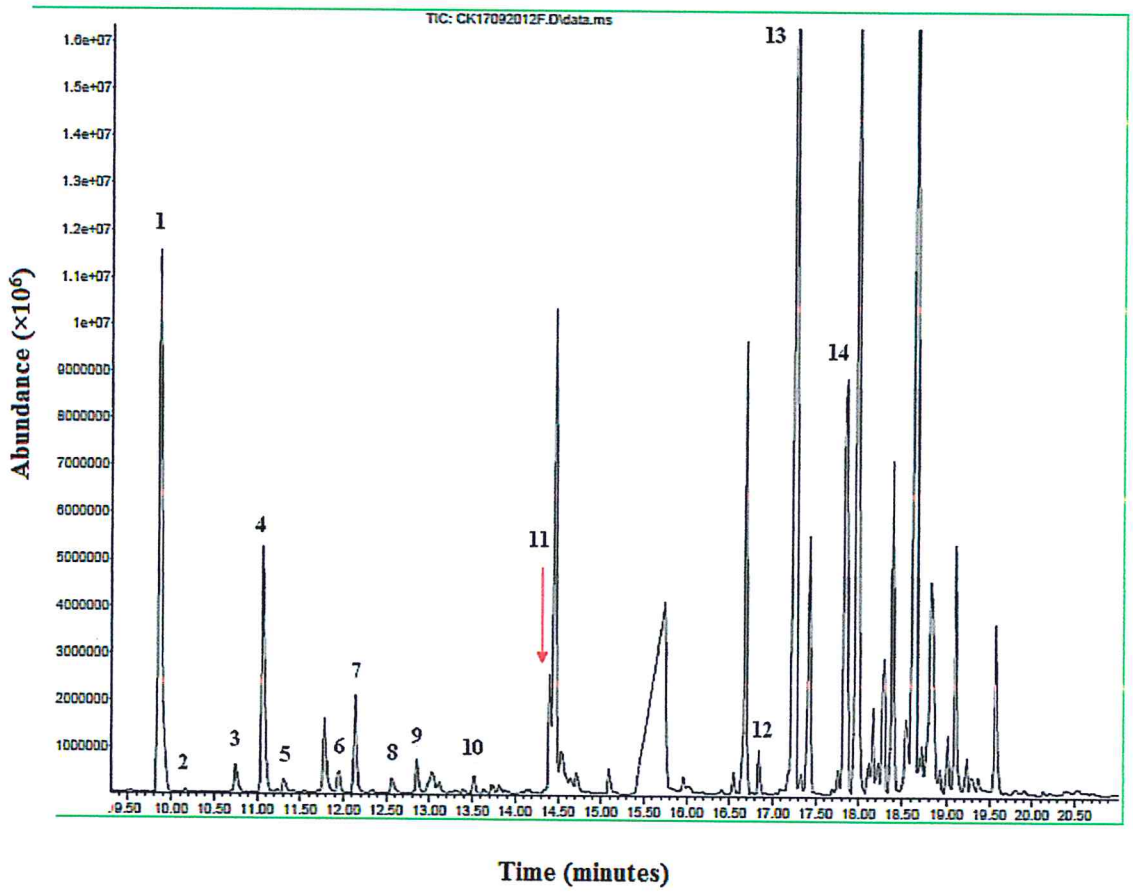


Figure 4.16: GC-MS chromatogram of the head space volatiles of cashew leaves; (1) α-pinene (2) Camphene (3) β-pinene (4) β-myrcene (5) α-phellandrene (6) (*E*)-β-ocimene (7) (*Z*)-β-ocimene (8) myrcenol (9) 4-carene (10) allo-ocimene (11) 3-hexenyl butanoate (12) α-cubebene (13) copaene (14) caryophyllene

4.5.5 Quantification of chemical components in the head space volatiles of the cashew plant

Chemical components of cashew volatiles (Table 4.7) were quantified using calibration curves generated from two representative external standards (Appendices 5 and 6) and expressed as nanograms per plant per hour ± standard error of means. Compounds 1-12 are monoterpenes while 13-17 are sesquiterpenes. Overall, the leaves, fruit and inflorescence shared a similarity in their chemical composition. The most abundant monoterpenes were α-pinene in the leaves and fruit and (*Z*)-β-ocimene in the inflorescence whereas, copaene was the most abundant sesquiterpene in the leaves and fruit and α-farnesene was most dominant in the inflorescence.

