DEVELOPMENT OF SEMIOCHEMICAL-BASED STRATEGIES FOR THE MANAGEMENT OF ANTESTIA BUG, *ANTESTIOPSIS THUNBERGII* (HETEROPTERA: PENTATOMIDAE)

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Development of semiochemical-based strategies for the management of Antestia bug, *Antestiopsis thunbergii* (Heteroptera:Pentatomidae)

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DECLARATION

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

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DEDICATION

To my family

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LIST OF ACRONYMS AND ABBREVIATIONS

A.S.L	Above sea level
ANODEV	Analysis of deviance
DAG	Dorsal abdominal gland
EAD	Electroantennographic detection
FID	Flame ionisation detector
GC/EAD	Gas chromatography/electroantennographic detection
GC/MS	Gas chromatography/mass spectrometer
GLM	Generalized linear model
IBMP	2-isobutyl-3-methoxypyrazine
icipe	International Centre of Insect Physiology and Ecology
IPMP	2-isopropyl-3-methoxypyrazine
MDT	Methyl (2,4,6)-decatrienoate
MDT-*EEZ*	Methyl (2*E*,4E,6Z)-decatrienoate
MTG	Metathoracic gland
PCA	Principal component analysis
RH	Relative humidity
SE	Standard error
SPME	Solid phase microextraction

ABSTRACT

Antestia bug *Antestiopsis thunbergii* (Gmelin) is a key insect pest of Arabica coffee associated with high losses in terms of quality, yield and export market of produce from the African region. Although the pest prefer feeding on unripe (mature) green berries than other coffee berry maturity stages or coffee parts, the cues that influence their host choice are unknown. In addition, *A. thunbergii* is gregarious in the field and produces a strong distinct smell while disturbed, which is characteristic of defence and alarm pheromones. However, little is known about the identity of the volatile chemical compounds that this pest produces neither their biological functions. Hence, this study sought to identify and examine the role of bioactive coffee berry derived volatiles and *A. thunbergii* pheromones and, explore their potential for the pest management. Various laboratory experiments were conducted comprising: behavioral assays in a dual choice Y-tube olfactometer; electrophysiology tests using Gas chromatography-electroantennographic detection (GC/EAD) and chemical analysis in GC-mass spectrometry, and field tests in a coffee plantation located at Kiambu County, in Central Kenya. Behavioral tests showed that volatile emissions from unripe coffee berries (mature green stage) were highly attractive to *A. thunbergii* nymphs and adults, whereas those from ripe berries induced repellence. Antennal activity recording with *A. thunbergii* nymphs isolated five and ten electrophysiologically active components in unripe berries and ripe berries respectively. Through behavioral tests with synthetic standards of the EAD-active compounds, a three-component blend derived from unripe berries comprised of anisole, methyl 3-ethyl-4-methylpentanoate and (5*S*,7*S*)-conophthorin was found to be highly attractive to the pest. On the contrary, a five-component blend derived from ripe berries comprising 3-hydroxy-2-butanone, 2-heptanone, 2-isopropyl-3-methoxypyrazine, 2-isobutyl-3-methoxypyrazine and (*E*)-β-caryophyllene was repellent to the pest. These results suggest that the two distinct blends likely contribute to *A. thunbergii* host food choice amongst unripe and ripe coffee berries and are candidate semiochemical based components that could be used either as lures or repellents to control the pest. Intraspecific communication assays established that *A. thunbergii* males were attractive to males and females, but females were unattractive to either sex. Through chemical analysis, a candidate male specific aggregation pheromone was identified as methyl (2*E*,4*E*,6*Z*)-decatrinoate (MDT-*EEZ*) in Antestia bug males. In addition, chemical analysis of dorsal abdominal glands of nymphs, and metathoracic glands of adults and headspace volatiles of disturbed adults led to the identification of various candidate alarm pheromones that are produced by the pest and dominated by alkanes and aldehydes. In field trials that evaluated the performance of candidate lures which were dispensed from delta® traps, the highest tested dose of MDT-*EEZ* (5 mg) was significantly attractive but no significant attraction was recorded with the kairomone at the tested doses. MDT-*EEZ* attracted *Antestiopsis* spp adults and nymphs (*A. thunbergii* and *A. facetoides*) with aggregations appearing on pheromone baited plants (7 bugs/ tree) unlike in unbaited plants (control) (1 bug/tree). However, delta® traps did not capture the bugs, as bugs stayed near traps baited with pheromone without entering inside. Hence, future studies should investigate different trap designs that are compatible with the

pheromone and as well as an effective formulation of kairomone. In conclusion, this study identified candidate chemical cues that govern host-food selection and avoidance; aggregation and alarm behaviors of *A. thunbergii*, and further explored the performance of attractive semiochemicals in the management of the pest in coffee plantations attaining promising findings. Optimization of attractant compounds identified in this study could lead to effective management of the pest through surveillance, mass trapping and 'attract and kill' strategies. Identified candidate repellent compounds may be used to expel the pest from coffee plantations or to mask susceptible host food (unripe berries). A push- pull system could also be developed comprised of attractive and repellent compounds to enhance suppression of the pest.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background of the study

Coffee is the second largest traded commodity after petroleum products (Mussatto *et al.,* 2011), with annual returns estimated at US $ 90 billion globally (Jaramillo *et al.,* 2011). The crop is grown in 80 tropical countries (Marcone, 2004) by an estimated 125 million coffee farmers who are mainly smallholder farmers (Osorio, 2002). Africa's coffee market share has been on a steady decline over the past three decades, currently at 12% (ICO, 2015), due to various constraints, key among them is crop loss due to pests and diseases. Irrespective of this, coffee still substantially supports the economy of many African countries by contributing a high proportion in export earnings, e.g. Kenya (6%), Rwanda (26%), Ethiopia (28%) and Burundi (70%) (ICO, 2015). It is therefore crucial to address the problems facing coffee production to boost the economy and alleviate poverty among coffee farmers in the region.

Antestia bug, *Antestiopsis* spp. (Hemiptera: Heteroptera: Pentatomidae) is a complex of various species that attack coffee in various regions of Africa; the two most common and economically important species are *A. thunbergii* (Gmelin) and *A. intricata* (Ghesquière and Carayon) (Babin *et al.,* 2018). The bugs attack various coffee parts, including flowers and berries, leading to yield losses in form of flower abortion and premature abscission of berries. They are also proposed to vector pathogenic micro-organisms, leading to coffee bean rot (Le Pelley 1942; Cilas et *al.,* 1998) and 'potato taste defect' PTD in processed coffee, rendering it unmarketable (Matsuura *et al.,* 2014). Because of their destructive nature, a population density of 1–2 bugs/tree is considered as the economic threshold level for pest control measures in Kenya (Mugo *et al.,* 2013). Currently, the pest is controlled using conventional methods such as pruning and pesticide application (Nyambo *et al.,* 1996). However, these methods are only partially effective and could harm non-target beneficial organisms; thus, alternative, eco-friendly pest control methods are needed. One

approach could be the use of semiochemicals, which can be integrated into existing management tools.

Semiochemicals are naturally occurring volatile organic compounds that mediate communication in insects. Thus, elucidation of specific roles of certain semiochemicals in mediating intraspecific and interspecific communication of insects, their synthesis and mass production has led to manipulation and successful management of many economically important insect pests through strategies such as; monitoring, mass trapping, mating disruption and repellence. Semiochemicals are preferred over pesticides due to their specificity, biodegradability and affordability (El-Sayed *et al.,* 2006; Witzgall *et al.,* 2010). Whereas, most semiochemicals that are utilized for pests' management are chemical cues that help them: (1) recognize suitable hosts and avoid unsuitable hosts (2) find mates or aggregate around suitable feeding/ ovipositional sites and, avoid natural enemies (predators and parasitoids); the existing information of *A. thunbergii* pertaining these aspects is quite limited and formed the basis of this research.

It is known that Antestia bugs prefer to feed on unripe berries rather than other berry maturity stages or coffee parts. Unripe berries are crucial for the pest survival and reproduction unlike ripe berries, which do not support their development (Le Pelley, 1942; Matsuura *et al.,* 2014). However, the cues contributing to the pest discriminatory feeding pattern remains unknown. In addition, *A. thunbergii* are known to aggregate, a behavior suspected to be governed by semiochemicals (Cilas *et al.,* 1998), but this has not been investigated up to date. Further, most pentatomids such as Antestia bugs often have well developed scent glands that expel irritant secretions that serve as effective chemical defences against predators and to warn conspecifics (Millar, 2005). This study therefore investigated the chemical communication basis of the above behavioral attributes by examining: (1) Host plant volatiles from different coffee berry maturity stages that mediate *A. thunbergii* host finding, location and selection behaviors (kairomones and allomones), (2) Antestia bug aggregation pheromone responsible for their gregarious distribution patterns in the field, (3) candidate alarm pheromones, (4) the performance of a candidate aggregation pheromone and kairomone in luring Antestia bugs in coffee plantations.

1.2 Statement of the problem

There has been a huge decline in the Africa's market share in coffee exports over the past three decades from 27% to 12% (ICO, 2015). Pests are among the key challenges facing coffee production in Africa, and dominated by Antestia bugs, *Antestiopsis thunbergii* in eastern Africa highland coffee (Jackels *et al.,* 2014). The pest transmits pathogens, causes flower abortion and premature abscission of unripe berries (Pelley, 1942). The pest is also associated with causing potato taste defect (PTD) in brewed coffee characterised with a poor aroma and taste (Jackels *et al.,* 2014). PTD in East Africa coffee cup quality rose from 18% in 2012 to 51% in 2013 (Crave, 2012), worsening the regions capacity to produce speciality coffee and threatening the livelihoods of millions coffee farmers in the region. The economic threshold level for the pest is only 1-2 bugs per coffee tree, which can cause up to 45% crop loss (Craves, 2012).

Application of pesticides is currently the most common management strategy for *A. thunbergii* (Babin *et al.,* 2018), but these have various limitations including toxicity and high cost (Deletre *et al.,* 2016) hence alternative management approaches should be pursued. Previously, the role of coffee volatiles in *A. thunbergii* host finding and selection processes were unknown, though they prefer to feed on unripe coffee berries rather than the ripe berries or other coffee parts (Pelley 1942; Matsuura *et al.,* 2014). Furthermore, Antestia bugs aggregate on certain coffee trees or sections of the farm (Cilas *et al.,* 1998) but no study has investigated the possible role of pheromones in the pest behavior. Hence, there is a need to identify semiochemicals (bioactive compounds) that are produced by coffee and *A. thunbergii,* and to ultimately formulate mimicry products with the ability to manipulate and manage the pest.

1.3 Justification

The use of semiochemicals is widely accepted as an effective and eco-friendly approach of managing pests (Cook et al., 2007; Witzgall *et al.,* 2010). Application of pesticides is currently the most common management strategy for *A. thunbergii,* a

major pest of coffee in the Africa region (Babin *et al.,* 2018), but these have various shortcomings such as toxicity to humans and beneficial organisms, environmental pollution and unaffordability. To promote sustainable agriculture, curb excessive use of pesticides and support farmers, organizations such as Fairtrade purchase organically grown farm produce at premium prices, including organic coffee, whose production is barely 1% globally (Bacon, 2005; Van der Vossen, 2005). Therefore, research and development of other management alternatives for *A. thunbergii* that are effective, affordable and safer such as semiochemicals is needed. The use of semiochemicals to replace or minimize use of pesticides in *A. thunbergii* management is expected to boost quality, yields and profitability of coffee, as well as preserve the ecosystem.

1.4 Objectives

1.4.1 General objective

To identify coffee berry volatiles and pheromones that mediate inter- and intra-specific communication of Antestia bugs, *Antestiopsis thunbergii* and evaluate their potential in monitoring and mass trapping of the pest.

1.4.2 Specific objectives

1. To identify coffee berry volatiles that influence host selection and discrimination behaviors of *Antestiopsis thunbergii.*
2. To identify pheromones that mediate conspecific communication in *Antestiopsis thunbergii.*
3. To evaluate the performance of candidate kairomones and aggregations pheromone in monitoring and trapping of *Antestiopsis thunbergii* in coffee plantations.

1.5 Hypotheses

1. Volatiles from different coffee berry maturity stages do not influence the host selection and discrimination behaviors of *Antestiopsis thunbergii*.

2. Conspecific communication of *Antestiopsis thunbergii* is not mediated by pheromones.
3. Candidate kairomones and aggregation pheromones are not useful in luring of *Antestiopsis thunbergii* for its control in coffee plantations.

CHAPTER TWO

LITERATURE REVIEW

2.1 Coffee

2.1.1 Taxonomy and distribution

Coffee, *Coffea* spp. (Gentianales: Rubiaceae) is a perennial plant, native to Africa where it evolved as an understorey tree (DaMatta, 2004). The genus *Coffea* is made up of 103 species (Davis *et al.,* 2006) but only *Coffea arabica* L. (Arabica coffee) and *Coffea canephora* Pierre ex A. Froehner (Robusta coffee) are economically important. The two species are cultivated in approximately 80 countries (FAO, 2013) distributed along the tropics in America, Africa and Asia. Whereas, Arabica coffee is suited for high altitude of about 1600 - 2800 m above sea level (asl), Robusta coffee is adopted for lower altitude ranging 0-1200 m asl (Vega *et al.,* 2009). Globally, *C. arabica* is the predominant species (70%), highly valued for its higher quality as compared to the better yielding *C. canephora* (Medina-Filho *et al.,* 2006).

2.1.2 Phenology of coffee

The coffee plant is a perennial crop which takes approximately 3 years to develop from seed germination to first fruit production. Its lifespan can be upto 80 years, but the economic lifespan is around 30 years (Wintgens, 2004; 2009). For matured trees, the duration from flowering to harvesting takes 6-8 months for Arabica coffee and 8-11 months for Robusta coffee depending on variety and site (Pohlan and Janssens, 2010). The fruit of the coffee tree known as a berry or cherry each contains two beans (endosperm) inside. Coffee beans are the valuable produce of the coffee crop, often processed after harvesting to make roast and ground coffee, soluble coffee powders, and coffee liquor (Wintgens, 2004).

During the fruiting cycle, coffee berries goes through different growth stages characterized by changes in berry size and color, and vitrification of the coffee bean (Wintgens, 2004; Da matta *et al.,* 2008). The main berry maturity stages are:

immature berries (small size, green color and coffee bean not vetrified); mature green berries (fully grown size, green color and vitrified coffee bean) and lastly; ripe berries which are harvestable and red in color (Giordanengo *et al.,* 1993; Ortiz et al. 2004). As the ripening period is long and can last up to 10 weeks (Da matta *et al.,* 2008), hand picking rather than mechanical method is a common harvesting method.

2.1.3 Production and importance of coffee

Coffee is the most profitable agricultural commodity in the globe with a retail value of $ 90 billion annually (Jaramillo *et al.,* 2011). The crop is cultivated by 125 million farmers who are mainly smallholder farmers (Lewin *et al.,* 2004). The total production of coffee is approximately 9.5 million tonnes annually (ICO, 2018). Brazil and Vietnam are the leading coffee producers and exporters, contributing about 50% of the total production (ICO, 2018). Overall Africa contributes about 12%, half its market share three decades ago (ICO, 2015). The East Africa region is the dominant producer of coffee from Africa (75%), led by Ethiopia, Uganda, Tanzania and Kenya (ICO, 2015; 2018). The crop is a major source of foreign earnings in the region, in the range of 4% - 70% depending on the country gross domestic product and coffee production levels (ICO, 2015).

Coffee is an important beverage with about 2 billion cups consumed daily (Diby *et al.,* 2016). It is a primary source of caffeine, popular due to its stimulatory effects in humans (Butt & Sultan, 2011). Coffee consumption is also associated with several health benefits, such as reduced risks of several diseases like type 2 diabetes (Taylor, 2007). However, moderate consumption is important as excessive coffee use may increase health risks in some individuals (Butt and Sultan, 2011; Taylor, 2007). Finland has the highest per capita coffee consumption (12.2 kg) followed by Brazil (5.9 kg) (Miranda *et al.,* 2017; Samoggia & Riedel, 2018).

2.1.4 Coffee production in Kenya

In Kenya, coffee is grown by around 700,000 smallholder farmers (<5 ha), and over 3000 small- and large-scale growers (USAID, 2010; Mugo *et al.,* 2013). Arabica coffee is the predominant cultivated species (90%) while the rest is robusta coffee

(Gichimu & Omondi, 2010). Annual coffee production is estimated at 47,000 tonnes per year (ICO, 2018). Low productivity is common amongst smallholder farmers who produce about 200 kg/ha/yr on average, due to low investment unlike large estates who produce up to tenfold more (USAID, 2010). The coffee producing regions are mainly in the Central and western Kenya (Figure 2.1). Common arabica coffee varieties under cultivation are Ruiru 11, Batian, SL28, SL34 and K7. There are two main coffee harvesting seasons, the low season/early crop in May-July and the high season/late crop in October-December (USAID, 2010). To promote sustainable coffee production, several certification agencies are operating in the country, including Fairtrade, Nestlé Nespresso and Starbuck C.A.F.E Practices (Kamau *et al.,* 2011).

Figure 2.1: Coffee producing regions in Kenya

Source: Kimani, *icipe*

2.1.5 Major pests of coffee and their control

Globally, the Coffee berry borer, *Hypothenemus hampei* Ferrari is the most notorious pest of coffee, associated with high infestation levels of up to 100% (Pereira, *et al.,* 2012) and losses globally estimated at $ 500 million annually (Vega *et al.,* 2009). Although, several management options exist for *H. hampei* control including pesticides, cultural methods (shading and sanitation), entomopathogens and alcohol baits dispensed through the Brocap® trap (Vega *et al.,* 2009), the pest continue to devastate many coffee farmers worldwide.

In Africa, in addition to the Coffee berry borer, other key pests of coffee include several species of stem borers, including *Monochamus leuconotus* and *Bixadus* spp., the Antestia bugs, *Antestiopsis* spp., the Green scale, *Coccus alpinus* and the Coffee thrips, *Diarthrothrips coffeae* (Mugo *et al.,* 2013; Nyambo *et al.,* 1996). Although, cultural methods and pesticide sprays comprise their main control tactics (Nyambo *et al.,* 1996); they are only partially effective. Besides, cultural methods are labor intensive whereas pesticides are expensive and are toxic to humans, non-target beneficial organisms and environment. Hence, efforts to develop safer, cheaper and gentler management options for the pests is required.

2.2 Antestia bugs

2.2.1 Taxonomy and distribution

The Antestia bugs, *Antestiopsis* spp. (Heteroptera: Pentatomidae) are a complex of various species and subspecies distributed in various African countries (Babin *et al.,* 2018). The key species include *A. thunbergii, A. intricata* and *A. facetoides* (Africa); *A. cruciata* (Asia) and *A. clymeneis* (Madagascar). Among the African species, *A. thunbergii* is common in East and South Africa; *A. intricata* in Central and West Africa; whereas *A. facetoides* is limited to Kenya and Tanzania (Figure 2.2; Greathead, 1966; Greathead and Neuenshwander, 2003; Babin *et al.,* 2018). The three species are present in Kenya: both *A. thunbergii* and *A. facetoides* in Eastern and Central, and *A. intricata* in western regions. However, *A. thunbergii* also commonly known as *A.orbitalis* is the most economically important species in East

Africa, including Kenya (Jackels *et al.,* 2014; Matsuuri *et al.,* 2014; Cilas *et al.,* 1998)

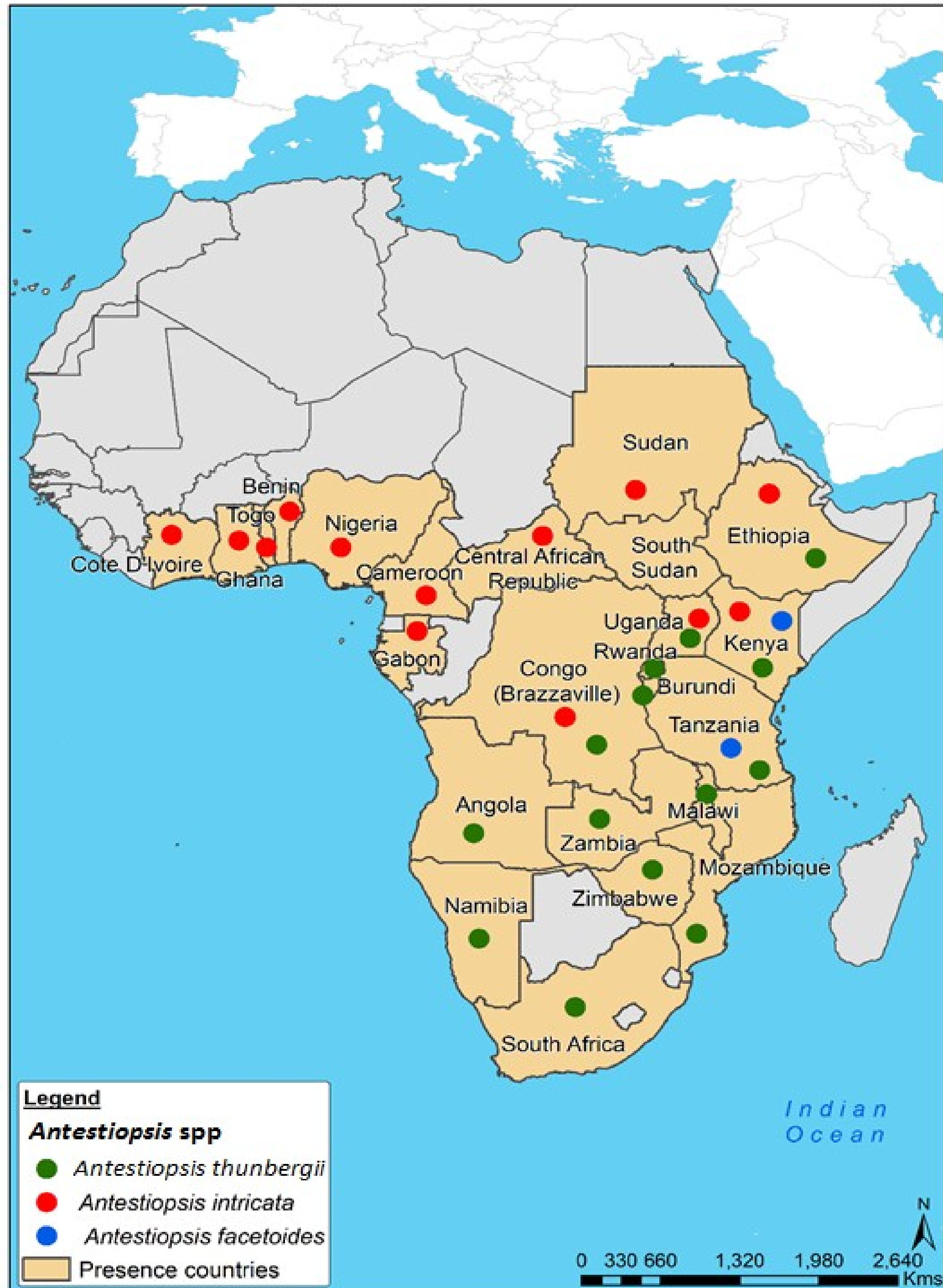

Figure 2.2: Distribution of Antestia bugs, *Antestiopsis* spp. in Africa

Source: Kimani, *icipe*

2.2.2 Lifecycle of Antestia bug, *Antestiopsis thunbergii*

Antestia bug, *Antestiopsis thunbergii* is a hemimetabolous insect, whose development from egg to adult stages takes about three months (Ahmed *et al.,* 2016). The females often lay eggs on the lower surface of *C. arabica* leaves in batches of twelve, but also on coffee berries and shoots. At a constant temperature of 20°C, the eggs hatch in 9 days into first instar nymphs who remain together displaying little or no movement or feeding activity (Kirkpatrick, 1937; Ahmed *et al.,* 2016). First nymphal instars last 8 days before molting into second instars. The second, third, fourth and fifth nymphal instars last 25, 16, 13 and 19 days respectively. The adults have a uniform sex ratio and they can live for up to three months (Ahmed *et al.,* 2016).

Plate 2.1: Antestia bugs, *Antestiopsis* spp. life stages and species.

Alphabets represent: *A. thunbergii:* (A) first instar nymphs (B) second instar nymphs (C) Adult (D) *A. facetoides* adult. © Copeland, *icipe*

2.2.3 Feeding habits and behavior of Antestia bugs

Coffee, especially Arabica coffee is the primary host of Antestia bug, *Antestiopsis* spp. However, some wild plant species in the same family as coffee (Rubiaceae) are alternative host plants for the pest and are considered reservoirs that support the pest during offseason (Le Pelley, 1942; Babin *et al.,* 2018). Although, they feed on both the vegetative and reproductive parts of coffee, unripe coffee berries are preferred and necessary for the pest survival and reproduction (Le Pelley, 1942; Matsuuri *et al.,* 2014). Contrary, ripe coffee berries do not support the pest development (Le Pelley, 1942). Feeding is often observed in the morning, and in the evening (Foucart & Brion, 1959). However, long flights by adults to colonize new plantations is common during warm weather (Babin *et al.,* 2018). *Antestiopsis thunbergii* is gregarious in the field, especially first instar nymphs and adults. Cilas *et al.,* (1998) suggested that this aggregation behavior could be mediated by semiochemicals. The pest also produces a strong odor while disturbed, a distinct characteristic of Pentatomids/ "stink bugs" the name illustrative of the odor the species produce to warn conspecifics of impending danger and repel natural enemies (Millar, 2005).

2.2.4 Damage and economic importance of Antestia bugs

Antestiopsis spp. feed on various coffee parts including leaves, shoots, flower buds and berries leading to a reduction in quality and quantity of coffee (Le Pelley 1942; Cilas *et al.,* 1998). Their infestation leads to flower abortion, distorted leaves, multiple branching, necrosis, rotting and premature berry fall and transmission of secondary pathogenic microorganisms (Le Pelley 1942; Cilas *et al.,* 1998). The pest is also associated with causing 'potato taste defect' in processed infested coffee, characterized by poor flavour, leading to major losses in export earnings (Jackels *et al.,* 2014). The economic threshold level for control in Kenya is 1-2 bugs per coffee tree (Mugo *et al.,* 2013). The incidence of potato taste defect has been on the rise in East Africa and was estimated at 51% in 2013 (SNV, 2014), prompting intense research from different stakeholders to curb the problem.

2.2.5 Management strategies for Antestia bugs

Chemical insecticides are commonly used to control Antestia bugs including organophosphates, carbamates, organochlorines and pyrethroids (Mbondji & Dina, 1992; Chichaybelu, 1993; Mugo *et al.,* 2011). However, most of them are highly toxic to humans and environment and their use have been banned in most developed countries (Babin *et al.,* 2018). Hence, their use in Africa continues to pose a health risk, which should be averted by replacing the insecticides with other effective and safer management methods.

Biopesticides are an alternative method for Antestia bug control, especially pyrethrum, which is cheaper and safer as compared to chemical insecticides. However, they have low persistence and effectiveness is dependent on precise application when the pest is active in field (Babin *et al.,* 2018).

A few biological control agents of Antestia bugs occur naturally in coffee growing regions. Although, high parasitism rates of 40-95% have been recorded in a predominant egg parasitoid species, *Telenomus sechellensis*, very low parasitism rates prevail for adults (Greathead, 1966). Currently, there are no commercially produced biological control agents (Babin *et al.,* 2018). Hence, future studies are required to identify an effective natural enemy against different developmental stages of Antestia bugs and, to develop a mass rearing method for their subsequent release in Antestia bug infested regions.

Cultural practices such as pruning, and shading are reported to reduce Antestia bug populations (Mugo *et al.,* 2013). However, shading favor coffee berry borer, another major pest of coffee (Jaramillo *et al.,* 2013b), hence the practice may be counterproductive as both pests often exist together in many coffee farms in East Africa.

2.3 Semiochemicals in pest management

Semiochemicals is a collective term that refers to specific volatiles produced by plants and insects that stimulate the olfaction of insects leading to a wide range of

behaviors (Foster & Harris, 1997). Plant volatiles are broadly classified into two: kairomones and allomones, which attract and repel recipient insects, respectively (Reddy and Guerrero, 2004). Insects produce odors known as pheromones with diverse roles including aggregation, anti-aggregation, host marking, alarm, or mating purposes within conspecifics and for defence against heterospecifics. In recent years, there has been a lot of research regarding utilization of pheromones and plant volatiles in pest management with notable successes. Key characteristics that make semiochemical-based pest strategies to be preferred over pesticides include efficiency in small amounts, specificity, affordability and biodegradability (El-Sayed *et al.,* 2006; Witzgall *et al.,* 2010).

A clear understanding of a pest biology and behavior is a fundamental step towards the development of semiochemical based approaches for pest management. Although these aspects have been studied widely (Babin *et al.,* 2018), very little is known about the chemical communication of Antestia bugs. The only study that attempted to examine Antestia bug pheromones was conducted by Jackels *et al.* (2014). The authors reported several compounds such as alkanes in the headspace volatiles of desiccated *A. thunbergii*, which were dominated by tridecane and dodecane, which are known defensive compounds produced by other pentatomid species. According to Millar *et al.* (2005), headspace volatile collection from live bugs is recommended for a clear representation of bug pheromones as dead bugs often produce high amounts of defensive compounds masking aggregative compounds. The behavior and feeding habits of *A. thunbergii* strongly suggest that the pest utilizes semiochemicals to choose food on host plant, to aggregate, to warn conspecifics and to defend against natural enemies. Hence, chemo-ecological studies are required to identify these semiochemicals, confirm their role and develop an alternative management option for the pest.

2.4 Semiochemical trapping systems for stink bugs

The use of pheromones in stink bug control is highly investigated but their performance varies between species. For instance, Phytophagous stink bugs are only weakly attracted to attractive pheromones. Though attracted to the pheromone source

through long range cues, a high proportion land close to the lure source but fail to enter inside the traps, as they require bug produced (species-specific) and substrate borne-vibrational cues at short range which most traps lack (Millar, 2005). “Lure and kill” approach is proposed to control such species whereby a few “lure” baited plants are sacrificed or used as trap crops where the pest converge for elimination through minimal spot spray of pesticides or biopesticide application.

On the other hand, some stink bug pheromones, especially aggregation and sex pheromones are highly effective and have been commercialized for use in pest management for surveillance and mass trapping (Leskey *et al.,* 2012; Morrison *et al.,* 2017). For example, pheromone baited traps are strongly effective in catching mirids (*Phytocoris* sp), than other sampling methods (Millar *et al.,* 1997).

CHAPTER THREE

IDENTIFICATION OF COFFEE BERRY VOLATILES THAT INFLUENCE FOOD SELECTION AND DISCRIMINATION BEHAVIORS OF ANTESTIOPSIS THUNBERGII

Abstract

Understanding the roles of volatile organic compounds (VOCs) in insect–plant interactions is a key component towards the development of safe pest management strategies and sustainable agriculture. Antestia bug *Antestiopsis thunbergii* Gmelin, a key pest of *Coffea arabica* in East Africa has a strong preference for unripe coffee berries (mature green), which are essential for the bug to complete its lifecycle, reproduce and enhance its longevity. On the contrary, ripe coffee berries are not preferred, neither do they support the pest development. Hence, it is likely that *A. thunbergii* relies on key volatile compounds emitted by the superior quality food and suboptimal food respectively for recognition and discrimination. This study tested this hypothesis using behavioral and electrophysiological assays, and chemical analysis. Volatile emissions from unripe coffee berries elicited strong attraction in second instar nymphs and adults of *A. thunbergii* whereas those from ripe coffee berries were repellent. Notably, the second instar nymphs were more behaviourally responsive to the volatiles followed by males and females respectively. Coupled gas chromatography–electroantennographic detection (GC/EAD) with second instar nymphs isolated five antenally-active components from unripe berry volatiles, four of which were identified by coupled GC–mass spectrometry as toluene, anisole, methyl 3-ethyl-4-methylpentanoate and (5*S*,7*S*)-conophthorin. In concentration assays, in which second instar nymphs did not respond to toluene, they were strongly attracted to anisole, methyl 3-ethyl-4-methylpentanoate and (5*S*,7*S*)-conophthorin, and a blend from the three compounds at concentrations lower or equivalent to the natural volatile extract. These results suggest that the blend from these three compounds allows host recognition and attraction towards unripe berries (suitable host food) in second instar nymphs of Antestia bug. The 3-component blend is thus a

candidate kairomone that could be incorporated for monitoring and trapping of the pest in coffee plantations.

On the other hand, coupled GC–mass spectrometry analysis of the headspace volatiles of ripe coffee berries (unsuitable host food) revealed a more complex blend of chemicals of which ten elicited electrophysiological activity in antennae of second instar nymphs. Five of these compounds including 3-hydroxy-2-butanone, 2-heptanone, 2-isopropyl-3-methoxypyrazine [IPMP], 2-isobutyl-3-methoxypyrazine [IBMP] and (*E*)-β-caryophyllene were identified as unique to the volatiles of ripe berries (not found in unripe berries) and they elicited avoidance behavior in second instar nymphs when tested singly and in a blend. In addition, their blend also inhibited responses of nymphs to a 3-component attractant blend, previously identified in unripe berries. These findings suggest that the blend of the five compounds can be exploited as repellents in the management of *A. thunbergii* to repel the pest from coffee plantations or interfere with the pest–host recognition process through masking of suitable host odors from the susceptible unripe coffee berry stage.

3.1 Introduction

Herbivorous insects identify and choose suitable host plants using "specific plant odors" or "specific ratios" of ubiquitous plant odors (Bruce *et al.,* 2005; Cook *et al.,* 2007). Repellent behaviors may be elicited if an insect perceives odors which signal unsuitable hosts (Cook *et al.,* 2007). Repellence is exhibited in various forms such as expellency, avoidance, antifeeding and odor masking (Cook *et al.,* 2007; Deletre *et al.,* 2016). The elucidation of the chemo-ecological roles of various plant volatiles in insect–plant communication is important for the development of novel crop protection strategies, for example, through engineering or screening crop plants for resistance against insect pests and exploiting semiochemicals that act as attractants or repellents of insect pests (Bruce *et al.,* 2005).

Most past studies examined kairomones (attractants) of many key pests of coffee. Subsequently, exploitation of kairomones as potential attractants has been reported

for most coffee pests including the coffee berry borer *Hypothenemus hampei* Ferrari (Vega *et al.,* 2009; Jaramillo *et al.,* 2013; Njihia et *al.,* 2014); the black twig borer *Xylosandrus compactus* Eichhoff (Egonyu & Torto, 2017); the Mediterranean fruit fly, *Ceratitis capitata* Wiedemann (Warthen *et al.,* 1997) but not for Antestia bug *Antestiopsis thunbergii* Gmelin. As a result, an alcohol bait incorporated in Brocap traps® is widely used commercially to monitor and mass trap *H. hampei* (Vega *et al.,* 2009).

However, there appears to be little information on repellents of major coffee pests except for *H. hampei* (Borbón *et al.,* 2000; Njihia *et al.,* 2014), for which (*Z*)-3-hexenol, 3-methylcyclohex-2-en-1-one and verbenone from green leaves are repellents (Borbón *et al.,* 2000). Another volatile organic compound, frontalin, which is emitted in higher amounts in infested ripe *C. arabica* berries, elicited avoidance in *H. hampei* females in laboratory assays and inhibited commercial attractant captures of the pest by 77% in field tests (Njihia *et al.,* 2014). Thus, there is clearly a need to investigate the nature of other host plant-derived repellents of coffee pests including *A. thunbergii*.

Antestiopsis thunbergii (Heteroptera: Pentatomidae) is a major pest of coffee in Africa (Greathead, 1966; Babin et al., 2018), particularly East Africa. This oligophagous pest attacks and causes damage to both the vegetative and reproductive parts of *C. arabica* (Le Pelley 1942) but prefers to feed on unripe berries (mature green) that are crucial for their survival and reproduction. By contrast, ripe (red) berries are not preferred and do not support *A. thunbergii* development (Le Pelley, 1942; Matsuura *et al.,* 2014). In the present study, the olfactory basis of host food selection and discrimination of *A. thunbergii* was investigated using behavioral and electrophysiological assays, and chemical analysis.

3.2 Materials and Methods

3.2.1 Study site

Laboratory studies were carried out at the International Centre of Insect Physiology and Ecology *(icipe)* in Nairobi, Kenya ($1^0$16’60’’S; $36^0$49’0’’E). Coffee berry

samples and Antestia bugs, *Antestiopsis thunbergii* used in laboratory experiments were obtained from a coffee plantation in Kiambu county, Central Kenya ($1^0$11'27.15''S; 36^0 49'23.03''E; altitude = 1722 m. a.s.l). The farm is a privately-owned large commercial coffee estate (~ 120 ha) known as Mbumi Coffee Estate and Mills.

3.2.2 Coffee berry samples

Different maturation stages of organically grown coffee berries *viz.* unripe (mature green) and ripe berries (red and harvestable) were obtained from a coffee plantation (farm described in section 3.3.1). The maturity index of unripe and ripe berries was based on full grown size and their characteristic green and red colors, respectively. Berries were carefully excised from coffee tree branches in the field using a sterile scalpel blade No. 21 and placed into 500 ml sterile cylindrical glass jars covered with quick fit lids (Sigma Scientific, Gainesville, FL, USA). Berry samples were promptly transferred to a laboratory at *icipe* which is 13 km from the farm, for use in subsequent assays.

3.2.3 Rearing of Antestia bugs, *Antestiopsis thunbergii*

A colony of *A. thunbergii* for use in tests was established at laboratories at *icipe* in the year 2015, using a rearing method developed by Ahmed *et al.* (2016). Briefly, *A. thunbergii* nymphs and adults were collected from a coffee plantation (described in section 3.3.1). The bugs were reared in 20 X 20 X 20 cm transparent plastic cages that were open but sealed with white muslin cloth on two opposite sides to provide aeration (plate 3.1). They were fed on unripe coffee berries (mature green) and young shoots, which were replenished every 3–4 days. A cotton ball moistened with distilled water was placed inside the cages to provide humidity and a water source. Once eggs were laid, a cluster of about 70 eggs was removed from the colony and placed in separate cages with similar food substrate described above, where they were closely monitored through the developmental stages. Whereas nymphs were maintained in these groups of similar age, adults were held individually by separating them as soon as they emerged (< 24 h post emergence) into smaller 20 ml plastic

containers. First or second-generation nymphs and adults of known age were used in the experiments. The colony was maintained in incubators set at 23 ± 1°C, relative humidity (RH) of 75 ± 5% and 12 h photoperiod.

