UFAL
INSTITUTO DE QUÍMICA E BIOTECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO
EM QUÍMICA E BIOTECNOLOGIA

IDENTIFICATION OF CHEMICAL ATTRACTANTS FOR *STOMOXYS CALCITRANS* (DIPTERA: MUSCIDAE) CONTROL AND MONITORING PURPOSES.

NADIA STEFANIA JELVEZ SERRA

Universidade Federal de Alagoas
Campus A. C. Simões
Tabuleiro do Martins
57072-970 - Maceió-AL

NADIA STEFANIA JELVEZ SERRA

IDENTIFICATION OF CHEMICAL ATTRACTANTS FOR *STOMOXYS CALCITRANS* (DIPTERA: MUSCIDAE) CONTROL AND MONITORING PURPOSES.

Theses for the Post-graduate program in Chemistry and Biotechnology at the Federal University of Alagoas, to obtain a doctorate degree in Chemical Ecology and Biotechnology.

Supervisor: Prof. Dr. Antonio Euzebio Goulart Santana

Maceio – AL
2016

Catalogação na fonte
Universidade Federal de Alagoas
Biblioteca Central
Bibliotecária Responsável: Janaina Xisto de Barros Lima

S487n Serra, Nadia Stefania Jelvez.
Identification of chemical attractants for *stomoxys calcitrans* (dipteral: muscidae) control and monitoring purposes / Nadia Stefania Jelvez Serra. – 2016.
122 f. : il. tabs e gráfs.

Orientador: Antonio Euzebio Goulart Santana.
Tese (Doutorado em Ciências) – Universidade Federal de Alagoas. Instituto de Química e Biotecnologia. Programa de Pós-Graduação em Química e Biotecnologia. Maceió, 2016.

Bibliografia: f. 112-114.
Apêndices: f. 115-121.

1. Relação vetor-hospedeiro. 2. Vinhaça. 3. Mosca de estábulo. 4. CG-EAG. 5. CG-MS. I. Título.

CDU: 661.7:565.77

UNIVERSIDADE FEDERAL DE ALAGOAS

INSTITUTO DE QUÍMICA E BIOTECNOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM QUÍMICA E BIOTECNOLOGIA

BR 104 Km14, Campus A. C. Simões
Cidade Universitária, Tabuleiro dos Martins
57072-970, Maceió-AL, Brasil
Fone: (82) 3214-1144
Email: ppgqb.ufal@gmail.com

FOLHA DE APROVAÇÃO

Membros da comissão julgadora da defesa de tese da doutoranda **Nadia Stefania Jelvez Serra** intitulada: **"IDENTIFICATION OF CHEMICAL ATTRACTANTS FOR *STOMOXYS CALCITRANS* (DIPTERA: MUSCIDAE) CONTROL AND MONITORING PURPOSES",** apresentada ao Programa de Pós-Graduação em Química e Biotecnologia da Universidade Federal de Alagoas no dia 30 de Junho de 2016, às 09h, na na Sala de Aulas do Renorbio, na Universidade Federal de Alagoas.

COMISSÃO JULGADORA

Prof. Dr. Antônio Euzébio Goulart Santana
Orientador (PPGQB/IQB/UFAL)

Prof. Dr. José Maurício Bento
(ESALQ-USP)

Profa. Dra. Ruth Rufino do Nascimento
(PPGQB/IQB/UFAL)

Profa. Dra. Angelina Bossi Fraga
(CECA/UFAL)

Prof. Dr. Henrique Fonseca Goulart
(CECA/UFAL)

Prof. Dr. Josealdo Tonholo
(PPGQB/IQB/UFAL)

ACKNOWLEDGEMENT

I believe that with the support and belief of several individuals, I have accomplished to pursue, prepare and execute this study. Moving to another country would not have been an easy decision without the love and support that I have received.

First of all I would like to thank my supervisor Prof. Antonio Euzebio Goulart Santana for always believing in me and encouraging me to pursue my career. Much of the confidence and professional skills that I have acquired today is because of his guidance and support. I have not only had the privilege to have a great supervisor but also a life-long mentor.

I would also like to thank all my friends and colleagues: Alessandro, Daniel, Henrique, Mariana, Merybeth, Paulo and Sheila who have helped me with my project and also settling in and made me feel part of this country.

I would never have made it without the unconditional love and support from my family who always encouraged me to aim for nothing else but the stars.

A special person who shares my ambitions and future goals and has always supported and given me a shoulder to lean on, is my loving husband Elton Melo. Thank you for being my person.

And last but certainly not least; I would like to thank CAPES for the funding and the Federal University of Alagoas and Chemistry and Biotechnology post-graduate program for the opportunity to conduct my research in Brazil.

RESUMO

A *Stomoxys calcitrans* (Diptera: Muscidae), também conhecida como a mosca de estábulo, é considerada uma das principais pragas do gado em todo o mundo, causando grande prejuízo financeiro na produção de carne e produtos lácteos. Nos últimos anos, a infestação por mosca de estábulo tem sido associada com a colheita e, portanto, tornou-se importante no agroecossistema. Ao longo desse estudo foram abordados dois aspectos principais sobre a infestação por mosca de estabulo: questões relacionadas ao hospedeiro e quanto a atração desses insetos por culturas agrícolas, em particular a cultura da cana-de-açúcar. Resultados primários que envolvem a procura de hospedeiro têm demonstrado que as fêmeas *Stomoxys calcitrans* são significativamente atraídas pelos compostos orgânicos voláteis emitidos pelos hospedeiros e estimulantes de oviposição associados ao seu ambiente, incluindo estrume e infusão de grama. Estudos de eletrofisiologia confirmaram atividade de vários compostos voláteis presentes nas amostras recolhidas. A análise química das amostras eletrofisiologicamente ativas identificou uma vasta gama de compostos, incluindo, álcoois primários, fenóis, e cetonas. A maior parte dos compostos identificados não foi anteriormente associada com a mosca de estábulo, proporcionando assim, novos compostos químicos que poderiam ser utilizados como iscas para aprimorar o controle da praga. Os compostos eletrofisiologicamente ativos diferiram entre equinos e bovinos e seus respectivos estrumes, sugerindo que a atração da mosca de estábulo para estes dois substratos poderiam ser de dois perfis de estimulantes químicos diferentes. Resultados secundários que envolvem a atração e recentes surtos de moscas de estábulo em cultura de cana-de-açúcar, revelaram através de estudos comportamentais que o fertilizante de vinhaça a base de cana-de-açúcar, atrai as moscas fêmeas acasaladas e não acasaladas. Estudos de campo demonstraram que as armadilhas com iscas de vinhaça pegaram seis vezes mais moscas de estábulo do que as armadilhas sem iscas. As análises eletrofisiológica e química das amostras de compostos orgânicos voláteis que foram recolhidos, identificou uma gama de possíveis atraentes químicos responsáveis pelos surtos graves de mosca de estábulo em cultura de cana-de-açúcar.

Palavras-chave: Relação vetor-hospedeiro. Vinhaça. Mosca de estabulo. CG-EAG. CG-MS

ABSTRACT

Stomoxys calcitrans L. (Diptera: Muscidae) also known as stable flies are considered to be a major livestock pest worldwide causing great financial loss within meat and dairy production. In recent years, stable flies have been associated with crop and have therefore become of importance in the agro-ecosystem. Throughout this study two major aspects: host-seeking behavior and attraction to agricultural crop in particularly sugarcane mills were investigated. Primary results involving host-seeking behavior demonstrated that female *Stomoxys calcitrans* are significantly attracted to emitted host volatile organic compounds and oviposition stimulants associated to their environment including manure and grass infusion. Electrophysiological studies confirmed various compounds of the collected volatile samples that elicited an electrophysiological response. Chemical analyses of electrophysiological active samples identified a wide range of compounds including, aliphatic alcohols, phenols, and ketones. Most of the identified compounds have not previously been associated with stable flies thus providing novel chemical compounds that could be used as lures for trapping enhancement. Electrophysiologically active compounds differed between equines and cattle and their respective manure suggesting that the attraction of stable flies to these two hosts could be of two different chemical stimulant profiles. Secondary results involving the attraction and recent outbreaks of stable flies in sugarcane mills revealed through behavioral studies that the sugarcane based fertilizer vinasse, attracts both mated and unmated female flies. Field studies demonstrated that vinasse baited traps caught six times more stable flies than non-baited traps. Electrophysiological and chemical analyses of collected volatile organic samples identified a wide range of possible chemical attractants responsible for the severe outbreaks of stable flies in sugarcane mills.