Table 4.7 : Composition of cashew volatiles, quantified in nanograms per plant per hour (ng/plant/hr) ± standard error of means (SEMs')

No.	Compound	Leaf (ng/plant/hr) ± SEM	Fruit (ng/plant/hr) ± SEM	Inflorescence (ng/plant/hr) ± SEM
1	α-pinene	24.041±0.100	22.25±0.351	0.754±0.032
2	camphene	0.083±0.001	0.583±0.010	0.042±0.000
3	β-pinene	1.166±0.05	0.792±0.013	0.125±0.002
4	β-myrcene	6±0.047	2.292±0.031	1.208±0.007
5	α-phellandrene	0.708±0.03	8.458±0.144	8.083±0.030
6	β-phellandrene	-	-	13.833±0.166
7	(*E*)β-ocimene	1.625±0.010	-	0.047±0.009
8	(*Z*)β-ocimene	42.958±0.461	1.083±0.018	77±0.384
9	(*Z*)Linalool oxide	-	-	0.617±0.002
10	myrcenol	1.083±0.03	-	-
11	4-carene	0.708±0.007	-	-
12	Allo-ocimene	1.25±0.010		2.25±0.013
13	α-cubebene	0.125±0.001	0.042±0.001	-
14	copaene	4.542±0.017	3.042±0.049	0.333±0.000
15	caryophyllene	1.666±0.010	2.958±0.049	0.0583±0.001
16	α-caryophyllene	-	0.292±0.005	0.083±0.000
17	α-farnesene	-	-	2.167±0.016

CHAPTER FIVE

DISCUSSION

5.1 *Pseudotheraptus wayi*

5.1.1 Life cycle

On the average, egg laying to emergence of adults in *Pseudotheraptus wayi* had been suggested to take about 41 days (25°C) but may take a shorter period at higher temperatures (Way, 1953). In the present study, the development time of *P.wayi* was ≈ 35 days at 27 to 28°C and 65 and 80% relative humidity. In a previous study by Egonyu (2012) the development time was ≈ 43days at a lower temperature of 24.6± 1°C and 80± 1.3%. Based on these findings it is apparent that temperature influences the growth of *P.wayi* significantly, in line with the assertion by Way (1953).

5.1.2 Survival of immature *P. wayi* in laboratory conditions

Monitoring the progression of the instars through to adulthood revealed that the highest mortalities occurred in the 1st and 3rd instars. While, almost 100% survivorship rates were recorded in the other stages, only about 60% and 80% survivorship was recorded in the 1st and 3rd instar respectively (Figure 4.1). The cause of the apparent vulnerability of these two stages is not very clear. However in a previous study on the morphometry of the immature *P.wayi* the transition of 1st and 3rd instars to the subsequent stages appeared to be characterized by a marked change in body size and weight compared to the other stages (Egonyu, in press).

In both invertebrates and vertebrates alike, growth is governed by the insulin like growth promoting factors as well as growth hormones (GH). In vertebrates these factors have been shown to have physiological implications on life span of organisms. In recent studies, GH deficient transgenic mice have been shown to outlive the wild type strain by nearly one year. The increased life span was attributed to fewer immunological and pathological lesions as well as increased efficiency of anti-oxidative enzymes, all of which are negatively correlated with growth hormones (Shimokawa *et al.*, 2002; Brown-Borg *et al.*, 2002). Similarly, in insects, the factor dSH2B in *Drosophila melanogaster* analogous to the mammalian insulin-like growth promoting factor SH2B was shown to be linked to a decrease in metabolic resources, resistance to starvation, oxidative stress and ultimately life span (Song *et al.*, 2010). It would be interesting to study whether similar physiological and immunological changes occur in the immature *P. wayi*. Furthermore, when the development time and survival rates of 1st and 3rd instars of *P. wayi* in this study (≈ 35 days and 60 and 80% respectively) are compared to the study by Egonyu (≈ 43 days and 77 and 98.5% respectively) it is apparent that faster growth (induced by increased rearing temperatures in this case) affects survival significantly.

5.2 *P. wayi- O. longinoda* interaction

5.2.1 Chemical signals in the compromise and combat response

Generally, the head space in the combat response of both *P. wayi* sexes had an abundance of volatile chemicals unlike the compromise interaction (Figures 4.2 and

4.3). The chemical composition of the head space volatiles in this response was also similar with the exception of heptyl hexanoate and octyl hexanoate that were only identified only in females and males respectively. Conspicuously, combat in immature *P. wayi* was dominated by a different set of volatile components ((*E*)-hex-2-enal, (*E*, *E*)-hex-2, 4-dienal, Oct-2-enal and 2-cyclohexen-1,4-dione) (Figure 4.4) from those identified in the interactions with adult insects.

In previous studies, hexanal (1), hexanol (2), hexyl acetate (3), hexyl hexanoate (8) and (*E*)-hex-2-enal had been identified in the defense and alarm secretions of several heteroptera species in the Coreidae family (Aldrich, 1988; Zarbin *et al.*, 2000; Millar, 2005; Raska, 2009; Hassani *et al.*, 2010). Interestingly, the analysis of volatiles from whole insect extracts of *O. longinoda* (Figure 4.5), also revealed the presence of hexanal, hexanol, hexyl acetate, hexyl hexanoate and undecane. In an elaborate study by Bradshaw *et al* (1979b) hexanal (1), hexanol (2) and hexyl acetate (3) were described as alarm pheromones (Bradshaw *et al.*, 1979b). Undecane and 2-butyl, oct-2-enal comprised the biting pheromone (Bradshaw *et al.*, 1979 b), suggesting a similarity in the defensive and alarm chemistry of coreids to that of *O. longinoda*. This sort of chemical mimicry is not unusual since the production of volatile irritants such as those identified in the defense secretions of coreids, has been shown to be mostly aimed at arthropods mainly ants (Pasteels and Grègoire, 1983). Given the small of ants it s almost always likely that they will come into contact with chemicals once in close proximity to the defended insect

(Pasteels and Grègoire, 1983). Therefore, the adaptive significance of these phenomena is to help the prey cause a temporary panic among the ants, allowing them to escape (Blum, 1996). Given the aggressive nature of predaceous *O. longinoda* ants, such an adaptation would serve *P. wayi* well.