Plate 3.1: Rearing cage for Antestia bug, *Antestiopsis thunbergii*

3.2.4 Insect samples

Antestiopsis thunbergii second instar nymphs that were 9 ± 3 days old and unmated adults that were 8 ± 2 days old were used in experiments. Their rearing conditions were as described previously in section 3.3.3. Second instar nymphs were selected because they consist the first actively feeding stage of the insect and are very mobile (Ahmed *et al.,* 2016). Hence, they may be considered as the most damaging of all the nymphal instars. On the other hand, adults of *A. thunbergii* are known to fly long distances in search of suitable host food to sustain their survival and reproduction.

Both nymphs and adults were starved for 16 h before use in behavioral and/ electrophysiological tests.

3.2.5 Olfactometer set up for conducting behavioral assays

A glass Y-tube olfactometer similar to that described by Jaramillo *et al.* (2013) and Njihia *et al.* (2014) was used, with minimal modifications. The Y-tube, which was positioned horizontally consisted of two 10-cm-long arms converging to a 16-cm long main arm at 80° inside angle and 2.5 cm overall diameter. The Y-tube was placed at the centre of a carton box (34 × 36 × 54 cm), which was lined inside with white printer paper, and open at the front and top side, in a dark room to minimize visual distraction. A 11 W fluorescent bulb placed on top of the box illuminated the olfactometer arena evenly. Respective treatments or controls were put in 50 ml glass chambers, placed outside the box and attached to either of the arms of the Y-tube using Teflon tubing. A pump (WOB-L pump 2522C, Monroe, Louisiana, USA) supplied charcoal-filtered and humidified air (80% RH) through each arm of the Y-tube at 100 ml min^{-1}. PVC tubing at the base of the olfactometer was connected to the vacuum source of the pump operating at 200 ml min^{-1}.

Behavioral tests were conducted by introducing individual *A. thunbergii* at the entrance of the main arm of the Y-tube with a fine camel hair brush and allowing up to 15 min to make a choice. Positive responses were recorded when an insect walked upstream and spent at least 20 s inside the left or right arm of the olfactometer. Insects that remained at the same spot after introduction into the Y-tube for 8 min or were mobile within the vertical arm of the olfactometer but did not make a choice in the allocated time (15 min) were considered as non- respondents and were not included in the statistical analysis. Each test insect was considered as a replicate. Assignment of odor source to each arm of the olfactometer was reversed in between tests to eliminate positional bias. The bioassays were conducted in a room maintained at 24 ± 1°C and 50 ± 5% RH, between 10:00 and 16:00 h, which is the time when the bugs are active in the field (Foucart & Brion, 1959).

3.2.6 Behavioral assays with different coffee berry maturity stages

These assays were conducted in a dual choice olfactometer arena previously described in section 3.3.5, to investigate behavioral responses of second instar nymphs towards unripe and ripe coffee berries. Prior to the tests, responses of the nymphs were compared amongst two blank odor sources to check for uniformity of both sides of the Y-tube olfactometer and eliminate any positional bias. Then, the following tests were conducted: (1) unripe berries vs. blank; (2) ripe berries vs. blank and (3) unripe berries vs. ripe berries. Each test comprised two berries of each maturity stage whose total weight was approximately 2 g. The berries were replaced after testing responses of every five nymphs. Forty nymphs (N=40) were used per test, conducted on different days.

3.2.7 Volatile collection from coffee berry samples

Volatiles were collected from unripe and ripe coffee berries using a headspace sampling system similar to that previously described by Jaramillo *et al.* (2013). Fresh berry samples (150 g each) were placed in 500-ml cylindrical glass jars (Sigma Scientific, Gainesville, FL, USA) and clean air blown through the sample at a flow rate of 348 ml min^{-1} using a battery-operated pump (PAS-500 Personal Air Sampler, Supelco, Bellefonte, PA, USA), which also pulled volatile organic compounds (VOCs) onto pre-cleaned charcoal filter adsorbents (5 mg, Brechbuhler, Schlierensee, Switzerland) for 24 h. Each filter was subsequently eluted with 150 µl dichloromethane for use in behavioral tests and analyses using gas chromatography electroantennographic detection (GC/EAD). In addition, eluates from three different samples were pooled; the 450 µl sample was reduced to 30 µl under a gentle stream of nitrogen for use in GC–mass spectrometry (GC/MS). Volatile collection was also done from empty chambers under the same conditions to serve as control. Samples were stored at – 80 °C until use. Several replicates were collected and used for the various experiments but three were examined in GC/MS analysis.

3.2.8 Behavioral assays with coffee berry volatiles and their mixtures with berries

Behavioral responses of *A. thunbergii* adults (both sexes) and second instar nymphs to unripe and ripe berry odors were each tested at three concentrations (i.e. emissions of berry samples per day): 3 g berries/day, 6 g berries/day and 12 g berries/day, respectively. They were tested by applying 3, 6 or 12 µl of the volatile extracts, respectively, onto 2 cm X 2 cm filter papers with a syringe. Vaporization of the solvent was allowed for 1 min before placing the impregnated filter papers into the odor source chambers. The controls were equivalent amounts of dichloromethane applied onto filter papers of matching size.

In addition, to investigate whether ripe berry odors also have a masking effect in addition to avoidance identified in the preceding assays, behavioral responses of *A. thunbergii* second instar nymphs to a mixture of ripe berry volatiles and unripe berries (preferred host food) were tested against blank, unripe berries and ripe berries. During assays, a filter paper impregnated with an optimum repellent concentration of ripe berry volatiles selected from preceding assay (6 g berries/day) was placed in the same odor source chamber with two unripe berries (~2 g). The control was blank, or two berries of each maturity stage. Treatments were replaced after every 30 min in both experiments. Total number of bugs tested in various tests ranged between 34 and 50.

3.2.9 Analysis of bioactive volatile components from coffee berries

To isolate coffee berry odor components that are detected by the antennae of *A. thunbergii* second instar nymphs, coupled gas chromatography–electroantennographic detection (GC/EAD) analysis was conducted on a Hewlett-Packard (HP) 5890 Series II gas chromatograph equipped with HP-1 column (30 m X 0.32-mm diameter X 0.25 µm film thickness (Agilent, Palo Alto, California, USA) using nitrogen as the carrier gas at 1.2 ml min^{-1} flow rate. Volatiles were analysed in the splitless mode at an injector temperature of 280 ^{0}C and a split valve delay of 3 min. The oven temperature was held at 35 ^{0}C for 3 min, programmed at 10 ^{0}C min^{-1}

to 280 ^{0}C and maintained at this temperature for 10 min. The column effluent was split 1:1 for simultaneous recording by a flame ionization detector (FID) and EAD. The antennal preparation was made by filling in two sharpened glass capillaries with ringer saline solution (Kugel 1977). One of the capillaries was used to pierce the abdomen of a second instar nymph and attached to a reference electrode, whereas the other connected the antenna end of the nymph to a recording electrode. The antennal signal was detected through an amplifier (INR-II, Syntech, Hilversum, The Netherlands), which was acquired and processed by a data acquisition controller (IDAC-4, Syntech, Hilversum, The Netherlands) and later analyzed with a GC/EAD software (EAD 2000, Syntech) to generate FID and EAD signals on a computer. Aliquots (5 µl) of headspace samples previously collected from unripe and ripe coffee berries (section 4.3.4) and 1–3 µl of 100 ng/µl of each corresponding EAD-active synthetic standard formulated in dichloromethane were analysed. EAD responses were considered when at least three antennal elicitations were recorded for each compound.

3.2.10 Identification of coffee berry volatiles

Identification of volatile organic compounds emitted by unripe and ripe coffee berries was achieved by GC/MS analysis on an Agilent Technologies 7890A GC linked to a 5795C MS, equipped with MSD ChemStation E.02.00.493 and Wiley 9th/NIST 2008 MS library. The GC/MS column and the temperature program used were like those described for GC/EAD analysis (section 3.3.9). Confirmation of the identity of compounds in samples was done by comparing their retention time and mass spectral fragmentation of corresponding authentic standards in the library (NIST/EPA/NIH Mass spectral Library 2005a, version V2.od). Quantification of EAD-active coffee odor components was based on peak area comparison with those of the authentic standards

3.2.11 Behavioral assays with synthetic standards

These bioassays were conducted to investigate the role of specific EAD-active compounds identified in second instar nymphs. First, responses of the nymphs to

four EAD active compounds, identified from unripe coffee berries namely toluene, anisole, methyl 3-ethyl-4-methylpentanoate and 5*S*, 7*S*-conophthorin were studied. Each of the compound was tested at three concentrations formulated from the natural amounts found in unripe berries. Hence, tested concentrations comprised natural concentration; one-tenth and tenfold the natural concentrations of each compound (Table 3.1 and Table 3.2). Treatments were dispensed by applying 10 µl of each concentration onto a 2 cm x 2 cm filter paper with a syringe and allowing the solvent to evaporate for 1 min before testing. The control was 10 µl dichloromethane. Then, a three-component blend comprising optimal attractive doses of anisole (0.067 ng/µl), methyl 3-ethyl-4-methylpentanoate (0.114 ng/µl) and (5*S*,7*S*)- conophthorin (0.39 ng/µl), chosen from previous assays results was also tested against solvent and 6 µl aliquot (6 g/day) of headspace sample from unripe berries. This blend was tested by applying 10 µl of the appropriate concentration level of each compound on separate filter papers, which were then placed in the same odor source chamber for dispensation as a blend.

Second, responses of nymphs to five EAD- active compounds unique in headspace samples of ripe berries (not detected in unripe berries), were tested at three concentrations, formulated from natural amounts as explained previously and as shown in Table 3.1 and Table 3.2. These compounds were 3-hydroxy-2-butanone, 2-heptanone, 2-isopropyl-3-methoxypyrazine, 1 2-isobutyl-3-methoxypyrazine and (*E*)-β-caryophyllene). Consequently, a five-component blend comprising each of the above five EAD active compounds was formulated at three concentrations in natural ratios. Still, the concentrations of the blend were tested at the natural concentration; one-tenth and tenfold the natural concentrations and tested against the solvent. To investigate masking, the five-component blend from ripe berries at natural amounts was mixed with the three-component blend from unripe berries; the blends had been found to repel and attract nymphs respectively. The mixture of the two different blends was tested against solvent, three-component blend and five-component blend alone to investigate whether the repellent blend could mask the attractant blend. Treatments were replaced after 30 min. Total number of insects tested for various treatments was either 40 or 50 nymphs.

Table 3.1: Concentrations of EAD-active compounds tested in behavioral tests

Source	Compounds	Tested concentrations (ng/µl)		
Unripe coffee berry odors	Toluene	0.070	0.70	7.0
	Anisole	0.067	0.67	6.7
	Methyl 3-ethyl-4–methylpentanoate	0.114	1.14	11.4
	(5*S*,7*S*)-Conophthorin	0.039	0.39	3.9
Ripe coffee berry odors	3-Hydroxy-2-butanone	2.39	23.9	239
	2-Heptanone	0.054	0.54	5.4
	2-Isopropyl-3-methoxypyrazine	0.083	0.83	8.3
	2-Isobutyl-3-methoxypyrazine	0.103	1.03	10.3
	(*E*)-β-caryophyllene	0.062	0.62	6.2

Values at the middle column are the natural concentrations produced by coffee berries

3.2.12 Source of synthetic standards

Methyl 3-ethyl-4-methylpentanoate (purity 97%), (5*S*,7*S*)-conophthorin (purity 99%) and rac.-chalcogran (mixture of four stereoisomers; purity 98%) were provided by Prof. Wittko Francke, University of Hamburg, Germany. β -ocimene (purity≥ 95) was purchased from Chemika whereas β-cedrene and linalool oxide (purity ≥ 98%) were from Fluka Analytical. All other standards were purchased from Sigma-Aldrich (purity ≥ 98%)

3.2.13 Data analysis

The number of *A. thunbergii* nymphs and adults responding to treatments i.e., unripe or ripe coffee berries, headspace volatiles from the coffee berries, synthetic EAD-active standards and their blends compared to control (e.g. blank/solvent) were analysed using Chi-square (χ^2) goodness-of-fit tests, assuming a distribution ratio of 1:1. Only responding insects (n) were considered in the analysis. The total amount of volatiles emitted by unripe and ripe coffee berries were analysed using GLM with Gamma distribution and inverse link function, followed by deviance analysis (ANODEV). The differences in the chemical profiles of unripe and ripe coffee berries were assessed by principal component analysis (PCA). The data were analyzed using R statistical software, version 3.1.2 (R Core Team, 2014).

3.3 Results

3.3.1 Responses of nymphs to different coffee berry maturity stages

There were no significant differences in responses of nymphs when tested against two blanks ($\chi^2 = 0.04$, df = 1, P = 0.841) indicating that the olfactometer system was not biased. In subsequent tests, the responses of nymphs were significantly greater to the volatile emissions of unripe berries than the blank (control) ($\chi^2 = 5.12$, df = 1, P = 0.023). On the contrary, nymphs significantly avoided volatiles from ripe berries when compared to the blank ($\chi^2 = 6.82$, df = 1, P = 0.009). In a no-choice assay amongst the two maturity stages, about five-fold more nymphs were attracted to unripe berries than ripe berries ($\chi^2 = 15.11$, df = 1, P = 0.001; Figure 3.1).

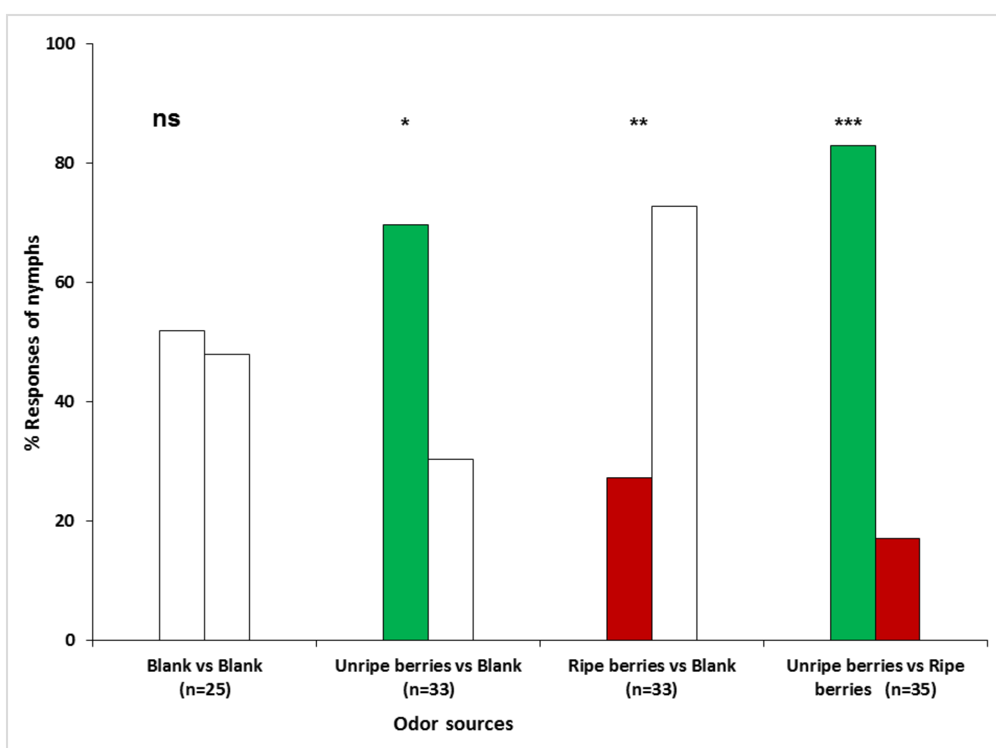

Figure 3.1: Behavioural responses of *Antestiopsis thunbergii* second instar nymphs to different maturity stages of coffee berries

Total insects tested per pairing was 40. "n" represents the number of respondents and asterisks indicate the significance levels; ns=not-significant, * p< 0.05, ** p< 0.01 and *** p< 0.001.

3.3.2 Responses of nymphs to coffee berry volatiles and their mixtures with berries

Headspace volatiles collected from unripe berries attracted approximately three times more nymphs than the solvent at all the concentration levels tested (3 g/day: χ^2=7.26, df = 1, P = 0.007; 6 g/day: χ^2=8.53, df = 1, P = 0.003; and 12 g/day: χ^2=6.13, df = 1, P = 0.013) (Figure 3.2A). By contrast, headspace volatiles from ripe berries elicited significant avoidance by nymphs at all the three concentrations tested (3 g/day: χ^2 = 5.49, df = 1, P = 0.019; 6 g/day: χ^2 =12.60, df = 1, P = 0.001; and 12 g/day: χ^2 = 6.40, df = 1, P < 0.011) (Figure 3.2B).

Further, a mixture of ripe berry volatiles and unripe berries (suitable host food) was significantly avoided by nymphs, which instead preferred blank (χ^2 =4.90, df = 1, P = 0.027) and unripe berries alone (χ^2 =7.05, df = 1, P = 0.008). However, there was no significant difference in nymph behavioral response when the same mixture was compared with ripe berries (χ^2 =0.02, df = 1, P = 0.876) (Figure 3.3).

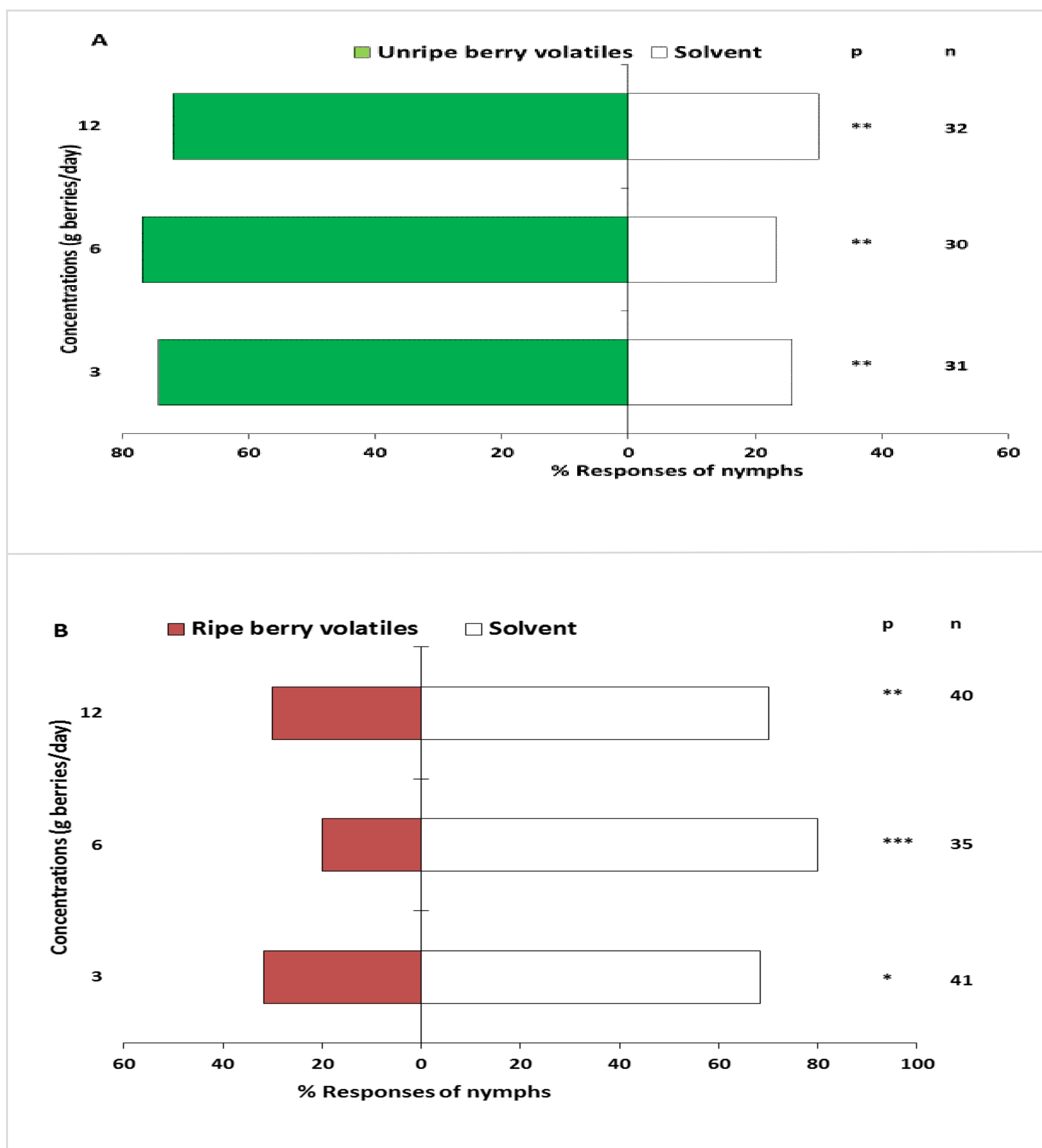

Figure 3.2: Behavioural responses of *Antestiopsis thunbergii* second instar nymphs to headspace volatiles obtained from: (A) Unripe coffee berries (B) Ripe coffee berries.

Total insects tested per pairing was 40 and 50 respectively; “n” is the number of respondents and asterisks indicate the significance levels * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

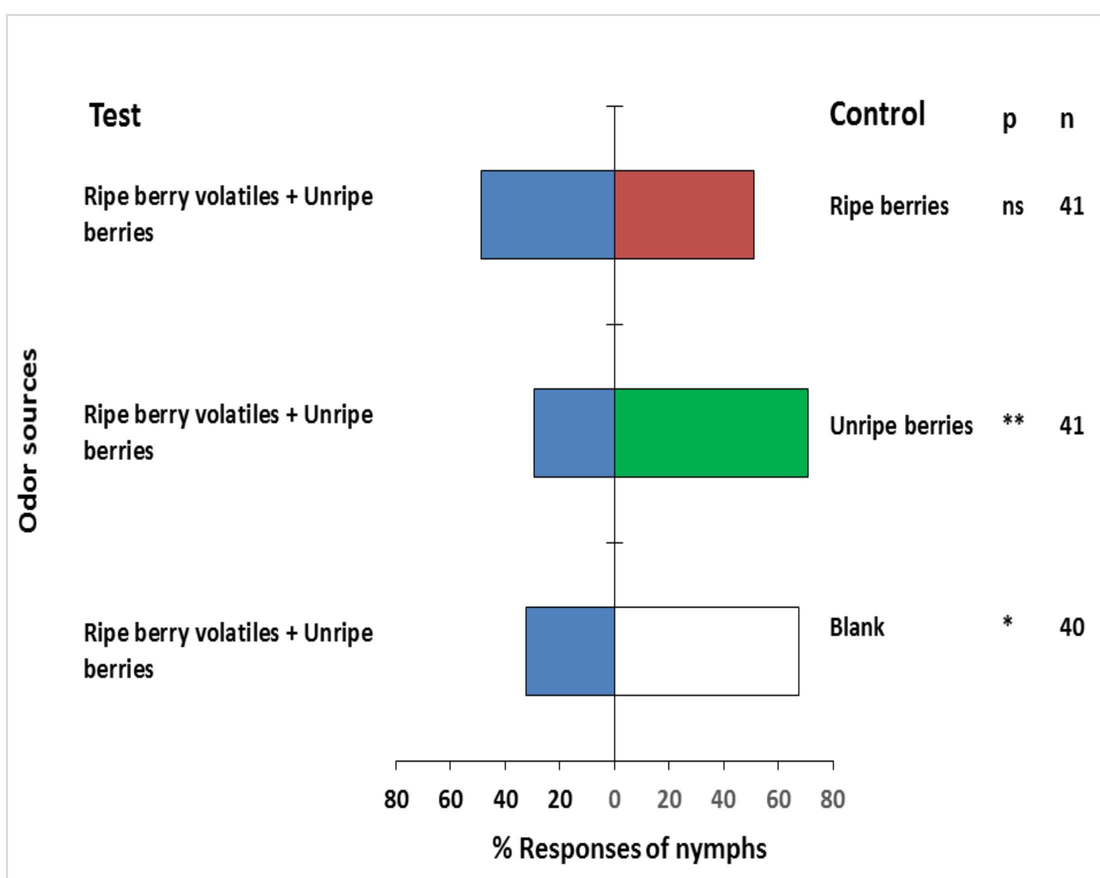

Figure 3.3: Behavioral responses of *Antestiopsis thunbergii* second instar nymphs to a mixture of ripe coffee berry volatiles and unripe coffee berries (suitable host) versus a blank, unripe or ripe coffee berries.

The total number of insects tested per pairing was 50; n represents the number of respondents. Asterisks represent the significant levels; ns=not-significant, *P<0.05 & **P<0.01.

3.3.3 Responses of adults to coffee berry volatiles

Unripe berry volatiles did not induce significant behavioural response in *A. thunbergii* males at the least tested concentration of 3 g/day (χ^2=2.13, df = 1, P = 0.140), but higher concentrations were highly preferred than control: 6 g/day (χ^2=6.53, df = 1, P = 0.010) and 12 g/day (χ^2=8.53, df = 1, P = 0.003). Although, females did not respond to the least and moderate concentrations of 3 g day^{-1} (χ^2 = 1.2, df = 1, P = 0.273) and 6 g/day (χ^2 = 2.13, df = 1,P = 0.144) they were

significantly attracted to the highest tested concentration of unripe berry volatiles of 12 g/day (χ^2 = 4.8, df = 1, P = 0.028) (Figure 3.4)

On the other hand, ripe berry volatiles did not elicit significant behavioral responses in males at the least and moderate tested concentrations of 3 g/day (χ^2 = 0, df = 1, P = 1.00) and 6 g/ day (χ^2=1.20, df = 1, P < 0.27), but they strongly avoided the highest tested concentration of 12 g/day (χ^2_1=6.53, df=1, P < 0.01). Although, females seemed to avoid volatiles from ripe berries (20% more females opted for the control (solvent) over ripe berry volatiles), these differences were not statistically significant for all the three tested concentrations of 3, 6 and 12 g/day (χ^2 = 2.13, df=1, P = 0.14) (Figure 3.5)

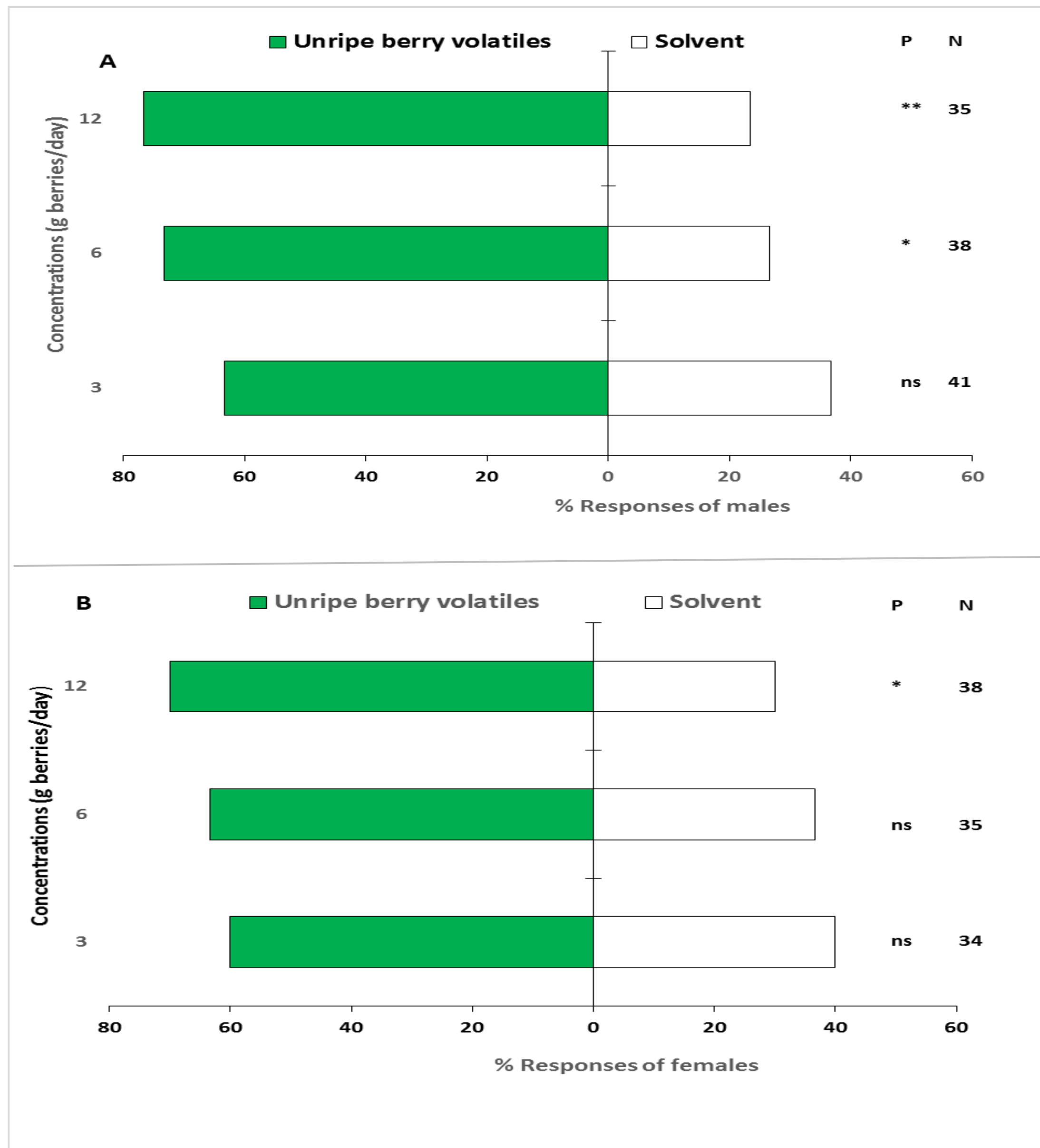

Figure 3.4: Behavioral responses of *Antestiopsis thunbergii* (A) males and (B) females to headspace volatiles obtained from unripe coffee berries.

The total number of insects tested per pairing is represented by “N”, while the total number of respondents (n) was 30 insects. Asterisks represent the significance levels; ns = non-significant, $^{*}P<0.05$ & $^{**}P<0.01$

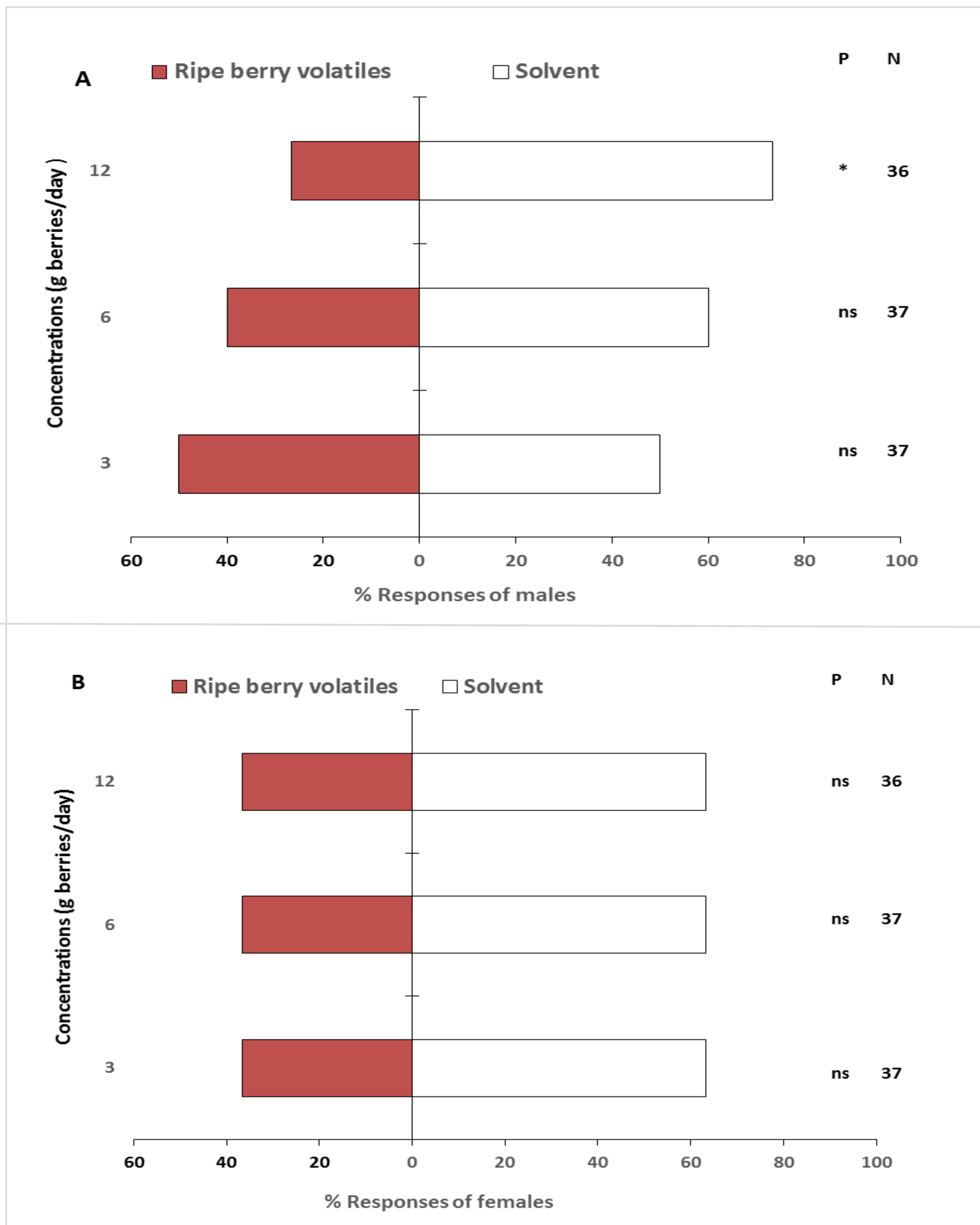

Figure 3.5: Behavioral responses of *Antestiopsis thunbergii* (A) males and (B) females to headspace volatiles obtained from ripe coffee berries

The total number of insects tested per pairing is represented by "N", while the total number of respondents (n) was 30 insects. Asterisks represent the significance levels; ns = non-significant, $^{*}P<0.05$ & $^{**}P<0.01$

3.3.4 Comparison of volatile components from unripe and ripe coffee berries

The total amount of volatiles emitted by unripe berries differed significantly from the amount emitted by ripe berries ($\chi^2 = 27.444$, df = 1, P = 0.001) (Figure 3.6). Ripe berries had a richer volatile blend complex than unripe berries, i.e. 37 and 20 compounds for the two maturity stages, respectively (Table 3.2). Some compounds were unique to each stage, while others were shared amongst the two phenology stages, in varied quantities. In total, 43 compounds were detected in either of the berry categories and consisted 15 sesquiterpenes, 7 monoterpenes, 5 aromatic hydrocarbons, 5 ketones, 4 spiroacetals, 3 pyrazines, 2 alcohols,1 ester and 1 aldehyde. Most aromatic hydrocarbons (toluene, anisole, 3-methylanisole and naphthalene) except one were found in both unripe and ripe coffee berries in varied amounts. All alcohols, pyrazines and ketones except 6-Ethenyldihydro-2,2,6-trimethyl-2H-pyran-3(4H)-one were specifically found in ripe berries only (Table 3.2).

Principal component analysis (PCA), which evaluated the influence of each compound on the blend, separated the chemical profile of unripe from that of ripe (Figure 3.7). More than 98% of the observed variation in the profiles was explained by the first two principal components (PCs). PC1, which accounted for 88.9% was highly influenced by compounds which were unique to each coffee berry maturity stage. The top contributors included (5*R*,7*S*)-conophthorin and α–humulene. The next highest variation (9.6%) was accounted for by PC2, which was highly impacted by 3-methylanisole and toluene.

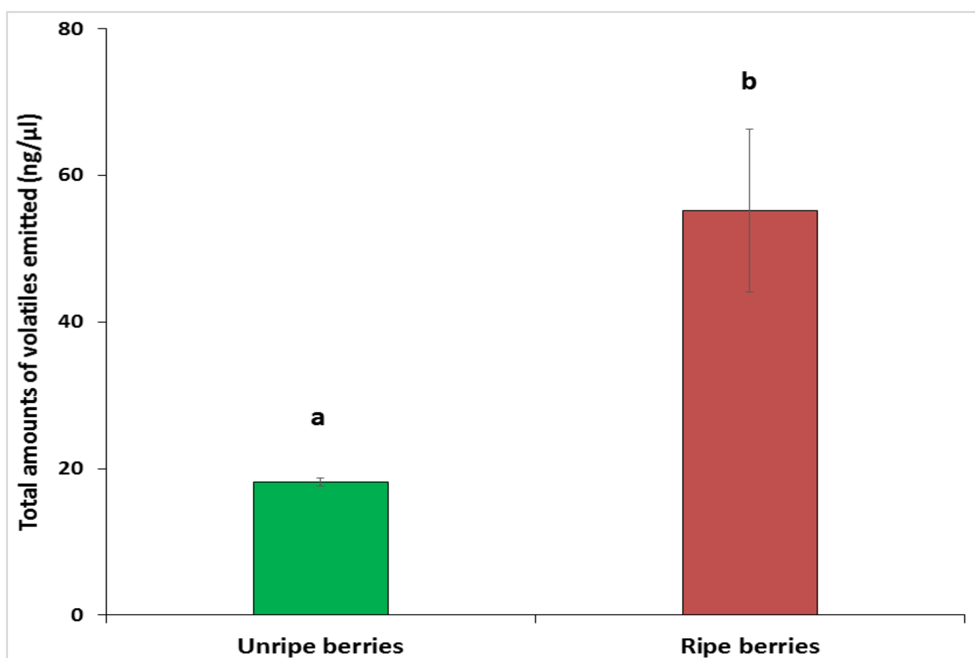

Figure 3.6: Total amount ± SE (ng/24 h/g of berry) of volatiles released by unripe and ripe coffee maturity stages.