Keywords: Host-seeking behavior. Vinasse. Stable flies. GC-EAG. GC-MS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ASPs	Antennal specific proteins
CF	Cattle feces
CV	Cattle volatile
CSPs	Chemosensory proteins
DAG	Diacylglycerol
EIAV	Equine Infectious Anemia Virus
EAG	Electroantennogram
EAD	Electroantennogram detector
FID	Flame Ionization Detector
FC	Filter cake
FC2	Filter cake after two days of fermenting
FC5	Filter cake after five days of fermenting
GC	Gas-chromatography
GC-MS	Gas chromatography coupled mass spectrometry
GC-EAG	Gas chromatography coupled electroantennogram
GI	Grass infusion
GOPBs	General odorant binding proteins
HPLC	High Performance Liquid Chromatography
HF	Horse feces
HV	Horse volatile
IP_3	Phosphatidyl mositol triphosphate
IPM	Integrated pest management
IVM	Integrated vector management
OBP	Odorant binding protein
OR	Olfactory receptor
OSN	Olfactory sensory neuron
PBPs	Pheromone binding proteins
PET	Poluethyleneterephthalate
PKC	Protein kinase
PLC	Phospholipase C
PTFE	Polytetrafluoroethylene

RI	Retention Index
SSR	Single sensillum recording
UV	Ultra violet
V	Vinasse
VEM	Vector ecology management
VOC	Volatile Organic Compound
VS	Vinasse mixed with sugarcane straw
VS2	Vinasse mixed with sugarcane straw after two days of fermenting
VS5	Vinasse mixed with sugarcane straw after five days of fermenting
WHO	World Health Organization

SUMMARY

1 INTRODUCTION

Stomoxys calcitrans L. (Diptera: Muscidae) commonly known as “stable flies” are a worldwide heamatophagous pest of livestock and other warm-blooded animals. Both male and female flies are obligate aggressive blood-feeders. Unlike other heamatophagous insects, stable flies have a saw-like proboscis responsible for its notorious painful bite. The saliva has a degree of toxicity that induces immunosuppression and contributes to stress and (BALDACCHINO et al., 2013). Sugar feeding from decaying fruits and plants is common for stable flies but in order for females to produce a viable batch of eggs, a complete blood meal is essential (SALEM et al., 2012; [a]JEANBOURQUIN, 2005). Although stable flies are habitual pests in livestock farms including dairy and feedlot it is not uncommon to find this nuisance pest in agricultural areas disrupting field workers. In warm climate countries such as Costa Rica and Brazil, stable flies have expanded from a livestock pest to also an agricultural concern, as severe outbreaks have occurred in pineapple and sugarcane plantations (SOLORZANO et al., 2015; BARROS et al., 2010). The underlying reasons for these severe outbreaks are not fully understood but it is believe that the expansion of sugar- alcohol refinery into grazing land has provided stable flies with adequate breeding environment.

This biting fly is of great importance due to the economical impact they cause. Female flies oviposit in decaying rotting organic matter including manure, consequently completing its lifecycle in close proximity to livestock. Severe biting activity causes great distress to the animal that in turn leads to weight decrease, reduced grazing time, increased heat stress, necrotic dermatitis and anemia. An estimated loss in the American cattle industry of US$ 2,2 million per year is due to stable flies alone (TAYLOR et al., 2012). In Brazil, another major meat and dairy producer estimated a potential loss of US$ 335 million due to stable flies (GRISI et al., 2014). This value is assumed to be an underestimate as the recent outbreaks in agricultural areas were not included.

In addition to being nuisance, stable flies are mechanical vectors of pathogen including bacteria, viruses, helminthes and protozoans present in the hosts’ blood (BALDACCHINO et al., 2013). Due to the induced pain upon biting, blood feeding is often interrupted through muscle twitching, tail flicking, and foot stomping. This requires finalizing its blood meal on a secondary host, which in turn

increases the probability of mechanical transmission of pathogens. Blood loss, energy loss and weight decrease caused by stable flies also decreases the animals immune response thus increasing the chances of infection.

Like many other heamatophagous dipteran, stable flies rely on its olfactory system to find its host. Host seeking is deemed to occurs in three stages: appetitive-searching, stimulation through chemical cues emitted by the host, and attraction upon host location and feeding (LEHANE et al., 1991). Exploring semiochemicals has provided a deeper understanding of breeding sites and host seeking behavior. Most studies have focused on cattle derived semiochemicals that attract stable flies (BIRKETT et al., 2004). Several compounds present in cattle urine and manure have also shown to be important cues for stable flies such as phenol, *m*-cresol, *p*-cresol and 1-octen-3-ol ([b]JEANBOURQUIN et al., 2007; TANGTRAKULWANICH et al., 2011). Dimethyl trisulphide, an oviposition stimulant for *Culex* mosquitoes has also been confirmed as an active stimulant to gravid *S.calcitrans* ([a]JEANBOURQUIN et al., 2007).

Current control methods rely upon maintenance and insecticides. Removal of potential breeding sites such as rotting hay, bedding and manure piles provide an efficient approach to control severe population outbreaks however it requires manpower and extensive working hours. Broad spectrums of insecticides are available against stable flies in the form of ear-tags, pour-ons, and sprays. Due to the increased insecticide resistance found in Stomoxys species and environmental impact caused by toxic insecticides, alternative methods are sought. The use of traps as a complementary strategy has been employed as it provides a cost-effective method. A strategy that has acquired a lot of attention in recent years is the use of chemical lures and visual stimuli to optimize trapping methods.

2 AIM AND OBJECTIVES

The aim of this thesis was to determine chemical compounds of olfactory importance to *Stomoxys calcitrans* in finding resources. In this study the main focus has been set on volatile organic compounds present in natural substrates of importance to *S.calcitrans.* The primary line of research was to further understand the attraction of *S.calcitrans* to its preferred hosts, cattle and equine and associated environmental stimulants. Secondly, various sources present in sugarcane mills will be investigated in order to identify chemical attractants that could be responsible for the recent outbreaks of *S.calcitrans* in sugarcane mills.

2.1 Specific objectives

- To determine behavioral responses of *Stomoxys calcitrans* to volatile organic compounds emitted by horses and cattle and their environmental related odorants including manure and grass infusion.

- To conduct electroantennogram analyses to document electrophysiological responses of *Stomoxys calcitrans* to horses and cattle and their environmental related odorants and use analytical methods to identify all chemical compounds.

- To determine behavioral responses of female *Stomoxys calcitrans* to volatile organic compounds found in sugarcane based fertilizers.

- To conduct field studies using sugarcane based fertilizers to enhance *Stomoxys calcitrans* catches.

- To conduct electroantennogram analyses to document electrophysiological responses of *Stomoxys calcitrans* to extracts of sugarcane based fertilizers.

- To identify all electrophysiologically active compounds and produce dose-response curve with active compounds.

REFERENCES

BALDACCHINO, F. et al. Tranmission of pathogens by *Stomoxys* flies (Diptera, Muscidae): a review. **Parasite,** v. 20, p. 26, 2013.