The remaining esters (hexyl butanoate, octyl acetate, heptyl hexanoate and octyl hexanoate) not identified in *O. longinoda* volatiles were associated to *P. wayi* based on their chemical relatedness to the other coreid produced candidate defense compounds. Similarly, the chemical relatedness of (*E*, *E*)-hex-2, 4-dienal, Oct-2-enal and 2-cyclohexen-1, 4- dione, to (*E*)-hex-2-enal suggest they constitute candidate defence compounds in immature *P. wayi*.

5.2.2 Behavioural observations during combat and compromise

One of the key behaviors observed in the combat response during the prey-predator interaction was snapping of the mandibles by *O. longinoda* before engaging *P. wayi* in a physical tussle (Table 4.4). In previous studies, this behaviour was shown to signify aggression (Hölldobler and Wilson, 1977; Bradshaw *et al.*, 1979 a; Newey, 2009). Additionally *O. longinoda* were also seen rubbing their mandibles using the forelegs as well as against the other conspecifics in the experimental chamber. Behaviour associated with nest mate recruitment in ants (Roux *et al.*, 2010). Ideally the display of aggression was likely for purposes of intimidating the prey while the mandibular rubbing communicated important information for the ants to co-ordinate a successful attack. Similarly, before the actual physical tussle, the *P. wayi*

would angle the posterior end of its body towards the *O. longinoda* which would responds by retreating. Surprisingly, this peculiar behaviour was only observed in adult *P. wayi* but not in the immature stages and although its significance is unclear, it appeared to help the adult prey intimidate the predator to some extent.

During combat, the *O. longinoda* subdued *P. wayi* by biting and pulling at its limbs which was followed by a potent irritant odor emanating from the experimental chamber (Table 4.4). A possible explanation of these events is that; *O. longinoda* has been shown to possess poison apparatus and other exocrine glands that produce chemicals that are useful in subduing prey (Bradshaw *et al.*, 1979 b; Morgan, 2008). Therefore it could have injected paralyzing toxins while biting on the *P. wayi* limbs. In turn, *P. wayi* produced noxious chemicals possibly from the defensive glands that have been suggested to occur in all insects of the order heteroptera. Since the defensive secretions described in coreids are highly irritant chemicals (Pasteels and Grègoire, 1983), it's possible that they could have caused the discomfort (curling and grooming) observed in *O. longinoda* (Table 4). Furthermore they have been shown to be injurious even to the producer (Pasteels and Grègoire, 1983) and therefore could have led to the death of both prey and predator. Conspicuously, combat between immature *P.wayi* and *O.longinoda* resulted in the death of the immature life forms but not the predator unlike the interaction with the adult stage. This finding suggests that the immature stages are more vulnerable to predation than the adults. The underlying cause of this

peculiarity was not very clear, however, it was presumed to be linked to the apparent dissimilarity in the chemical composition of the candidate defense compounds between both stages.

5.3 Scent glands

5.3.1 Metathoracic glands (MTGs') of *Pseudotheraptus wayi*

Glandular secretions in both adult sexes were comprised of four main compounds; hexanal, hexanol, hexyl acetate and hexyl hexanoate (Figure 4.6 and Table 4.5). These compounds had previously been reported to occur in the defensive secretions of various coreids (Aldrich, 1988; Zarbin *et al.*, 2000; Yung-Ho and Millar, 2001; Millar, 2005; Raska, 2009; Hassani *et al.*, 2010). In this current study they were detected in the metathoracic glands of *P. wayi*, confirming that their presence in the head space volatiles during the prey-predator interaction was defense related. However the corresponding esters (hexyl butanoate, octyl acetate, heptyl hexanoate and octyl hexanoate) were absent in the MTG secretions. However, it has been shown that, mixtures of defensive chemicals are mostly toxic even to the producer (Pasteels and Grègoire, 1983). Consequently, the final biosynthetic steps take place in extracellular spaces and therefore, the initial constituents may only be the precursors of the final reactions (Pasteels and Grègoire, 1983). Based on this premise, it is likely that hexanal, hexyl acetate and hexyl hexanoate are synthesized and stored as precursors to the other related compounds that are only produced on demand, hence their absence in the MTGs'.

5.3.2 Dorsal abdominal glands (DAGs') of *Pseudotheraptus wayi*

Chemical analysis of DAGs' (Figure 4.7 and Table 4.6) showed that (*E*)-hex-2-enal was the only component in these glands. This compound had been identified in several coreid species and shown to mediate both alarm and defense, an indication that it also mediates defense in the immature *P. wayi* (Aldrich, 1988; Millar, 2005). Interestingly, the compound was absent in MTG secretions and none of the MTG components were in DAGs' either, suggesting that although both glands share a primary role, defensive chemistry in *P. wayi* is stage specific. Similar to the adults, the three additional components identified in the head space volatiles during the interaction between immature *P. wayi* and the predator were also absent in the DAGs'. Again, their chemically related to (*E*)-hex-2-enal, the main DAG component suggests that, they are possibly synthesized and stored as a precursors and the entire array of defense compounds is only produced on demand.