Different letters above bars represent significant differences between treatments.

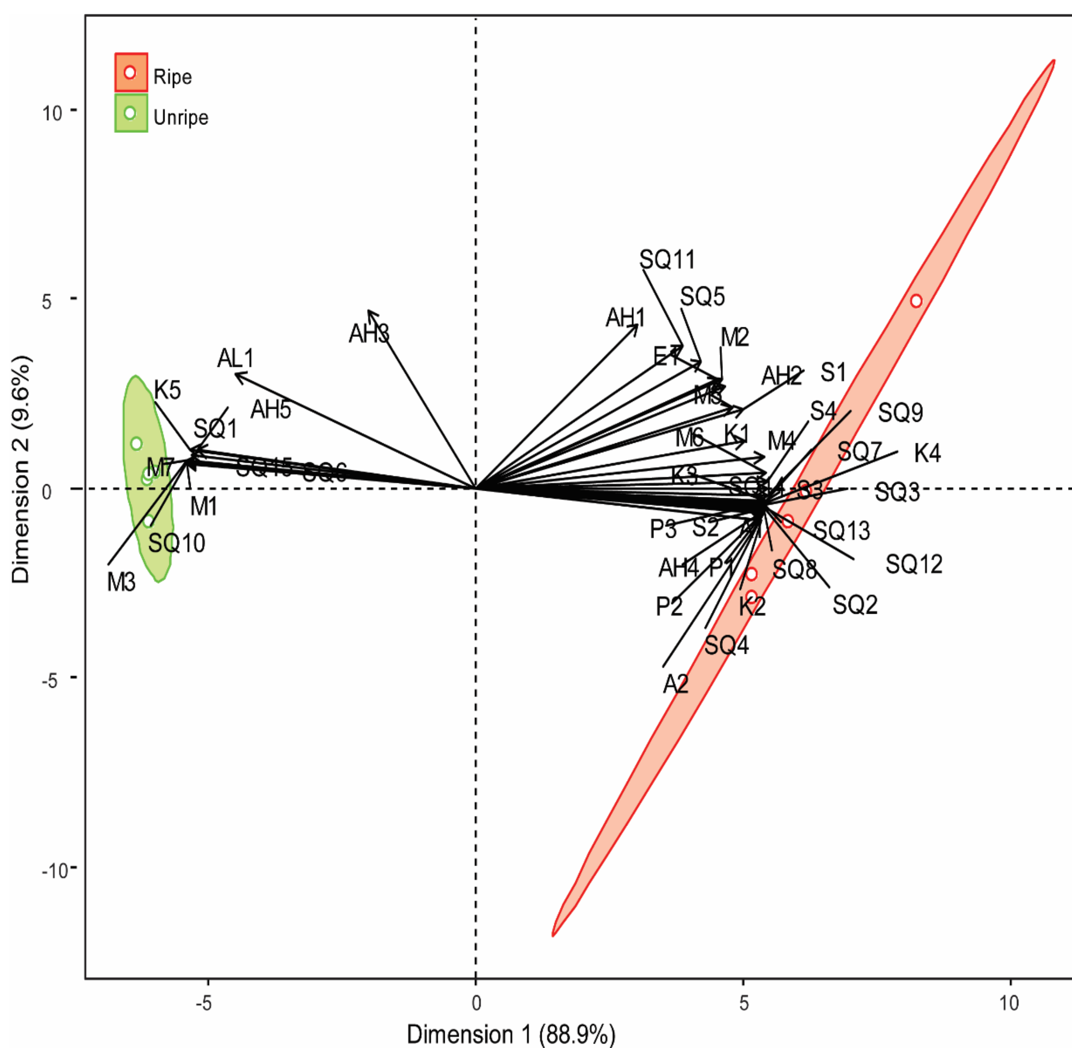

Figure 3.7: Principal component analysis showing the separation of volatile blends collected from unripe and ripe coffee berries.

The vectors of each compound, whose numbers match those in Table 3.2.

Table 3.2: Headspace volatiles identified from unripe and ripe coffee berries (*Coffea arabica* var. Ruiru 11) through GC-MS analysis

Class of compounds	Compounds name	Concentration (ng/µl)	
		Unripe berries	Ripe berries
Alcohol	A1. 2,3-Butanediol (unidentified isomer)	-	0.61±0.06
	A2. 2,3-Butanediol (unidentified isomer)	-	0.59±0.06
Aldehyde	AL. Nonanal	1.08±0.05	0.46±0.21
Aromatic hydrocarbon	AR1. Toluene[a]	0.70±0.01	0.72±0.02
	AR2. Anisole[a]	0.67±0.00	1.63±0.46
	AR3. 3-Methylanisole[a]	1.26±0.09	1.12±0.18
	AR4. 3,4-Dimethoxy toluene	-	0.69±0.01
	AR5. Naphthalene	1.08±0.07	0.69±0.01
Ester	E. Methyl 3-ethyl-4-methylpentanoate[a]	1.14±0.03	1.99±0.45
Ketone	K1. 3-Hydroxy-2-butanone[a]		23.92±8.19
	K2. 4-Hydroxy-4-methyl-2-pentanone		0.56±0.04
	K3. 2- Heptanone[a]	-	0.54±0.24
	K4. 3-Octanone	-	0.52±0.02
	K5. 6-Ethenyldihydro-2,2,6-trimethyl-2H-pyran-3(4H)-one	1.14±0.05	0.55±0.02
Monoterpene	M1. Unidentified monoterpene[a]	0.99±0.01	-
	M2. Limonene	1.05±0.02	1.89±0.42
	M3. 1,8-Cineole	1.12±0.04	-
	M4. (*E*)- Linalool oxide	1.01±0.02	2.71±0.31
	M5. (*Z*)-linalool oxide	1.11±0.04	1.49±0.16
	M6. δ-3-carene	-	1.92±0.06

	M7. β-ocimene	1.17±0.04	-
Pyrazine	P1. 2-Isopropyl-3-methoxypyrazine[a]	-	0.83±0.01
	P2. 3-Sec-butyl-2-methoxypyrazine	-	0.90±0.01
	P3. 2-Isobutyl-3-methoxypyrazine[a]	-	1.03±0.4
Spiroacetal	S1. (5*S*,7*S*)-Conophthorin[a]	0.39±0.02	1.87±0.54
	S2. (5*R*,7*S*)-Conophthorin	-	0.43±0.04
	S3. Chalcogran	-	0.36±0.01
	S4. Rac-chalcogran	-	0.37±0.01
Sesquiterpene	SQ1. (*Z*)-4,8-Dimethylnona-1,3-7 triene	1.18±0.05	-
	SQ2. α – Copaene	-	0.59±0.01
	SQ3. β – Elemene	-	0.61±0.02
	SQ4. Himachala-1,4-diene	-	0.60±0.01
	SQ5. α – Cedrene	0.59±0.00	0.62±0.02
	SQ6. b-Cedrene	0.58±0.00	-
	SQ7. (*E*)-β- Caryophyllene[a]	-	0.62±0.03
	SQ8. β – Copaene	-	0.59±0.00
	SQ9. α – Humulene	-	0.64±0.04
	SQ10. Prezizaene	0.58±0.00	-
	SQ11. β –Acoradiene	0.61±0.03	0.75±0.10
	SQ12. δ - cadinene	-	0.59±0.01
	SQ13. Unidentified sesquiterpene	-	0.60±0.02
	SQ14. (*Z*)-α- Damascone	-	0.63±0.05
	SQ15. (*E,E*)-4,8,12-Trime- thyl-1,3,7,11-tridecatetraene	0.67±0.09	

Vectors for each compound match those in figure 3.7

[a] Represents compounds found to be EAD-active in *Antestiopsis thunbergii*

3.3.5 Identification of electrophysiologically-active volatile components in unripe and ripe berries

Electrophysiology analysis showed that five compounds from the headspace volatiles of unripe berries consistently stimulated the antennae of second instar nymphs (Figure 3.8). These specific compounds were: toluene, anisole, methyl 3-ethyl-4-methylpentanoate and *(5S,7S)*-conophthorin. It was not possible to identify an EAD-active monoterpene that eluted adjacent to anisole and with weak fragmentation ions at *m/z* 77, 79 and 93. Electrophysiological activity of the four identified compounds was confirmed by recording positive responses with their respective synthetic standards (Figure 3.8).

Further, ten volatile compounds produced by ripe coffee berries, stimulated the antennae of second instar nymphs and included: 3-hydroxy-2-butanone, toluene, 2-heptanone, anisole, 3-methylanisole, methyl 3-ethyl-4-methylpentanoate, (5*S*,7*S*)-conophthorin, 2-isopropyl-3-methoxypyrazine, 2-isobutyl-3-methoxypyrazine and *(E)*-β-caryophyllene (Figure 3.9). Five of these, comprising two ketones (3-hydroxy-2-butanone and 2-heptanone), two pyrazines (2-isopropyl-3-methoxypyrazine and 2-isobutyl-3-methoxypyrazine) and a sesquiterpene (*(E)*-β-caryophyllene) were specifically found in ripe berries only (i.e. not been detected in unripe berries; Table 3.2). Electrophysiological activity of these five unique compounds from ripe berries was further confirmed by positive antennal recordings with their respective synthetic standards (Figure 3.9).

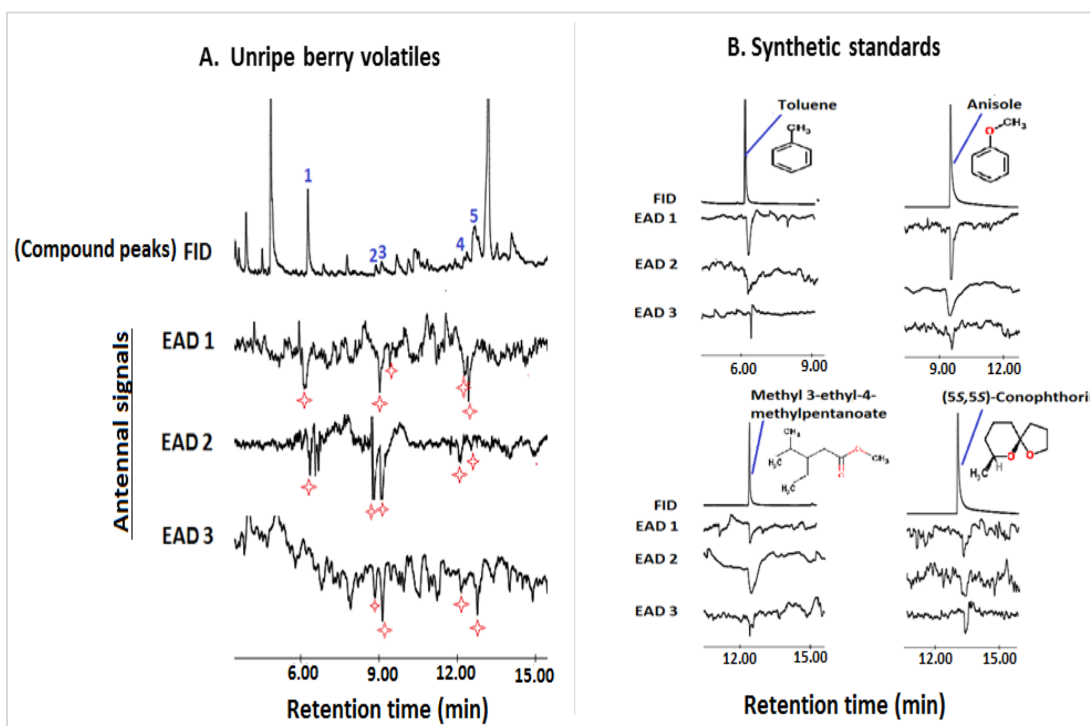

Figure 3.8: GC/EAD responses of *Antestiopsis thunbergii* second instar nymphs to: (A) charcoal-trapped headspace volatiles from unripe berries, (B) corresponding synthetic standards.

The EAD-active compounds include: (1) toluene (2) unidentified (3) anisole (4) methyl 3-ethyl-4-methylpentanoate (5) *(5S,7S)*-conophthorin.

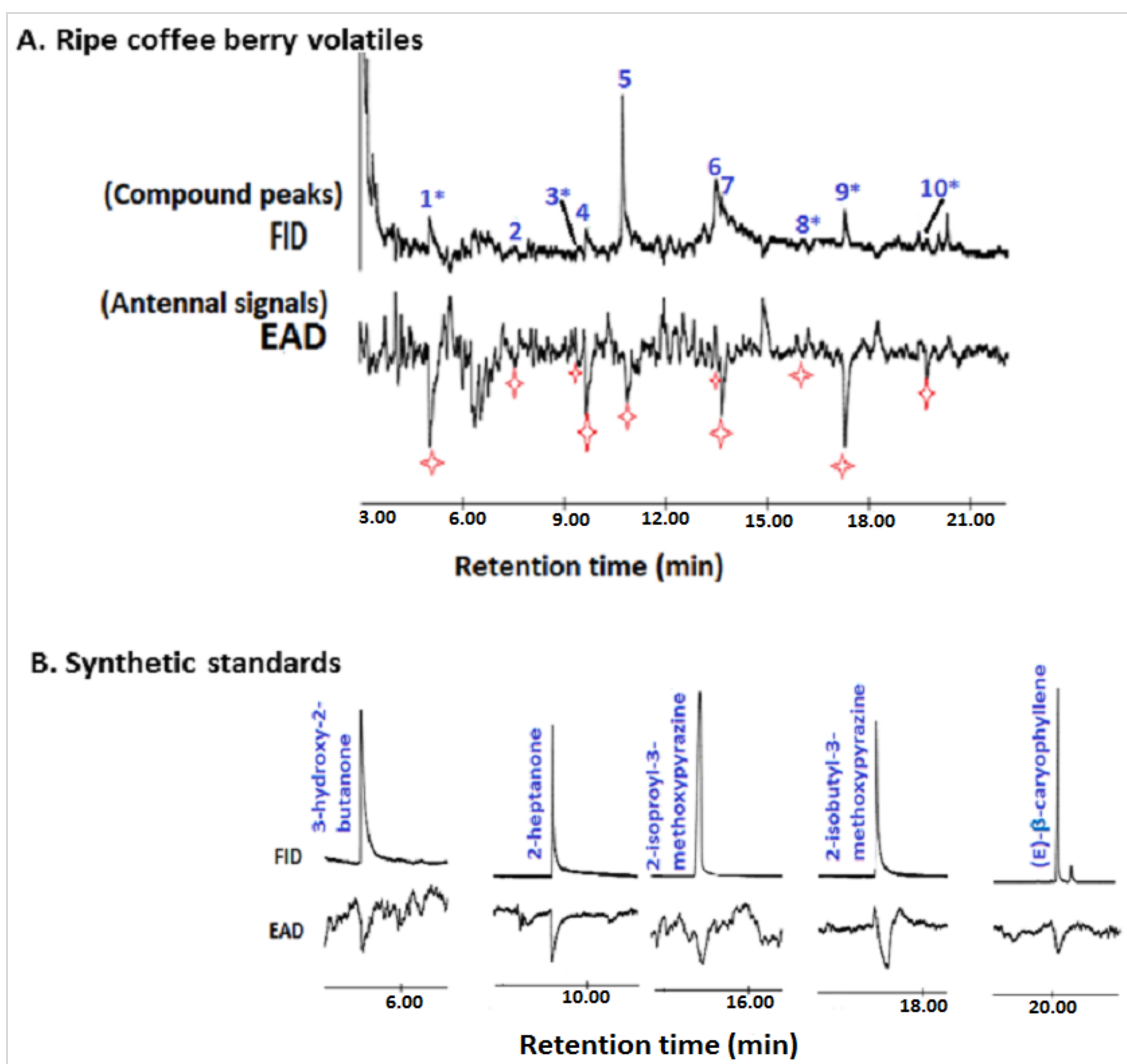

Figure 3.9: GC/EAD responses of *Antestiopsis thunbergii* second instar nymphs to: (A) headspace volatiles from ripe coffee berries, (B) Synthetic standards of compounds that were unique to the ripe berry stage.

The EAD-active compounds were: (1^*) 3-hydroxy-2-butanone, (2) toluene, (3^*) 2-heptanone, (4) anisole, (5) *3*-methylanisole, (6) methyl 3-ethyl-4-methylpentanoate, (7) (5*S*,7*S*)-conophthorin, (8^*) 2-isopropyl-3-methoxypyrazine, (9^*) 2-isobutyl-3-methoxypyrazine, (10^*) *(E)*-β-caryophyllene. Numbers followed by asterisks represent the compounds only found in the ripe berry stage.

3.3.6 Behavioral role of synthetic standards

3.3.6.1 Behavioral effect of synthetic standards from unripe coffee berries

Of the four identified EAD-active compounds, only toluene did not elicit behavioral responses in nymphs (Figure 3.10). Anisole strongly attracted nymphs at the lowest concentration, while the highest concentration elicited an avoidance response. There was however, no significant response to the intermediate (natural) concentration. Methyl 3-ethyl-4-methylpentanoate attracted three times more nymphs at the lowest concentration than the control, whereas there were no significant responses at the intermediate and highest concentrations. (5*S*,7*S*)-conophthorin attracted almost three-fold more nymphs at the intermediate concentration compared to control, while the lowest and highest concentrations elicited no significant responses from the nymphs (Figure 3.10).

The proportion of nymphs that selected a three-component blend of anisole, methyl 3-ethyl-4-methylpentanoate and (5*S*,7*S*)-conophthorin was approximately three times higher compared to the solvent control (χ^2=7.52, df = 1, P = 0.006). However, there were no significant differences between proportion of nymphs that selected the three-component blend and headspace volatiles from unripe berries (χ^2=0.47, df = 1, P = 0.493; Figure 3.11).

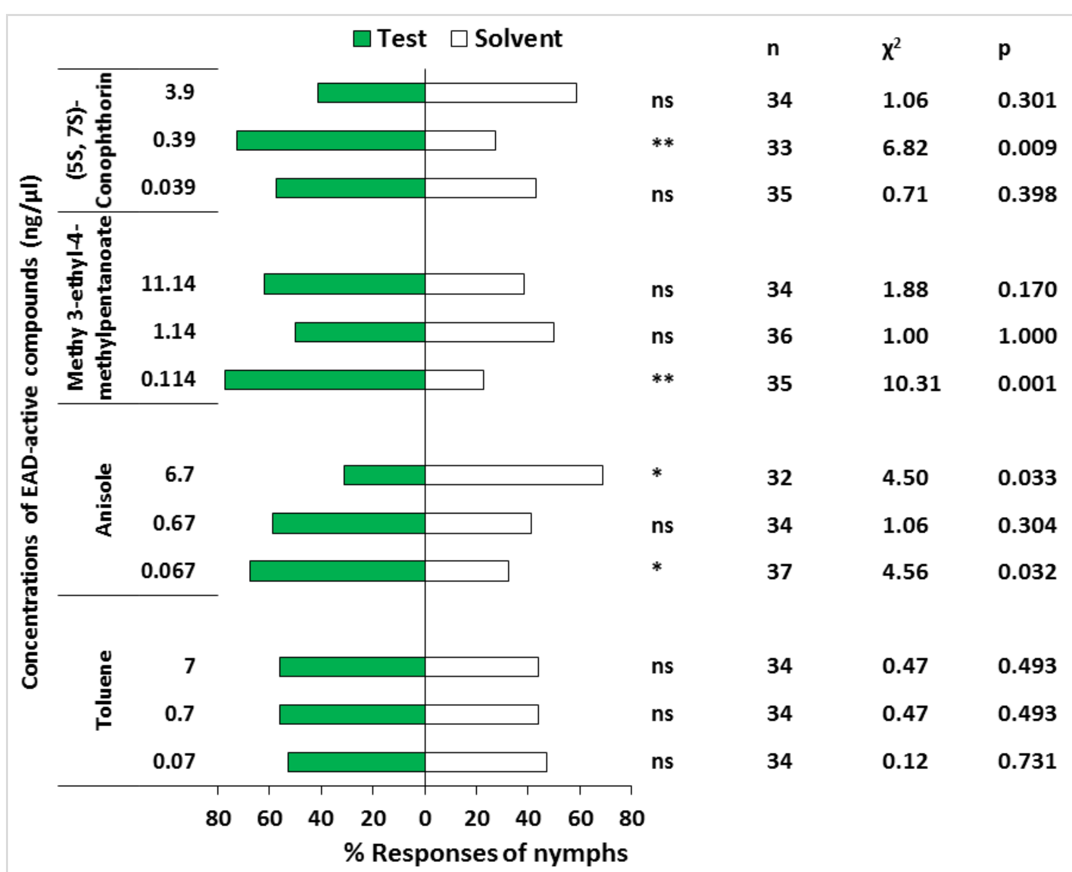

Figure 3.10: Behavioural responses of *Antestiopsis thunbergii* second instar nymphs to different concentrations of EAD-active compounds identified from headspace volatiles of unripe coffee berries.

The total number of insects tested per pairing was 40. “n” is the number of respondents and asterisks indicate the significance levels; ns=not significant, *$p < 0.05$ & **$p < 0.01$.

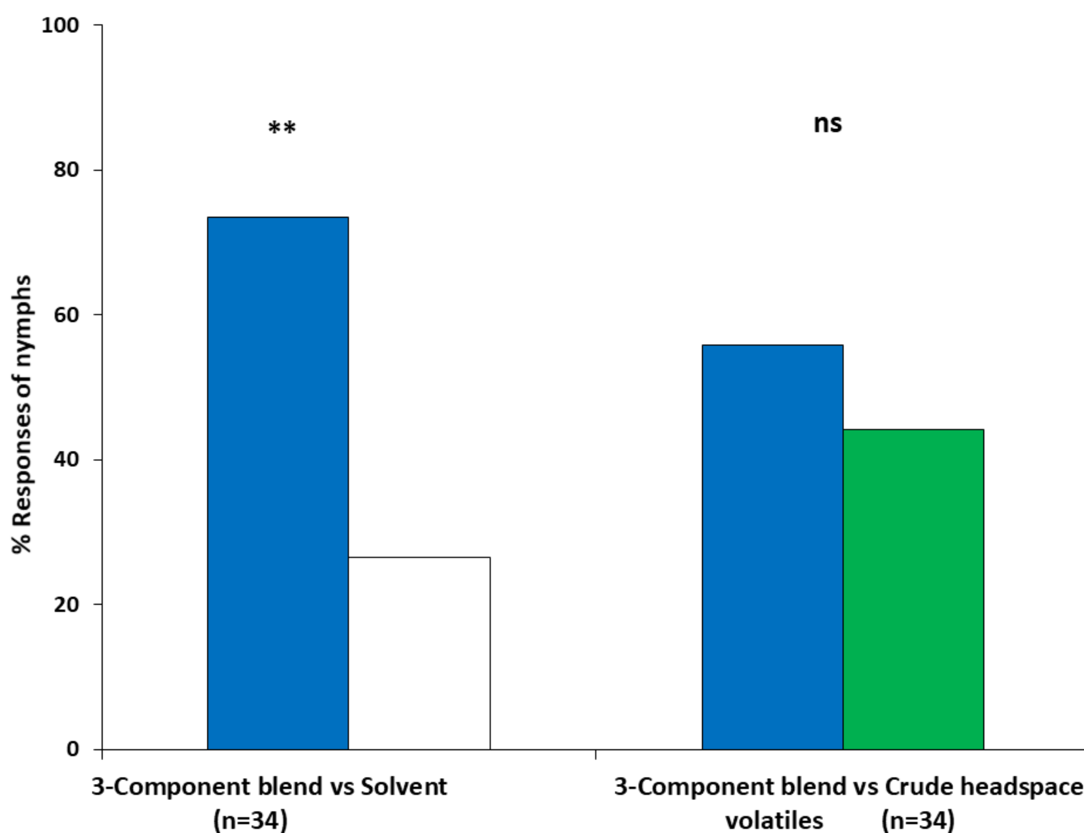

Figure 3.11: Behavioral responses of *Antestiopsis thunbergii* second instar nymphs to a 3-component blend comprising anisole, methyl 3-ethyl-4-methylpentanoate and *(*5*S*,7*S)*-conophthorin against solvent (control) and crude headspace volatiles from unripe coffee berries.

The total number of insects tested per pairing was 40. "n" is the number of respondents and asterisks indicate the significance levels; ** p< 0.01.

3.3.6.2 Behavioral effect of synthetic standards unique to ripe coffee berries

Nymphs significantly avoided four of the five EAD-active compounds found only in ripe berries at two of the three concentrations tested and for 2-heptanone at all three concentrations (Figure 4.12). The other four compounds namely 3-hydroxy-2-butanone, 2-isopropyl-3-methoxypyrazine (IPMP), 2-isobutyl-3-methoxypyrazine (IBMP) and (*E*)-β-caryophyllene did not elicit significant behavioral responses at the lowest concentration tested (Figure 3.12). A five-component blend comprising 3-

hydroxy-2-butanone, 2-heptanone, 2-isopropyl-3-methoxypyrazine, 2-isobutyl-3-methoxypyrazine and *(E)*-β-caryophyllene was significantly avoided by the nymphs at all the three concentrations tested: lowest (χ^2 =4.90, df = 1, P = 0.026; intermediate/natural (χ^2 =14.70, df = 1, P = 0.001) and highest (χ^2 =14.53, df = 1, P = 0.001). Nymphs avoidance by the blend was enhanced with increasing concentration, i.e. 67% > 78% >79% respectively (Figure 3.13).

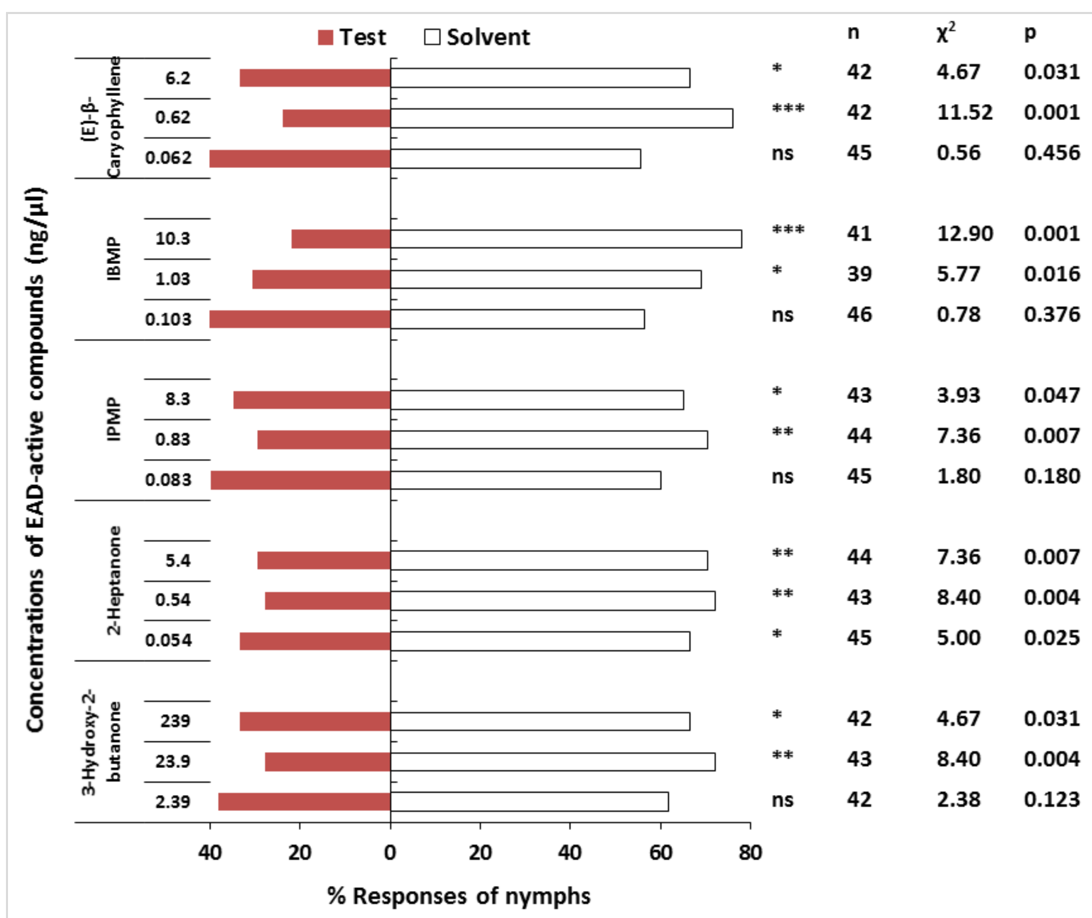

Figure 3.12: Behavioral responses of *Antestiopsis thunbergii* second instar nymphs to EAD-active compounds identified from headspace volatiles of ripe berries

IPMP =2-isopropyl-3-methoxypyrazine; IBMP= 2-isobutyl-3-methoxypyrazine.

The total number of insects tested per pairing was 50; n represents the number of respondents. Asterisks indicate the significance levels; *p< 0.05, **p< 0.01& ***P<0.001.

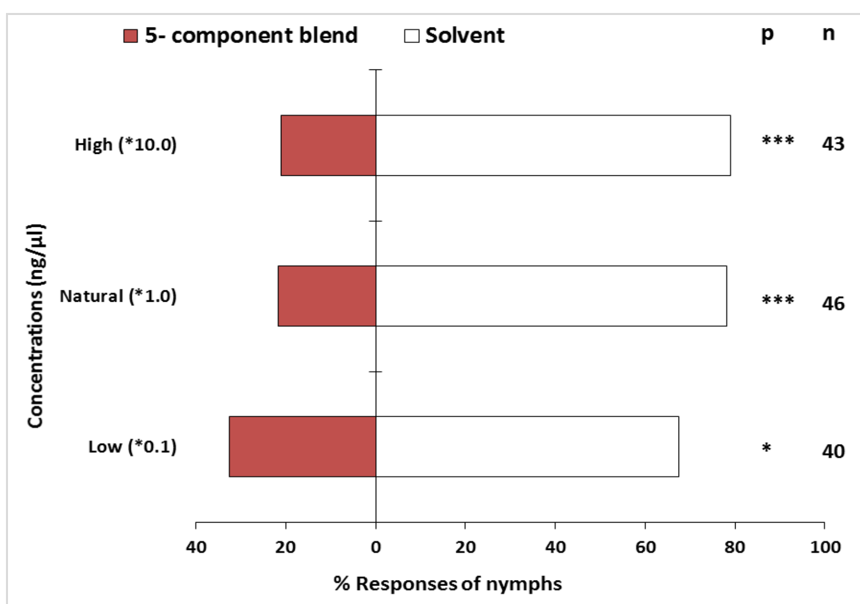

Figure 3.13: Behavioral responses of *Antestiopsis thunbergii* second instar nymphs to a 5-component blend comprising 3-hydroxy-2-butanone, 2-heptanone, 2-isopropyl-3-methoxypyrazine, 2-isobutyl-3-methoxypyrazine and *(E)*-β-caryophyllene.

The total number of insects tested per pairing was 50; n represents the number of respondents. Asterisks represent significant levels; *P<0.05 & ***P<0.001.

Mixing the five-component blend (intermediate concentration) with a known attractant of *A. thunbergii* nymphs {three-component blend of 0.067 ng/µl anisole, 0.114 ng/µl methyl 3-ethyl-4-methylpentanoate and 0.39 ng/µl (*5S,7S*)-conophthorin} led to masking of the attractant. Nymphs significantly avoided the now 8-compounds mixture, with a high proportion preferring solvent (73%; χ^2 =9.80, df = 1, P = 0.002) and 3-component attractant blend (76%; χ^2 =12.52, df = 1, P = 0.001). However, there was no significant difference in responses of nymphs while

comparison was amongst the 8-compounds mixture and 5-component blend (χ^2 =0.03, df = 1, P = 0.872) (Figure 3.14)

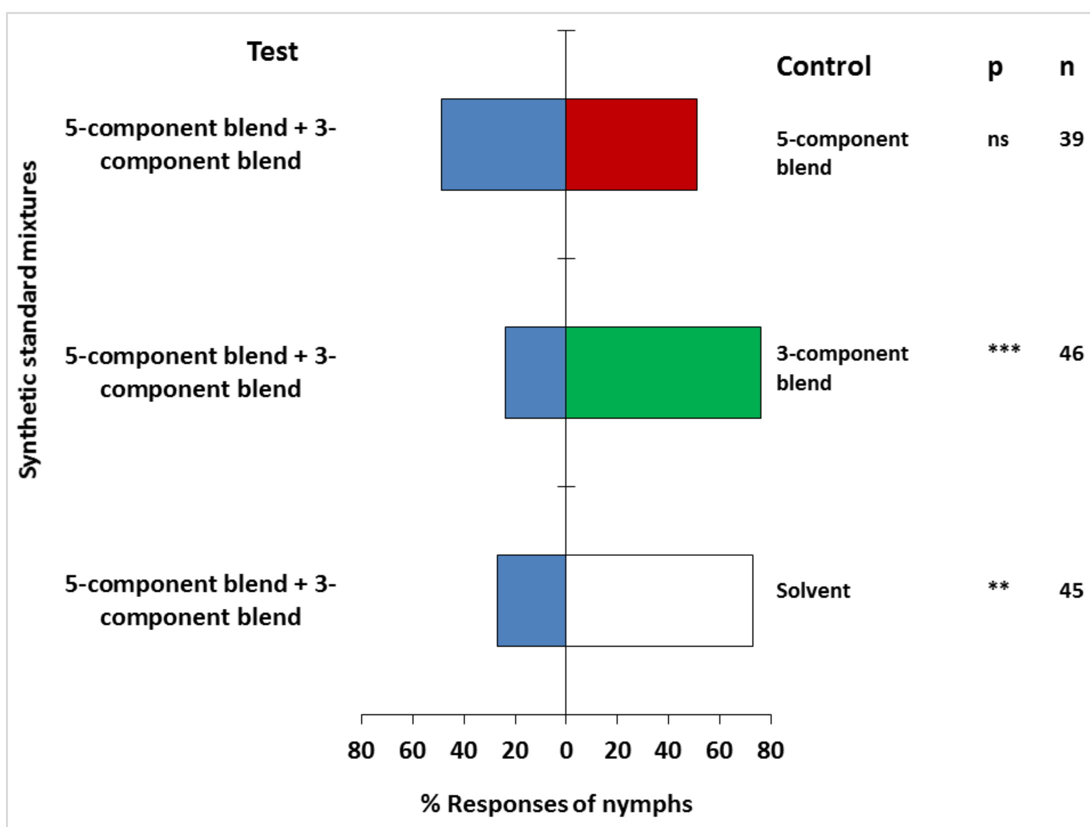

Figure 3.14: Behavioral responses of *Antestiopsis thunbergii* second instar nymphs to eight synthetic standard mixture.

Mixture formulated from 5-component repellent blend comprising 3-hydroxy-2-butanone, 2-heptanone, 2-isopropyl-3-methoxypyrazine (IPMP), 2-isobutyl-3-methoxypyrazine (IBMP) and (E)-β-caryophyllene and 3-component attractant blend comprising anisole, methyl 3-ethyl-4-methylpentanoate and (*5S,7S)*-conophthorin. The total number of insects tested per pairing was 50; n represents the number of respondents. Asterisks represent significant level, with ns=not-significant, **P<0.01 & ***P<0.001.

3.4 Discussion

3.4.1 Differential responses of nymphs and adults to host odors

Though the literature reports that unripe (mature green) berries are the most preferred for feeding, survival and reproduction of the Antestia bug, *A. thunbergii* (Le Pelley, 1942; Matsuura *et al.,* 2014) unlike ripe berries, no studies had elucidated the olfactory cues that mediate host recognition in this pest. Clear differences were found in the chemical composition of the two phenological stages of coffee berries in quality and amounts. In addition, adults and second instar nymphs of *A. thunbergii* were strongly attracted to headspace volatiles from unripe berries and avoided those from ripe berries suggesting that olfaction plays a key role in host recognition and discrimination. However, second instar nymphs were more behaviorally responsive towards the optimal or away from suboptimal host substrates followed by males and females. Since the nymphs are the first actively feeding stage of the pest, it is likely that their olfaction is adopted to quickly identify an optimal food for its survival as well as avoid suboptimal food. According to Knolhoff and Heckel (2014), larvae must quickly make choices that have immediate consequences for their survival mainly mediated by plant cues; whereas adults may rely on additional cues such as sex or aggregation pheromones. During the colonization process in Heteropterans, males are the initial colonizers who search for food and they produce aggregation pheromones to attract females (Aldrich *et al.,* 1999). Hence, our results corroborate this in that males were more responsive to food odors than females who possibly requires both aggregation and host odors. Although, subsequent in-depth studies associated with plant cues were conducted with second instar nymphs, it is recommended that future studies examine adults and whether kairomones (attractive host odors) and aggregation pheromones are synergistic.

3.4.2 Identification of *A. thunbergii kairomones* from preferred host food-unripe berries

Screening of bioactive volatile components through GC/EAD showed that the antennae of second instar nymphs of *A. thunbergii* detected five compounds present

in the volatiles emitted by unripe coffee berries, four of which were identified as toluene, anisole, methyl 3-ethyl-4-methylpentanoate and (5*S*, 7*S*)-conophthorin. Subsequent behavioral assays were conducted to investigate the function of each of the antennally active compounds and their blends. Although toluene elicited no detectable attractive response in second instar nymphs, anisole, methyl 3-ethyl-4-methylpentanoate, and (5*S*,7*S*)-conophthorin were attractive at concentrations lower or equivalent to their natural concentrations in the extract of the coffee berry volatiles. In addition, anisole elicited avoidance behavior in second instar nymphs at a concentration higher than its natural concentration in the volatile extract. Our results agree with those of previous studies showing that extremely high concentrations of 'attractant' compounds above their natural occurrence in hosts, may result in inhibitory interactions or repellence (Webster *et al.,* 2010; Bruce *et al.,* 2011; Njihia *et al.,* 2014). The blend of the three attractive components mimicked the attractiveness of the natural volatile extract. These results highlight the differential role played by specific compounds at different concentrations and when they are presented in a blend to explain the host recognition process in an insect. Bruce *et al.,* (2005) proposed two hypotheses that explain host recognition and location in herbivores among a background of many VOCs in the ecosystem to involve host derived unique VOCs and specific ratios of ubiquitous VOCs. The results of the current study support both hypotheses, as specific concentration levels of a few compounds (three) attracted second instars of *A. thunbergii*. Besides variation in host plant volatiles among individual plants of the same genotype (Bruce *et al.,* 2011) and seasonal variability (Vallat & Dorn, 2005), it would be interesting to investigate how the coffee bug responds to volatiles of the same plant genotype during different seasons and obtained from different geographic locations (Suinyuy *et al.,* 2015). These would reveal potential intra-specific variation in volatile chemistry determined by these environmental factors, and whether the olfactory system of the bug has the plasticity to recognize suitable hosts producing varied odor ratios/amounts.