BARROS, A. T. M. et al. *Stomoxys calcitrans* outbreaks in pastured beef cattle in the state of Mato Grosso do Sul, Brazil. **Pesquisa Veterinaria Brasileir***a*. v. 30. P. 945–952. 2010.

BIRKETT, M.A. et al. The role of volatile semiochemicals in mediating host location and selection by nuisance and disease-transmitting cattle flies. **Medical and Veterinary Entomology,** v. 18, p. 313-322, 2004.

GRISI, L. et al. Reassessment of the potential economic impact of cattle parasites in Brazil. **Brazilian Journal of Veterinary Parasitology,** v. 23, p. 150-156, 2014.

JEANBOURQUIN, P. The role of odour perception in the sensory ecology of the stable fly, *Stomoxys calcitrans* L. 2005. 107 p. Dissertation for the Degree of Doctor of Philosophy, University of Neuchatel, 2005.

——; GUERIN, P.M. Chemostimuli implicated in selection of oviposition substrates by the stable fly *Stomoxys calcitrans.* **Medical and Veterinary Entomology,** v. 21, p. 209-216, 2007.

_____; _____. Sensory and behavioral responses of the stable fly *Stomoxys calcitrans* to rumen volatiles. **Medical and Veterinary Entomology,** v. 21, p. 217-224, 2007.

LEHANE, M. J. **The biology of blood-sucking insects**. 2nd ed. Liverpool: Cambridge University Press**.** 1991.

SALEM, A. et al. Feeding and breeding aspects of *Stomoxys calcitrans* (Diptera: Muscidae) under laboratory conditions. **Parasite,** v. 19, p. 309-317, 2012.

SOLORZANO, J. A.; GILLES, J.; BRAVO, O. et al. Biology and trapping of stable flies (Diptera : Muscidae) Developing in Pineapple Residues (Ananas comosus) in Costa Rica. **Journal of Insect Science**, v. 15, 2015.

TAYLOR, D.B.; MOON, R.D.; MARK, D.R. Economic impact of stable flies (Diptera: Muscidae) on dairy and beef cattle production. **Journal of Medical Entomology,** v. 49, p. 198-209, 2012.

TANGTRAKULWANICH, K. et al. Characterization of olfactory sensilla of *Stomoxys calcitrans* and electrophysiological responses to odorant compounds associated with hosts and oviposition media. **Medical and Veterinary Entomology,** v. 25, p. 327-336, 2011.

3 CHAPTER I: HEAMATOPHAGOUS INSECTS AND THEIR RELATION TO CHEMICAL ECOLOGY

In 1878, Manson first confirmed that pathogens could be transmitted through insects when it was found that *Culex quinquefasciatus* was able to successfully develop the filarial worm *Wucheria bancroftii.*

Today, vector-borne diseases account for more than 17% of all infectious diseases (WHO, 2016). It is estimated that there are 14 000 species of insects that feed on blood, 300-400 of them are of great importance (LEHANE, 2005). It is hypothesized that heamatophagous insects developed from a prolonged association between vertebrates and insects, benefitting them to become blood-sucking dependent. Environmental factors such as temperature and precipitation benefits the development pathogen and prolonged lifespan of vectors (HUNTER, 2003). Even though pharmaceuticals and vaccines are commercially available to some vector-borne diseases such as malaria and yellow fever both transmitted by mosquitoes, the demand for new drugs and knowledge regarding newly emerged pathogens is still great. Vector-borne diseases are particularly difficult to predict, prevent and control as one needs to take into consideration social-economical factors in addition to geographical, environmental and epidemiological factors.

3.1 Vectors of human and animal diseases

Transmission of pathogen from blood-sucking insects can occur through two routes: mechanical transmission or biological transmission. Mechanical transmission includes little involvement from the insect, this occurs through the bite from one host to another with contaminated mouthparts. Due to the small relationship between the vector and the pathogen upon transmission, most blood-sucking insects are potential mechanical transmitters. However, an important factor is time of feeding and survival span of the pathogen outside the host's body. Muscids and Tabanids cause great pain upon biting therefore inducing host defense behavior that interrupts feeding. The fly must therefore complete their blood meal on a secondary host increasing the probability of mechanical transmission (TRAVERSA et al., 2008; DESQUENES et al., 2003).

Biological transmission is when there is a dependency between the pathogen and the vector. This involves replication and development of pathogen

within the vector before transmission. *Aedes aegypti* (Diptera: Culicidae) responsible for transmission of Chikungunya, Yellow Fever, Dengue, and Zika and phlebotomine flies responsible for transmitting leishmaniasis are two main examples in which biological transmission occurs. Arboviruses are also ingested through blood meals, however, in order for the insect to get infected it must ingest an abundant virus concentration.

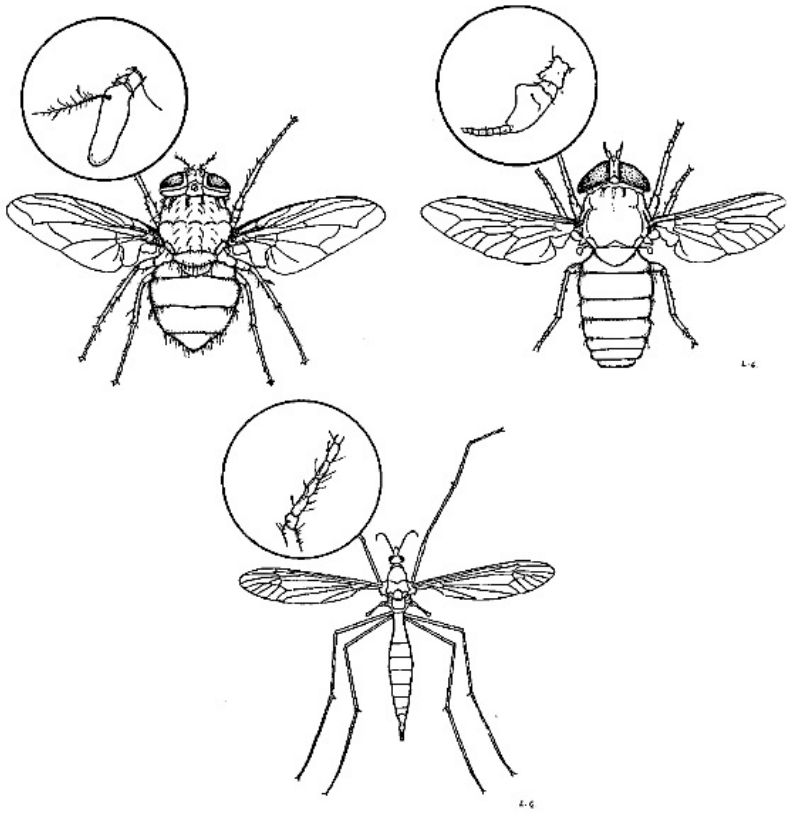
3.2 Heamatophagous insects

The classification Insecta includes 29 orders, in which five of them: Lepidoptera, Siphonaptera, Diptera, Hemiptera, and Phthiraptera include blood-sucking insects. Diptera is the order that contains the most important blood-sucking insects to man and animals causing great economical and health impacts across the world. The order Diptera can be divided into three suborders: (1) Nematocera including mosquitoes, sandflies, and blackflies; (2) Brachycera including tabanids and rhagionids; (3) Cyclorrhapha including the commonly known “flies” (Figure 1). The characteristic that distinguishes Dipteran from other insect orders is their possession of a single pair of wings. The second pair of wings also known as hindmost, that most dipteran posses are for balancing purposes. Other characteristics are the suctorial mouthparts and large developed eyes.

Culicidae is probably the most well known family that contains three subfamilies, two of which contain blood-sucking insects: Anophelinae and Culicinae. The most common genera are *Culex* and *Aedes*, which include the vectors responsible for transmitting malaria, dengue fever, zika, chikungunya, and yellow fever amongst other pathogens. *Anopheles gambiae,* the malaria-transmitting mosquito is responsible for putting 3.4 billion of people at risk of infection in 106 different countries (Center of Disease Control, 2016).