Comparatively, the chemical composition of the DAGs' secretions was less sophisticated than that the MTGs'. The relevance of the chemical variation in the two *P. wayi* stages is not very clear. Intraspecific polymorphism in the defense chemistry of heteroptera has been suggested to confer an advantage especially with regard to avian predators. The birds learn to avoid those with diverse chemical profiles better than those with few chemicals (Skelhorne and Rowe, 2005). In this study the variation in defense chemistry did not seem to benefit the prey entirely, since only combat with the adult *P. wayi* resulted in death of the predator,

suggesting that the polymorphism could be a handicap rather than strength. Given the fitness cost of such a polymorphism it would be interesting to investigate the adaptive significance. However, it is possible that the immature stage lacks the biochemical sophistication to synthesize a complex array of compounds *de novo* like the adults. Moreover, (*E*)-hex-2-enal the major DAG component is a leaf aldehyde associated with either mechanical or herbivore damage (Arimura *et al.*, 2005). It is likely that the compound may be sequestrated by the nymphs while feeding, then stored and finally modified into other components when the need arises. Furthermore, sequestration of plant compounds has been documented in heteroptera and even though not very well investigated in the Coreidae family, the phenomenon cannot be ruled out entirely (Aldrich *et al.*, 1990; Raska, 2009; Wink *et al.*, 2000).

5.4 Sexual signaling in *Pseudotheraptus wayi*

Copulation/mating is the most important act in the life of an organism that reproduces sexually. Therefore sexual behaviour revolves around stimulus-response mechanisms that ensure successful copulation (Barth and Lester, 1973). The stimulus is in the form of sex pheromones and the response is the actual copulation. Sex pheromones ensure proper co-ordination of sexual behaviour before, during and after mating (Barth and Lester, 1973). Generally they are produced in small doses and therefore in heteroptera they are often overwhelmed by defence chemicals (Aldrich, 1988; Millar, 2005). However, it has been shown that it is possible to make a distinction between the two components by collecting

emissions from live insects and comparing them to secretions of the scent glands (Durak and Kalender, 2007a). A similar approach was adopted for this study and it was found that; emissions of live *P. wayi* were predominantly C_6 aldehydes and their corresponding esters. Four compounds (hexanal, hexanol, hexyl acetate and hexyl hexanoate) stood out since they were consistently produced by both sexes over the 40 day period of volatile collection (Figures 4.8 and 4.9).

The onset of volatile production (8 days) coincides with the age at which the first mating pairs were observed (Figure 4.10). Volatile production then follows the same trend as the mating pattern. Peaking on the same days when increments in mating pairs were observed.

In a previous study by Egonyu (2012), hexanal, hexanol, hexyl acetate and hexyl hexanoate were identified as candidate *P.wayi* sex pheromones. In this study the similarity in mating pattern to the trend in volatile production suggests that these components coordinate mating, lending support to the assertion by Egonyu (2012)

Ideally, sex pheromones are produced by one sex and intended for the opposite sex (Moraes *et al.*, 2008). However in this study the candidate sex pheromones were produced by both sexes varying only in quantity and timing. Production began earlier in females (8 days) and peaked up in males (16 days) at a time when it was waning off in females. Assuming that communication between sexes is hinged on

variations in the quantity of candidate pheromone produced, these findings suggest that; early onset of candidate pheromone production in females is possibly for purposes of signaling /calling males. Once the males have aggregated pheromone production wanes off in the females and begins in the males to ensure that they secure a mate. At around 28 days another similar cycle begins where production is sustained only between 28 and 32 days in females and all the way through to 40 days and possibly beyond in males. Finally, the similarities of the candidate sex pheromones (Figures 4.8 and 4.9) to the components in the MTG secretions (Figure 4.6) suggest that the *P.wayi* metathoracic gland may possess dual roles. Such phenomenon where defensive glands adapt communication roles is not unusual as it has been described in other studies and is regarded as an extreme form of semiochemical parsimony (Blum, 1996).

5.5 Cashew– *O. longinoda* interaction

5.5.1 *Oecophylla longinoda* responses to cashew volatiles

In the Y-tube olfactometer experiment, *O. longinoda* ants walked back forth within both arms of the olfactometer but spent a significantly higher amount of time in the test arm as opposed to the control arm for all the test odors (Figure 4.11). Odor preference has been documented in a variety of insects that includes moths, honey bees as well as ants and has been linked to associative learning (Smith and Getz, 1994; Farooqui *et al.*, 2003; Cunningham *et al.*, 2003; Reuven, 2008). In ants, responses to host plant volatiles have been documented in several species (Edwards

et al, 2006; Dáttilo *et al*, 2009) but never before for the *O. longinoda* to cashew plant system and therefore it is reported in this study for the first time.