The four EAD-active compounds reported in unripe berries play a role in the host recognition in various insects. For example, the benzenoid toluene is a kairomone of the olive fruit fly *Dacus oleae* Gmelin (Scarpati *et al.,* 1993). Also, the benzenoid anisole serves as a kairomone for various scolytid species as well as a sex pheromone (Leal *et al.,* 1996; Vrkočová *et al.,* 2000; Ward *et al.,* 2002). It is also a constituent of the volatiles of *C. arabica* coffee berries whose amount increases as the fruit ripens (Ortiz *et al.,* 2004). Since *A. thunbergii* prefers to feed on unripe coffee berries and cannot fully complete its life cycle when fed only on berries at a later stage of maturation (Le Pelley 1942), our findings suggest that coffee bugs use low levels of anisole in the volatiles as an indicator of the presence of unripe berries (suitable food) and to discriminate them ripe coffee berries (non-suitable food). The ester methyl 3-ethyl-4-methylpentanoate has been reported in the volatiles released by *C. arabica* as well (Jaramillo *et al.,* 2013). Here, it elicits both attraction and repellence in the coffee berry borer, *Hypothenemus hampei* at various concentration levels (Jaramillo *et al.,* 2013). The spiroacetal *(5S, 7S)*-conophthorin is a common semiochemical mediating communication in several scolytid species, as an aggregation and anti-aggregation pheromone as well as a non-host volatile constituent of some angiosperm trees (Byers *et al.,* 1998; Huber *et al.,* 1999; Morewood *et al.,* 2003; Francke & Kitching, 2001). In addition, it is a key component in the volatiles of both ripe and unripe *C. arabica* berries, whose amounts are higher in ripe berries (Jaramillo *et al.,* 2013). The presence of spiroacetal *(5S, 7S)*-conophthorin and the ester methyl 3-ethyl-4-methylpentanoate in the blend that elicited attraction in *A. thunbergii* is interesting as the two have also been reported to elicit attraction in the coffee berry borer (Jaramillo *et al.,* 2013). Hence, future studies should explore using the two compounds as part of a bait for trapping both pests simultaneously.

3.4.3 Identification of *A. thunbergii* allomones from unsuitable host food-ripe berries

In addition to avoidance in second instar nymphs away from headspace volatiles collected from ripe berries, the nymphs also significantly avoided a mixture of ripe

berry volatiles and unripe berries (optimal food) opting for blank or unripe berries alone. Thus, volatiles from the ripe berries appeared to induce repellence as well as mask those emitted from unripe berries, thereby inhibiting attraction of second instar nymphs to the mixture of volatiles. Ahmed *et al* (2016) observed that *A. thunbergii* populations in the field were favored by several factors, including availability of unripe berries. The current results suggest that *A. thunbergii* populations are not only affected by the availability of food, but that olfactory cues also play a key role in the host finding process. These attract or repel the bug in coffee farms with a high proportion of unripe berries and ripe berries respectively. Provided that the same olfactory cues lead to the same behavior for *A. thunbergii* adults, this behavior can be exploited in pest control by intercropping coffee with hosts that interfere with host location by potential pests or use of synthetic repellent lures derived from these volatile organic compounds (VOCs) (Vallat & Dorn 2005; Cook *et al.,* 2007).

Electrophysiological tests to screen for ecologically relevant VOCs in the headspace volatiles of ripe berries isolated ten compounds; five (toluene, anisole, 3-methylanisole, methyl 3-ethyl-4-methylpentanoate and (5*S*,7*S*)-conophthorin) are common to both unripe and ripe berries (Jaramillo *et al.,* 2013; Njihia *et al.,* 2017). The other five, two ketones (3-hydroxy-2-butanone and 2-heptanone), two pyrazines (2-isopropyl-3-methoxypyrazine (IPMP) and 2-isobutyl-3-methoxypyrazine (IBMP) and the sesquiterpene *(E)*-β-caryophyllene) were only detected in ripe berries. Compounds that are common to both ripe and unripe berries can contribute to the bug avoidance behavior (Bruce & Pickett, 2011). It should be noted that mixing compounds in this context can lead to redundancy, synergism, and antagonistic or additive effects (Bruce & Pickett, 2011). However, by focusing only on compounds found in ripe berries, potential bioactive repellents can be less complex and more economical to develop. Behavioral assays showed that each of the five compounds present in ripe berries alone as well as their blend were avoided by second instar nymphs. In addition, mixing the blend with an attractant blend of *A. thunbergii* second instar nymphs, led to avoidance of the mixture, indicating that the 5-component blend had masked the suitable host odors. Thus, the five-component blend contributes to the pest discrimination against ripe berries as previously

demonstrated in both laboratory and field tests (Le Pelley, 1942; Matsuura *et al.,* 2014; Njihia *et al.,* 2017). Whether it is possible to formulate a blend that is less complex but at least as effective as the five-component blend should be examined.

The five-component repellent blend has previously been reported as constituents of headspace volatiles produced by ripe berries of both *C. arabica* and *C. canephora* (Mathieu *et al.,* 1998; Ortiz *et al.,* 2004; Jaramillo *et al.,* 2013; Cruz & Malo, 2013; Cruz-López *et al.,* 2016). 3-Hydroxy-2-butanone is a compound often associated with ripening and fermentation, and as a kairomone of *Drosophila melanogaster* that feeds on fermenting fruits (Becher *et al.,* 2010), unlike *A. thunbergii* that prefers to feed on unripe coffee berries. Thus, it appears that second instar nymphs of *A. thunbergii* associate this compound with ripe berries and avoid them. 2-Heptanone, the only compound that demonstrated repellent properties at all concentrations tested, has been reported as an alarm pheromone of social insects such as bees and ants (Blum, 1969), and a repellent for the granary weevil *Sitophilus granarius* (Germinara *et al.,* 2015). This is the first report that 2-heptanone can play an allomonal role for a pentatomid species. The two pyrazines (IPMP + IBMP) have previously been reported as universal warning odors of various insects, including heteropteran species (Moore *et al.,* 1990; Aldrich *et al.,* 1996). Both pyrazines are also responsible for the bad taste and smell produced by coffee with “potato taste defect” but these likely occurs when their amounts are inflated above a certain threshold (Scheidig *et al.,* 2007; Jackels *et al.,* 2014). Jackels *et al.,* (2014) detected IBMP in healthy and *A. thunbergii-* infested unripe *C. arabica* beans that had been ground into powder, but IPMP was detected in samples that were infested only. These and our findings suggest that IPMP could be a repellent compound for the pest that is produced constitutively in ripe berries but induced in unripe berries after Antestia bug infestation. The role of pyrazines especially, IPMP in the ecology of *A. thunbergii,* and whether *A. thunbergii* herbivory on unripe berries (suitable diet) induces production of more defence compounds against the pest (Freeman & Beattie 2008; Fürstenberg-Hägg *et al.,* 2013) should be investigated. *(E)*-β-caryophyllene is an attractant of many insects including aphids, beetles, bugs, moths and weevils (Fujii *et al.,* 2010). This sesquiterpene also provide both direct and indirect resistance to

various crops against pests through inhibition of growth and survival of some pests and microbial pathogens (Langenheim, 1994; Huang *et al.,* 2012), and attraction of their biological control agents such as the Aphid parasitoid *Aphidius ervi* Haliday (Heuskin *et al.,* 2012), and the Entomopathogenic nematode *Heterorhabditis megidis* (Degenhardt *et al.,* 2009; Tamiru & Khan, 2017). Hence the potential role of *(E)*-β-caryophyllene in the inter-trophic interactions amongst the coffee plant, *A. thunbergii* and its natural enemies should be investigated.

3.5 Conclusion

This study has showed that host location in *A. thunbergii* is triggered by a 3-component blend of volatile organic compounds derived from unripe coffee berries. The 3-component blend is a candidate kairomone for monitoring populations and mass trapping of *A. thunbergii*. In addition, ripe berries produce compounds that repel *A. thunbergii,* which likely contributes to lower infestation than the unripe berry stage. These compounds can be exploited as candidate repellents to expel Antestia bugs or mask suitable host food (unripe berries). The development of coffee varieties that produce these compounds in the early maturity stages should also be explored. The attractants and repellents identified in this study may also be deployed in a "push-pull" pattern to enhance suppression of *A. thunbergii* in coffee plantations.

CHAPTER FOUR

IDENTIFICATION OF PHEROMONES THAT MEDIATE CONSPECIFIC COMMUNICATION IN ANTESTIOPSIS THUNBERGII

Abstract

Antestia bug *Antestiopsis thunbergii* (Gmelin) poses a major threat to international trade of coffee from Africa due to its contribution in diminishing the quality and yield of coffee. The pest has a low economic injury threshold level of 1-2 bugs/tree complicating its management through existing cultural and chemical methods, necessitating the search for other alternatives such as pheromones. Although Antestia bugs pheromones are largely unknown, the pest has certain characteristics that suggest that they utilize them for communication. For instance, they are gregarious and belong to the "stink bug" family, often characterised by the production of distinct odorous compounds with an irritating smell to warn conspecifics of impending danger and as a defence mechanism to intimidate their natural enemies. Therefore, this study investigated whether *A. thunbergii* produces aggregation/sex and alarm/defence pheromones that likely contribute to their distribution patterns, proliferation and survival in coffee plantations. Dual choice tests were conducted in a Y-tube olfactometer and headspace odors collected from live *A. thunbergii* adults using different sampling techniques. Super Q, charcoal filter and solid phase micro extraction (SPME) fibre adsorbents were used to collect headspace volatiles from undisturbed bugs, targeting to identify sex specific aggregation or sex pheromones. To identify candidate alarm/defence pheromones, SPME technique was used to sample headspace volatiles from disturbed adult bugs. In addition, metathoracic glands (MTG) contents of adults and dorsal abdominal glands (DAG) of nymphs (1st, 3rd and 5th instar nymphs) were examined. The pheromones were analysed by gas chromatography/mass spectrometry (GC/MS) and/ GC-electroantennographic detection. In dual choice bioassays, sexually mature unmated males of *A. thunbergii* attracted both sexes, but females were unattractive to either sex, suggesting the presence of a male-produced aggregation pheromone. Chemical analyses of

undisturbed bugs trapped by Super Q identified 22 compounds, which were identical to volatiles emitted by both sexes of *A. thunbergii* and dominated by alkanes (9), aldehydes (8), and typical defence and alarm pheromones. Fewer compounds (18) were identified through charcoal filter adsorbent, which also identified the ester methyl (2*E*,4E,6*Z*)-decatrinoate (MDT-*EEZ*) as a male-specific pheromone, Interestingly, SPME analysis did not detect any compounds in undisturbed females but detected selected compounds previously sampled by either Super Q or charcoal filter including MDT-*EEZ* in males. MTG of adults had a more complex blend than DAG of nymphs suggesting that adults were more chemically protected than the immatures. Male-specific synthetic MDT-*EEZ* elicited antennal response in both sexes, indicating that the compound could be serve as an aggregation pheromone. In conclusion, this study identified candidate aggregation and alarm pheromones, which could be exploited for *A. thunbergii* control through surveillance/mass trapping and repellence, respectively.

4.1 Introduction

Pheromones provide chemical cues that mediate communication in insects. They directly impact the success of herbivores by facilitating mating, resource allocation and partitioning, warning conspecifics against impending danger, and defence against natural enemies. As a result, the use of pheromones in crop protection is widely accepted globally as a safe, specific, eco-friendly and affordable means of managing pests through surveillance, mass trapping, and mating disruption (Angeli *et al.,* 2007; Cook *et al.,* 2007; Foster & Harris, 1997).

Although Antestia bugs, *Antestiopsis thunbergii* are gregarious, more so in the adults (Cilas *et al.,* 1998), existing information on likely chemical stimuli involved is limited. Njihia *et al.,* (2017) found that Antestia bugs are attracted to volatiles from unripe coffee berries and identified the candidate kairomone that mediate the pest host recognition and selection. Since host plant volatiles and pheromones can often act alone or synergistically in attracting herbivores (Landolt, 1997; Reddy & Guerrero, 2004), it is likely that Antestia bug aggregation is mediated by both

kairomones and pheromones. Indeed, most heteropteran males after finding habitats with suitable food produce aggregation pheromones to attract conspecifics (both sexes and nymphs) (Aldrich et al., 1999). The only study that had so far attempted to study Antestia bug pheromones was by Jackels *et al.* (2014), who detected alkanes such as tridecane and dodecane as candidate pheromones in the headspace volatiles of desiccated Antestia bugs. The identified compounds are mainly known for defence and alarm in related species (Fávaro *et al.,* 2012; Jackel *et al.,* 2014). To identify a wide range of pheromones especially aggregation and sex pheromones, analysis of odors from live insects is crucial (Millar, 2005).

Aggregation pheromones have been identified for many closely related species of Antestia bugs and incorporated in pest management programs (Millar, 2005; Laumann *et al.,* 2011; Leskey *et al.,* 2012). For instance, methyl (2,4,6)-decatrinoate (MDT) is an important and commercialized stink bug pheromone, which is unstable and comprise various isomers, although only two have been reported to occur naturally *(EEZ &EZZ)* (Khrimian, 2005). The pheromone may isomerize during analysis or under exposure to sunlight, but this does not diminish its performance in the field (Khrimian *et al.,* 2007).

Alarm and defence pheromones are found in metathoracic glands (MTG) of adults and dorsal abdominal glands (DAG) of nymphs in stink bugs, (Fávaro *et al.,* 2012). Analysis of freshly moulted exuviae is a standard method of identifying DAG contents as they are shed along with the molt (Fávaro et *al.,* 2012). Sex pheromone and aggregation pheromones are hard to locate and identify because they are not produced by specific glands (Millar, 2005), hence headspace sampling of bug odors is preferred. There are different sampling adsorbents for volatiles such as activated charcoal or a porous polymeric phase such as Porapak-Q, Haysep-Q, Super-Q or Tenax-TA (Jones and Oldham, 1999). Since different adsorbents have different affinities, comparison of different adsorbents is a recommended strategy to ensure that all volatiles are sampled (Tholl *et al.,* 2006). However, most studies report one method, that is available, selected, or with desired output.

The present study investigated attractiveness of sexually mature *A. thunbergii* adults towards each other to confirm the existence of an aggregation or sex pheromone. Attractiveness of males and females were examined in Y-tube olfactometer bioassays and headspace volatiles of undisturbed bugs (both sexes) were compared by coupled gas chromatography/mass spectrometry (GC/MS) analysis. In addition, pheromones produced by the pest under stress (alarm/defence) were evaluated by analysing the headspace volatiles of disturbed bugs, and direct extraction from the DAG and MTG of nymphs and adults respectively.

4.2 Materials and Methods

4.2.1 Study location

The experiments were conducted at the laboratories of the International Centre of Insect Physiology and Ecology, *icipe* in Nairobi, Kenya ($1^0$16'60''S; $36^0$49'0''E).

4.2.2 Insects

Sexually mature-unmated adults [28 ± 3 days] and 1st, 3rd and 5th instar nymphs of *A. thunbergii* were evaluated in the experiments. Their rearing method was previously described in section 3.3.3.

4.2.3 Olfactometer assays with adults

To identify possible producers of attractant pheromones (aggregation/sex), dual choice assays were conducted in a Y-tube olfactometer set up previously described (section 3.3.5). A total of four possible combinations of potential attractant pheromones producers and responders were bioassayed and comprised of attraction of odors of: (1) females to males, (2) males to males, (3) males to females, and (4) females to females. The tests were conducted by placing a single A. thunbergii of known sex into a 50 ml glass chamber (the treatment insect); a similar but empty chamber was used as the control. The chambers were attached, one to each arm of the Y-tube, using Teflon tubing. A pump (WOB-L pump 2522C, Monroe, Louisiana, USA) supplied charcoal-filtered and humidified air (80% RH) through each arm of

the Y-tube at 100 ml/min; PVC tubing at the base of the olfactometer was connected to the vacuum source of the pump and operated at 200 ml/min. Before entering the olfactometer arms, each air stream passed through the treatment and control odor sources. Responses were recorded by introducing an individual *A. thunbergii* (the test insect) at the entrance of the stem of the Y-tube olfactometer to make a choice between the treatment and control. A positive response entailed the test insect walking past a third of one or the other arm of the olfactometer and spending at least 20 s in that arm. Test insects that did not make a choice in 15 min were recorded as non-respondents and removed from the olfactometer. Every 1 h, the insect in the treatment odor source chamber was replaced and clean glassware used. We tested the responses of 30 different individuals for each treatment.

To compare the chemical profile of *A. thunbergii* males and females, pheromones were collected from *A. thunbergii* in their natural state (undisturbed) with Super Q, charcoal filter and solid phase microextraction (SPME) adsorbents. These tests targeted identification of sex-specific aggregation or sex pheromones. To investigate stress-associated pheromones, headspace samples from disturbed *A. thunbergii* were sampled by SPME and direct extraction of metathoracic glands (MTG) and dorsal abdominal glands (DAG) of adults and nymphs, respectively

4.3.3.1 Headspace odors of undisturbed bugs sampled by Super Q and charcoal filter adsorbent

Treatments comprised ten (10) unmated females and males of *A. thunbergii* which were separately placed into one each of two 50 ml glass aeration chambers. To protect the insects from starving and minimize the production of stress-related pheromones, two unripe coffee berries (~2 g) and a moist cotton wool ball were added as a source of food and water, respectively. Thus, odors from two unripe berries and moist cotton wool were also sampled to serve as the control. To sample the headspace odors, humidified and charcoal-filtered air was drawn through the glass chambers (200 ml/min) and entrained odors collected on charcoal filter adsorbent (5 mg, Brechbuhler, Schlierensee, Switzerland) for 48 h (Njihia et al.,

2017). Each adsorbent was eluted with 150 µl of hexane. Three replications were conducted for each sex and control. Eluates were stored in a -80°C freezer until analysis. The same trapping procedure was repeated using charcoal filter adsorbent (5 mg, Brechbuhler, Schlierensee, Switzerland) for comparison. Individual insects were used only once.

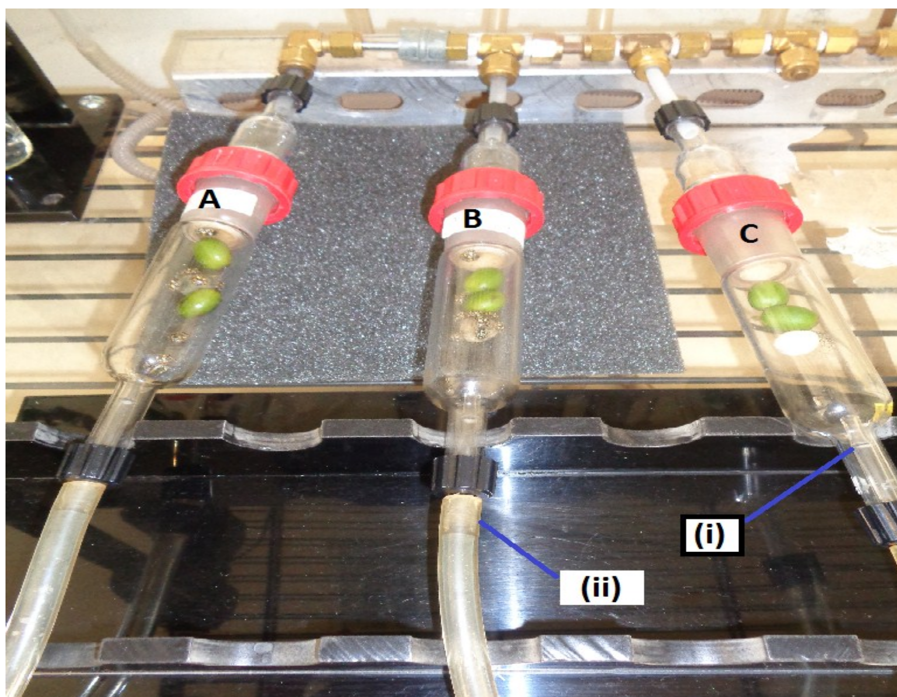

Plate 4.1: Headspace pheromone collection set up by charcoal filter method.

Treatments comprised 10 Antestia bug, *Antestiopsis thunbergii* (A) males (B) females. The control was two unripe coffee berries and a wet cotton ball. Roman numbers represent (i) charcoal filter adsorbent (ii) vacuum tube pulling headspace volatiles on to the adsorbent.

4.2.3.2 Headspace odors of undisturbed and disturbed bugs sampled by SPME adsorbent

Five (5) unmated *A. thunbergii* of each sex were gently placed into each of two 10 ml glass vials whose top was covered with aluminium foil. The tip of a clean preconditioned solid phase micro-extraction SPME fibre, (PD-MS DVB 65µL) that

pierced the foil cover was used to adsorb bug odors for 3 h and analyzed immediately by GC/MS (see below). To investigate the effect of disturbance on *A. thunbergii* emissions, each of the same samples were covered with new aluminium foil and then shaken by hand for 3 min. The odors from the disturbed bugs were adsorbed on a clean preconditioned SPME fibre for 15 min and similarly analyzed. The tests were conducted during the day between 9 am and 4 pm. Three replicates using different batches of insects were conducted.

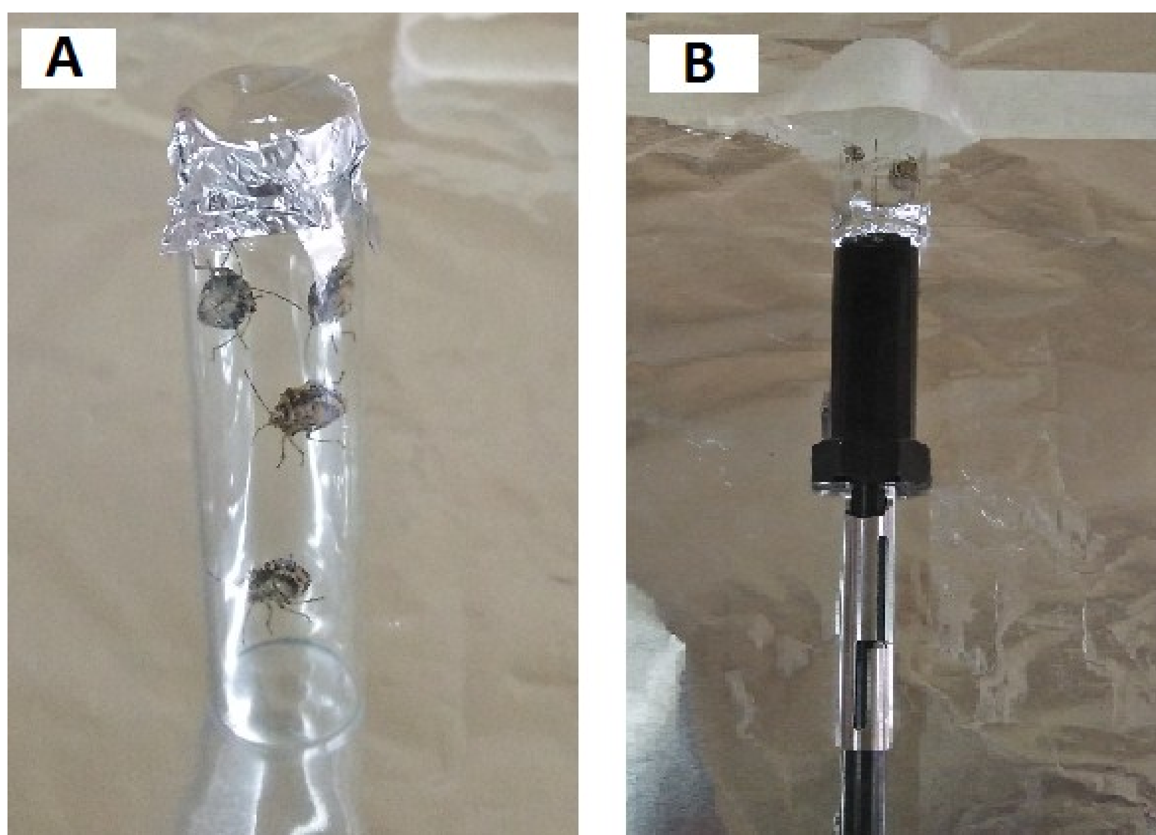

Plate 4.2: Headspace pheromone collection set up by solid phase microextraction (SPME) method.

(A) Five *Antestiopsis thunbergii* of same sex in a 10 ml vial, (B) Pheromone collection with an extracted SPME fibre.

4.2.3.3 Metathoracic gland extraction from adults

Pheromones were extracted from the metathoracic glands using capillary tubes that were sharpened at one end using a micropipette puller (Moraes et al., 2008). Each *A. thunbergii* insect was first immobilized and frozen at -8 °C for 5 min before placement on a dissecting kit under a compound microscope in a ventral position to expose its rear side. The thorax which harbours the metathoracic gland was pierced with the tip of the sharpened tube; green fluid ascended the tube by capillary action. These contents were emptied to a 100 µl insert placed in a vial and dissolved in 50 µl hexane. The mixture was gently vortexed to obtain a homogenous sample and stored at -80°C prior to analysis. Four replicates (glands) were separately analyzed for each sex.

4.2.3.4 Dorsal abdominal glands contents from nymphs

Pheromones were extracted from the dorsal abdominal glands (DAG) by analyzing fresh molts (Moraes *et al.,* 2008) of *A. thunbergii* immatures. Fresh molts (0- 12 h) from nymphs, that is, 10 from 1st instars, 4 from 3rd instars, and 1 from 5th instar, were picked with clean forceps and dissolved in 150 µl of hexane in a 1.5 ml vial to extract pheromones. The samples were kept in airtight vials at room temperature (25 ±3 °C) for 24 h. The hexane extracts were transferred with a syringe to a clean vial and concentrated under gentle nitrogen flow to 30 µl before GC/MS analysis. Three replicates were evaluated for every nymphal stage. Samples were stored in -80^{0}C freezer until use.

4.2.4 Identification of pheromones by GC/MS

To identify headspace volatiles components of *A. thunbergii* adults and metathoracic and dorsal abdominal gland contents of adults and nymphs, respectively, analysis was conducted on coupled gas chromatography-mass spectrometry (GC-MS) on an Agilent Technologies 7890A GC linked to a 5795C MS, equipped with MSD ChemStation E.02.00.493, and Wiley 9th/NIST 2008 MS library and a HP5 MS column (30 m x 0.25 mm iD). Headspace samples collected by Super Q and charcoal

filter in undisturbed bugs were analysed with a temperature program of 5 min at 35°C, then an increase of 10°C/min to 280°C. A sample [1 μl] from the eluates was analysed in the splitless mode, using helium as a carrier gas at a flow rate of 1.0 ml/min. Spectra were recorded at 70 eV in the electron impact (EI) ionisation mode, with emission current of 34.6 μA. The SPME-trapped odors were analyzed by inserting each impregnated fibre on to the GC-MS injection port and thermally desorbing adsorbed volatiles at 220°C for 2 min, followed by analysis as above. An additional temperature program (5 min at 70°C, then 10°C/min to 230°C; see Khrimian et al., 2007) was also used to analyze SPME trapped-odors from undisturbed males and various isomers of synthetic methyl (2,4,6)-decatrienoate (MDT). To confirm the MDT isomer that is produced by males, the fragmentation ions and retention time of the MDT was compared with that of the *EEZ* isomer and a four-isomer blend of MDT (*ZEZ, ZEE, EEZ, EEE*). Other pheromones in A. thunbergii samples were also identified by comparing their mass spectra fragmentation and retention time with that of the corresponding authentic standards in the library (NIST/EPA/NIH Mass spectral Library 2005a version V2.od). Quantification of the pheromones was based on peak area comparison to those of the authentic standards.

4.2.5 Electrophysiology assays with a male-produced specific pheromone

Antennal responses of *A. thunbergii* males and females to a male-specific pheromone, that is, synthetic methyl (2*E*,4*E*,6*Z*)-decatrienoate (MDT-*EEZ*) was analysed using coupled gas chromatography-electroantennogram detection (GC/EAD), on a Hewlett-Packard (HP) 5890 Series II gas chromatograph equipped with HP-1 column (30 m X 0.32-mm diameter X 0.25 μm film thickness) (Agilent, Palo Alto, California, USA) using nitrogen as the carrier gas at 1.2 ml min^{-1} flow rate. The antennal preparation was made by filling in two sharpened glass capillaries with Ringer saline solution. One of the capillaries was connected to the basal end of an antenna of a male/ female and attached to a reference electrode, whereas the other connected the antenna tip to a recording electrode. The antennal signal was detected through an amplifier (INR-II, Syntech, Hilversum, The Netherlands), which was

acquired and processed by a data acquisition controller (IDAC-4, Syntech, Hilversum, The Netherlands) and later analysed with a GC/EAD software (EAD 2000, Syntech) to generate FID and EAD signals on a computer. An aliquot, 2µl of 100 ng μl^{-1} of MDT-*EEZ* was analysed using a temperature programme as follows: 35°C at 5 min, then an increase of 10°C/min to 280°C. Three replicates were conducted with antennae of *A. thunbergii* males and females.

4.2.6 Chemicals

The ester methyl (2,4,6)-decatrienoate i.e *EEZ* isomer and a blend of four isomers (*ZEZ*, *ZEE*, *EEZ* and *EEE*) (purity 95%) was synthesized and provided by Dr. Ashot Khrimian, Agricultural Research Service, US Department of Agriculture, Beltsville. Other standards were purchased from sigma Aldrich (purity > 98%).

4.2.7 Data analysis

Data on dual choice tests were analysed by Chi Square (χ^2) goodness-of-fit tests, assuming an equal distribution of insects choosing treatment (male/ female) or control (blank). Only responding insects (n) were considered in the analysis. The data were analysed using R version 3.1.2 software (R Core Team, 2014). The differences in the chemical profiles of metathoracic glands (MTG) and dorsal abdominal glands (DAG) of adults and nymphs were assessed by heat maps using PAST version 2.17.

4.3 Results

4.3.1 Responses to odors of conspecifics

Males of *Antestiopsis thunbergii* significantly attracted both males (χ^2=9.78, df=1, P = 0.002) and females (χ^2=8.05, df=1, P = 0.005). Four times more insects of each sex chose the olfactometer arm with a male as compared to blank (control). However, no significant behavioral responses were found when *A. thunbergii* females were used as treatment, indicating that females were unattractive to both sexes (i.e male: χ^2=0, df=1, P = 1.000 and female: χ^2=0.25, df=1, P = 0.617) (Figure 4.1).

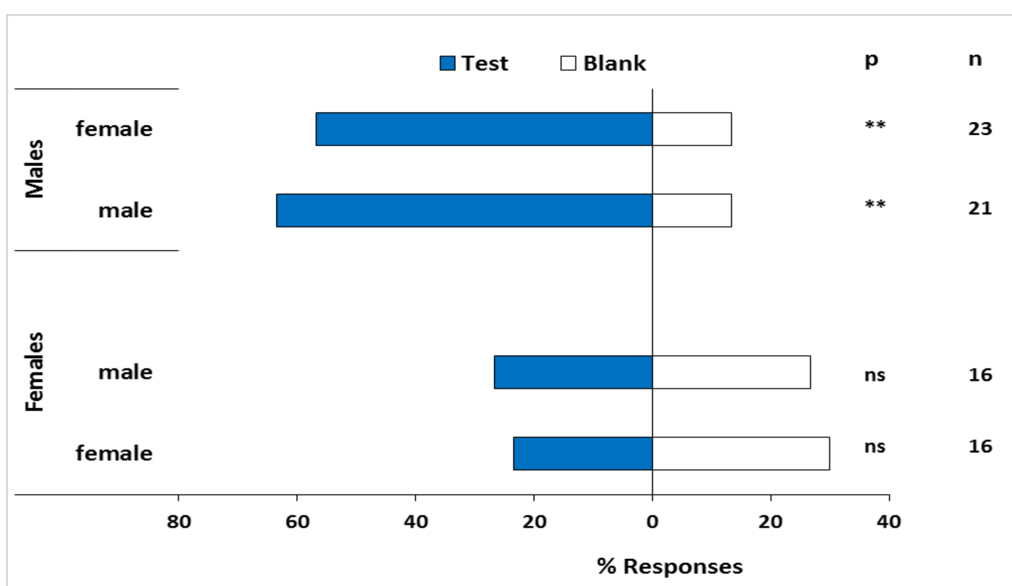

Figure 4.1: Comparison of behavioral responses of unmated sexually mature Antestia bug, *Antestiopsis thunbergii* adults against each sex.

Total insects tested per pairing was 30. n represents the number of respondents and asterisks indicate the significance levels, with $^{**}p < 0.01$.

4.3.2 Candidate attractive pheromones

4.3.2.1 Headspace odors of undisturbed adults sampled by Super Q and charcoal filter

Twenty-two components were identified in the headspace volatile emissions of undisturbed *Antestiopsis thunbergii* males and females trapped on Super Q adsorbents (Figure 4.2; Table 4.1). The compounds identified were identical for both sexes and dominated by tridecane. Their chemical group classification comprised [9] alkanes, [8] aldehydes, [2] esters, [1] alkene, [1] ketone and [1] monoterpene.

On the other hand, fewer compounds were detected in charcoal trapped volatile emissions from Antestia bugs as compared to those sampled by Super Q adsorbents for the same period (48 h) (Table 4.1). However, the charcoal filter trapped additional components (not found on the Super Q), identified as 5-methyl-6-hepten-2-one and 2-ethyl-1-hexanol which was common for both sexes; and three

compounds that were unique to males only namely methyl (2,4,6)-decatrienoate (MDT), (*Z*)-methyl ether-4-decen-1-ol and dodecanoic acid. MDT eluted thrice in different retention times, which could indicate that Antestia bugs produce different isomers of MDT; or isomerization of the ester before or during analysis.

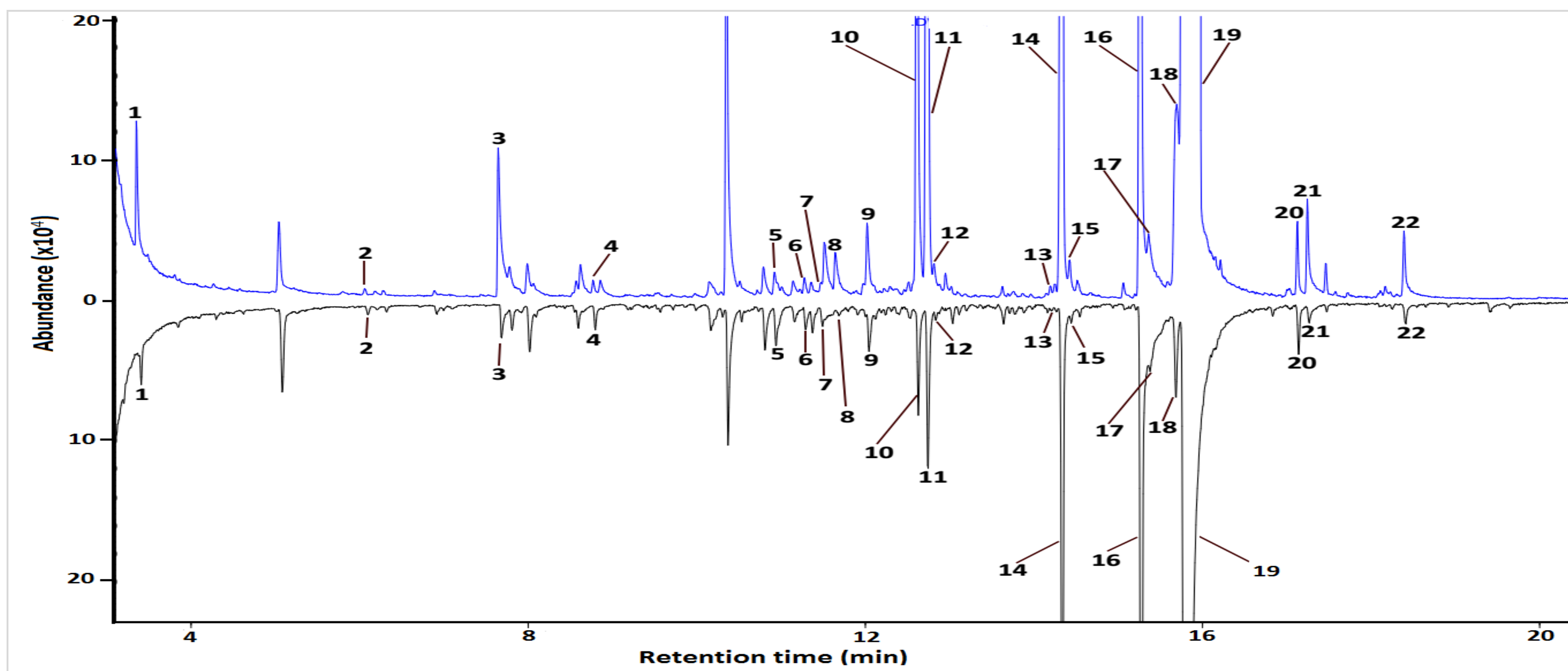

Figure 4.2: Representative chromatogram of headspace volatiles of *Antestiopsis thunbergii* males and females collected by Super Q sampling method for 48 h.