Another major disease transmitting subfamily is Phlebotominae that contain two major vector species: *Lutzomyia* and *Phlebotomus*. Phlebotomine sandflies transmit the protozoan parasite that causes leishmaniasis, which affects both humans and domesticated animals. The Word Health Organization estimated in 2016 over 20 000 deaths annually due to visceral leishmaniasis and one millions cases of cutaneous leishmaniasis reported in the last five years.

Figure 1 - Dipteran suborders. (Left) Cyclorrhaphan. (Right) Brachyceran. (Bottom) Nematoceran.



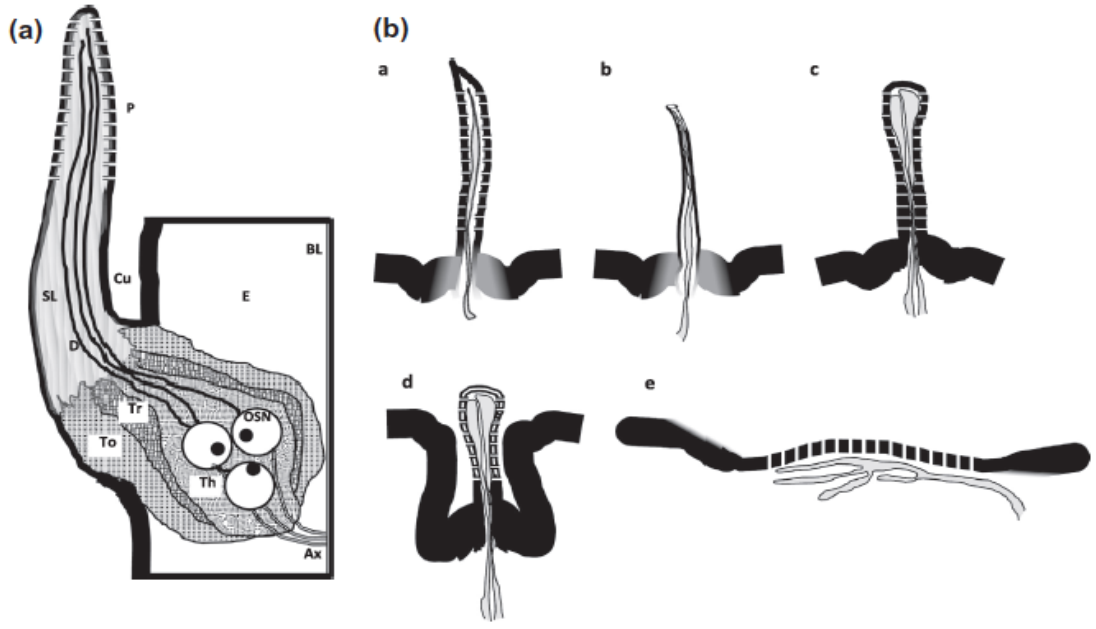
Source: LEHANE, 2012.

3.3 Insect Olfaction

Insects have a sensitive olfactory system that is of crucial importance, especially for heamatophagous insects. Understanding the sensory biology allows the development of control tools to manipulate their behavior. The main olfactory organ is the antenna but the maxillary- and labial palps, legs, genitalia and wings can also serve as odor detectors. Certain insects such as *Anopheles gambiae* (Diptera: Culicidae) and *Manduca sexta* (Lepidoptera: Sphingidae) use their maxillary palps to detect carbon dioxide. Unlike, the previously mentioned insects; the vinegar fly uses instead the palps for taste enhancement (HANSSON et al., 2011). The antenna is covered in olfactory sensillas, these can be of various shapes and size but with the same objective, to encapsulate and protect the olfactory sensory neurons (OSNs) (Figure 2). There are two main types of sensilla; single walled and double walled sensillas. The single walled sensillum includes

trichoid and basiconic forms and the double walled sensillum include coeloconic and styloconic forms. The role of this structural diversity is not fully determined however it is believed that it can be related to the odorant binding proteins (OBPs) (STEINBRECHT 1996). The olfactory sensillum also contains three cells named: thecogen, trichogen, and tormogen cells (GUIDOBALDI, et al., 2014). Trichogen and tormogen are responsible for the maintenance of an adequate ionic concentration and protein quantity in the lymph. The olfactory system is of great morphological diversity depending on species, the importance of this diversity is still undergoing research. Sexual dimorphism also plays a role in diversity of the olfactory system in certain species e.g. the female *A.gambiae* has three to four times more olfactory sensilla than the male (McIVER, 1982). It is argued that this kind of sexual dimorphism is related to the females' crucial perception of olfactory cues to locate its host (ZWIEBEL and TAKKEN, 2004). The specific role of this great diversity is still unclear.

Figure 2 - Structure of the sensillum. (a) Internal structure of olfactory sensillum – demonstrating the olfactory neurons (OSNs), its three accessory cells: thechogen (Th), trichogen (Tr) and tormogen (To), P = pores, Cu = cuticule, D = dendrites, E = epidermic cell, Ax = axon, BL = basal lamine, SL = sensillum lymph. (b) Various types of chemosensory sensilla: a = multiporous trichoid sensillum (olfactory), b = uniporous trichoid sensillum (gustatory), c = basiconical sensillum (olfactory), d = coeloconical sensillum (olfactory), and e = hair plate (olfactory).



Source: GUIDOBALDI et al., 2014.

3.4 Molecular process of insect odor perception

When an odor molecule reaches the antennae of an insect it passes through pores or slits found on the sensillum cuticle. Once inside they migrate through the sensillum lymph where odorant receptors (ORs) are found. Migration through the sensillum lymph is assisted by odor binding proteins OBPs. The exact function of OBPs is only completely determined for pheromone communication, but is believed that they play a general role of transporting the odor ligands to the receptor sites situated in the dendritic membrane of the odor sensory neurons (OSNs). In addition to carrying odor molecules they are able to transport hydrophobic ligands. In general, OBPs have been divided into four groups according to their primary protein sequences (Table 1) (FAN et al., 2014). In insects, OBPs are usually divided into three groups: pheromone- binding proteins (PBPs), general odorant binding proteins (GOPBs), and antennal specific proteins (ASPs) (ZHOU, 2010). Certain insects have two different types of proteins in the lymph; odorant binding proteins (OBPs) and chemosensory proteins (CSPs). The number of OBPs present in different species is still not fully determined but currently ranges from 1 to 51 per species, Table 2 (LEAL, 2005; XU et al., 2009). Even though the role of these two proteins is the same, the structure differs. Odor binding proteins have a pattern of six cysteine residues whilst chemosensory proteins have four cysteine residues. In addition to serving as transporters, it is believed that these carrier proteins could play different roles as they are expressed in gustatory organs and outside chemosensory tissues.

Insects have the incredible ability to distinguish between the thousands of chemicals that they encounter. The exact manner in how they manage to distinguish and respond to only certain compounds is believed to be cause of the thousands of ORs that they possess. The ORs of the *Anopheles* mosquito has almost been completely identified and has revealed that some ORs display a varying degree of selectivity and specificity whilst others respond to a broad spectrum of compounds. The number of identified odorant receptors range significantly in size e.g. from 10 in Phthiraptera (KIRKNESS et al., 2010) to approximately 200-400 in Hymenopteras (WURM et al., 2011) (Figure 3).