In the olfactometer the ants were seen probing the glass walls with their mouth parts and their antennae as if searching for something. This behaviour was not very clear at that time. Howver, odor preference had been shown to be linked to associative learning (Smith and Getz, 1994; Farooqui *et al.*, 2003; Cunningham *et al.*, 2003; Reuven, 2008) and well demonstrated for *Oecophylla* species (Hölldobler and Wilson, 1977; Newey, 2009). As such, it was presumed that the ants were relating the cashew odor to something else. In ant-plant interactions, provision of rewards by plants to ants is fundamental to the sustenance of these associations (Heil and Mckey, 2003). For the cashew plant, food rewards in the form of extrafloral nectar, to drive the associations between the plant and ants (Wijit, 1991; Rickson and Rickson, 1998). On this basis, the searching behaviour was presumed to be 'reward seeking', motivating the inclusion of sugar rewards in the subsequent experiment.

5.5.2 *Oecophylla longinoda* responses to cashew volatiles paired with a sugar reward

When sugar droplets were offered together with the cashew odor, the searching behaviour was seen to facilitate successfully reward discovery. Since the sugar reward was non-volatile this finding clearly demonstrated that *O. longinoda* relates cashew volatiles to a food item. Given that, the ants had not been conditioned to

either the odor or the sugar reward prior to the experiment, it is likely that the odor-reward association was acquired in the wild and retained to memory. Furthermore, odor processing pathways and reward learning pathways are linked through associative learning (Farooqui *et al.*, 2003; Perry and Barron, 2012). This paradigm is clearly demonstrated in social insects such as ants and bees using the maxilla-labium extension response (MaLER) and Proboscis extension response (PER) (Menzel, 2009; Guerrieri and d'Ettore, 2010).

After sugar feeding in the test quadrant the ants could be seen walking around the arena with their mandibles open and gaster raised. This display was reminiscent of the aggressive behaviour described in *Oecophylla* spp. by Hölldobler and Wilson (1977), Bradshaw *et al.*, (1979 b) and Newey (2009). The relationship between the reward pathway suggested in these findings and aggressive displays observed was not very clear. However, from a neurobiology point of view, insects have been found to have a specialized set of neurons (VUMmx1) in the antennal lobe that respond to sugar stimulus (Menzel, 2009; Riffel, 2011; Perry and Baron 2012). This mechanism is modulated by the neuro chemical octopamine, also shown to regulate aggression in the cricket *Gryllus bimaculatas*, and the fly *Drosophila melanogaster* (Adamo *et al.*, 1995; Baier *et al.*, 2002; Kravitz and Huber, 2003; Rillich and Stevenson, 2011). On that basis, it is plausible to hypothesize that sugar stimulation possibly elicits aggression in *O. longinoda*.

Finally, the ants spent almost twice as much time in the vicinity of the odor when the odor was paired with a sugar reward (Figure 4.12) than when presented alone (Figure 4.11). This corroborates the assertion that 'rewards are important motivators of animal behaviour' (Perry and Baron, 2012). These findings suggest that, in the natural set up food rewards motivate ant constancy around the plant which is critical for effective anti-herbivore defense.

Evidently the frequency of feeding was significantly higher in the presence of cashew odor paired with a sugar reward than when the rewards were presented singly (Figure 4.13). This indicates that *O. longinoda* relies on cashew volatiles are reliably predict food rewards

5.6 Chemistry of cashew volatiles

Cashew volatiles were predominantly monoterpenes (≈ 60%) and a few sesquiterpenes. Generally, there were striking similarities in the composition of leaves, inflorescence and fruit (Table 4.7). This may be the reason why there were no significant differences in *O. longinoda* preference across the odors in all the assays (Figures 4.11, 4.12 and 4.13). In other studies, terpenoids have been shown to be useful in attracting various species of natural enemies (Arimura *et al.*, 2005; Cheng *et al.*, 2007; Mithöffer and Boland, 2012) and based on the findings in this current study *O. longinoda* has joined the klatch.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

Based on the *P. wayi* rearing data, it is apparent that faster growth may be correlated to reduced survival rates as observed in the 1st and 3rd instars. These findings require investigation not only to further understand the biology of the insect but also to determine whether it is a loophole in the insects' biology, which can be exploited in pest control.

Pseudotheraptus wayi candidate defense compounds constitute a complex, potent and irritant mixture of chemicals as observed during the experiments. Such properties have the potential to be exploited as insect repellants or even pesticides necessitating further study and testing. Additionally, the chemical polymorphisms in defense chemistry and their biosynthesis in immature and mature adult insects also require further investigations. Finally, the role of hexanal, hexanol, hexyl acetate and hexyl hexanoate in sexual signaling, is not only helpful in understanding the biology of *P. wayi* but other heteropterans as well. More importantly, it provides a framework for future research into how these pheromones can be used, possibly in the form of lures or otherwise.

With regard to the host plant- predator interaction, cashew volatiles have proven to be important cues for the African weaver ant more so when enhanced with food

rewards. This is an important finding because there are indications that plants allelochemicals can be used in the enhancement of the performance of bio-control agents by; increasing the residence times of natural enemies on crops, manipulating their foraging activities, breeding plants that express the chemicals attractive to the natural enemy in high amounts, among other applications (Dicke *et al.*, 1990). It therefore, provides a framework for the development of tools engineered to manipulate and enhance the performance of *O. longinoda* in crop protection.