Peak numbers are identical to those in table 4.1. Unlabelled peaks were either unidentified or present in the control sample (2 g unripe berries and wet cotton wool)

Table 4.1: Average amount ± SE (ng/24h) of *Antestiopsis thunbergii* pheromones trapped by Super Q or charcoal filter adsorbents for each sex in 48h.

Peak No.	Retention time	Compound name	Compound class	Super Q adsorbent		Charcoal filter adsorbent	
				Male	Female	Male	Female
1	3.36	Heptane	Alkane	0.37±0.21	0.31±0.11	-	-
2	6.06	Octane	Alkane	0.08±0.05	0.81±0.58	-	-
3	7.65	(*E*)-2-Hexenal	Aldehyde	3.16±1.82	2.12±1.17	4.03±2.08	0.31±0.07
4	8.77	Nonane	Alkane	0.20±0.11	0.23±0.02	-	-
5	10.93	Decane	Alkane	0.40±0.23	0.48±0.14	-	-
	10.88	5-Methyl-6-epten-2-one	Ketone	-	-	0.07±0.01	0.05±0.01
6	11.28	(*E*)-2-Hexenyl acetate	Ester	0.71±0.41	1.98±1.17	-	-
7	11.48	Limonene	Monoterpene	0.19±0.11	0.74±0.16	-	-
8	11.65	(*E*)-4-Oxohex-2-enal	Aldehyde	0.89±0.51	1.42±0.76		-
9	12.02	(*E*)-2-Octen-1-al	Aldehyde	0.98±0.57	2.05±0.47	3.39±2.37	0.94±0.66
10	12.61	2-Nonanone	Ketone	7.42±4.28	2.33±0.28	0.68±0.40	0.24±0.09
11	12.74	Undecane	Alkane	9.06±5.23	2.91±1.56	2.00±0.58	0.97±0.54
12	12.82	Nonanal	Aldehyde	1.16±0.67	1.72±0.67		-
13	14.20	(*Z*)-4-Decenal	Aldehyde	0.21±0.12	0.57±0.00		-
14	14.34	Dodecane	Alkane	19.41±11.21	7.55±3.65	14.80±4.88	5.80±3.97
15	14.43	Decanal	Aldehyde	1.22±0.70	1.70±0.90		-
16	15.27	(*E*)-2-Decenal	Aldehyde	21.32±12.31	33.04±19.00	50.12±30.70	17.93±10.41
	15.58	MDT	Ester	-	-	4.50±1.33	-
	15.63	MDT	Ester	-	-	2.71±0.08	-
17	15.36	(*E*)-Dodecenal	Aldehyde	2.31±1.34	6.08±1.35		-
18	15.70	1-Tridecene	Alkane	3.33±1.92	1.90±0.74	6.29± 2.50	2.04±1.24
19	15.96	Tridecane	Alkane	354.22±204.51	166.73±66.20	410.43±83.76	190.85±76.04
20	17.13	Tetradecane (C14)	Alkane	0.71±0.41	0.83±0.26	1.04±0.51	0.63±0.26
	17.16	(*Z*)-4-Decen-1-ol, methyl ether	-	-	-	1.28±0.22	-
21	17.25	(E)-2-Decenyl acetate	Ester	1.62±0.93	3.21±2.13	17.45±0.84	0.84±0.31
	17.85	MDT	-	-	-	1.95±1.00	-
22	18.39	Pentadecane	Alkane	0.517±0.30	0.478±0.20	1.48±0.83	0.35±0.16
	19.27	Dodecanoic acid	Acid	-	-	0.21±0.08	-

4.3.2.2 Headspace odors of undisturbed adults sampled by SPME adsorbent

Pheromones were only detected in *A. thunbergii* males but not females when the collection was conducted on undisturbed bugs for 3 h with solid phase microextraction (SPME) fibre (Figure 4.3). About fifteen compounds were found in males (Figure 4.3). The ester methyl (2,4,6)-decatrienoate (MDT) was also detected in males only as previously (Table 4.1) in two peaks of unidentified isomers at 15.60 min and 18.05 min. Analysis of synthetic MDT-*EEZ* isomer using the same temperature programme that was used to analyse insect headspace volatiles led to elution of the synthetic sample in multiple peaks with two main elution times similar to those of the MDT from male volatiles (Figure 4.3; Figure 4.4). Some of the prominent fragmentation ions of MDT amongst *A. thunbergii* males and synthetic MDT-*EEZ* isomer were (*m/z*) 39, 43, 79, 91,105,107,119 121,137 and 149 (Figure 4.5).

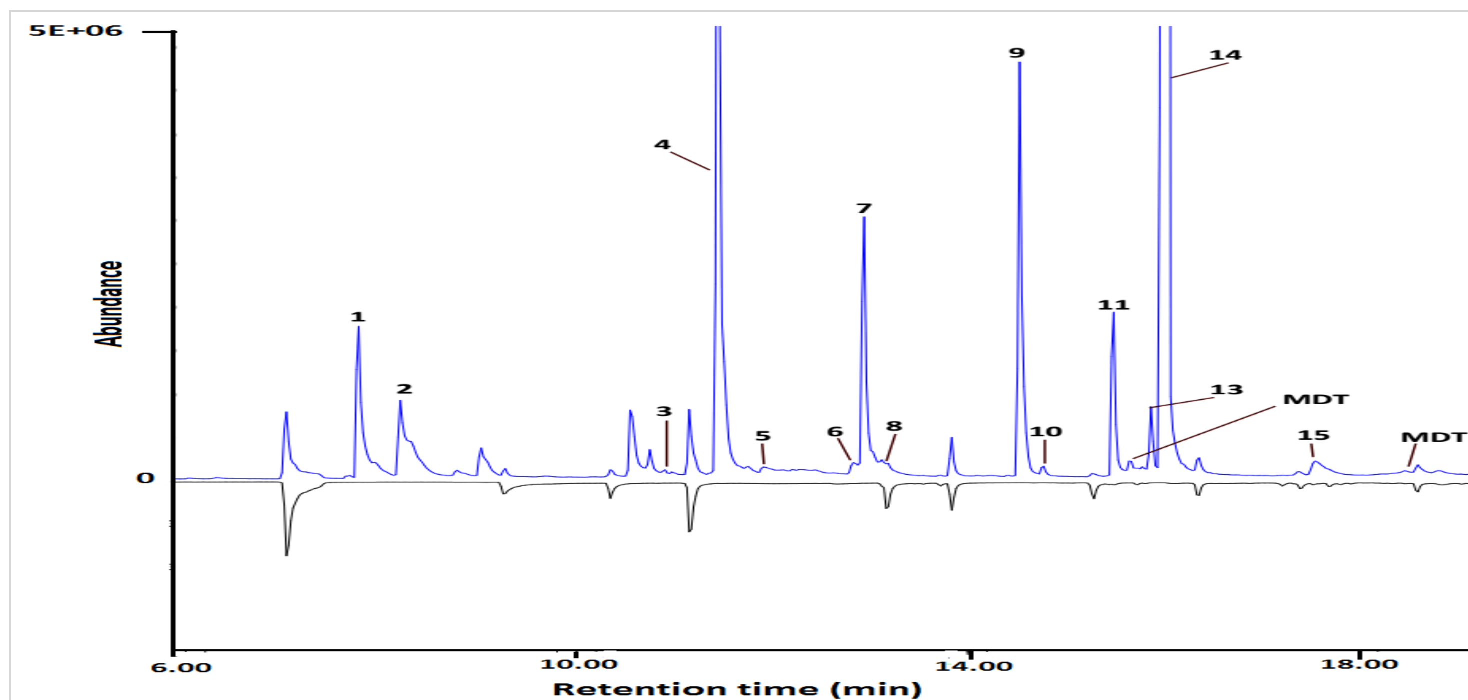

Figure 4.3: Representative chromatogram of chemical compounds detected in headspace odors of *Antestiopsis thunbergii* males (top) and females (bottom) collected by solid phase microextraction fibre from undisturbed insects for 3 h.

Compounds were only detected in males and comprised [1] (*E*)-2-Hexenal [2] (*E*)-2-Hexenol [3] 5-Methyl-6-hepten-2-one [4] (*E*)-2-Hexenyl acetate [5] (*E*)-4-Oxohex-2-enal [6] 2-Nonanone [7] Undecane [8] Nonanal [9] Dodecane [10] (*E*)-2- Octenol acetate [11] (*E*)-2-Decenal [12] Methyl (2,4,6)-decatrienoate (MDT-unknown isomer) [13] 1- Tridecene [14] Tridecane [15] (*E*)-2-Decenyl acetate [16] Methyl (2,4,6)-decatrienoate (MDT-unknown isomer)

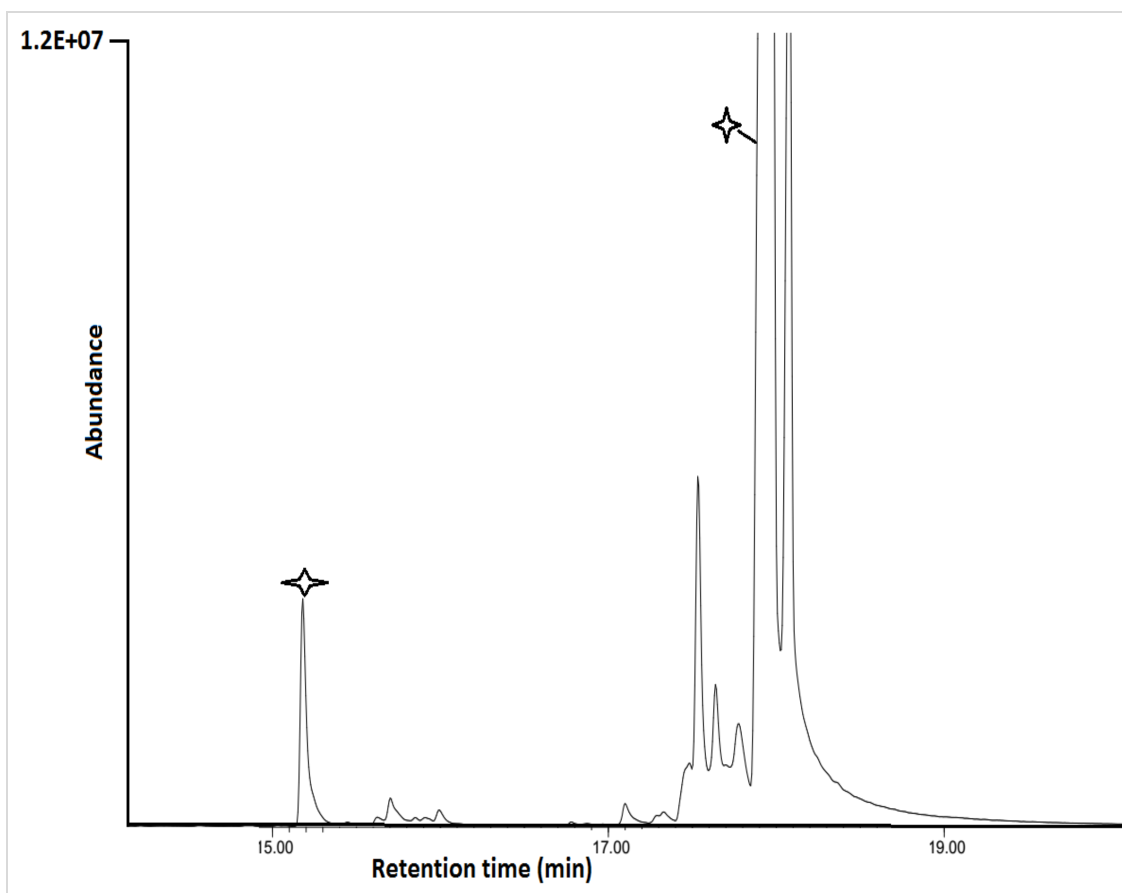

Figure 4.4: Representative chromatogram of synthetic methyl (2*E*,4*E*,6*Z*)-decatrienoate.

Multiple peaks at around 15 min and 18 min.

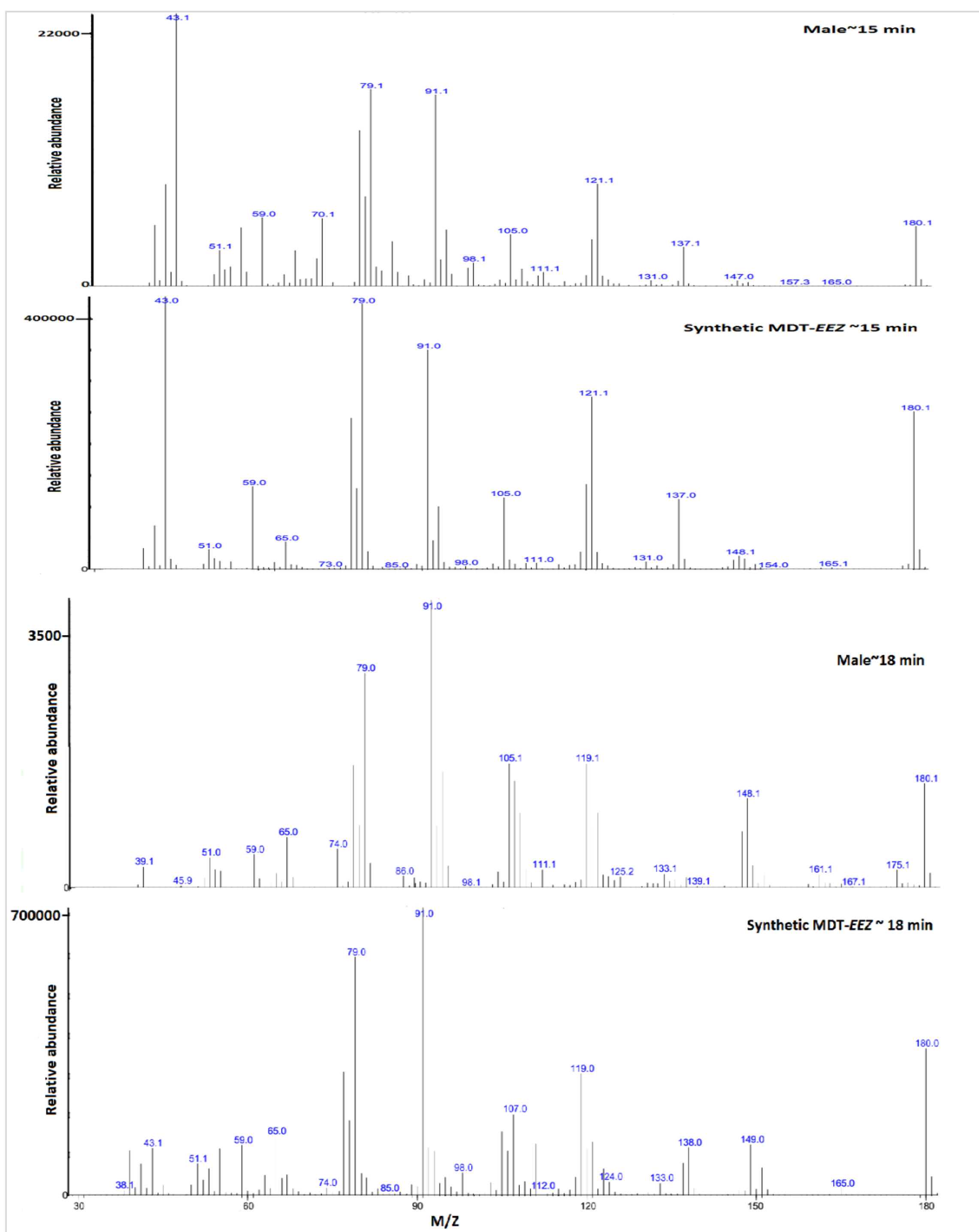

Figure 4.5: Mass spectra of the naturally occurring ester in Antestia bug male volatiles and synthetic standard of methyl (2*E*,4*E*,6*Z*)-decatrienoate.

The spectra is for dominant peaks occurring at around 15 min and 18 min.

4.3.2.3 Analysis of male volatiles and different isomers of synthetic MDT using a modified GC/MS programme

Analysis of a four-component blend of mixed isomers of methyl (2,4,6)-decatrienoate led to elution of the compound in four elution peaks. The order of elution of the isomers was *ZEZ*, *ZEE*, *EEZ* and *EEE* (Figure 4.6). The MDT peak in volatiles of males eluted at 14.46 min matching the elution time of *EEZ* isomer (Figure 4.7).

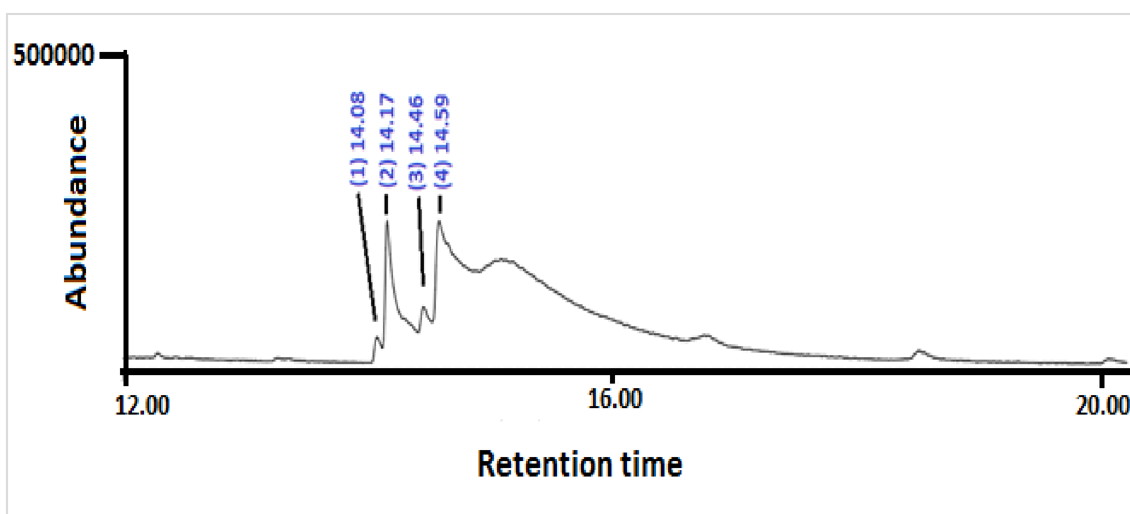

Figure 4.6: GC/MS elution sequence of a 4-component isomer blend of methyl-(2,4,6)-decatrienoate. (1) *ZEZ* (2) *ZEE* (3) *EEZ* (4) *EEE*

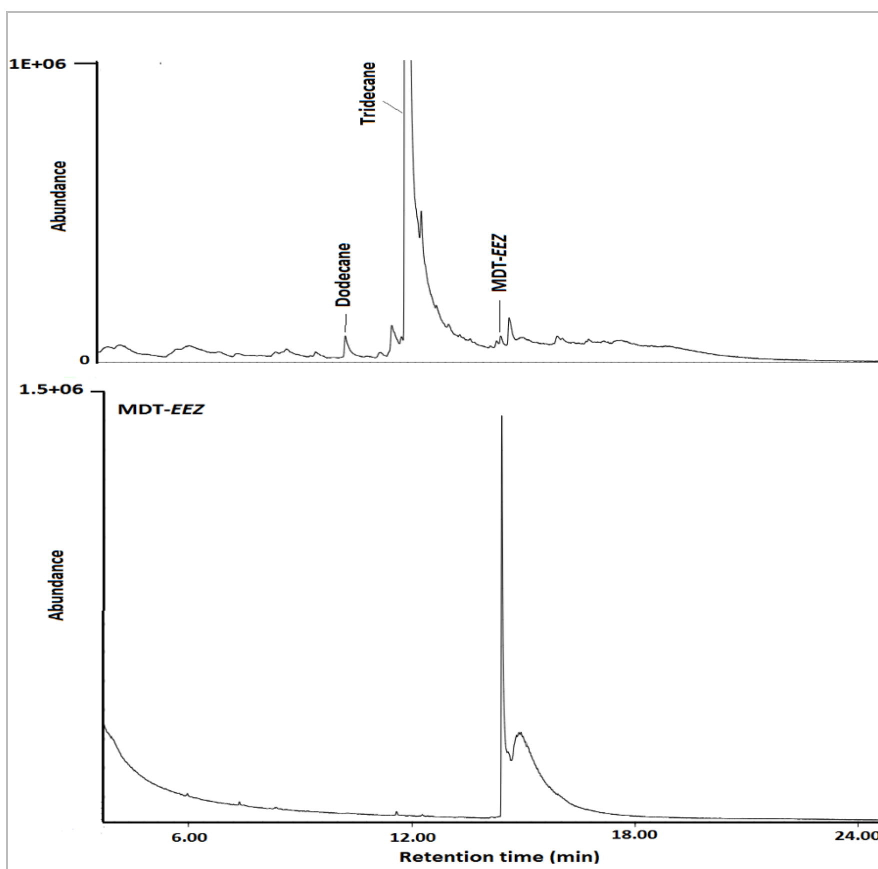

Figure 4.7: Chromatogram representation of male volatiles including methyl (2,4,6)-decatrienoate (MDT) and synthetic MDT-*EEZ* isomer that eluted at the same time.

4.3.3 Candidate alarm and defence pheromones

4.3.3.1 SPME trapped odors from disturbed adults

Disturbance led to high production of pheromones in both sexes sampled for only 15 min (Figure 4.8). Disturbed Antestia bugs produced ten key common compounds amongst the sexes which were dominated by tridecance, (*E*)-2 -decenal and dodecane (Figure 4.8). Other minor constituents were also detected in both sexes including (*Z*)-methyl ether-4-decen-1-ol and dodecanoic acid.

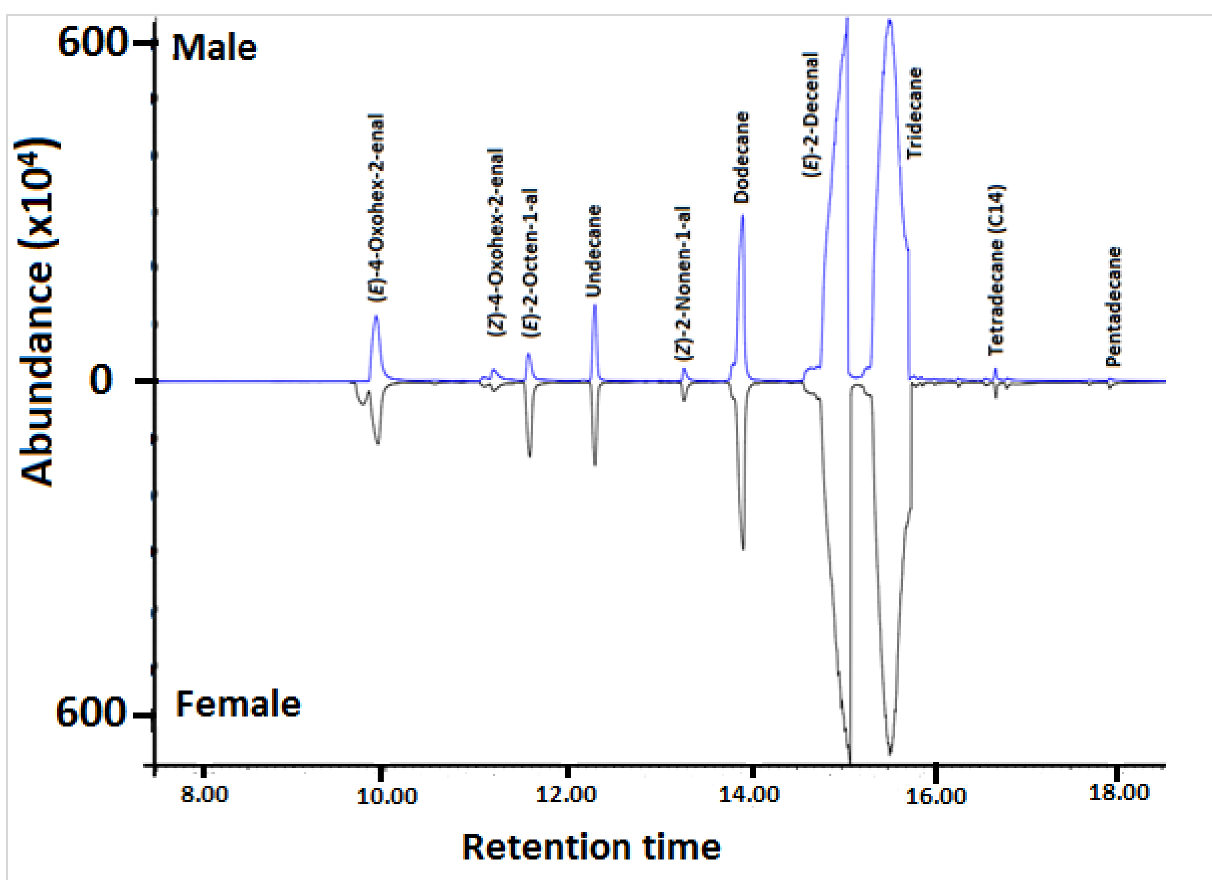

Figure 4.8: Representative chromatogram of dominant chemical compounds sampled by solid phase microextraction fibre from headspace odors of disturbed *Antestiopsis thunbergii* males and females collected for 15 min.

4.3.3.2 Gland contents of adults and nymphs

Twenty-two compounds were found in the metathoracic glands (MTG) of adults (both sexes) whereas only eight compounds were found in exuviae of 1st, 3rd and 5th instar nymphs, representing dorsal abdominal glands (DAG) contents (Table 4.2). Quantitative rather than qualitative differences were observed in the chemical composition within each maturity stage (amongst nymphs or adults) (Table 4.2). Tridecane, (*E*)-2-decenal, (*E*)-2-octen-1-al and (*E*)-4-oxohex-2-enal were amongst the predominant compounds identified in the MTG and DAG. Age appeared to be directly correlated with increasing production of most pheromones (Figure 4.9; Table 4.2). Dimorphism was also observed in selected compounds such as (*E*)-2-Hexenal and (*E*)-2-decenyl acetate whose amounts exceeded100 times in males than females,

whereas tridecane was in higher amounts in females than males (Figure 4.9; Table 4.2)

4.3.4 Antennal responses of adults to male specific pheromone

Synthetic methyl (2*E*,4*E*,6*Z*)-decatrienoate elicited positive antennal responses in *Antestiopsis thunbergii* males and females in GC/EAD analysis (Figure 4.10).

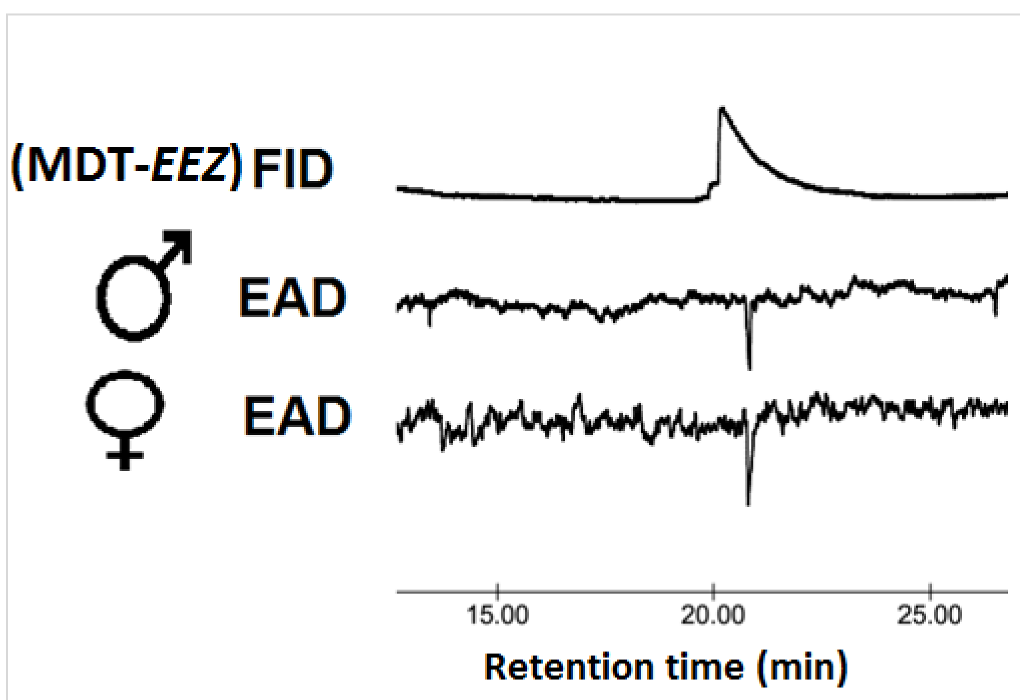

Figure 4.9: Electrophysiological responses of *Antestiopsis thunbergii* adult antennae to methyl (2*E*,4*E*,6*Z*)-decatrienoate

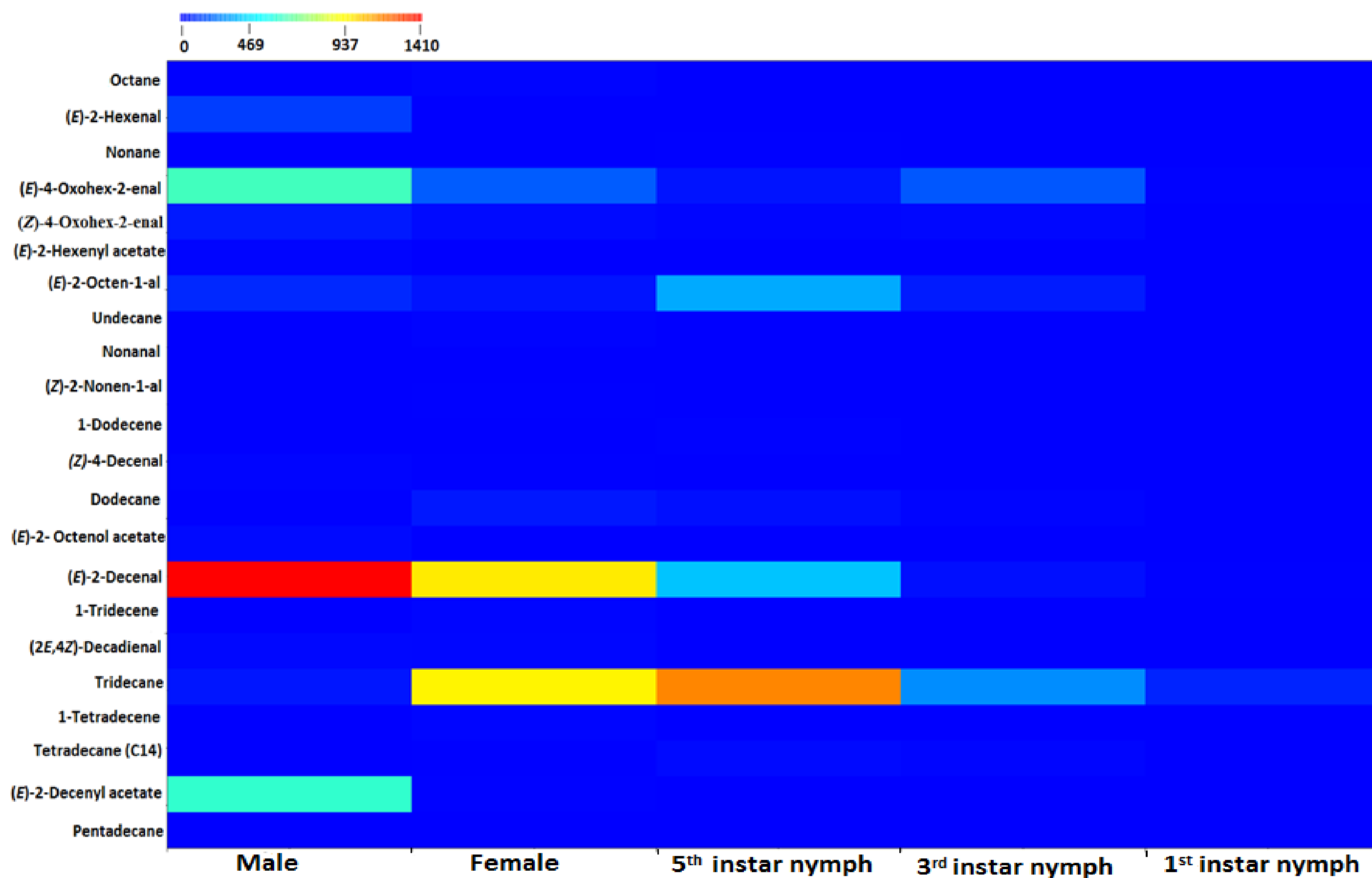

Figure 4.10: Heat map showing differences in the chemical composition of the metathoracic glands of adults and dorsal abdominal glands of nymphs of *Antestiopsis thunbergii*

Table 4.2: Average concentration ± SE (ng/µl) of metathoracic and dorsal abdominal glands contents of *Antestiopsis thunbergii* adults and nymphs respectively

Retention time	Compound name	Compound class	Male	Female	5th instar	3rd instar	1st instar
6.19	Octane	Alkane	0.30±0.01	8.37±0.67	1.72±0.09	1.45±0.07	0.21±0.07
7.65	(*E*)-2-Hexenal	Aldehyde	111.81±33.24	1.80±0.36	-	-	
8.86	Nonane	Alkane	0.06±0.03	1.24±0.33	1.87±0.15	1.31±0.07	0.22±0.06
10.17	(*E*)-4-Oxohex-2-enal	Aldehyde	588.06±56.53	170.19±32.11	34.89±0.67	160.70±33.89	4.20±0.29
10.38	(*Z*)-4-Oxohex-2-enal	Aldehyde	46.65±0.05	19.92±8.44	9.61±0.82	14.40±1.33	0.59±0.21
11.32	(*E*)-2-Hexenyl acetate	Ester	8.87±2.18	0.90±0.11	-	-	-
12.07	(*E*)-2-Octen-1-al	Aldehyde	74.99±1.16	36.22±16.20	312.71±109.80	49.59±21.42	0.43±0.02
12.77	Undecane	Alkane	0.53±0.34	6.05±0.69	-	-	-
12.81	Nonanal	Aldehyde	0.92±0.00	0.52±0.04	-	-	-
13.76	(*Z*)-2-Nonen-1-al	Aldehyde	-	2.29±1.76	-	-	-
14.23	1-Dodecene	Alkene	0.07±0.00	0.83±0.41	1.88±0.39	-	0.09±0.05
14.30	*(Z)*-4-Decenal	Aldehyde	8.72±0.03	3.22±0.64	-	-	-
14.36	Dodecane	Alkane	2.51±0.56	43.75±18.12	26.63±3.43	7.61±1.44	1.62±0.06
14.56	(*E*)-2- Octenol acetate	Ester	17.67±6.44	0.84±0.09	-	-	-
15.29	(*E*)-2-Decenal	Aldehyde	1405.93±17.06	974.14±312.03	359.88±16.80	26.79±8.45	3.50±0.33
15.70	1-Tridecene	Alkane	0.55±0.13	9.68±2.93	-	-	-
15.76	(2*E*,4*Z*)-Decadienal	Aldehyde	13.66±3.40	11.78±0.77	-	-	-
15.82	Tridecane	Alkane	39.89±8.11	956±327.41	1158.77±170.45	259.87±55.07	65.69±2.36
16.42	1-Tetradecene	Alkane	0.03±0.02	10.64±7.48	-	-	-
17.06	Tetradecane (C14)	Alkane	0.11±0.01	2.34±1.23	16.71±5.02	9.65±0.94	-
17.28	(*E*)-2-Decenyl acetate	Ester	558.55±238.68	4.37±1.12	-	-	-
18.43	Pentadecane	Alkane	0.14±0.03	0.88±0.56	-	-	-

4.4 Discussion

4.4.1 Headspace pheromone sampling techniques

The chemical profile of *A. thunbergii* was studied using different headspace sampling techniques. Super Q sampled a wide range of compounds, whereas charcoal filter sampled a few compounds but had a higher level of specificity; it detected a male specific compound, later established to be an aggregation pheromone in subsequent behavioral assays. However, the two methods required a long sampling period of 48 h, which is a setback associated with the two conventional extraction methods (Prosen & Kralj, 1999). It is possible that the male-specific pheromones could be detected even with Super Q after a longer trapping time and more insects (Moraes *et al.,* 2005), but this was not possible in our situation due to constraints of maintaining a large colony of insects. Long collection hours may have resulted in high production of stress-related pheromones to mask aggregation/ sex pheromones (Millar, 2005).

Solid phase microextraction (SPME) technique was developed to address the need for rapid sampling (Pawliszyn, 2001). In this study, it was the most important method of sampling pheromones in terms of required sample size and sensitivity, within a short time. In addition to trapping the male-specific candidate aggregation pheromone in 3 h, SPME also trapped other minor components such as (Z)-methyl ether-4-decen-1-ol and dodecanoic acid in both sexes, previously detected in the odor of males only in charcoal trapped samples. SPME also sampled excessive amounts of pheromones in disturbed bugs within 15 min. However, since its analysis is by thermal desorption of the SPME fibre, it is impossible to store adsorbed volatiles for later use (Pawliszyn, 2001; Prosen & Kralj, 1999) unlike in other adsorbents. Our findings emphasize the importance of comparing different adsorbents in the analysis of semiochemicals particularly in inaugural studies.

4.4.2 Candidate aggregation pheromone

Headspace volatiles of Antestia bug, *A. thunbergii* males attracted both sexes, indicating the involvement of an aggregation pheromone. Males of Heteropterans are known to be the initial colonizers, who search and find host food, and then produce aggregation pheromones to attract conspecifics (Aldrich *et al.,* 1999). This is the first study to demonstrate that *A. thunbergii* males also produce aggregation pheromones. Aggregation pheromones may have a dual function, including bringing both sexes together for mating, hence are sometimes referred to as aggregation-sex pheromones (Carde, 2014), but this aspect would require further investigation in *A. thunbergii*.