The odorant receptors are G proteins with seven transmembrane regions (BREER 1994). Binding to the odor receptor site initiates a cascade of events that

leads to the nervous activity. Firstly, it activates the G protein that in turn couples to a phospholipase C (PLC). The PLC then cleaves a membrane phospatidyl inositol triphosphate (IP_3) and diacylglycerol (DAG) (BRUCH 1996). As IP_3 is released in the cell it induces an increase of Ca^{2+} levels. Calcium activates a protein kinase (PKC) that phosphorylates the ion channel involved in the depolarization of the chemosensory cell (HANSSON 1995; JACQUIN-JOLY et al., 2004) (Figure 4). Once the dendrite is depolarized, the receptor potential spreads towards the nerve impulse generator on the soma of the receptor cells, generating action potentials that are sent via the axon to the brain (KELLING 2001). In order to detect subsequent stimuli, the odor molecule is removed from the receptor sites otherwise perception of change in odor concentration and quality would not be possible (STENGL et al., 1999). Deactivation of the odor occurs the oxidation of the odorant-OBP complex.

Table 1 - Shows the four groups of insect OPBs classification based on the primary protein sequences.

Classification	Cysteine residues	Other description	Example
Classical OBPs	6	3 disulfide bonds formed by 6 cysteine residues ≈14 kDa	BmorPBP (Vogt and Riddiford, 1981)
Plus-C OBPs	6 ± 2/3	1 highly conserved proline residue at least 2 conserved cysteines 17-25 kDa	AgamOBP-48 Putative Plus-C alignment GenBank[-2] accession No., ALIGN_000581 (Zhou et al, 2004a)
Minues-C OBPs	<6	-	*Drosophila* Obp99a, Obp99b and Obp99d (Hekmat-Scafe et al., 2002)
Atypical OBPs	≥6	A long C-terminus up to 38 kDa	*Anopheles* OBP35 (XU et al., 2003).

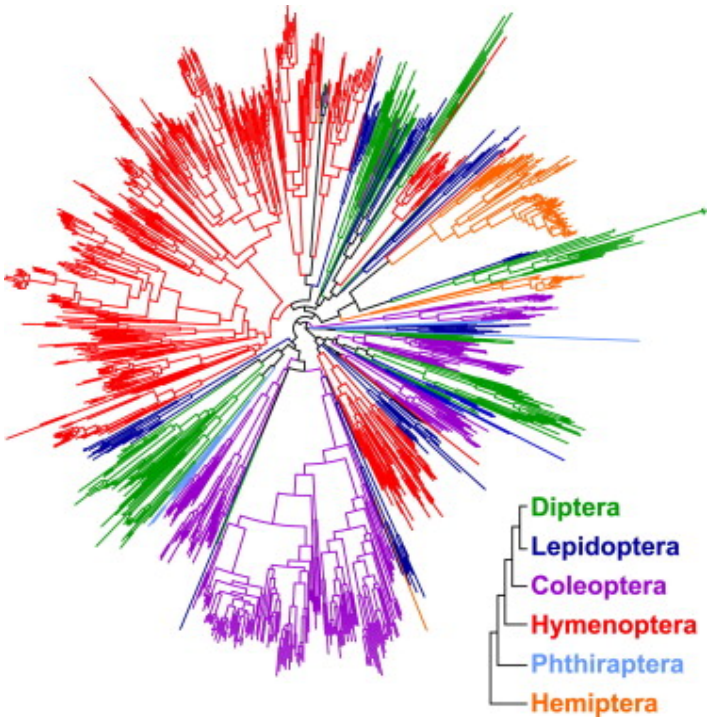
Source, FAN et al., 2011.

Table 2 – Shows the number of OBPs and CSPs from 38 and 37 insect species, respectively.

Orders	Species	OBP_number	CSP_number
Blattaria	*Periplaneta americana*	3(3)	4(3)
Coleoptera	*Diabrotica virgifera virgifera*	29(29)	8(8)
	Diaprepes abbreviatus	3(3)	1(1)
	Hypothenemus hampei	2(2)	0
	Leptinotarsa decemlineata	4(4)	2(2)
Diptera	*Anopheles funestus*	0	3(3)
	Chironomus tentans	0	1(1)
	Culicoides sonorensis	3(0)	0
	Glossina morsitans	1(1)	0
	Haematobia irritans irritans	6(6)	0
	Lutzomyia longipalpis	1(1)	1(1)
	Mayetiola destructor	1(1)	0
	Oncometopia nigricans	3(3)	3(3)
	Phlebotomus papatasi	2(2)	1(1)
	Rhynchosciara americana	1(1)	0
	Orseolia oryzae	0	0
	Sitodiplosis mosellana	1(1)	0
Hemiptera	*Acyrthosiphon pisum*	4(4)	10(4)
	Aphis gossypii	1	4(4)
	Bemisia tabaci	0	1(1)
	Diaphorina citri	1	2(2)
	Graphocephala atropunctata	2(2)	4(4)
	Homalodisca coagulata	4(4)	5(5)
	Myzus persicae	4(4)	5(3)
	Nilaparvata lugens	3(3)	9(9)
	Rhodnius prolixus	4(4)	2(2)
	Oncopeltus fasciatus	0	0
	Toxoptera citricida	2(2)	0
Hymenoptera	*Lysiphlebus testaceipes*	7(7)	4(4)
	Nasonia giraulti	10(10)	8(8)
	Nasonia vitripennis	3	2
	Solenopsis invicta	8(7)	15(14)
	Microctonus hyperodae	0	0
	Vespula squamosa	3(3)	4(4)
Lepidoptera	*Agrotis segetum*	2(1)	7(7)
	Bicyclus anynana	0	5(5)
	Danaus plexippus	1(1)	2(2)
	Heliconius melpomene	0	6(3)
	Lonomia obliqua	1(1)	5(3)
	Manduca sexta	14	12
	Heliconius erato/himera mixed	1	0
	Plutella xylostella	0	1
	Plodia interpunctella	1(1)	2(2)
	Spodoptera frugiperda	0	2(1)
	Antheraea mylitta	0	3(3)
	Helicoverpa armigera	0	0
	Heliothis virescens	0	0
	Ostrinia nubilalis	0	0
	Trichoplusia ni cabbage	2(2)	1
Orthoptera	*Gryllus bimaculatus*	3(3)	10(10)
	Laupala kohalensis	0	4(4)
	Locusta migratoria	1(1)	18(2)
Phthiraptera	*Pediculus humanus capitis*	0	0
Total	54	142(117)	177(129)

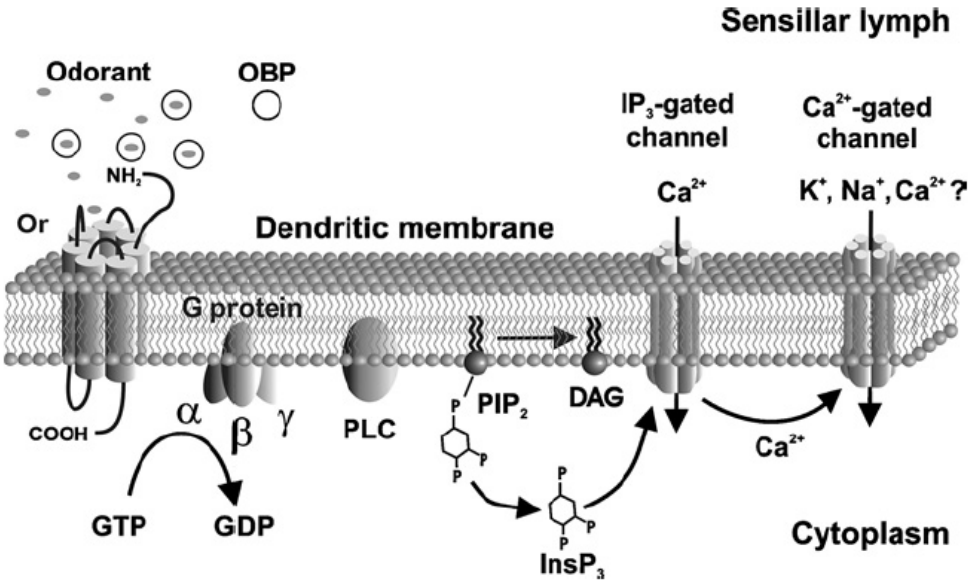
Source, XU et al., 2009.