In conclusion, this study has demonstrated that indeed, chemical signals mediate the tri-trophic interaction under investigation but more importantly, they define potential loopholes that can be exploited for pest control.

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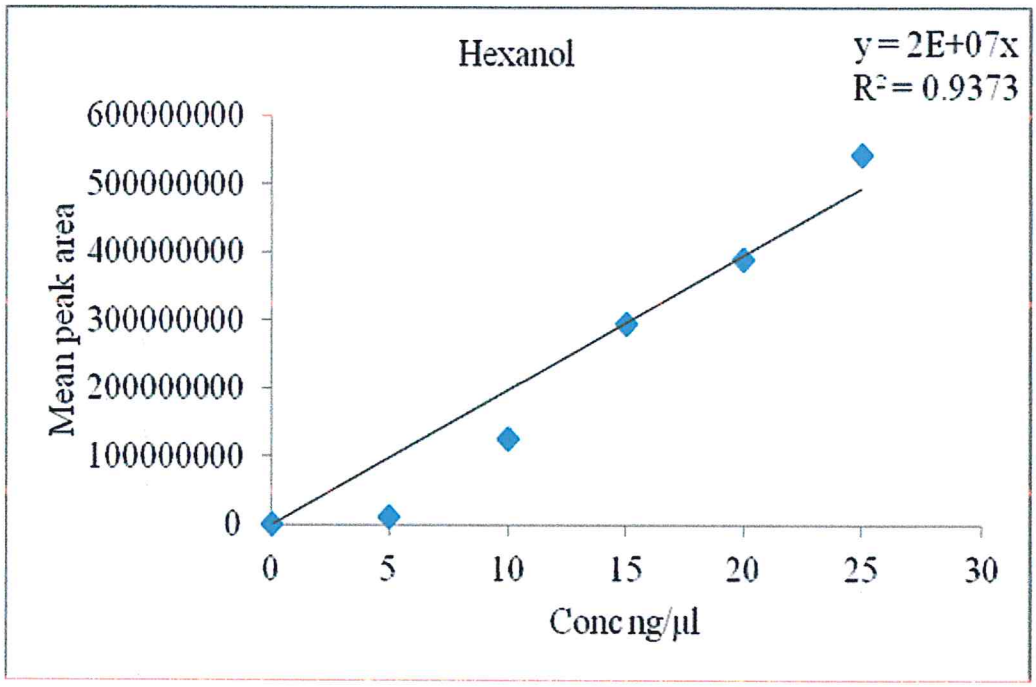
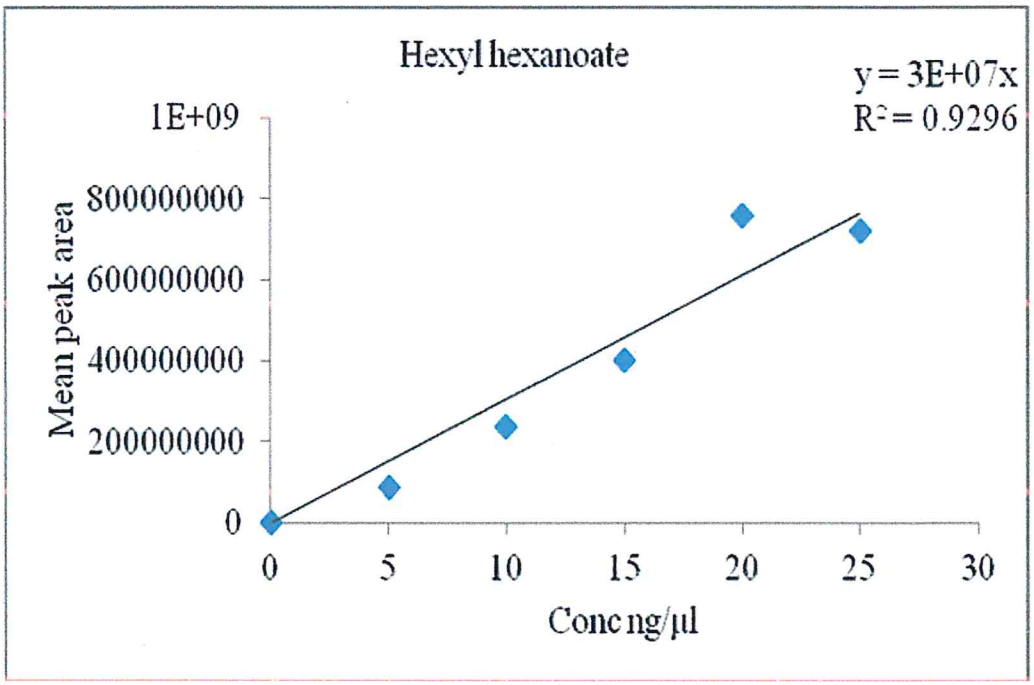
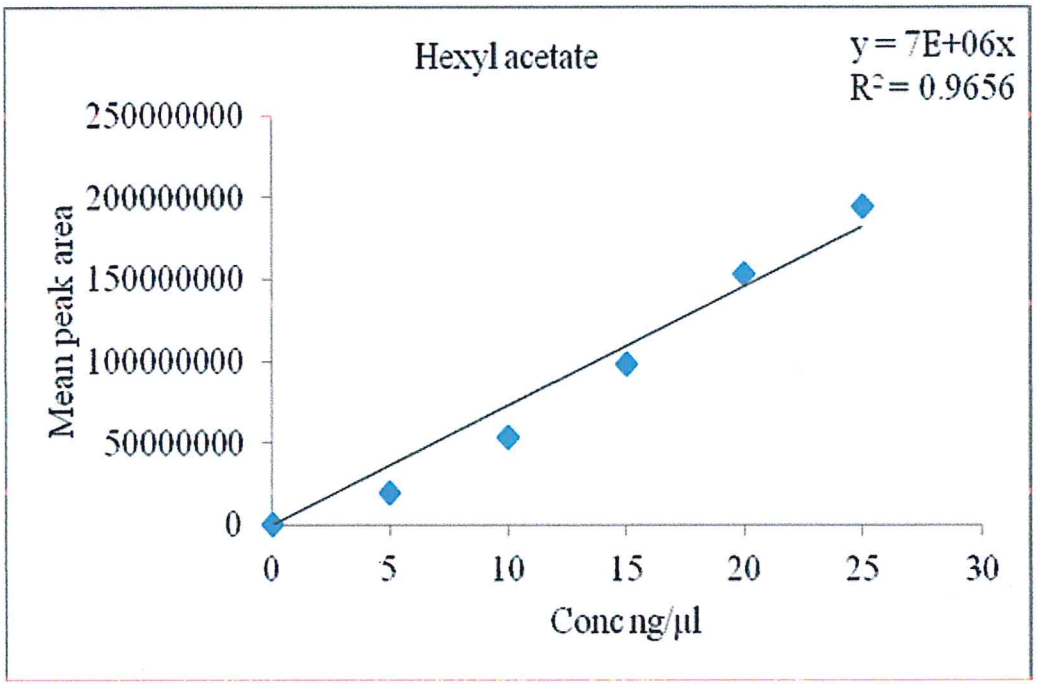
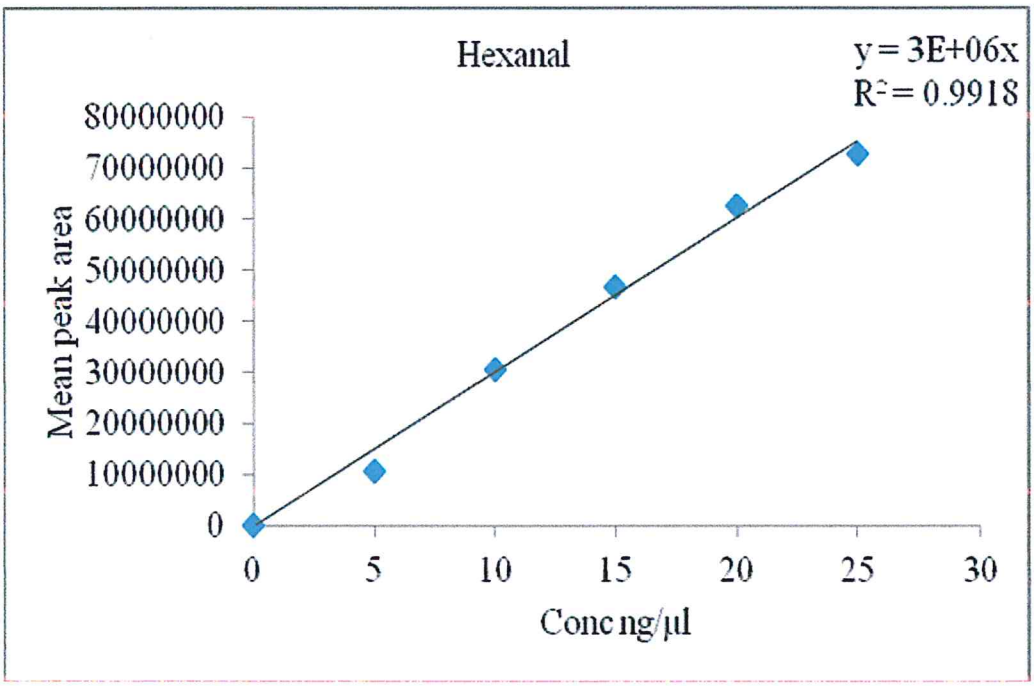
APPENDIX 1