Methyl (2,4,6)-decatrienoate (MDT) was identified as a male-specific pheromone of *A. thunbergii* and its *EEZ* isomer elicited antennal response in both males and females, suggesting that the compound it is a candidate aggregation pheromone. Although eight isomers of MDT exist, only two have been reported previously to occur naturally and are biologically active (Khrimian, 2005). MDT-*EZZ* has been reported as a male-produced sex pheromone of the red-shouldered stink bug, *Thyanta perditor* (Moraes *et al.,* 2005) and MDT-*EEZ* isolated from males of the brown-winged green bug, *Plautia stali*, as an aggregation pheromone (Millar, 1997). Although, the two species occur in the temperate region unlike *A. thunbergii*, they belong to the same family (Pentatomidae) and tribe (Antestiini) as *A. thunbergii*. Hence, it appears that closely related species, irrespective of their habitats, may use similar chemical cues for communication. The aggregation pheromone MDT-*EEZ* is commercially produced to lure many stink bug species in the USA and Europe (Short *et al.,* 2017; Weber *et al.,* 2014; Aldrich *et al.,* 2006). It has also been found to be a kairomone that mediates host location for a tachinid parasitoid (Aldrich *et al.,* 2007), which is a natural enemy for stink bugs. Our results and literature data strongly suggest that MDT-*EEZ* could also be a male-produced aggregation pheromone in *A. thunbergii,* with potential for use in monitoring and mass trapping of the pest in coffee plantations. The next chapter (chapter five) evaluates the potential of MDT-*EEZ* in luring this species in coffee plantations.

4.4.3 Candidate alarm and defence pheromones

Analysis of metathoracic glands (MTG) in adults and abdominal glands (DAG) / exuviae in nymphs is a standard method of screening for alarm and defence pheromones, as these glands are their reservoirs in stink bugs (Millar, 2005; Fávaro *et al.,* 2012).Additionally, collecting headspace volatiles of disturbed insects has also been previously reported in screening stress-induced compounds in stink bugs (Krall, 1999) and coleopterans (Bonacci *et al.,* 2011). Overall, the quantity of pheromones found in the glands of adults was more than in nymphs. Similarly, DAG contents amongst nymphs varied in amounts, mainly as a function of age. Lower amounts of alarm pheromones found in *A. thunbergii* nymphs than adults could translate to high mortalities in nymphs as they are less protected from predators. (*E*)-4-Oxo-2-hexenal, tridecane and (*E*)-2-Octenal were dominant compounds in *A. thunbergii* nymphs as was found in stink bug*, Agroecus griseus* nymphs (Fávaro *et al.,* 2012).

In adults, (*E*)-4-Oxo-2-hexenal, (*E*)-2-decanal, (*E*)-*2*-Decenyl acetate and tridecane were major components in both sexes. These have also been reported as alarm and defence pheromones for stink bugs, ants and beetles (Millar, 2005; Staples *et al.,* 2002; Krall *et al.,* 1999). Interestingly, dimorphism was also observed with most compounds found in higher amounts in males than females. For instance, (*E*)-2-hexenal was a hundred-fold more in males than females. Since the males are the early colonizers and produce the aggregation pheromone, then they may be at a higher risk of being found by predators, hence would need higher reservoirs of the defence and alarm pheromones. The quantitative and ratio differences could also be a tactic used by the pest to differentiate among sexes. Some alarm or defence pheromones may also function as sex or attractive pheromones (Krall *et al.,* 1999). Several studies have shown that (*E*)-2-hexenal and (*E*)-2-octenal might function as part of the aggregation pheromone blend of Bed bugs *Cimex lectularius* (Choe *et al.,* 2016). Whether *A. thunbergii* uses MDT in synergy with other selected pheromones to attract conspecifics would require further investigations by performing dose-response assays with selected pheromones separately and in blends.

Headspace volatiles sampled by solid phase micro extraction (SPME) included similar compounds in disturbed bugs as identified in the glands emphasizing, they could be indeed candidate alarm and defence compounds. These were dominated by (*E*)-2-decanal and tridecane. Jackels *et al.,* (2014) found several alkanes such as tridecane and dodecane in the headspace volatiles of *A. thunbergii* that had been killed and suggested that they could be defence compounds. Our results corroborate this finding, using live insects and further reports additional compounds previously reported in other stink bugs. The alarm pheromones could serve as candidate semiochemical-based options for repelling the pest away from host plants or protecting host plants from invasion. They could also be incorporated in the development of genetically modified coffee plants resistant to *A. thunbergii* through production of compounds with alarm functions.

4.5 Conclusion

This study evaluated different pheromone screening methods and highlighted their pros and cons, knowledge that could be applied while studying the chemical ecology of other insects especially stink bugs. The solid phase micro extraction sampling method was simple, sensitive and replicable within a short period. Pheromones that likely have alarm or defence function were reported from headspace volatiles of disturbed bugs and metathoracic glands of adults and dorsal abdominal glands of nymphs. A candidate aggregation pheromone was identified, in males, which was found to attract both sexes in behavioral assays. Hence, future studies should investigate its performance in coffee plantations. This study provides a blue print of the identities of pheromones produced by *A. thunbergii*, opening avenues to further research to deduce the biological functions of specific pheromones and how they could be utilized in pest management programmes. There were striking similarities of the pheromones produced by *A. thunbergii* with related pentatomid species occurring in the temperate region, which have been widely studied. Therefore, researchers could explore optimization of existing knowledge and techniques to fast track development of pest control strategies of emerging or neglected related pests such as *A. thunbergii*, particularly in developing countries.

CHAPTER FIVE

EVALUATION OF THE PERFORMANCE OF CANDIDATE KAIROMONE AND AGGREGATION PHEROMONE IN MONITORING AND MASS TRAPPING OF ANTESTIOPSIS THUNBERGII IN COFFEE PLANTATIONS

Abstract

This study was conducted to evaluate the performance of candidate attractants previously identified in laboratory assays for the management of Antestia bugs in coffee plantations. The treatments comprised a 3-component kairomone blend of anisole, methyl 3-ethyl-4-methylpentanoate and (5*S*,7*S*)-conophthorin from unripe coffee berries, and methyl (2*E*,4*E*,6*Z*)-decatrienoate (MDT-*EEZ*), a candidate aggregation pheromone produced by males of *A. thunbergii*. The performance of three doses of each candidate lure (blend and MDT-*EEZ*), and control (solvent only) was investigated in field tests. The treatments were dispensed in rubber septa placed inside delta traps® and were replaced every three days with fresh ones. In a subsequent test, the number of Antestia bugs found on pheromone-baited plants (5 mg) were compared to those of unbaited plants (control) over a period of one week without replenishing the treatments. Dose response tests showed that MDT-*EEZ* was significantly attractive to two *Antestiopsis* species namely *A. thunbergii* and *A. facetoides* at the highest tested dose (5 mg), unlike the kairomone, which did not induce significant responses at the tested doses. In subsequent tests with 5 mg MDT-*EEZ*, Antestia bug commenced aggregations on coffee trees within 24 h of pheromone placement and peaked on the fourth day with up to a mean of 7.3 bugs per tree as compared to control trees without the pheromone, which had 1 bug/ tree on average. Notably, Antestia bugs rested on baited plants without being captured by the delta traps®. Our results confirm that MDT-*EEZ* is an aggregation pheromone of Antestia bugs with potential for use in surveillance and mass trapping of the pest. MDT-*EEZ* could also be used in “attract and kill strategy” to restrict control measures at selected portion of the farm. Future studies should investigate an

appropriate trap design that is compatible with MDT-*EEZ* in capturing bugs. Further studies are also required to establish a kairomone formulation and dose that is effective in luring Antestia bugs in the field.

5.1 Introduction

Kairomones (attractants) derived from host plant volatiles have been used widely to lure insect pests (Szendrei & Rodriguez-Saona, 2010; Gregg *et al.,* 2010; Vega *et al.,* 2009; Cook et *al.,* 2007; Prokopy *et al.,* 1990). Also, aggregation pheromones are often incorporated in pest management programs. For instance, methyl-2*E*,4*E*,6*Z*-decatrinoate (MDT-*EEZ*), an aggregation pheromone of various stink bug species is widely used for monitoring or mass trapping in USA and Europe (Short *et al.,* 2017; Weber *et al.,* 2014; Aldrich *et al.,* 2006). Trap design and color also play a crucial role in determining efficiency of a trapping system (Leskey *et al.,* 2012; Joseph, 2014). Whereas mass trapping of various insect pests using pheromones especially beetles and moths is usually very successful (Witzgal *et al.,* 2010), poor catches are reported in most stink bugs, which are arrested around the pheromone source but fail to enter traps possibly because traps lack short range vibrational cues (Virant-Doberlet *et al.,* 2004; Millar, 2005). Thus, use of "trap/ sacrificial" crops is a common strategy that concentrates a pest on lure-baited plants for elimination (Cook *et al.,* 2007; Shelton & Badenes-Perez, 2006; Krupe *et al.,* 2001) hence minimizing damage and control costs.

Antestia bug, *A. thunbergii* is a key pest of Arabica coffee in many African countries (Babin *et al.,* 2018). Its association with high yield losses (Mugo *et al.,* 2013) and "potato taste" defect in infested coffee beans (Jackels *et al.,* 2018) currently threatens coffee production and trade in affected countries. Due to the severity of the damage caused by the pest, a population density of 1-2 bugs/ tree meets the recommended threshold for control in Kenya (Mugo *et al.,* 2013). The pest is currently controlled by cultural methods and use of pesticides (Nyambo *et al.,* 1996) which are only partially effective. Furthermore, use of pesticides is discouraged due to their toxicity to the ecosystem, development of resistance and unaffordability by most farmers

(Cook *et al.,* 2007; Liedtke *et al.,* 2011). This study evaluated the use of semiochemicals in *A. thunbergii* management, as they are regarded as safer, cheaper, specific and effective in tiny amounts (El-Sayed *et al.,* 2006; Witzgall *et al.,* 2010).

To utilize semiochemicals in pest management, knowledge of the biology and behavior of the pest is paramount. Although Antestia bugs feed on different coffee parts and maturity stages, unripe coffee berries are preferred and crucial for the pest survival and development (Le Pelley, 1942; Matsuura *et al.,* 2014). In addition, Antestia bugs usually aggregate on certain coffee trees (Cilas *et al.,* 1998). These behaviors suggest involvement of semiochemicals, which was indeed confirmed in previous chapters of this study. A 3- component kairomone blend comprised of anisole, methyl 3-ethyl-4-methylpentanoate and (5*S*,7*S*)-conophthorin from unripe coffee berries was significantly attractive to second instar nymphs of *A. thunbergii* suggesting that the blend mediate host recognition (chapter three). In addition, a candidate aggregation pheromone (MDT-*EEZ*) was reported in males, which were attractive to conspecifics in laboratory bioassays (chapter four). Subsequently, the current study was conducted to establish the attractiveness of identified kairomone blend and pheromone paired with delta traps® in coffee plantations.

5.2 Materials and Methods

5.2.1 Study site

The study was conducted at Mbumi Coffee Estate, located in Kiambu County, Kenya (for coordinates see section 3.1), between 8th May and 25th May 2018. The farm size is about 120 hectares, with coffee trees planted uniformly, with approx. 2 m by 2 m spacing between plants. No chemicals or insecticides were sprayed during the study period. Three plots were selected within the farm (Figure 5.1) for conducting subsequent experiments after an initial sampling that established that the sites were infested by two Antestia bugs species, i.e *Antestiopsis thunbergii* and *Antestiopsis facetoides*. A data logger was installed in the farm to record temperature and humidity.

The plots selected were mostly shaded or near trees, such as *Grevillea* sp.. Shading has been shown to favor Antestia bug populations (Mugo *et al.,* 2013). During the time of study, the temperatures were very low (average 18^{0}C) and it rained on a few days which may have negatively impacted the results obtained as Antestia bugs require sunny and warm weather to actively fly between coffee trees in the field (Babin *et al.,* 2018). The phenological state of coffee during tests was dominated by unripe coffee berries (mix of young and mature green) and very few ripe coffee berries.

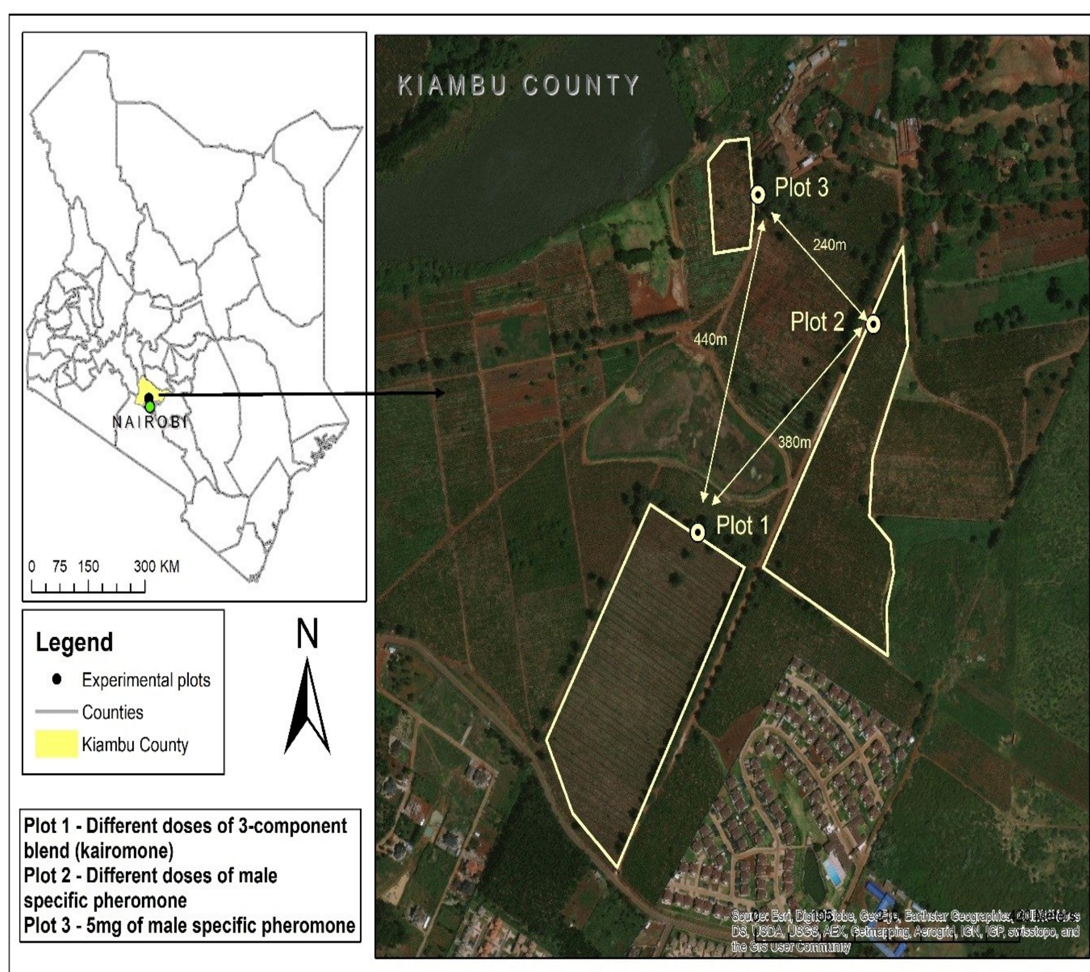

Figure 5.1: Study sites where the performance of candidate lures was evaluated for the management of Antestia bug.

The coffee farm is suited at Kiambu County, Central Kenya.

5.2.2 Treatments

First, candidate kairomone and aggregation pheromone were evaluated independently at three different doses each, with the solvent as control. The doses of a 3-component kairomone blend was formulated from concentrations that were previously found to be attractive in laboratory assays (Njihia *et al.,* 2017), i.e. anisole, methyl 3-ethyl-4-methylpentanoate and (5*S*,7*S*)-conophthorin, hereby abbreviated as A, MP and C, respectively. The doses were formulated in dichloromethane by multiplying the dose that was effective in laboratory assays by 10, 100 and 1000 to constitute the following amounts in µg: blend A = 0.067 A + 0.114 MP + 0.039 C, blend B = 0.67 A + 1.14 MP + 0.39 C and blend C = 6.7 A + 11.4 MP + 3.9 C. Dichloromethane was used as negative control. Methyl (2*E*,4*E*,6*Z*)-decatrienoate (pheromone) doses were selected based on literature of related pentatomid species in the temperate region (Aldrich *et al.,* 2006; 2009; Laumann *et al.,* 2011). These comprised 1.25 mg, 2.5 mg and 5 mg, formulated in hexane, and thus, an addition hexane solvent was used as negative control.

In a second experiment, responses of Antestia bugs to 5 mg of methyl (2*E*,4*E*,6*Z*)-decatrienoate and control (hexane) were assessed.

5.2.3 Study design for dose response tests

To compare the performance of various doses of candidate kairomone and pheromone, tests were conducted in plots 1 and 2, respectively (Figure 6.1), each with a replicated Latin square design of 4x4 (4 doses and 4 replicates). The experiment lasted 12 days, in which the position of the treatments in the Latin square was changed after 3 days (4 replications). Each plot size measured 30 m x 30 m, and treatments were 10 m apart. Each treatment dose (shown in section 5.3.2) was formulated in 2 ml of solvent and put in a white rubber septum (Suba seal size 25; internal diameter 1.5 cm x 2.5 cm depth; Sigma-Aldrich) to dispense volatiles. Each impregnated rubber septum was affixed on the sticky surface of a green delta trap® (size: 10 cm x 10 cm X 18cm) (Trécé Incorporated, Adair, Oklahoma, USA) before hanging on coffee trees at approx. 1.5 m above ground (plate 5.2). Delta trap® was

chosen because it is a relatively cheap trap design (US $ 2/trap) used to trap many pests including plant bugs, *Phytocoris difficilis* (Heteroptera) (Zhang, Q.H. and Aldrich, 2003) and is also locally available. Traps were evaluated every day in the afternoon between 12.00 pm – 5.00 pm). During evaluation, the sticky surface of the delta trap® was observed for captured Antestia bugs. In addition, Antestia bugs found on the coffee trees where the traps were hanged were assessed through careful observation of the coffee berries and vegetative parts. The insects found inside trap or on the trap tree were identified by species, development stage (nymph/ adult) and sex for adults, counted and then removed from the trap/tree at each observation. Only mobile nymphs (2nd instars to 5th instars) were recorded. Replacement of lures with fresh ones was done every 3 days, upon which the treatment positions were alternated and randomized.

Plate 5.1: Delta trap® used to dispense lures suspended on a coffee tree

5.2.4 Study design to evaluate aggregation

This study was performed to evaluate the performance of methyl (2*E*,4*E*,6*Z*)-decatrienoate (MDT) in attracting Antestia bugs over a period of one week without replenishing it or removing lured Antestia bugs. The study was conducted in the plot 3 (Figure 5.1) measuring 30 m x 10 m, with treatment and control arranged on two separate rows parallel to each other. The treatment comprised 5 mg of MDT, whereas control was hexane solvent each replicated four times, and 10 m apart. As in previous assays (section 6.3.2), treatments were dispensed on rubber septa, which was put inside green delta trap® and hanged on coffee trees approx. 1.5 m above ground. Evaluation was done daily, in the afternoon by checking traps and trap-trees for the presence of Antestia bugs, which were recorded depending on species and maturity stage, without removal for seven days. Differentiation of sex and nymphs' species was not done to avoid disturbing the bugs.

5.2.5 Source of chemicals

Synthetic methyl (2*E*,4*E*,6*Z*)-decatrienoate (purity 95%) was synthesized and provided by Ashot Khrimian. Methyl 3-ethyl-4-methylpentanoate (purity 97%) and (5*S*,7*S*)-conophthorin (purity 99%) were a gift from Prof. Wittko Francke, University of Hamburg, Germany. Anisole, hexane and dichloromethane were bought from Sigma Aldrich (purity > 98%).

5.2.6 Data analysis

Total numbers of Antestia bugs found on lure (pheromone/kairomone) baited trees at different doses or control were compared by a negative binomial regression, followed by the test of Analysis of deviance (Anodev), and then means were separated using a pairwise Tukey test. Different maturity stages (nymphs, females and males) of lured bugs were compared by χ^2 test. The data was analysed using R version 3.1.2 software (R Core Team, 2014).

5.3 Results

5.3.1 Responses of Antestia bugs to different doses of kairomone and pheromone

No bugs were captured by the delta trap® during the entire experimental period. Although, no significant differences were found in the mean number of Antestia bugs on the kairomone baited trees when compared to unbaited trees (control) ($F_{3,15}$=7.71, P=0.195; Figure 5.1); dose of kairomone was inversely associated with number of Antestia bugs. Contrary, Antestia bugs were significantly attracted by pheromone baited trees than unbaited trees ($F_{3,15}$=9.35, P=0.025) (Figure 5.2). Although, greater doses of methyl (2*E*,4*E*,6*Z*)-decatrienoate (MDT-*EEZ*) led to an increase in Antestia bug numbers found on the pheromone baited trees, statistical differences were detected between control (hexane solvent) and highest tested dose (5 mg MDT) only. The ratio of males and females attracted to 5 mg of MDT-*EEZ* was 1:1.2. The proportion of individuals was directly correlated with increasing dose of the pheromone, for both species, sexes and all development stages (nymphs and adults) (Table 5.1).

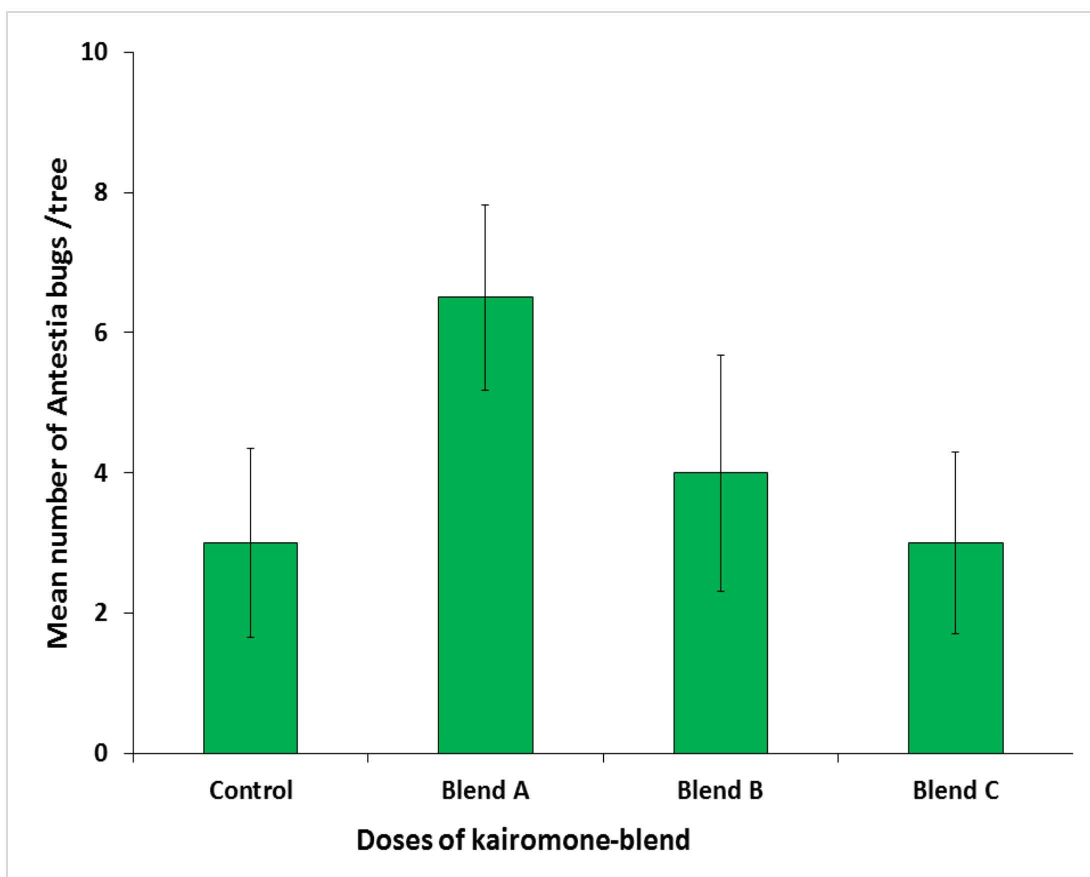

Figure 5.2: Mean number of Antestia bugs on coffee trees baited with varying concentrations of a 3-component kairomone blend comprised of varied doses of anisole, methyl 3-ethyl-4-methylpentanoate and (5*S*,7*S*)-conophthorin, and control (solvent)

Insects were removed from trees after each observation (daily) during evaluation which was conducted for 12 days. No significant difference was recorded between the treatments.

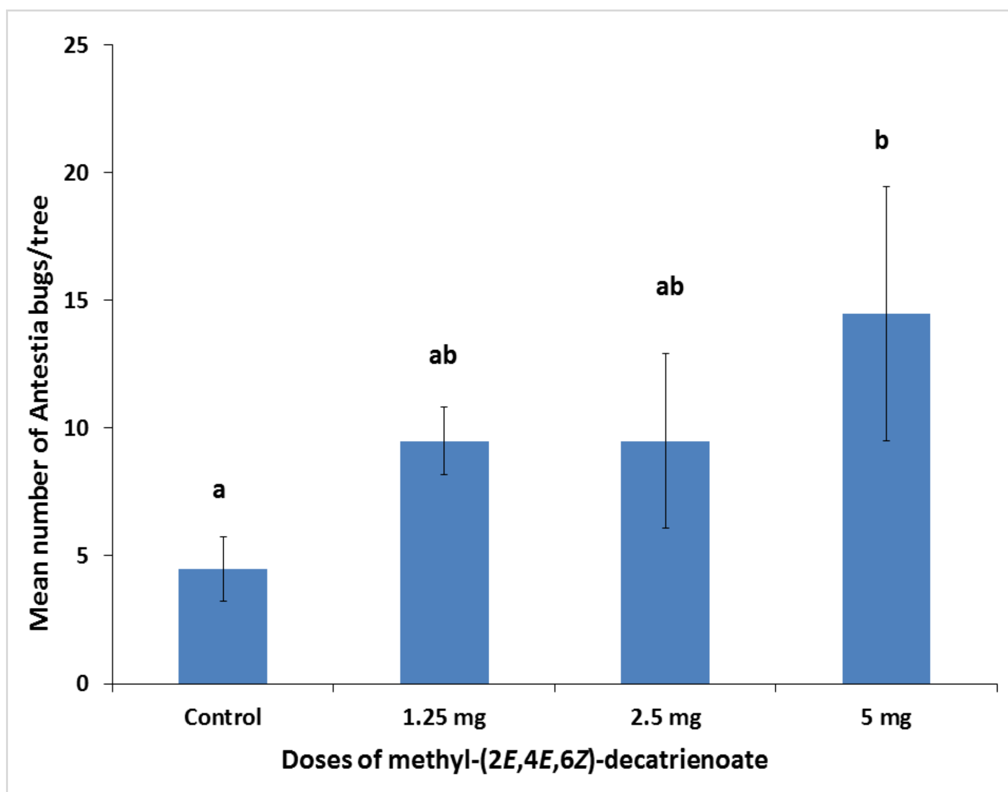

Figure 5.3: Mean number of Antestia bugs on coffee trees baited with varying concentrations of synthetic methyl (2*E*,4*E*,6*Z*)-decatrienoate, and control (solvent).

Insects were removed from trees after each observation (daily) during evaluation which was conducted for 12 days. Alphabets above error bars represent significant differences among treatments.

Table 5.1: Total number of Antestia bugs found on trees baited with varying concentrations of methyl (2*E*,4*E*,6*Z*)-decatrienoate for the different species, life stages, and sexes amongst adults.

	Antestiopsis thunbergii				*Antestiopsis facetoides*			
Treatments	**Male**	**Female**	**Nymph**	***Total***	**Male**	**Female**	**Nymph**	***Total***
Control	4(13)a	5(12)a	4(11)a	**13(12)a**	2(15)a	1(6)a	2(13)a	**5(11)a**
1.25 mg	4(13)a	11(27)ab	7(21)ab	**22(21)ab**	6(47)a	6(35)ab	3(19)ab	**15(32)b**
2.5 mg	7(23)ab	11(27)ab	10(30)ab	**28(27)b**	2(15)a	3(18)ab	4(25)ab	**9(20)ab**
5 mg	15(50)b	14 (34)b	13(38)b	**42(40)c**	3(23)a	7(41)b	7(43)b	**17(37)b**

Control was no methyl (2*E*,4*E*,6*Z*)-decatrienoate

Values in brackets are proportions calculated in columns.

Different letters within columns represent significant differences between treatments.

5.3.2 Aggregation assay

At the commencement of the experiment, the average population was ~1 bug/ tree. However, 24 hrs after the pheromone [methyl (2*E*,4*E*,6*Z*)-decatrienoate] was deployed, the abundance of the bugs increased on pheromone baited trees to 3.5 bugs/ tree, whereas no significant changes were observed in the control plants (without pheromone) through the study period. The numbers of bugs found on pheromone baited trees continued to gradually increase and peaked on the 4th day with 7.3 bugs/ tree, which coincided with the warmest day (23 °C) recorded during the study period. Thereafter, reduced populations of about ~2 bugs/tree were recorded from the 5th day onwards (Figure 5.3).

In total 137 bugs were assessed in the study i.e 105 on pheromone baited and 32 on unbaited plants respectively. They comprised 53 adults of *A. thunbergii,* 8 adults of *A. facetoides* and 76 nymphs.

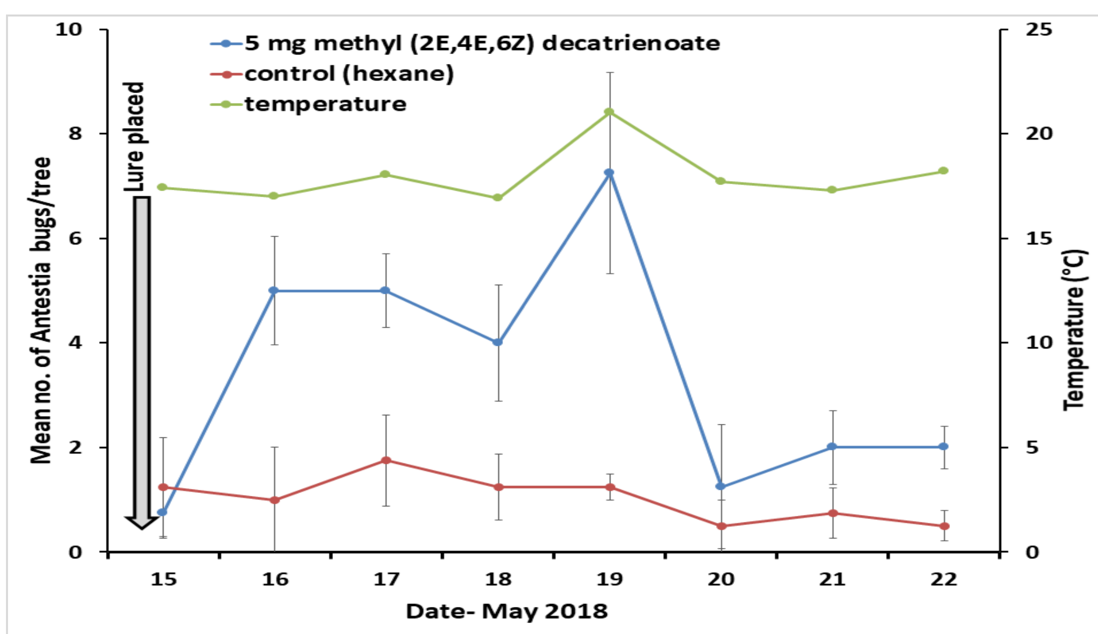

Figure 5.4: Mean numbers of Antestia bugs attracted to pheromone-baited (methyl (2*E*,4*E*,6*Z*)-decatrienoate) or unbaited coffee plants over a period of one week.

Bugs were counted without removal and without replenishing treatments.

5.4 Discussion

The 3-component kairomone blend did not significantly attract Antestia bugs in any of the tested doses, which could be due to a number of factors: (1) It is likely that the correct dose was not evaluated. Although the results indicated that doses were not different, increasing doses was associated with less numbers of Antestia bugs (Figure 5.1), hence it is likely that testing lower doses could have led to significant attraction. It has been shown that high doses of attractive compounds may have inhibitory effects on the responses of herbivores (Renou *et al.,* 2000) as the neurons may clog. In fact, all the compounds in the blend, while tested individually at high doses were either repellent or failed to induce any behavioral responses in *A. thunbergii* in laboratory assays (Njihia *et al.,* 2017). However, it is a common practice to test higher doses in field assays as experimental areas are vast, but there is no rule of by how much the laboratory-tested doses should be increased. Thus, the multiplying coefficient highly varies between studies. In our case, the lowest dose was formulated by multiplying the dose that was attractive in the laboratory by ten, but it appears that a lower multiplying coefficient should have been used. (2) Mixing of the three-component blend could have also led to antagonistic reactions (Bruce & Pickett, 2011). In previous laboratory assays, the blend components were dispensed from different filter paper dispensers, whereas they were mixed in a single rubber septum dispenser for the field assays, for simplicity of use and to evaluate a formulation that can easily be adopted by farmers.

On the other hand, Antestia bugs were attracted to the male-produced aggregation pheromone (MDT-*EEZ*) as higher numbers of the pest were recorded on pheromone-baited plants than unbaited plants. Unfortunately, the bugs did not enter delta traps® but stayed close to the lure source (pheromone baited traps). The issue of low or no catches while using attractants is not new for stink bugs. Low performance of traps associated with stink bugs is common because several species often require close range substrate vibrational cues that most traps lack (Virant-Doberlet *et al.,* 2004; Millar, 2005; Polajnar *et al.,* 2015). In addition, unlike sex pheromones, the function of which should be succeeded by contact between individuals, aggregation pheromones bring conspecifics close together, hence it is not a must for the attracted

insects to be in contact, implying that they do not have to enter traps. Trap design and color also have a significant effect on the potency of the trapping system, as black pyramid traps have been found to be more attractive to the Brown marmorated stink bug, *Halyomorpha halys* (Stål) (Leskey *et al.,* 2012) and Bagrada bug, *Bagrada hilaris* (Burmeister) (Joseph, 2014) than green, yellow, clear, white and yellow traps. Hence, future studies should investigate the effect of trap design and color (visual cues) in trapping Antestia bugs.

MDT (especially the *EEZ and EZZ* isomers) has been studied vastly and either has been found to lure several stink bug species, including *Halyomorpha halys, Acrosternum hilare* and *Thyanta* spp. (Weber *et al.,* 2014; Laumann *et al.,* 2011; Aldrich *et al.,* 2009; Khrimian *et al.,* 2002; McBrien *et al.,* 2002). In the current study, MDT-*EEZ* attracted two *Antestiopsis* species, i.e *A. thunbergii* and *A. facetoides*, which often occur in similar habitats especially in the central region of Kenya (Babin *et al.,* 2018). Therefore, it is likely that both *Antestiopsis* species produce a similar aggregation pheromone, or *A. facetoides* "eavesdrop" its counterpart pheromones to optimize its exploitation of resources. Various doses of MDT have been shown to work depending on different habitats. In the current study, the highest tested dose (5 mg) was significantly attractive but it would be interesting to investigate whether higher doses would perform better. A commercial formulation of MDT--*EEZ* (60-66 mg), with regulated release rates, to last up to two months without replenishing (Weber *et al.,* 2014) should also be evaluated for *Antestiopsis* spp management.

Though our study obtained promising results, optimization of the pheromone lure and effective trap design is required. The technology could be used in surveillance (Leskey *et al.,* 2012), to determine if the pest populations have reached the economic threshold level (Short *et al.,* 2016), or for mass trapping (Morrison *et al.,* 2017) of Antestia bugs. In addition, it could be used in "attract and kill" strategies by luring and confining the pest in a certain perimeter for spot spraying, instead of treating the entire farm with pesticides (Mfuti *et al.,* 2017).

5.5 Conclusion

The male-produced aggregation pheromone was attractive to *Antestiopsis* spp., indicating that it is a potent lure that could be useful in the pest management. However, a suitable formula that is more effective and long lasting, and a compatible trap design should be developed for the successful management of the pest in coffee plantations. More tests are required for formulating the kairomone blend and identifying a dose that is effective in the field.

CHAPTER SIX

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

6.1 General discussion

Contrary to visual and tactile cues, which are only valuable at short range, chemical stimuli are important in providing herbivores with long range signals to identify and select suitable hosts, avoid sub-optimal or non-hosts, locate mates e.t.c (Bruce *et al.,* 2005; Cook *et al.,* 2007). This is the first study to show that Antestia bugs, *A. thunbergii* are attracted to headspace volatiles from unripe coffee berries and avoid those from ripe coffee berries. Thereby confirming that olfaction plays a key role in the pest host plant selection and discrimination processes in the field. Differential responses to host odors amongst immatures and adults was an interesting finding. Nymphs were more responsive, followed by males and lastly females, possibly because whereas nymphs majorly require food to survive, adults may require additional cues, such as those for locating mates or oviposition sites. Indeed, it has been shown that heteropteran males often look for food and produce aggregation pheromones that attract females (Aldrich *et al.,* 1999).

PCA analysis separated headspace volatiles from unripe and ripe coffee berries, confirming that they are indeed distinct in quality and quantity, which would influence host choice by coffee pests. Behavioral assays with antennally-active components from unripe coffee berries identified a candidate blend used by the pest for host recognition and a repellent blend from ripe coffee berries. The repellent blend was characterized by compounds associated with ripened fragrance (Njihia *et al,* 2017), hence it is possible that *A. thunbergii* uses the compounds to distinguish them from preferred unripe coffee fruits. Since ripe berries do not support Antestia bug development (LePelley 1942; Matsuura *et al.,* 2014) and their populations drop while ripe berries are prevailing in the field (Ahmed *et al.,* 2016), it is likely that the identified compounds are also toxic to the bugs or cause antifeedant effects (Deletre *et al.,* 2016; Isman, 2006; Faccoli *et al.,* 2005), but this would require further

investigations. Identification of key host derived candidate attractants and repellents of the pest in this study opens avenues for developing semiochemical-based options for management of *A. thunbergii*, in the form of baits or repellents, respectively. In addition, the repellent odors could be used as markers in screening resistant cultivars.