Figure 3 – Phylogenetic relationship within the insect odorant receptor family. Phylogenetic tree represents 1069 OR genes from nine species belonging to six different orders. Illustration shows that insect ORs form a large and highly divergent gene family, with no close orthologies or subfamily structure conserved across insect orders.



Source: HANSSON et al., 2011.

Figure 4 - Hypothetical olfactory transduction cascade in insect olfactory neurons.



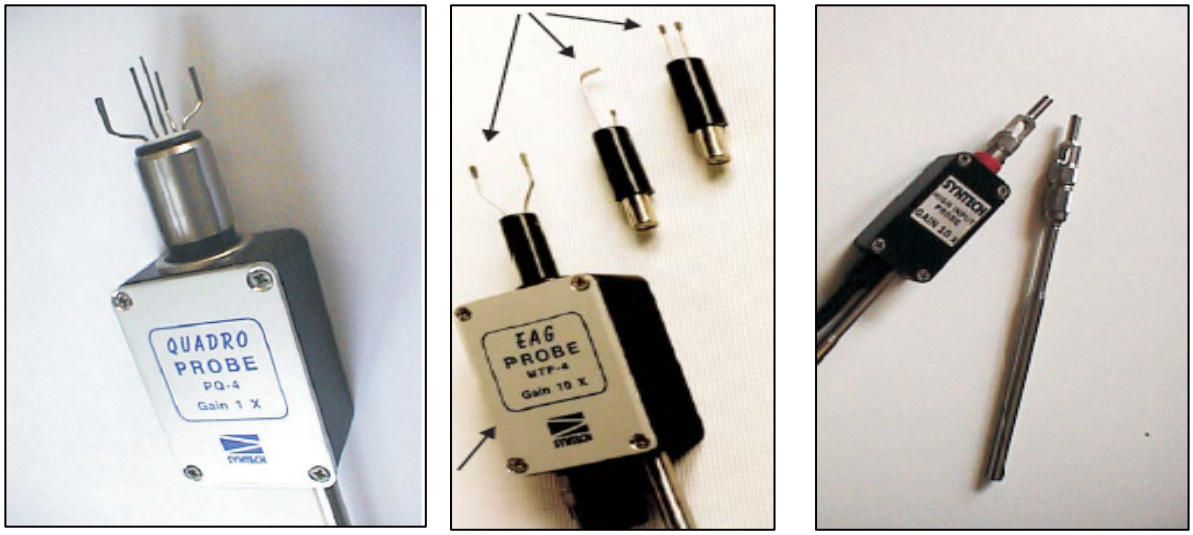
Source: JAQUIN-JOLY et al., 2004.

3.5 Electroantennography (EAG)

In 1957, Diectrich Schneider, a German biologist invented a technique called electroantennography (EAG). This is a technique that has been of invaluable importance to the understanding of chemistry of insects' odor perception (ARN et al, 1975). It manages to record potential changes between the base and the tip of the antenna upon chemical stimulation. Thanks to this technique it was possible to demonstrate that ORs are present on the antennae (CHAPMAN 1998). Toshio Nagai, 1981, got electrophysiological responses along the length of the antenna of the corn borer *Ostrinia nubilalis*, suggesting a summed potential of several ORNs lying in series. On the other hand, when electroantennogram recordings where made on the olive fly *Dacus oleae*, the responses varied according to the location of the antennal surface thus contradicting the idea of all the ORNs lying in series (CRNJAR et al., 1989). The amplitude of an electrophysiological response correlates to the concentration of the stimulus. Saturation of ORs depends on the nature of the stimulus but also physiological factors of the insect in question such as sex, species, age etc. This technique permits the monitoring of biological activity of samples obtained from natural sources. With time different probes have been developed to assist EAG recording depending on desired objective (Figure 5).

Single sensillum recording (SSR) is a technique that measures action potentials generated by OSNs within a single sensillum on the insect antenna. This is conducted by allowing an electrode to make contact with the extracellular receptor lymph. Single sensillum recording provides a quantitative response of the olfactory reception (OLSSON et al., 2013). It allows assessment of individual OSNs through separation of action potential amplitude. It is a technique commonly used to map OSN response profiles.

Figure 5 - Electroantennogram probes used to detect of volatiles perceived by antennal olfaction. (Left) Quadroprobe in which four antennae can be mounted and the signal of each antenna can be recorded individually. (Middle) Standard EAG probes for different antennae sizes. (Right) Universal AC/DC probe and indifferent holder.



Source, Synthech Research & Equipment, 2016.

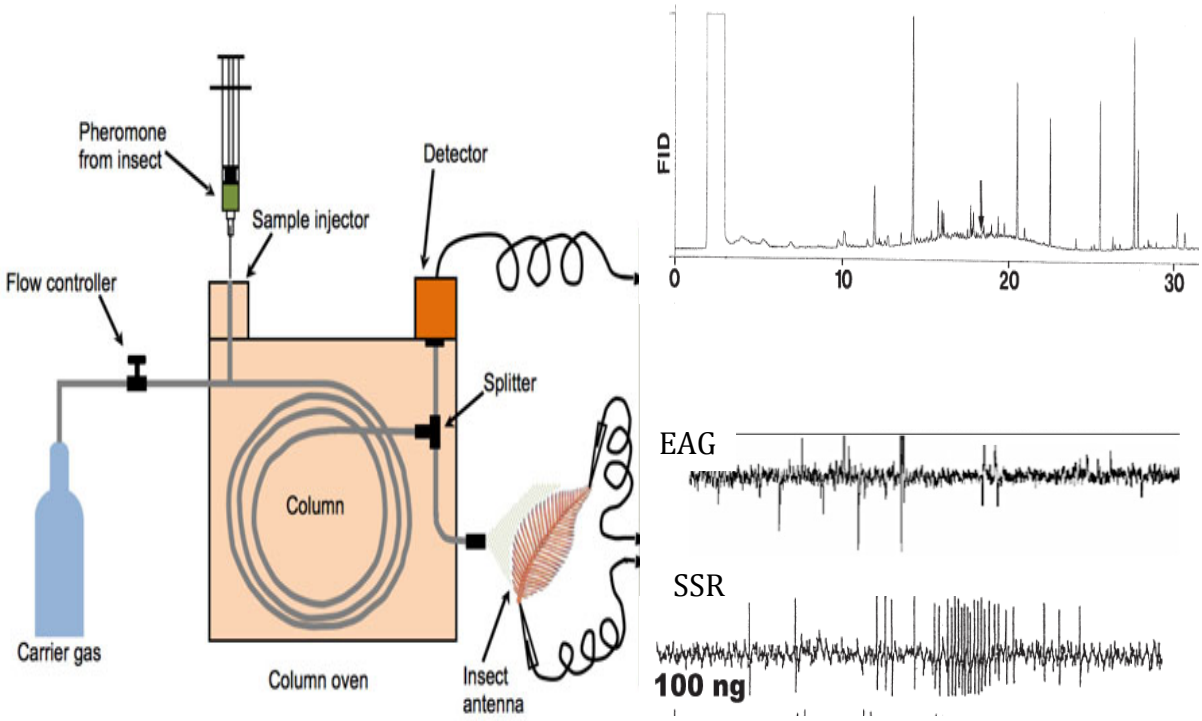
3.6 Gas chromatography (GC) and mass spectrometry (MS)

Gas chromatography (GC) and gas chromatography coupled with mass spectrometry (GC-MS) are two analytical chemistry instruments that can be used to quantify and accurately identify compounds of interest. A gas chromatograph consists of a flowing mobile phase, an injection port, a separation column containing a stationary phase, a detector and a data recording system. Compounds are analyzed through evaporation without decomposition. Organic compounds have different partitioning behavior between the mobile and stationary phase in the column. This causes each compound to elute at a different time, known as the retention time of the compound. Gas chromatography coupled mass spectrometry is a combination of chromatography apparatus which as explained separates the compounds according to their relative affinity for the stationary phase and the mass spectrometry that captures the released compounds, ionize, accelerate, and detect the ionized molecules separately. The ionized fragment and the mass-to-charge ratio (*m/z*) of each compound can be obtained.