Calibration curves used in the quantification of chemical components identified in *P. wayi* head space volatiles.



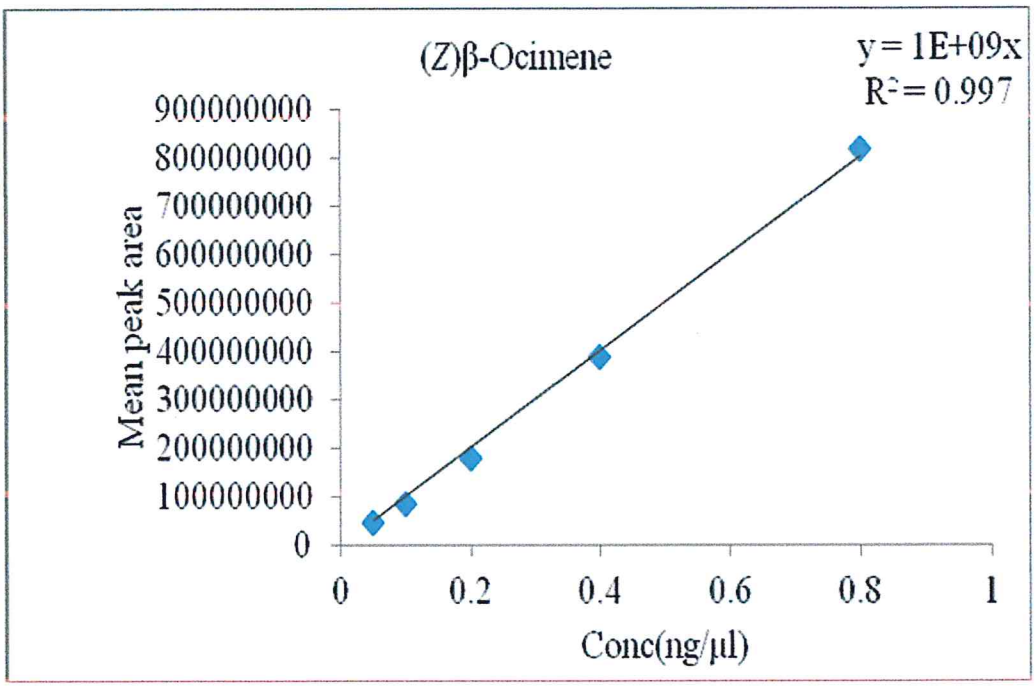

Hexyl hexanoate
y = 3E+07x
R² = 0.9296
1E+09
800000000
600000000
400000000
200000000
0
Mean peak area
0
5
10
15
20
25
30
Conc ng/µl

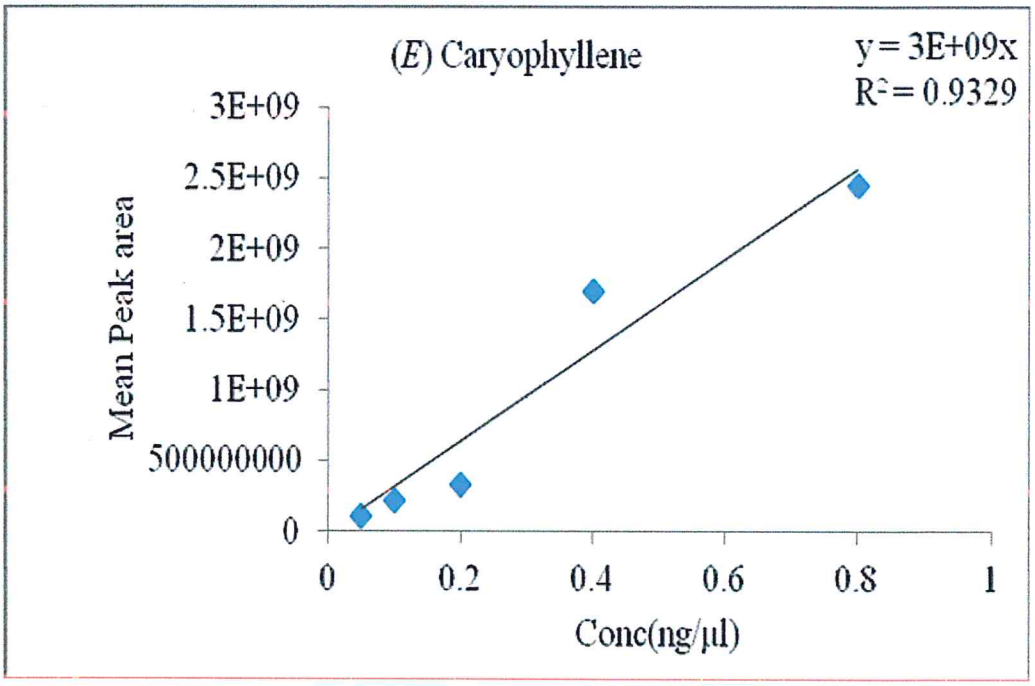

Hexyl acetate
y = 7E+06x
R² = 0.9656
Mean peak area
250000000
200000000
150000000
100000000
50000000
0
0
5
10
15
20
25
30
Conc ng/µl


Hexanal
y = 3E+06x
R² = 0.9918
Mean peak area
80000000
70000000
60000000
50000000
40000000
30000000
20000000
10000000
0
0
5
10
15
20
25
30
Conc ng/µl

APPENDIX II

Calibration curves used in the quantification of chemical components identified in the head space volatiles cashew volatiles.




(E) Caryophyllene
y = 3E+09x
R² = 0.9329
Mean Peak area
3E+09
2.5E+09
2E+09
1.5E+09
1E+09
500000000
0
0
0.2
0.4
0.6
0.8
1
Conc(ng/µl)