Pheromones play a crucial role in pest success by mediating behaviors that promote their survival and reproduction. This study is the first to document the chemical profile of *A. thunbergii* through extensive analysis of their headspace volatiles using different adsorbents with varied affinities for specific compounds (Tholl *et al.,* 2006; Jones & Oldham, 2006) as well as their direct extraction from glands. Super Q had a high adsorbency for many compounds; charcoal filter was selective whereas solid phase microextraction fibre was highly sensitive and selective. Methyl (2,4,6) decatrienoate (MDT-*EEZ*) was identified as a *A. thunbergii* male-specific pheromone. MDT-*EEZ* has previously been reported as an aggregation pheromone for various stink bugs occurring in northern countries and has already been commercialized as a lure for surveillance and mass trapping (Weber et al., 2014; Laumann et al., 2011; Aldrich et al., 2009; Khrimian et al., 2002; McBrien et al., 2002). The implication of related species in the same or different habitats using a universal pheromone for communication in terms of reproductive isolation (Deisig *et al.,* 2014) and dispersal is worth investigating.

Dominant pheromones detected either in the headspace volatiles of disturbed bugs and metathoracic glands of adults and dorsal abdominal glands of nymphs, have potential use as alarm pheromones to disperse conspecifics. Key among them was (*E*)-4-oxo-2-hexenal, (*E*)-2-decanal, (*E*)-*2*-decenyl acetate, tridecane and dodecane. Dimorphism in pheromone reserves found among sexes suggest that amounts and ratios of common compounds may also play a role in sex differentiation, rather than insects necessarily relying on a specific sex pheromone. Indeed, over a brief period (3 h), no pheromones were detected in the headspace of females whereas considerable amounts were found in males, confirming that not only are their reservoir different but also the release rates differ amongst sexes. Hence, further studies are required to understand the role of amounts and ratios in *A. thunbergii* intraspecific interactions.

In brief field assays, the 3-component kairomone blend did not elicit significant attraction in any of the tested doses. However, a dose-response trend showed that increasing dose was associated with less Antestia bugs, suggesting that lower doses may have been effective in luring the pest, which would require further tests to ascertain. It has been shown that doses, ratios and their formulation play a crucial role in bioactivity (Webster *et al.,* 2010; Bruce & Pickett, 2011; Jaramillo et al., 2013; Njihia *et al.,* 2014). Amongst the many candidate semiochemicals found to be bioactive in a laboratory-controlled setting, only a few have been shown to work in the field due to complications of development and differences in laboratory versus field environment (Cai *et al.,* 2017). Fortunately, the male-produced aggregation pheromone was effective in luring two *Antestiopsis* species *(A. thunbergii* and *A. facetoides)*. Its cross attraction of distinct species ultimately suggest that this compound could be a universal attractant for multiple species of stink bugs. Failure of the lured bugs to enter traps could be associated with several factors including trap design and color (Leskey *et al.,* 2012), hence comparing compatibility of MDT with several trap types such as the pyramid trap® is worth investigating. It would be also important to evaluate the performance of the existing commercial MDT developed for other stink bugs (Weber *et al.,* 2014; Morrison *et al.,* 2017), in *A. thunbergii* management prior to embarking on developing a new product that is durable.

6.2 Conclusions

Through various behavioral, olfactory, chemical analysis conducted in the laboratory tests and/ field experiments, this study found that:

1. A 3-component attractant (kairomone) blend comprised of anisole, methyl 3-ethyl-4-methylpentanoate and (5*S*,7*S*)-conophthorin contributes to host recognition and preference of *Antestiopsis thunbergii* towards unripe coffee berries (susceptible host food), and a candidate for use to lure Antestia bugs.
2. A 5-component repellent (allomone) blend comprised of compounds unique to ripe coffee berries (3-hydroxy-2-butanone, 2-heptanone, 2-isopropyl-3-methoxypyrazine [IPMP], 2-isobutyl-3-methoxypyrazine [IBMP] and (*E*)-β-caryophyllene) contributes to insusceptibility of the ripe berries against

Antestiopsis thunbergii. The blend is a candidate for use in repelling Antestia bugs.

3. Methyl (2*E*,4*E*,6*Z*)-decatrienoate, is a male produced aggregation pheromone of *Antestiopsis thunbergii* capable of luring both sexes and nymphs, including *A. facetoides* in coffee plantations and a good candidate for the pest management.
4. Disturbed *Antestiopsis thunbergii* produce excessive amounts of alarm pheromones, which are also found in metathoracic glands of adults and dorsal abdominal glands of nymphs, and could be developed for use in the pest control.
5. Delta trap design is not effective in capturing lured *Antestiopsis* spp.

6.3 Recommendations

The following recommendations emerge from this study for future research

1. Candidate repellents identified in ripe coffee berries (unsuitable host food) should be evaluated in repelling *A. thunbergii* in coffee plantations.
2. Further studies should be conducted to optimize the kairomone blend derived from unripe berries to effectively lure *A. thunbergii* under field set up i.e. coffee plantations.
3. Dose response studies should be conducted with dominant compounds (alkanes and aldehydes) identified in the headspace of disturbed *A. thunbergii* and metathoracic gland (MTG) contents of adults and dorsal abdominal gland (DAG) of nymphs, as they are candidate alarm and defence pheromones with potential for use in the pest management.
4. Existing commercial lure of MTD-*EEZ* or an improved formulation that is long lasting should be tested for *A. thunbergii* management.
5. Likely synergism of candidate attractants (kairomone and aggregation pheromone) and candidate repellents (alarm and allomones) in suppressing Antestia bugs should be investigated.
6. Further field studies should be conducted in highly infested fields for longer periods of time.
7. An effective trap design that is compatible with *A. thunbergii* lure is needed.

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APPENDICES

Appendix I: Publications

Chemoecology
DOI 10.1007/s00049-017-0248-y

CHEMOECOLOGY

ORIGINAL ARTICLE

Identification of kairomones of second instar nymphs of the variegated coffee bug *Antestiopsis thunbergii* (Heteroptera: Pentatomidae)

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Abstract The variegated coffee bug *Antestiopsis thunbergii* Gmelin is a key pest of *Coffea arabica* in East Africa. Although the bug feeds on various parts of the coffee plant, it has a strong preference for mature green berries which are essential for the bug to complete its life cycle, reproduce and enhance its longevity. To locate mature green coffee berries, we hypothesized that second instar nymphs, which are the most mobile and active feeding immature stage of the bug, must rely on key volatile compounds emitted by the host for recognition. We tested this hypothesis using behavioral and electrophysiological assays and chemical analysis. In olfactometer assays, the second instar nymphs were strongly attracted to volatiles emitted from mature green berries but avoided those from ripe coffee berries of *C. arabica.* Coupled gas chromatography–electroantennographic detection (GC/EAD) isolated five antenally active components from mature green berries volatiles, four of which were identified by coupled GC–mass spectrometry as toluene, anisole, methyl 3-ethyl-4-methylpentanoate and (5*S*,7*S*)-conophthorin. In concentration assays, in which second instar nymphs did not respond to toluene, they were strongly attracted to anisole, methyl 3-ethyl-4-methylpentanoate and (5*S*,7*S*)-conophthorin, and a blend from the three compounds at concentrations lower or equivalent to the natural volatile extract. Our results suggest that the blend from these three compounds allows host recognition in second instar nymphs of the variegated coffee bug and is a candidate kairomone for monitoring the pest in coffee plantations.

Keywords *Antestiopsis orbitalis* · Antestia bugs · Coffee · *Coffea arabica* · Kairomones

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Introduction

The variegated coffee bug, *Antestiopsis thunbergii* (Heteroptera: Pentatomidae) is a hemimetabolous insect, whose development from egg to adult stages takes about three months (Ahmed et al. 2016). The bug lays eggs in batches of about twelve mainly on the lower leaf surfaces of *Coffea arabica* (Arabica coffee) but also on its berries and shoots. After hatching, the first instar nymphs remain together on egg chorions displaying little or no movement or feeding activity (Kirkpatrick 1937; Ahmed et al. 2016). By contrast, second instars of this bug are very mobile, actively feed and have the longest lifespan (≈ 25 days) (Ahmed et al. 2016). Hence, they may be considered as the most damaging of all the nymphal instars. Although both nymphs and adults attack various coffee plant parts including flowers, leaves, shoots, unripe and ripe berries, the bugs strongly prefer to feed on mature green (unripe) berries (Le Pelley 1942; Matsuura et al. 2014). Their infestation leads to flower abortion, distorted leaves, multiple branching, necrosis and premature berry fall and

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transmission of secondary pathogenic microorganisms (Le Pelley 1942; Cilas et al. 1998). Furthermore, *A. thunbergii* is suspected to introduce microorganisms in coffee beans which produce certain pyrazines making the beans unpalatable, a condition referred to as 'potato taste defect' (Jackels et al. 2014). The defect affects the coffee bean's quality, resulting in poor prices and hence lower profit margins (Jackels et al. 2014; Matsuura et al. 2014). Because of their destructive nature, a population density of 1–2 bugs/tree is considered as the economic threshold level for pest control measures in Kenya (Mugo et al. 2013). Currently, the pest is controlled using cultural methods such as pruning, and pesticide application (Nyambo et al. 1996). However, these methods are only partially effective and could harm non-target beneficial organisms; thus, alternative, gentler pest control methods are needed. One approach could be the use of semiochemicals, which can be integrated into existing management tools.

Recent studies have identified semiochemicals for key pests of coffee including the coffee berry borer, *Hypothenemus hampei* Ferrari (Jaramillo et al. 2013), and the black twig borer, *Xylosandrus compactus* Eichhoff (Egonyu and Torto 2017). The dominant semiochemicals identified were mainly alcohols (Ortiz et al. 2004; Egonyu and Torto 2017) and spiroacetals (Jaramillo et al. 2013). Subsequently, ethanol or its mixtures with methanol have successfully been explored as an attractive bait for surveillance and mass trapping of both pests (Burbano et al. 2012; Njihia et al. 2014; Egonyu and Torto 2017). In the present study, we investigated the olfactory basis of host recognition in second instar nymphs of *A. thunbergii*. Our results suggest that a three-component blend derived from the volatiles of mature green berries allows host recognition in second instar nymphs of *A. thunbergii* and is a candidate kairomone for monitoring the pest in coffee plantations.

Materials and methods

Coffee berries

Mature green berries and ripe berries of organically grown *Coffea arabica* var. Ruiru 11, a release by the Kenya Agricultural and Livestock Research Organization (KALRO), were obtained from a privately owned commercial coffee estate in Kiambu County, Central Kenya (1°11′27.15″S; 36° 49′23.03″E; altitude = 1722 m. a.s.l). Both maturity stages were distinguished using the following criteria: mature green berries; fully grown size, vitrified endosperm and green color and ripe berries; harvestable and red color (Giordanengo et al. 1993; Ortiz et al. 2004). The berries were carefully excised without hand contact from coffee tree branches in the field using a sterile scalpel blade no. 21, and placed into 500-ml sterile cylindrical glass jars covered with quick fit lids (Sigma Scientific, Gainesville, FL, USA). The berries were immediately transported to the laboratory at the International Centre of Insect Physiology and Ecology (*icipe*), Duduville Campus, Nairobi, Kenya, 13 km from the farm, for subsequent experiments.

Insects

Second instar nymphs of the variegated coffee bug, *A. thunbergii* were obtained from a culture maintained at *icipe* using a rearing method described earlier (Ahmed et al. 2016). In brief, the colony was established in 2015 on coffee berries obtained from the same plantation described above. The bugs were reared in 20 × 20 × 20 cm transparent plastic cages that were open, but sealed with white muslin cloth on two opposite sides to provide aeration. They were fed on mature green berries and young shoots, which were replenished every 3–4 days. A cotton ball moistened with distilled water was placed inside the cages to provide humidity and also a water source. Once eggs were laid by newly emerged adults, a cluster of about 70 eggs was removed from the colony and placed in separate cages with similar food substrate described above until the second instar nymphs developed. The insects were kept in an incubator set at 23 ± 1 °C, a relative humidity (RH) of 75 ± 5% and 12 h photoperiod.

Collection of volatiles

Volatiles were collected from mature green berries using a headspace sampling system similar to that previously described by Jaramillo et al. (2013). Samples of fresh berries (150 g each) were placed in 500-ml cylindrical glass jars (Sigma Scientific, Gainesville, FL, USA) and clean air blown through the sample at a flow rate of 348 ml/min using a battery-operated pump (PAS-500 Personal Air Sampler, Supelco, Bellefonte, PA, USA), which also pulled volatile organic compounds (VOCs) onto pre-cleaned charcoal filter adsorbents (5 mg, Brechbuhler, Schlierensee, Switzerland) for 24 h. Each filter was subsequently eluted with 150 µl dichloromethane for use in behavioral tests and analyses using gas chromatography–electroantennographic detection (GC/EAD). In addition, eluates from three different samples were pooled; the 450 µl sample was reduced to 30 µl under a gentle stream of nitrogen for use in GC–mass spectrometry (GC/MS). Volatile collection was also done from empty chambers under the same conditions to serve as control. Samples were stored at − 80 °C until use.

Analysis of volatiles

Coupled gas chromatography–electroantennographic detection (GC/EAD) analysis of volatiles was conducted on a Hewlett-Packard (HP) 5890 Series II gas chromatograph equipped with HP-1 column (30 m × 0.32-mm diameter × 0.25 μm film thickness (Agilent, Palo Alto, California, USA) using nitrogen as the carrier gas at 1.2 ml min^{-1} flow rate. Volatiles were analyzed in the splitless mode at an injector temperature of 280 °C and a split valve delay of 3 min. The oven temperature was held at 35 °C for 3 min, programmed at 10 °C min^{-1} to 280 °C and maintained at this temperature for 10 min. The column effluent was split 1:1 for simultaneous recording by a flame ionization detector (FID) and EAD. The antennal preparation was made by filling in two sharpened glass capillaries with ringer saline solution (Kugel 1977). One of the capillaries was used to pierce the abdomen of a second instar and attached to a reference electrode, whereas the other connected the antenna end of the nymph to a recording electrode. The antennal signal was detected through an amplifier (INR-II, Syntech, Hilversum, The Netherlands), which was acquired and processed by a data acquisition controller (IDAC-4, Syntech, Hilversum, The Netherlands) and later analyzed with a GC/EAD software (EAG 2000, Syntech) to generate FID and EAD signals on a computer. Aliquots (5 μl) of headspace sample from the mature green coffee berries and 1–3 μl of 100 ng μl^{-1} of each corresponding EAD-active synthetic standard formulated in dichloromethane were analyzed. EAD responses were considered when at least three antennal elicitations were recorded for each compound.

Identification of volatile organic compounds from mature green coffee berry volatiles was achieved by GC/MS analysis on an Agilent Technologies 7890A GC linked to a 5795C MS, equipped with MSD ChemStation E.02.00.493 and Wiley 9th/NIST 2008 MS library. The GC/MS column and the temperature program used were similar to those described above for GC/EAD analysis. Confirmation of the identity of compounds in samples was done by comparing their retention time and mass spectral fragmentation of corresponding authentic standards in the library (NIST/EPA/NIH Mass spectral Library 2005a, version V2.od). Quantification of EAD-active coffee odor components was based on peak area comparison with those of the authentic standards.

Bioassays

A glass Y-tube olfactometer was used to study responses of second instar nymphs of *Antestiopsis thunbergii* to different maturity stages of coffee berries, volatiles of mature green coffee berries, and individual synthetic standards and blends of electrophysiologically active compounds (see below). Y-tube olfactometer comprised of two 10-cm-long arms which converged into a vertical 16-cm-long main arm. It had an 80° inside angle and 2.5 cm overall diameter. The treatments (or respective controls) were placed in each of the two glass odor source chambers, each of an internal volume of 50 ml, which were connected with Teflon tubing to the two arms of the Y-tube. A pump (WOB-L pump 2522C, Monroe, Louisiana, USA) supplied charcoal-filtered and humidified air (80% RH) through each arm of the Y-tube at 100 ml min^{-1}. PVC tubing at the base of the olfactometer was connected to the vacuum source of the pump at 200 ml min^{-1} to avoid odor build up in the system.

For all the assays, second instar nymphs (9 ± 3 days old), starved for 16 h, were individually introduced with a fine camel brush at the entrance of the main arm and allowed up to 15 min to make a choice. However, nymphs that remained at the same spot after introduction into the Y-tube for 8 min or were mobile within the vertical arm of the olfactometer but did not make a choice in the allocated time (15 min) were not included in the statistical analysis. Positive responses were recorded when a nymph walked upstream and spent at least 20 s inside the left or right arm of the olfactometer. Assignment of odor source to each arm of the olfactometer was reversed in between five tests to eliminate positional bias. The total number of insects tested (N) was 40 per test. The bioassays were conducted in a dark room maintained at 24 ± 1° C and 50 ± 5% RH in an arena illuminated by a 11-W fluorescent bulb. The tests were conducted during the day between 10:00 and 16:00 h, which is the time when the bugs are active in the field (Foucart and Brion 1959). The glassware and Teflon tubes were exchanged with clean ones in between trials to avoid contamination which could lead to bias. After finishing each day, they were washed with the detergent Teepol® (Teepol® products, Kent, UK) and rinsed with acetone and then with distilled water. The glassware was baked in an oven at 100°C overnight.

The initial behavioral tests compared responses of second instar nymphs to two different maturity stages of coffee berries. The tests conducted were: (1) blank vs. blank (2) mature green berries (2 g) vs. blank, (3) ripe berries (2 g) vs. blank and (4) mature green berries (2 g) vs. ripe berries (2 g). The berries were replaced after testing every five nymphs.

In subsequent bioassays, responses of *A. thunbergii* second instar nymphs to mature green berry odors were tested at three concentrations: 3 g berries/day, 6 g berries/day and 12 g berries/day, respectively. They were tested by applying 3, 6 or 12 μl of the volatile extracts, respectively, onto 2 cm × 2 cm filter papers with a syringe. Vaporization of the solvent was allowed for 1 min

before placing the impregnated filter papers into the odor source chambers. The controls were equivalent amounts of dichloromethane applied onto filter papers of similar size. Treatments were replaced after every 30 min.

Finally, bioassays that tested responses of the nymphs to four electrophysiologically active compounds, identified from the headspace volatiles of mature green coffee berries were tested at three concentrations. The natural amounts of EAD-active components in mature green berries (i.e., 0.07 ng μl^{-1} toluene, 0.67 ng μl^{-1} anisole, 1.14 ng μl^{-1} methyl 3-ethyl-4-methylpentanoate and 0.39 ng μl^{-1} 5S, 7S-conophthorin; see Table 1) were prepared and tested against the solvent. In addition, concentration assays were conducted by testing one-tenth and tenfold the natural amounts of each compound. Treatments were dispensed by applying 10 µl of each concentration onto a 2 cm × 2 cm filter paper with a syringe and allowing the solvent to evaporate for 1 min before testing. The control was 10 µl dichloromethane. A three-component blend comprising optimal attractive doses of anisole (0.067 ng μl^{-1}), methyl 3-ethyl-4-methylpentanoate (0.0114 ng μl^{-1}) and (5*S*,7*S*)-conophthorin (0.39 ng μl^{-1}) was also tested against solvent and 6 µl aliquot (≈ 6 g/day) of headspace sample from mature green berries. This blend was tested by applying 10 µl of the appropriate concentration level of each compound on separate filter papers, which were then placed in the same odor source chamber for dispensation as a blend.

Chemicals

EAD-active synthetic standards, i.e., toluene and anisole were purchased from Sigma-Aldrich (purity ≥ 98%), while methyl 3-ethyl-4-methylpentanoate (purity 97%) and (5*S*,7*S*)-conophthorin (purity 99%) were a gift from Prof. Wittko Francke, University of Hamburg, Germany. Other standards used were dichloromethane, nonanal and (R)-(+) limonene (Sigma-Aldrich); β-cedrene and linalool oxide (Fluka Analytical) and β-ocimene (Chemika) (purity ≥ 95).

Data analysis

The number of *A. thunbergii* nymphs responding to treatments, i.e., different maturity stages of coffee berries, headspace volatiles from mature green berries or synthetic EAD-active standards compared to control (blank/solvent) were analyzed using Chi-square (χ^2) goodness-of-fit tests, assuming a distribution ratio of 1:1. Although total insects used in the tests ($N = 40$) were recorded, only respondents (n) were considered in the analysis. The data were analyzed using R version 3.1.2 software (R Core Team 2014).

Table 1 GC-MS analysis of volatile compounds emitted by mature green berries (*Coffea arabica*)

Retention time (min)	Compound	Mean concentration (ng μl^{-1}) (± SD error)
4.86	Toluene[a]	0.70 ± 0.01
9.19	Unidentified monoterpene[a]	0.99 ± 0.01
9.23	Anisole[a]	0.67 ± 0.00
11.18	3-Methylanisole[b]	0.70 ± 0.01
11.3	Limonene	1.05 ± 0.02
11.36	1,8-Cineole	1.12 ± 0.04
11.77	Methyl 3-ethyl-4-methylpentanoate[a]	1.14 ± 0.03
11.84	(5*S*,7*S*)-conophthorin[a]	0.39 ± 0.02
12.13	(*E*)- Linalool oxide	1.01 ± 0.02
12.41	(*Z*)-Linalool oxide	1.11 ± 0.04
12.52	(Z)-4,8-Dimethylnona-1,3-7 triene[b]	0.59 ± 0.01
12.59	β-Ocimene	1.17 ± 0.04
12.68	Nonanal	0.17 ± 0.03
12.72	6-Ethenyldihydro-2,2,6-trimethyl-2H-pyran-3(4H)-one[b]	0.52 ± 0.01
13.99	Naphthalene[b]	0.68 ± 0.01
17.28	α-Cedrene[b]	0.59 ± 0.00
17.39	β-Cedrene	0.58 ± 0.00
17.80	Prezizaene[b]	0.58 ± 0.00
18.06	β-Acoradiene[b]	0.61 ± 0.03

[a] EAD-active compounds

[b] Identification by mass spectral match

Results

Analysis of volatiles

In total, nineteen compounds were detected in the headspace volatiles of mature green berries (Table 1). These comprised five monoterpenes (limonene, 1,8-cineole, (*E*)-linalool oxide, (*Z*)-linalool oxide and β-ocimene), five sesquiterpenes ((*Z*)-4,8-dimethylnona-1,3-7triene, α-cedrene, β-cedrene, prezizaene and β-acoradiene), four benzenoids (toluene, anisole, 3-methylanisole and naphthalene) and one; aldehyde (nonanal), ester (methyl 3-ethyl-4-methylpentanoate), spiroacetal ((5*S*,7*S*)-conophthorin) and ketone (6-ethenyldihydro-2,2,6-trimethyl-2H-pyran-3(4H)-one). Electrophysiology analysis showed that five compounds from the headspace volatiles of mature green berries consistently stimulated the antennae of second instar nymphs (Fig. 1). These specific compounds were toluene, anisole, methyl 3-ethyl-4-methylpentanoate and (5*S*,7*S*)-conophthorin. It was not possible to identify an EAD-active monoterpene that eluted adjacent to anisole and with weak fragmentation ions at *m/z* 77, 79 and 93. Electrophysiological activity of the identified compounds was confirmed by GC/EADs with their corresponding synthetic standards (Fig. 2).

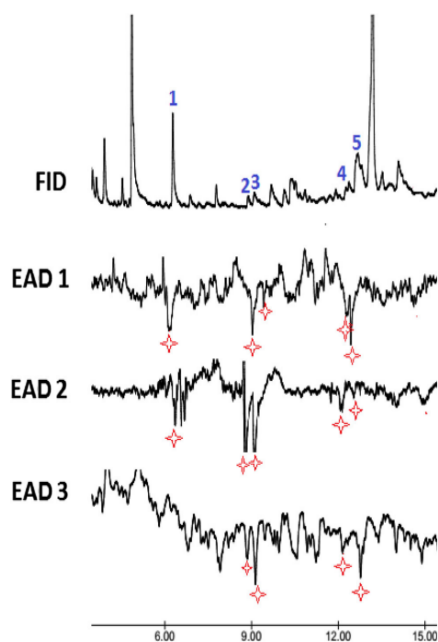

Fig. 1 Electrophysiological responses of second instar nymphs of *Antestiopsis thunbergii* to charcoal-trapped headspace volatiles from green mature coffee berries. The EAD-active compounds include: (1) toluene, (2) unidentified, (3) anisole, (4) methyl 3-ethyl-4-methylpentanoate (5) (5*S*,7*S*)-conophthorin

Bioassays

There were no significant differences when nymphs were tested against two blanks ($\chi^2 = 0.04$, $df = 1$, $P = 0.841$) showing that the olfactometer system was not biased. The response of nymphs was significantly greater to the volatile emissions of mature green berries than the blank (control) ($\chi^2 = 5.12$, $df = 1$, $P = 0.023$). On the contrary, nymphs significantly avoided volatiles from ripe berries when compared to blank ($\chi^2 = 6.82$, $df = 1$, $P = 0.009$). In a no-choice assay among the two maturity stages, about fivefold more nymphs were attracted to mature green berries than ripe berries ($\chi^2 = 15.11$, $df = 1$, $P = 0.001$; Fig. 3).

In subsequent assays, the volatile extract from mature green berries attracted approximately three times more nymphs than the solvent at all the concentration levels tested (3 g/day: $\chi^2 = 7.26$, $df = 1$, $P = 0.007$, 6 g/day: $\chi^2 = 8.53$, $df = 1$, $P = 0.003$ and 12 g/day: $\chi^2 = 6.13$, $df = 1$, $P = 0.013$; Fig. 4).

Of the four identified EAD-active compounds, only toluene did not elicit behavioral responses in nymphs (0.007 ng μl^{-1}: $\chi^2 = 0.12$, $df = 1$, $P = 0.731$; 0.07 ng μl^{-1} and 0.7 ng μl^{-1}: $\chi^2 = 0.47$, $df = 1$, $P = 0.493$; Fig. 5). Anisole strongly attracted nymphs at the lowest concentration (0.067 ng μl^{-1}: $\chi^2 = 4.56$, $P = 0.032$), while the highest concentration elicited an avoidance response (6.7 ng μl^{-1}: $\chi^2 = 4.5$, $df = 1$, $P = 0.033$). There was, however, no significant response to the intermediate (natural) concentration (0.67 ng μl^{-1}: $\chi^2 = 1.06$, $df = 1$, $P = 0.304$). Methyl 3-ethyl-4-methylpentanoate attracted three times more nymphs at the lowest concentration than the control (0.0114 ng μl^{-1}: $\chi^2 = 10.31$, $df = 1$, $P = 0.001$), whereas there were no significant responses at the intermediate and highest concentrations (0.114 and 1.14 ng μl^{-1}: $\chi^2 = 1.88$, $df = 1$, $P = 0.170$). (5*S*,7*S*)-conophthorin attracted almost threefold more nymphs at the intermediate concentration compared to control (0.39 ng μl^{-1}: $\chi^2 = 6.82$, $df = 1$, $P = 0.009$), while the lowest and highest concentrations elicited no significant responses from the nymphs (0.039 ng μl^{-1}: $\chi^2 = 0.71$, $df = 1$, $P = 0.398$ & 3.9 ng μl^{-1}: $\chi^2 = 1.06$, $df = 1$, $P = 0.304$; Fig. 5). The proportion of nymphs that selected the three-component blend of anisole, methyl 3-ethyl-4-methylpentanoate and (5*S*,7*S*)-conophthorin was approximately three times higher compared to the solvent control ($\chi^2 = 7.52$, $df = 1$, $P = 0.006$). However, there were no significant differences between proportion of nymphs that selected the three-component blend and crude headspace volatiles from

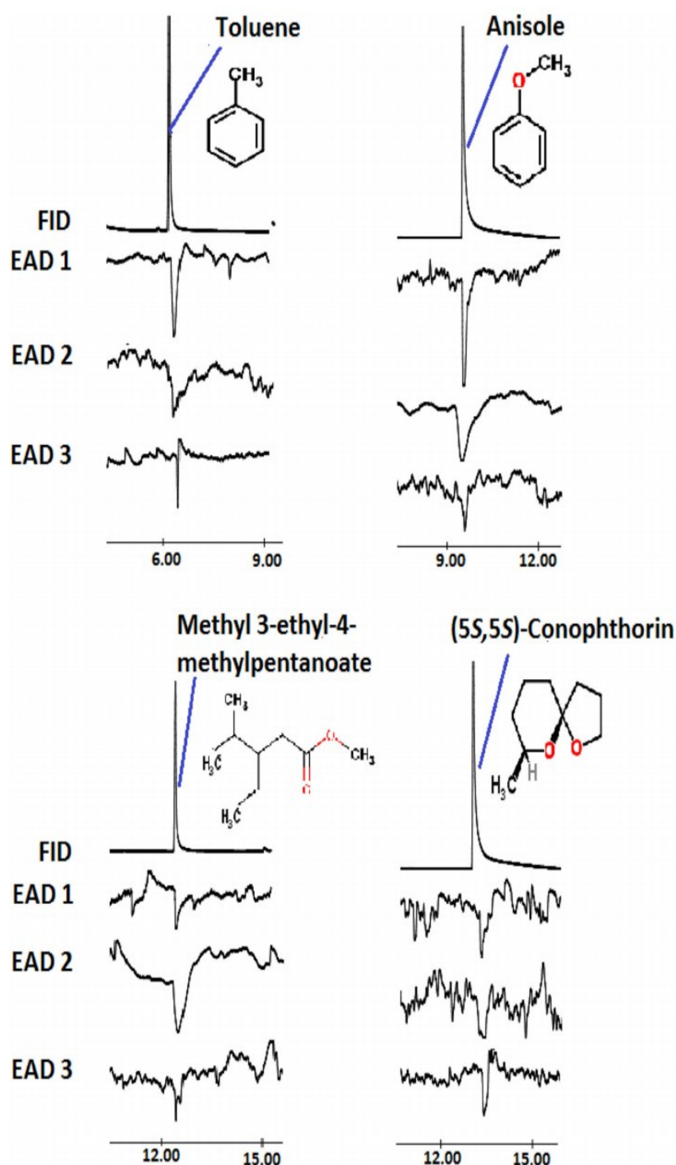

Fig. 2 GC/EAD responses of second instar nymphs of *Antestiopsis thunbergii* to synthetic standards identified from the headspace samples of mature green coffee berries

mature green berries ($\chi^2 = 0.47$, $df = 1$, $P = 0.493$; Fig. 6).

Discussion

While the literature reports that the mature green berries are the most preferred for feeding, survival and reproduction of the variegated coffee bug, *A. thunbergii* (Le Pelley 1942; Matsuura et al. 2014), no studies had elucidated the olfactory cues that mediate host recognition in this pest. Our results show that second instar nymphs of *A. thunbergii* were strongly attracted to headspace emissions of mature green berries and avoided those from ripe berries. Headspace extracts from mature green berries were also significantly attractive to the nymphs suggesting that olfaction plays a key role in host recognition. The antennae of second instar nymphs of *A. thunbergii* detected five of the components present in the volatiles emitted by mature green coffee berries four of which were identified as

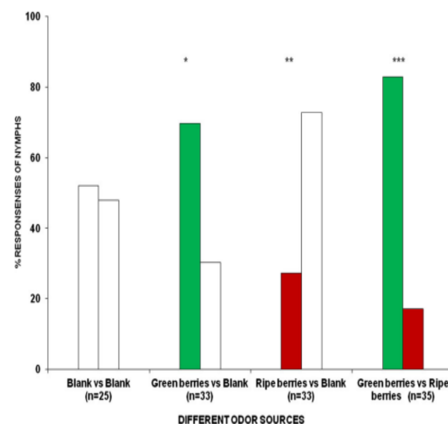

Fig. 3 Behavioral responses of second instar nymphs of *Antestiopsis thunbergii* to different maturity stages of coffee berries. Total number of insects tested was 40. '*n*' represents the number of respondents, and asterisks indicate the significance levels $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$

toluene, anisole, methyl 3-ethyl-4-methylpentanoate and (5*S*, 7*S*)-conophthorin.

Although toluene elicited no detectable attractive response in second instar nymphs, anisole, methyl 3-ethyl-4-methylpentanoate and (5*S*,7*S*)-conophthorin were attractive at concentrations lower or equivalent to their natural concentrations in the extract of the coffee berry volatiles. In addition, anisole elicited avoidance behavior in second instar nymphs at a concentration higher than its natural concentration in the volatile extract. Our results agree with those of previous studies showing that extremely high concentrations of 'attractant' compounds above their natural occurrence in hosts may result in inhibitory interactions or repellence (Webster et al. 2010; Bruce and Pickett 2011; Njihia et al. 2014). The blend of the three attractive components mimicked the attractiveness of the natural volatile extract. These results highlight the differential role played by specific compounds at different concentrations and when they are presented in a blend to explain the host recognition process in an insect. Bruce et al. (2005) proposed two hypotheses that explain host recognition and location in herbivores among a background of many VOCs in the ecosystem to involve host-derived unique VOCs and specific ratios of ubiquitous VOCs. The results of the current study support both hypotheses, as specific concentration levels of a few compounds (three) attracted second instars of *A. thunbergii*. Besides variation in host plant volatiles among individual plants of the same genotype (Bruce and Pickett 2011) and seasonal variability (Vallat and Dorn 2005), it would be interesting to investigate how the coffee bug responds to volatiles of the same plant genotype during different seasons and obtained from different geographic locations (Suinyuy et al. 2015). These would reveal potential intraspecific variation in volatile chemistry determined by these environmental factors, and whether the olfactory system of the bug has the plasticity to recognize suitable hosts producing varied odor ratios/amounts.

The four EAD-active compounds reported in the present study play a role in the host recognition in various insects. For example, the benzenoid toluene is a kairomone of the

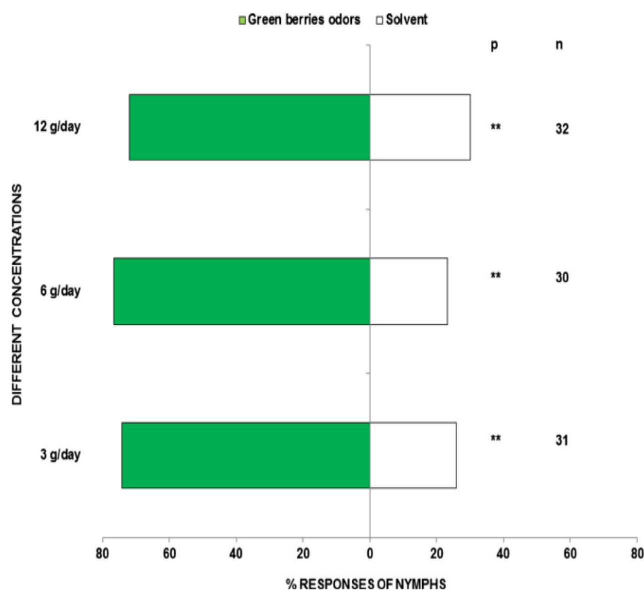

Fig. 4 Behavioral responses of second instar nymphs of *Antestiopsis thunbergii* to headspace volatiles obtained from mature green coffee berries. Total insects tested for each concentration level were 40; '*n*' is number of respondents, and asterisks indicate the significance levels $**P < 0.01$

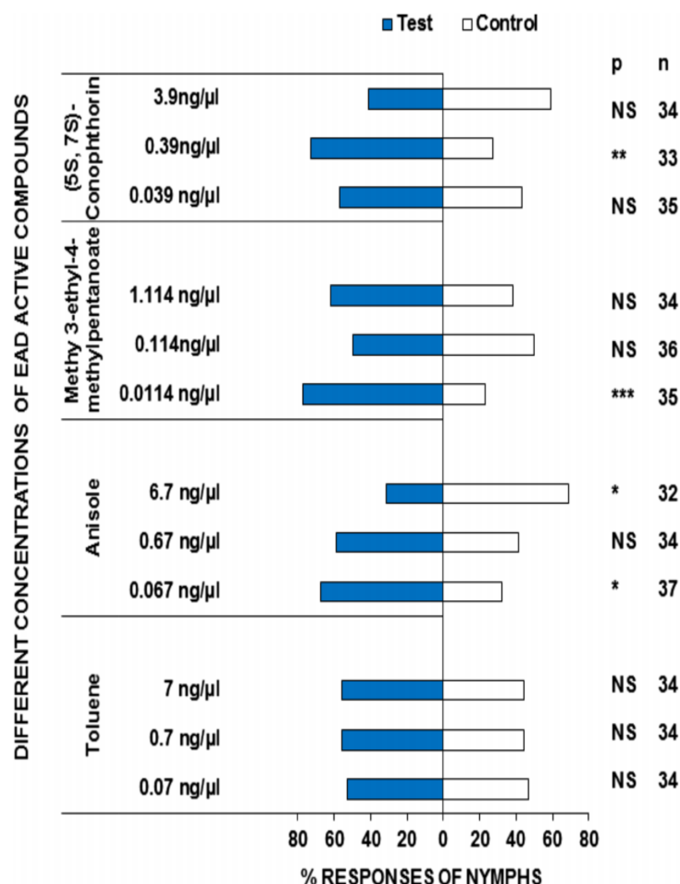

Fig. 5 Behavioural responses of second instar nymphs of *Antestiopsis thunbergii* to different concentrations of electrophysiologicaly- active compounds identified from headspace volatiles of mature green coffee berries. Total number of insects tested for each treatment level were 40. "*n*" is number of respondents and asterisks indicate the significance levels $*p < 0.05$, $**p < 0.01$ & $***p < 0.001$

olive fruit fly *Dacus oleae* Gmelin (Scarpati et al. 1993). Also, the benzenoid anisole serves as a kairomone for various scolytid species as well as a sex pheromone (Leal et al. 1996; Vrkočová et al. 2000; Ward et al. 2002). It is also a constituent of the volatiles of *C. arabica* coffee berries whose amount increases as the fruit ripens (Ortiz et al. 2004). Since *A. thunbergii* prefers to feed on mature green coffee berries and cannot fully complete its life cycle when fed only on berries at a later stage of maturation (Le Pelley 1942), our findings suggest that coffee bugs use low levels of anisole in the volatiles as an indicator of the presence of mature green berries (suitable food) and to discriminate them from ripe coffee berries (non suitable food). The ester methyl 3-ethyl-4-methylpentanoate has been reported in the volatiles released by *C. arabica* as well (Jaramillo et al. 2013); here, it elicits both attraction and repellence in the coffee berry borer, *Hypothenemus hampei* at various concentration levels (Jaramillo et al. 2013). The spiroacetal (5*S*, 7*S*)-conophthorin is a common semiochemical mediating communication in several scolytid species, as an aggregation and anti-aggregation pheromone as well as a non-host volatile constituent of some angiosperm trees (Byers et al. 1998; Huber et al. 1999; Morewood et al. 2003; Francke and Kitching 2001). Also, it is a key component in the volatiles of both ripe and unripe *C. arabica* berries, whose amounts are higher in ripe berries (Jaramillo et al. 2013). The presence of spiroacetal (5*S*, 7*S*)-conophthorin and the ester methyl 3-ethyl-4-methylpentanoate in the blend that elicited attraction in *A. thunbergii* is interesting as the two have also been reported to elicit attraction in the coffee berry borer (Jaramillo et al. 2013). Hence, future studies should explore using the two compounds as part of a bait for trapping both pests simultaneously.