Both electroantennogram apparatus and single cell recorder can be coupled to a gas chromatograph. Through the coupling of these instruments one can identify

and record action potentials in the antenna simultaneously whilst exposing it to natural headspace extracts (Figure 6). The effluent from the GC column is split and sent towards a flame ionization detector (FID) and the EAG or SSR.

Figure 6 - (Left) Gas chromatography coupled to electroantennogram (GC). (Top right) Chromatogram obtained through GC analyses. (Bottom) Single sensillum recording (SSR). (Middle right) Electroantennogram recording (EAG).



Source: Author, 2016 - Adapted from LEAL et al., 2001.

3.7 Semiochemicals

Semiochemicals are small organic compounds that transmit chemical messages that modify the behavior of an insect. Insect used them for both intra- and interspecies communication purposes. There are two classifications of semiochemicals: (1) Pheromones – and (2) Allelochemicals. Pheromones are compounds responsible for intraspecies communication. There are five groups of pheromones: Aggregation pheromones, alarm pheromones, sex pheromones, trail pheromones, and marking pheromones. Allelochemicals are those who are not required for metabolism and can be divided into three categories. (1) Allomones – compounds that are beneficial for the producer and not the receiver. These are

often used as chemical defense. (2) Kairomones – compounds that are beneficial for the receiver and not the producer e.g. host kairomones. (3) Synomones – compounds that are beneficial for both the receiver and the producer. Allelochemicals and pheromones are often referred to as attractants, arrestants, repellents, deterrents and stimulants in order to indicate the behavior that they cause on the insect.

Semiochemicals can provide information about breeding, location of host, and/or breeding sites, it is therefore a constantly researched area.

3.8 The use of semiochemicals

Heamatophagous insects use several semiochemicals upon host-location, location of oviposition site, mating, and aggregation. This is why research has focused on the use of semiochemicals to target specific species and life stages as a form of control and/or monitoring approach.

In the 70's, farmers began for the first time to use pheromones for monitoring insect pests in order to reduce insecticide use. Pheromones are so called "elegant" compounds to use as tools (ARN 1990). In order to substitute insecticide treatments, the use of pheromones against vectors and pests needs to be more cost-effective and efficient than current methods. In Europe there has been a successful application of a mating disruption technique against the horticultural crops, codling moth *Cydia pomonella,* and the grape berry moth *Eupoecilia ambiguella* and *Lobesiabotrana* (WITZGALL 2001).

Field trials using the oviposition pheromone of *Culex quinquefasciatus,* (5*R*, 6*S*)-6-acetoxy-5-hexadecanolide, have shown promising results especially when used in conjunction with oviposition kairomones (OLAGBEMIRO et al., 2004; MBOERA et al., 2000). Sandflies are ideal vectors to target with sex-pheromones because unlike other vectors, the sex pheromone targets the female insect and are species specific. The use of a synthetic pheromone (±) – 9-methylgermacrene-B against *Lutzomyia longipalpis* on sticky traps catches a significantly higher amount of sandflies in comparison to untreated traps (BRAY et al., 2010). For heamatophagous insects, host-derived semiochemicals play a direct role in the process of a host getting bitten and possibly infected. They would therefore be of great potential to use as a control approach, however, creating a lure that matches

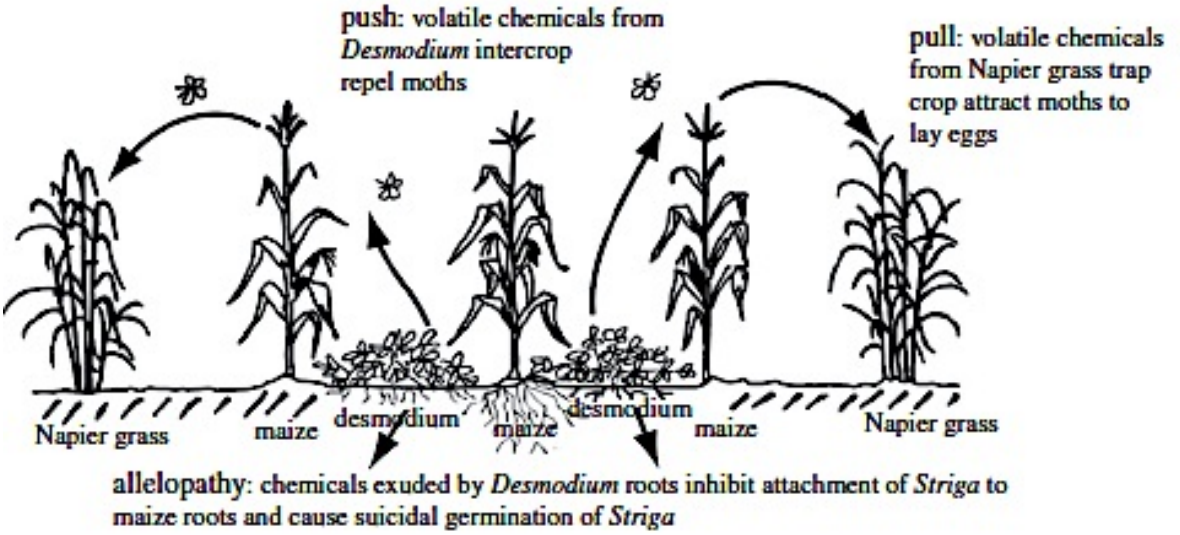
or ideally exceeds the attractiveness of a natural host is one of the greatest challenges. The World Health Organization (WHO, 2016) has generated a concept that involves "*a rational decision making process for the optimal use of resources for vector control"*, also known as Integrated Vector Management (IVM). The main objective of this concept is to prevent transmission of vector-borne diseases. Within this concept one also finds vector ecology and management (VEM). These campaigns include the use of semiochemicals against vectors in combination with pesticides under suitable management.

Integrated pest management (IPM) came from the concern of pesticides. The basic principle of IPM is to integrate various approaches and techniques to combat crop pests. This involves simple approaches such as increased sanitation, season clean up and also more technical approaches like the use of semiochemicals to target the behavior of insect pests. The use of semiochemicals instead of pesticides is a more environmental friendly approach and it spares harm on other insect species. From this strategy came the term Push-Pull. In 1987, Pyke et al., used both an attractant and a repellent to manipulate the distribution of *Helicoverpa* spp. in cotton plantations. However, Miller & Cowles in 1990, used this concept against the onion maggot *Delia antiqua*, and called it stimulo-deterrent diversion. The general strategy of the Push-Pull concept is to use semiochemicals to repel insect pests from crops (push) and attract them to other trap crops (pull) also known as habitat diversification. Good results were shown when this was implemented in order to increase grain yield in sub-Saharan Africa (KHAN et al., 2014). A repellent intercrop, desmodium, is known to emit volatiles that are unpleasant to the stemborer moth but attracts parasitic wasps. Napier grass was chosen as the trap crop as it attracts stemborers but the larva has an 80 percent mortality (Figure 7). The outcome of this approach was both economically beneficial and consistent over the years. Regardless of the sources used to push and pull, the principle of this technique is to maximize control efficiency, efficiency sustainability, and output, while decreasing negative environmental effects (COOK et al., 2007). Integrated pest management involves the use of several techniques in order achieve good results, this includes visual cues, synthetic repellent, antifeedants, alarm pheromones, aggregation pheromones etc.