In summary, we showed that host location in the variegated coffee bug is triggered by a 3-component blend of volatile organic compounds derived from mature green coffee berries. The 3-component blend is a candidate kairomone for monitoring populations of second instar nymphs of *A. thunbergii*. Since ripe coffee berries elicited

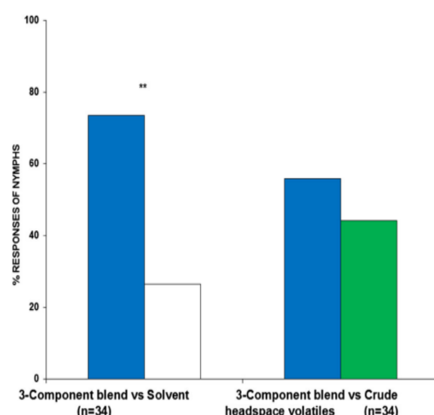

Fig. 6 Behavioral responses of second instar nymphs of *Antestiopsis thunbergii* to a 3-component blend comprising anisole, methyl 3-ethyl-4-methylpentanoate and (5*S*,7*S*)-conophthorin against solvent (control) and crude headspace volatiles from mature green berries. The total number of insects tested for every test was 40. '*n*' is the number of respondents, and asterisks indicate the significance levels $**P < 0.01$

avoidance, our future studies will investigate whether the stage produces allomones or specific ratios of certain compounds that repel the coffee bug with a goal of developing a 'push–pull' system for the pest management.

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Chemoecology
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CHEMOECOLOGY

ORIGINAL ARTICLE

Ripe coffee berry volatiles repel second instar nymphs of Antestia bugs (Heteroptera: Pentatomidae: *Antestiopsis thunbergii*)

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Abstract

Understanding the roles of volatile organic compounds (VOCs) in insect–plant interactions is a key component towards the development of safe pest management strategies and sustainable agriculture. Kairomones in unripe berries of *Coffea arabica* mediate host recognition of second instar nymphs of Antestia bugs, *Antestiopsis thunbergii* Gmelin, and are good candidates for the pest management. In the current study, we investigated the VOCs that contribute to deterring the pest from ripe berries. Behavioral assays showed that headspace volatiles collected from ripe berries were significantly avoided by second instar nymphs. A mixture of the ripe berry volatiles and unripe berries (known preferred diet) was also significantly avoided when tested against blank or unripe berries, thereby confirming that VOCs from ripe berries had altered the host recognition process and had an odor masking effect. Coupled gas chromatography/mass spectrometric (GC/MS) analysis of the headspace volatiles revealed a blend of chemicals of which ten elicited electrophysiological activity in antennae of second instar nymphs. Five of these compounds including; 3-hydroxy-2-butanone, 2-heptanone, 2-isopropyl-3-methoxypyrazine [IPMP], 2-isobutyl-3-methoxypyrazine [IBMP] and (*E*)-β-caryophyllene were identified as unique to the volatiles of ripe berries and they elicited avoidance behavior in second instar nymphs when tested singly and in a blend. In addition, their blend also inhibited responses of nymphs to a synthetic attractant blend (kairomone). Our results suggest that the blend of the five compounds can be exploited as repellents in the management of *A. thunbergii* by pushing the pest away from coffee plantations or interfering with the pest–host recognition process through masking of suitable host odors.

Keywords *Antestiopsis thunbergii* · *Coffea arabica* · Coffee berry · Variegated coffee bug · Volatiles · Repellent

Introduction

Insects identify suitable hosts using specific volatiles that are often present at specific ratios (Bruce et al. 2005; Cook et al. 2007). Repellent behaviors may be elicited if an insect perceives odors which signal unsuitable hosts (Cook et al. 2007). Repellence is exhibited in various forms such as expellency, avoidance, antifeeding and odor masking (Cook et al. 2007; Deletre et al. 2016). The elucidation of the chemo-ecological roles of various plant volatiles in insect–plant communication is important for the development of novel crop protection strategies, for example, through engineering or screening crop plants for resistance against insect pests, and exploiting semiochemicals that act as attractants or repellents of insect pests (Bruce et al. 2005).

The universal need to use environmentally friendly pest control strategies and the development of pesticide resistance has led to enhanced interest in research that exploits semiochemicals in pest management (Cook et al. 2007;

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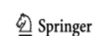

Deletre et al. 2016), as they are biodegradable, non-toxic, target specific and effective in tiny amounts. Most of the relevant research to date has focused on insect attractants (Peterson and Coats 2001). For example, exploitation of kairomones as potential attractants has been reported for most coffee pests including the Coffee berry borer *Hypothenemus hampei* Ferrari (Vega et al. 2009; Jaramillo et al. 2013; Njihia et al. 2014); the Black twig borer *Xylosandrus compactus* Eichhoff (Egonyu and Torto 2017); the Antestia bugs *Antestiopsis thunbergii* Gmelin (Njihia et al. 2017) and the Mediterranean fruit fly *Ceratitis capitata* Wiedemann (Warthen et al. 1997). However, there appears to be little information on repellents of major coffee pests except for *H. hampei* (Borbón et al. 2000; Njihia et al. 2014), for which (*Z*)-3-hexenol, 3-methylcyclohex-2-en-1-one and verbenone from green leaves are repellents (Borbón et al. 2000). Another volatile organic compound frontalin, which is emitted in higher amounts in infested ripe *C. arabica* berries, elicited avoidance in *H. hampei* females in laboratory assays and inhibited commercial attractant captures of the pest by 77% in field tests (Njihia et al. 2014). Thus, there is clearly a need to investigate the nature of other host plant-derived repellents of coffee pests. Previously, we have shown that second instar nymphs of Antestia bugs avoid volatile emissions from ripe *C. arabica* berries, which justifies the need for further studies to deduce the chemical cues involved (Njihia et al. 2017).

Antestiopsis thunbergii (Heteroptera:Pentatomidae) is a major pest of coffee in Africa (Greathead 1966; Babin et al. 2018), particularly East Africa. This oligophagous pest attacks and causes damage to both the vegetative and reproductive parts of *C. arabica* (Le Pelley 1942) but prefers to feed on unripe berries (mature green) that are crucial for their survival and reproduction. By contrast, ripe (red) berries do not support *A. thunbergii* development (Le Pelley 1942; Matsuura et al. 2014). Yield losses associated with pest damage in East Africa are estimated at 45% on average (Craves 2012). This pest could also cause coffee 'potato taste' defect in infested coffee beans, resulting in coffee of low quality and international trade value (Jackels et al. 2014; Matsuura et al. 2014).

In this study, we hypothesized that (1) ripe berries produce volatiles that repel nymphs of *A. thunbergii;* (2) these repellent volatiles can elicit avoidance and mask suitable host odors. We tested these hypotheses using behavioral and electrophysiological assays, and chemical analysis. Second instar nymphs were used because they are the first actively feeding nymphal stage of the pest, have a long lifespan, and thus are a good target for disrupting pest development and population growth (Ahmed et al. 2016; Njihia et al. 2017).

Materials and methods

Coffee berries

Ripe, red berries and unripe mature green berries of organically grown *Coffea arabica* var. Ruiru 11 were obtained from a privately owned commercial coffee plantation in Kiambu County, Central Kenya (1°11′ 27.15″S; 36°49′ 23.03″E; altitude 1722 m above sea level). The maturity index of ripe and unripe berries was based on full grown size and their characteristic red and green colors, respectively. Berries were carefully excised from coffee tree branches in the field using a sterile scalpel blade No. 21, and placed into 500-ml sterile cylindrical glass jars covered with quick fit lids (Sigma Scientific, Gainesville, FL, USA). Berry samples were transferred to a laboratory at the International Centre of Insect Physiology and Ecology (*icipe*), Duduville Campus, Nairobi, Kenya, which is 13 km from the farm within ~30 min.

Insects

Second instar Antestia bug nymphs were obtained from a colony maintained at icipe using a previously described rearing method (Ahmed et al. 2016; Njihia et al. 2017). The colony was kept in transparent plastic cages (20 × 20 × 20 cm) and fed on unripe coffee berries and young shoots that were replenished every 3–4 days. After oviposition, clusters of about 70 eggs were removed from the colony and placed in separate cages until second instar nymphs developed. The insects were maintained in incubators at 23 ± 1 °C temperature, 75 ± 5% relative humidity (RH) and 12:12 h light: dark photoperiod. Only nymphs of the first generation were used in experiments.

Olfactometer set up

A glass Y-tube olfactometer (Njihia et al. 2017) was used in dual choice tests. The Y-tube, which was positioned horizontally consisted of two 10-cm-long arms converging to a 16-cm-long main arm at 80° inside angle and 2.5 cm overall diameter. The Y-tube was placed at the center of a carton box (34 × 36 × 54 cm) which was lined inside with white printer paper, and open at the front and top side, in a dark room to minimize visual distraction. A 11 W fluorescent bulb placed on top of the box illuminated the olfactometer arena evenly. Respective treatments or controls were put in 50 ml glass chambers, placed outside the box and attached to either of the arms of the Y-tube using Teflon tubing. A pump (WOB-L pump 2522C, Monroe, Louisiana, USA) supplied charcoal-filtered and humidified

air (80% RH) through each arm of the Y-tube at 100 ml min^{-1}. PVC tubing at the base of the olfactometer was connected to the vacuum source of the pump operating at 200 ml min^{-1}.

Behavioral tests were conducted using second instar nymphs (9 ± 3 days old) that had been starved for 16 h. For every test, an individual nymph was introduced with a fine camel brush at the entrance of the long main arm, and allowed to make a choice for up to 15 min. Positive responses were recorded when a nymph walked upstream and spent at least 20 s in either arm of the olfactometer. Nymphs that did not make a choice within 15 min were recorded as non-respondents. Assignment of odor source to each arm of the olfactometer was reversed after every five tests to eliminate positional bias. A total of 50 insects were tested per treatment. The temperature and humidity in the bioassay room were maintained at 24 ± 1 °C and 50 ± 5% RH, respectively. The tests were conducted during the day between 10:00 and 16:00 h, which is the time when the bugs are active in laboratory assays (Njihia et al. 2017). Teflon tubes, Y-tubes and glass chambers were exchanged with clean ones in between trials. Thereafter, they were washed with detergent Teepol® (Teepol® products, Kent, UK), rinsed with acetone and then with distilled water and baked in an oven at 100 °C overnight.

Bioassays with ripe berry volatiles and their mixtures with berries

To investigate whether ripe berries produce repellent odors that influence the host seeking behavior of the pest, behavioral responses of second instar nymphs were evaluated in two experiments. First, responses of *A. thunbergii* second instar nymphs to headspace volatile samples derived from ripe berries (collection method presented in section below) were tested at three concentrations: 3 g berries day^{-1}, 6 g berries day^{-1}, 12 g berries day^{-1} by applying 3 µl, 6 µl and 12 µl of the volatile extracts, onto 2 cm × 2 cm filter papers, respectively. Vaporization of the solvent (dichloromethane) was allowed for 1 min before placing the impregnated filter papers into the odor source chambers. Similar amounts of the solvent were applied onto filter papers of equivalent size as a control. Second, responses of *A. thunbergii* second instar nymphs to a mixture of ripe berry volatiles and unripe berries (suitable host food) were tested against blank, unripe berries and ripe berries. During assays, a filter paper impregnated with an optimum concentration of ripe berry volatiles selected from preceding assay (6 g berries day^{-1}) was placed in the same odor source chamber with two unripe berries. The control was blank, or two berries of each maturity stage. Treatments were replaced after every 30 min in both experiments.

Collection of volatiles

Volatiles were collected from ripe berries using a headspace sampling system as previously described (Njihia et al. 2017). Fresh berry samples (150 g) were separately placed in 500-ml cylindrical glass jars (Sigma Scientific, Gainesville, FL, USA). Clean air was passed through the samples at a flow rate of 348 ml min^{-1} using a battery-operated pump (PAS-500 Personal Air Sampler, Supelco, Bellefonte, PA, USA), which also pulled volatile organic compounds (VOCs) onto pre-cleaned charcoal filter adsorbents (5 mg, Brechbuhler, Schlierensee, Switzerland) for 24 h. Each filter was subsequently eluted with 150 µl dichloromethane for use in behavioral tests and analyses using gas chromatography-electroantennographic detection (GC/EAD). In addition, eluates from three different samples were pooled; the 450 µl sample was reduced to 30 µl under a gentle stream of nitrogen for use in GC mass spectrometry (GC/MS). Samples were stored at −80 °C until use.

Electrophysiology and volatile identification

Coupled gas chromatography-electroantennographic detection (GC/EAD) analysis was conducted on a Hewlett–Packard (HP) 5890 Series II gas chromatograph equipped with a HP-1 column (30 m × 0.32-mm-diameter × 0.25-µm film thickness; Agilent, Palo Alto, California, USA), using nitrogen as the carrier gas at 1.2 ml min^{-1} flow rate. Volatiles were analyzed in the splitless mode at an injector temperature of 280 °C and a split valve delay of 3 min. The oven temperature was held at 35 °C for 3 min, programmed to increase by 10 °C min^{-1} to 280 °C and maintained at this temperature for 10 min. The column effluent was split 1:1 for simultaneous recording by a flame ionization detector (FID) and EAD. The antenna was prepared by sharpening two glass capillaries with a micropipette puller and filling them with ringer saline solution. One of the capillaries was used to pierce the abdomen of a second instar nymph and attached to a reference electrode, whereas the other connected the antenna end of the nymph to a recording electrode. The antennal signal was detected through an amplifier (INR-II, Syntech, Hilversum, The Netherlands), acquired and processed by a data acquisition controller (IDAC-4, Syntech, Hilversum, The Netherlands), and analyzed with a GC/EAD software (EAG 2000, Syntech). Analysis was conducted with headspace volatiles (5 µl) derived from ripe coffee berries and the corresponding EAD-active synthetic standards (100 ng µl^{-1}) unique to ripe berries. Three replicates were conducted for every test.

Identification of the VOCs from ripe coffee berries was achieved by gas chromatography-mass spectrometry (GC/MS) analysis on an Agilent Technologies 7890A GC linked to a 5795C MS, equipped with MSD ChemStation

E.02.00.493 and Wiley 9th/NIST 2008 MS library. The GC/MS column and the temperature program used were similar to those described above for GC/EAD analysis. Confirmation of the identity of compounds in samples was accomplished by comparing their retention time and mass spectral fragmentation of the corresponding authentic standards in the library (NIST/EPA/NIH Mass spectral Library 2005a, version V2.od). Quantification of EAD-active coffee odor components was based on peak area comparison with those of the authentic standards.

Bioassays with synthetic standards

To investigate the role of identified EAD- active compounds unique in headspace samples of ripe berries, behavioral response of *A. thunbergii* nymphs to five compounds at three concentrations was evaluated. The concentrations were formulated from the natural amounts of each compound in ripe berries and multiplied by *0.1, *1, *10 (i.e. natural amounts 23.92 ng μl^{-1} 3-hydroxy-2-butanone, 0.54 ng μl^{-1} 2-heptanone, 0.83 ng μl^{-1} 2-isopropyl-3-methoxypyrazine, 1.03 ng μl^{-1} 2-isobutyl-3-methoxypyrazine and 0.62 ng μl^{-1} (*E*)-β-caryophyllene). Concentration assays were conducted by putting10 µl of each concentration of every compound on filter papers (2 cm × 2 cm). Controls were 10 µl solvent. Consequently, a five-component blend comprising each of the above five EAD active compounds was formulated at three concentrations (*0.1, *1, *10) and tested against solvent. Blends were prepared by applying 10 µl of every compound on separate filter papers (0.5 cm × 0.5 cm) and placing them in the same odor source chamber.

In addition, the five-component blend comprising each EAD active compound at natural amounts (*1) was mixed with a three-component blend earlier derived from unripe berries and reported as a kairomone and an attractant of second instar nymphs of *A. thunbergii* (Njihia et al. 2017); the mixture of the two different blends was tested against solvent, three-component blend and five-component blend alone. The concentrations used for the three-component blend were those reported in Njihia et al. 2017 and comprised; 0.067 ng μl^{-1} anisole, 0.0114 ng μl^{-1} methyl 3-ethyl-4-methylpentanoate and 0.39 ng μl^{-1} (*5S,7S*)-conophthorin. Treatments were replaced after 30 min.

Chemicals

The synthetic standards of methyl 3-ethyl-4-methylpentanoate (purity 97%) and (*5S,7S*)-conophthorin (purity 99%), rac.-chalcogran (mixture of four stereoisomers; purity 98%) were a gift from Prof. Wittko Francke, University of Hamburg, Germany. Other standards were purchased from Sigma Aldrich Chemical Company (purity ≥ 98%).

Data analysis

The number of *A. thunbergii* nymphs responding to treatments with headspace volatiles from ripe berries, mixtures of ripe berry volatiles and unripe berries, synthetic EAD-active standards and their blends against respective controls were compared by Chi-Square (χ^2) goodness-of-fit tests, assuming equal distribution. Only responding nymphs (*n*) were considered in the analysis. The data were analyzed using R version 3.1.2 software (R Core Team, 2014).

Results

Behavioral effect of ripe berry volatiles

Headspace volatiles from ripe berries elicited significant avoidance by nymphs at all the three concentrations tested: 3 g day^{-1} ($\chi^2 = 5.49$, $df = 1$, $P = 0.019$), 6 g day^{-1} ($\chi^2 = 12.60$, $df = 1$, $P = 0.001$), and 12 g day^{-1} ($\chi^2 = 6.40$, $df = 1$, $P < 0.011$) (Fig. 1). In addition, a mixture of ripe berry volatiles and unripe berries (suitable host food) was significantly avoided by nymphs which instead preferred blank ($\chi^2 = 4.90$, $df = 1$, $P = 0.027$) and unripe berries alone ($\chi^2 = 7.05$, $df = 1$, $P = 0.008$). However, there was no significant difference in nymph behavioral response when the same mixture was compared with ripe berries ($\chi^2 = 0.02$, $df = 1$, $P = 0.876$) (Fig. 2).

Identification of ripe berry volatiles and electrophysiologically active components

Thirty-seven compounds, two unidentified, were found in the headspace volatiles of ripe berries (Supplementary Table 1). Of these ten, 3-hydroxy-2-butanone, toluene, 2-heptanone, anisole, 3-methylanisole, methyl 3-ethyl-4-methylpentanoate, (*5S,7S*)-conophthorin, 2-isopropyl-3-methoxypyrazine, 2-isobutyl-3-methoxypyrazine and (*E*)-β-caryophyllene stimulated the antennae of second instar nymphs (Fig. 3). Five of these, 3-hydroxy-2-butanone, 2-heptanone, 2-isopropyl-3-methoxypyrazine, 2-isobutyl-3-methoxypyrazine and (*E*)-β-caryophyllene were found in ripe berries only, i.e., they had not previously been detected in unripe berries (Njihia et al. 2017). Electrophysiological activity of the five later compounds that were unique to ripe berries was further confirmed by positive antennal recordings with their respective synthetic standards (Fig. 4).

Behavioral effect of synthetic standards

Nymphs significantly avoided four of the five EAG-active compounds found only in ripe berries at two of the three concentrations tested; and for 2-heptanone at all three

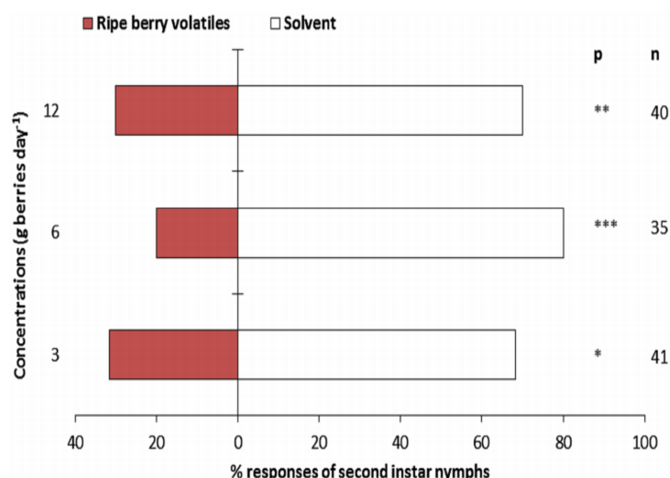

Fig. 1 Responses of *Antestiopsis thunbergii* second instar nymphs to different concentrations of headspace volatiles obtained from ripe coffee berries versus control (solvent). The total number of insects tested per pairing was 50; *n* represents number of respondents. Asterisks represent significant level; $^{*}P<0.05$, $^{**}P<0.01$ and $^{**}P<0.001$

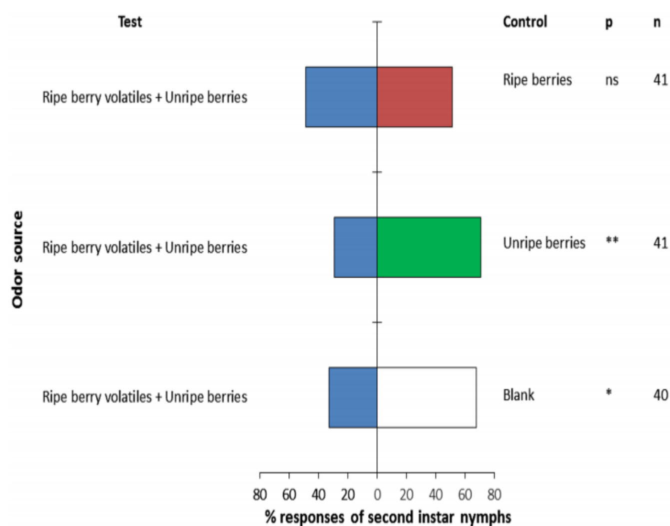

Fig. 2 Responses of *Antestiopsis thunbergii* second instar nymphs to a mixture of ripe coffee berry volatiles and unripe coffee berries (suitable host) versus a blank, unripe or ripe coffee berries. The total number of insects tested per pairing was 50; *n* represents number of respondents. Asterisks represent significant level; $^{*}P<0.05$ and $^{**}P<0.01$

concentrations (Fig. 5). The other four compounds, namely 3-hydroxy-2-butanone, 2-isopropyl-3-methoxypyrazine (IPMP), 2-isobutyl-3-methoxypyrazine (IBMP) and (*E*)-β-caryophyllene did not elicit significant behavioral responses at the lowest concentration tested (Fig. 5). A five-component blend comprising 3-hydroxy-2-butanone, 2-heptanone, 2-isopropyl-3-methoxypyrazine, 2-isobutyl-3-methoxypyrazine and (*E*)-β-caryophyllene was significantly avoided by the nymphs at all the three concentrations tested: lowest ($\chi^2=4.90$, $df=1$, $P=0.026$); intermediate/natural ($\chi^2=14.70$, $df=1$, $P=0.001$) and highest ($\chi^2=14.53$, $df=1$, $P=0.001$). Nymphs avoidance by the blend was enhanced with increasing concentration, i.e., 67% > 78% >79%, respectively (Fig. 6).

Mixing the five-component blend (intermediate concentration) with a known attractant of *A. thunbergii* nymphs (three-component blend of 0.067 ng μl^{-1} anisole, 0.0114 ng μl^{-1} methyl 3-ethyl-4-methylpentanoate and 0.39 ng μl^{-1} (*5S,7S*)-conophthorin) led to masking of the attractant. Nymphs significantly avoided the now eight-compound mixture, with a high proportion preferring solvent (73%; $\chi^2=9.80$, $df=1$, $P=0.002$) and three-component attractant blend (76%; $\chi^2=12.52$, $df=1$, $P=0.001$). However, there was no significant difference in responses of nymphs while comparison was amongst the eight-compound mixture and five-component blend ($\chi^2=0.03$, $df=1$, $P=0.872$) (Fig. 7).

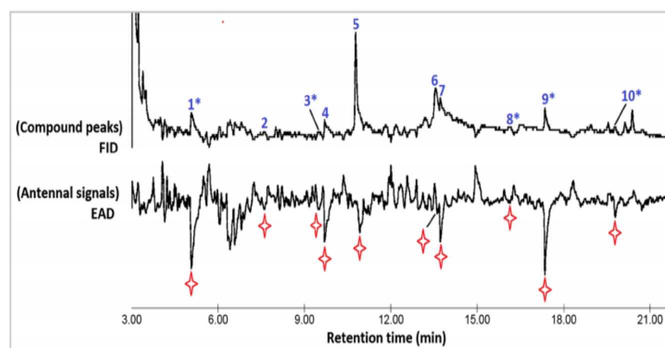

Fig. 3 GC/EAD responses of *Antestiopsis thunbergii* second instar nymphs to headspace volatiles from ripe coffee berries. The EAD-active compounds were: (1*) 3-hydroxy-2-butanone, (2) toluene, (3*) 2-heptanone, (4) anisole, (5) *3*-methylanisole, (6) methyl 3-ethyl-4-methylpentanoate, (7) (5*S*,7*S*)-conophthorin, (8*) 2-isopropyl-3-methoxypyrazine, (9*) 2-isobutyl-3-methoxypyrazine, (10*) (*E*)-β-caryophyllene. Numbers followed by asterisks represent the compounds only found in the ripe berry stage

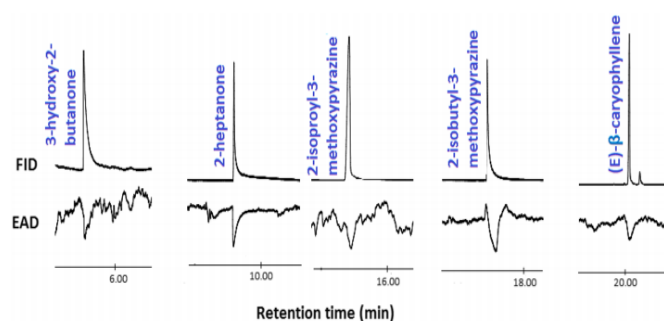

Fig. 4 GC/EAD responses of *Antestiopsis thunbergii* second instar nymphs to synthetic standards of the five EAD-active compounds that match those identified from ripe berries

Discussion

Our findings confirm that the olfactory system of the second instar nymphs of *Antestiopsis thunbergii* is adapted to locate suitable (unripe coffee berries) and avoid unsuitable (ripe coffee berries) host food. Significant avoidance was observed in second instar nymphs when the responses were compared between headspace volatiles collected from ripe berries and solvent (control). Moreover, the nymphs significantly avoided a mixture of ripe berry volatiles and unripe berries opting for blank or unripe berries alone. Thus, volatiles from the ripe berries appeared to induce repellence as well as mask those emitted from unripe berries, thereby inhibiting attraction of second instar nymphs to the mixture of volatiles. Ahmed et al. (2016) observed that *A. thunbergii* populations in the field are favored by several factors, including availability of unripe berries. From our findings, we propose that *A. thunbergii* populations are not only affected by the availability of food, but that olfactory cues also play a key role in the host-finding process. These attract or repel the bug in coffee farms with a high proportion of unripe berries and ripe berries, respectively. Provided that the same olfactory cues lead to the same behavior for *A. thunbergii* adults, this behavior can be exploited in pest control by intercropping coffee with hosts that interfere with host location by potential pests or use of synthetic repellent lures derived from these VOCs (Vallat and Dorn 2005; Cook et al. 2007).

Electrophysiological tests to screen for ecologically relevant VOCs in the headspace volatiles of ripe berries isolated ten compounds; five (toluene, anisole, 3-methylanisole, methyl 3-ethyl-4-methylpentanoate and (5*S*,7*S*)-conophthorin) are common to both unripe and ripe berries (Jaramillo et al. 2013; Njihia et al. 2017). The other five; two ketones (3-hydroxy-2-butanone and 2-heptanone), two pyrazines (2-isopropyl-3-methoxypyrazine (IPMP) and 2-isobutyl-3-methoxypyrazine (IBMP) and the sesquiterpene (*E*)-β-caryophyllene) were only detected in ripe berries. Compounds that are common to both ripe and unripe berries can contribute to the bug avoidance behavior, but mixing compounds in this context can lead to redundancy,

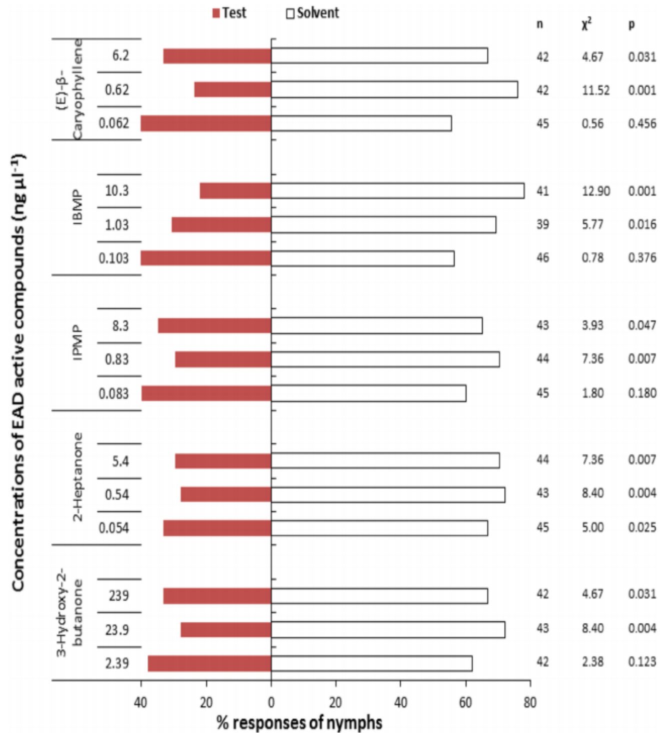

Fig. 5 Responses of *Antestiopsis thunbergii* second instar nymphs to electrophysiologically active compounds identified from headspace volatiles of ripe berries (IPMP = 2-isopropyl-3-methoxypyrazine; IBMP = 2-isobutyl-3-methoxypyrazine). Total number of insects tested per pairing was 50; n represents number of respondents

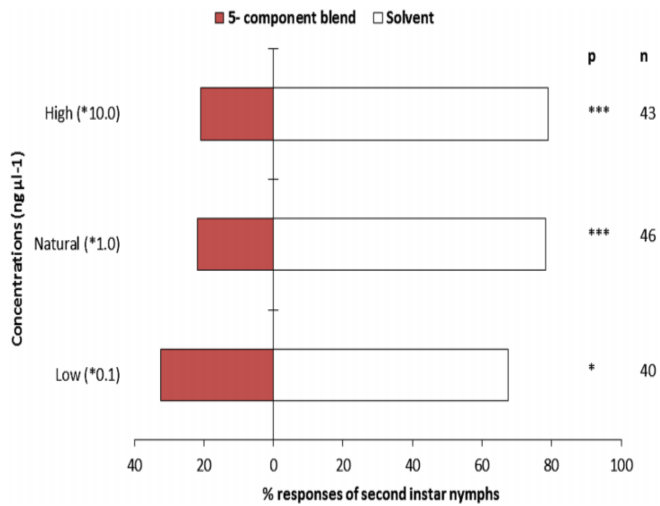

Fig. 6 Responses of *Antestiopsis thunbergii* second instar nymphs to a 5-component blend comprising; 3-hydroxy-2-butanone, 2-heptanone, 2-isopropyl-3-methoxypyrazine, 2-isobutyl-3-methoxypyrazine and (*E*)-β-caryophyllene. Total number of insects tested per pairing was 50; *n* represents number of respondents. Asterisks represent significant level; $^{*}P < 0.05$ and $^{***}P < 0.001$

synergism, and antagonistic or additive effects (Bruce and Pickett 2011). However, by focusing only on compounds found in ripe berries, potential bioactive repellents can be less complex and more economical to develop. Behavioral assays showed that each of the five compounds present in ripe berries alone as well as their blend were avoided by second instar nymphs. In addition, mixing the blend with an attractant blend of *A. thunbergii* second instar nymphs, led to avoidance of the mixture, indicating that the five-component blend had masked the suitable host odors. Thus,

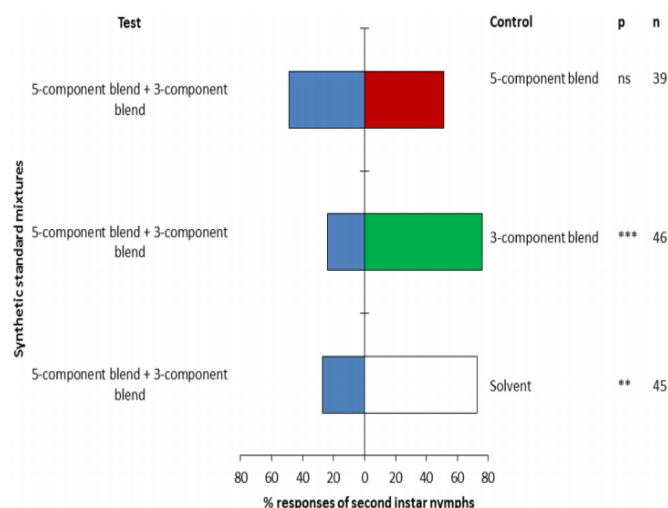

Fig. 7 Responses of *Antestiopsis thunbergii* second instar nymphs to an eight synthetic standards mixture formulated from 5-component blend comprising; 3-hydroxy-2-butanone, 2-heptanone, 2-isopropyl-3-methoxypyrazine (IPMP), 2-isobutyl-3-methoxypyrazine (IBMP) and (*E*)-β-caryophyllene and 3-component attractant blend comprising anisole, methyl 3-ethyl-4-methylpentanoate and (*5S,7S*)-conophthorin. Total number of insects tested per pairing was 50; *n* represents number of respondents. Asterisks represent significant level; $^{**}P<0.01$ and $^{***}P<0.001$

the five-component blend contributes to the pest discrimination against ripe berries as previously demonstrated in both laboratory and field tests (Le Pelley 1942; Matsuura et a. 2014; Njihia et al. 2017). Whether it is possible to formulate a blend that is less complex, but at least as effective as the five-component blend should be examined.

The five-component repellent blend has previously been reported as constituents of headspace volatiles produced by ripe berries of both *C. arabica* and *C. canephora* (Mathieu et al. 1998; Ortiz et al. 2004; Jaramillo et al. 2013; Cruz Roblero and Malo 2013; Cruz-López et al. 2016). 3-Hydroxy-2-butanone is a compound often associated with ripening and fermentation, and as a kairomone of *Drosophila melanogaster* that attacks fermenting fruits (Becher et al. 2010, 2012), unlike *A. thunbergii* that prefers to feed on unripe coffee berries. Thus, it appears that second instar nymphs of *A. thunbergii* associates this compound with ripe berries, and avoids them. 2-Heptanone, the only compound that demonstrated repellent properties at all concentrations tested, has been reported as an alarm pheromone of social insects such as bees and ants (Blum 1969), and a repellent for the granary weevil *Sitophilus granarius* (Germinara et al. 2015). This is the first report that 2-heptanone can play an allomonal role for a pentatomid species. The two pyrazines (IPMP + IBMP) have previously been reported as universal warning odors of various insects, including heteropteran species (Moore et al. 1990; Aldrich et al. 1996). Both pyrazines are also responsible for the bad taste and smell produced by coffee with “potato taste defect” but these likely occurs when their amounts are inflated above a certain threshold (Scheidig et al. 2007; Jackel et al. 2014). Jackels et al. (2014) detected IBMP in healthy and *A. thunbergii*-infested unripe *C. arabica* beans that had been ground into powder, but IPMP was detected in samples that were infested only. These and our findings suggest that IPMP could be a repellent compound for the pest that is produced constitutively in ripe berries but induced in unripe berries after Antestia bug infestation. The role of pyrazines especially, IPMP in the ecology of *A. thunbergii*, and whether *A. thunbergii* herbivory on unripe berries (suitable diet) induces production of more defense compounds against the pest (Freeman and Beattie 2008; Fürstenberg-Hägg et al. 2013) should be investigated. (*E*)-β-caryophyllene is an attractant of many insects including aphids, beetles, bugs, moths and weevils (Fujii et al. 2010). This sesquiterpene also provide both direct and indirect resistance to various crops against pests through inhibition of growth and survival of some pests and microbial pathogens (Langenheim 1994; Huang et al. 2012); and attraction of their biological control agents such as the Aphid parasite *Aphidius ervi* Haliday (Heuskin et al. 2012), and the Entomopathogenic nematode *Heterorhabditis megidis* (Degenhardt et al. 2009; Tamiru and Khan 2017). Hence, the potential role of (*E*)-β-caryophyllene in the inter-trophic interaction amongst the coffee plant, *A. thunbergii* and its natural enemies should be investigated.

In conclusion, this study has shown that ripe berries produce compounds that repel second instar nymphs of the Antestia bug *A. thunbergii*, which likely contributes to lower infestation than the unripe immaturity stage. These compounds can be exploited as candidate repellents to repel the pest or mask suitable host (unripe berries). The development of coffee varieties that produces these compounds in the early maturity stages would be beneficial.

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