Push-Pull can also be applied for vector management. *Heamatobia irritans* a blood sucking Muscidae also known as horn fly is a major cattle pest causing great economical losses. Studies showed that by introducing fly-resistant heifers into a highly susceptible herd decreased the fly load significantly (JENSEN et al., 2004). Other vectors of medicinal importance such as mosquitoes and biting midges are good candidates in which the push-pull strategy could be implemented, as this would reduce the use of insecticide in close proximity to humans. The use of natural repellents (push) and host-derived kairomones (pull) are potential tools for vector management. A recent study on the malaria mosquito tested a kairomone baited trap (pull) and a microencapsulated repellent (push) in houses of malaria-endemic areas. It was simulated that implementing such Push-Pull strategy would result in a 20-fold reduction in the entomological rate (MENGER et al., 2015). The number of studies using the Push-Pull technique as vector control is more limited than for pest control but this is a growing interest under constant research.

The Push-Pull strategy has several advantages in comparison to conventional control strategies. It is a targeted species-specific approach that is environmental friendly and long-term cost-effective. There are limitations to this strategy as there are to all control approaches. Broad knowledge of the behavior and chemical ecology of the host-plant/ vector-host is required which is not always available. Also the cost of semiochemical registration and production can be quite high but is still a promising and powerful strategy that needs to be further explored.

Figure 7 - Push-Pull strategy using trap crop. (Top) Schematic demonstration of -Pull strategy in maize crop. (Bottom) Push-Pull strategy used in maize crop in Suba district, Ethiopia. Maize (pull) and *Desmodium unicatum* push) at 1:1.



Source: (Tope picture) KHAN et al., 2014; (bottom picture) HASSALANI et al., 2008..

REFERENCES

ARN, H.,; STÄDLER, E.; RAUSCHER, S. The electroannographic detector – a selective and sensitive tool in the gas chromatographic analysis of insect pheromones. **Naturforsch,** v. 30, p. 722-725, 1975.

ARN, H. Pheromones: prophecies, economics, and the ground swell. In: _____. **Behavior modifying chemicals for insect management,** New York, 1990. p. 717-722.

BRAY, D. P et al. Synthetic sex pheromone attracts the leishmaniasis vector *Lutzomyia longipalpis* to experimental chicken sheds treated with insecticides. **Parasite & Vectors,** v. 3, p. 16. 2010.

BREER, H. Odor recognition and second messenger signaling in olfactory receptor neurons. **Cell Biology,** v. 5, p. 25-32, 1994.

BRUCH, R. C. Phosphoinositide second messenger in olfaction. **Comparative Biochemistry and Physiology,** v. 113, p. 451-459,1996.

CHAPMAN, R. F. **The insects, structure and function**. 5th ed. Cambridge: Cambridge University Press, 1998.

CENTER FOR DISEASE CONTROL AND PREVENTION. Available at: <http://www.cdc.gov/malaria/about/index.html>. Accessed on the: 31st of May 2016.

COOK, S. M.; KHAN, Z. R.; PICKETT, J. A. The use of Push-Pull strategies in Integrated Pest Management. **The Annual Review of Entomology,** v. 52, p. 375-400, 2007.

CRNJAR, R. et al. Morphology and EAG mapping of antennal olfactory receptors in *Dacus olea.* **Entomologia Experimentalis et Applicata,** v. 51, p. 77-85, 1989.

DESQUESNES, M.; DIA, M. L. *Trypanosomiasis vivax:* mechanical transmission in cattle by one of the most common African tabanids, *Atylotus agrestis.* **Experimental Parasitology**, v. 103, p. 35-43, 2003.

FAN, J. et al. An overview of odorant-binding protein functions in insects peripheral olfactory reception. **Genetic and Molecular Research,** v. 10, p. 3056-3069, 2011.

GUIDOBALDI, F.; MAY-CONCHA, I. J.; GUERENSTEIN, P. G. Morphology and physiology of the olfactory system of blood-feeding insects. **Journal of Physiology**, Paris, v. 108, p. 96-111, 2014.

HANSSON, B. A. Olfaction in lepidoptera. **Experientia,** v. 51, p. 1003-1027, 1995.

_____; STENSMYR, M. C. Evolution of insect olfaction. **Neuron,** v. 72, p. 698-711, 2011.

HUNTER, P. R. Climate change and waterborne and vector-borne disease. **Journal of applied microbiology,** v. 94, p. 37-46, 2003.

JACQUIN-JOLY, E.; MERLIN, C. Insect olfactory receptors: contributions of molecular biology to chemical ecology. **Journal of Chemical Ecology,** v. 30, p. 2359-2397, 2004.

JENSEN, K-.M. V. et al. Variation in the load of the horn fly, *Haematobia irritans,* in cattle herds is determined by the presence or absence of individual heifers. **Medical and Veterinary Entomology,** v. 18, p. 275-280, 2004.

KELLING-JOHANNES, F. Olfaction in houseflies: morphology and electrophysiology. Dissertation for the Degree of Doctor of Philosophy, University of Groningen. 2001.

KIRKNESS, E. F. et al. Genome sequences of the human body louse and its primary endosymbiont provide insights into the permanent parasitic lifestyle. **Pnas,** v. 107, p. 12168-12173, 2010.

KHAN, Z. R. et al. Achieving food security for one million sub-Saharan African poor through push-pull innovation by 2010. **Philosophical transaction of the Royal Society B,** v. 369, p. 1-12, 2014.

LEAL, W. S. Pheromone Reception. **Topics in Current Chemistry,** v. 240, p. 1-36, 2005.

LEHANE, M. J. The biology of blood-sucking in insects. Cambridge, **Cambridge University Press.** 2nd ed. 2005.

MENGER, D.J. et al. Field evaluation of a Push-Pull system to reduce malaria transmission. **Plos One,** v 14, p. 1-20, 2015.

MILLER, J. R.; COWLES, R. S. Stimulo-deterrent diversion: a concept and its possible application to onion maggot control. **Journal of Chemical Ecology,** v. 16, p. 3197-212, 1990.

MBOERA, L. E. G. et al. Oviposition and behavioral responses of *Culex quinquefasciatus* to skatole and synthetic oviposition pheromone in Tanzania. **Journal of Chemical Ecology,** v. 26, 2000.

NAGAI, T. Electroantennogram response gradient on the antenna of the European corn borer, *Ostrinia nubilalis.* **Journal of Insect Physiology,** v. 27, p. 889-894, 1981.

OLAGBEMIRO, T. O. et al. Laboratory and field responses of the mosquito, *Culex quinquefasciatus,* to plant derived *Culex* spp. Oviposition pheromone and the oviposition cue skatole. **Journal of Chemical Ecology,** v. 30, 2004.

OLSSON, S. B.; HANSSON, B.S. Electroantennogram and single sensillum recording in insect antenna. **Methods in Molecular Biology,** v. 1068, 2013.

PYKE, B. et al. The Push-Pull strategy – behavioral control of the *Heliothis* Australian Cotton Grower. 1987.

STEINBRECHT, R. A. Structure and function of insect olfactory sensilla. **Ciba Foundation Symposium,** v. 200, p. 158-74, 1996.

TRAVERSA, D. et al. Identification of intermediate hosts of Habronema microstoma and Habronema muscae under field conditions. **Medical and Veterinary Entomology,** v. 22, p. 283-287, 2008.

WITZGALL, P. Pheromones – future techniques for insect control? **IOBC-WPRC Bulletin,** v. 24, p. 114-122, 2001.

WORLD HEALTH ORGANIZATION. Available at: <http://www.who.int/mediacentre/factsheets/fs387/en/>. Accessed on the: 31 of May. 2016.

WURM, Y. et al. The genome of the fire ant *Solenopsis invicta.* **Pnas, v.** 108, p. 5679-5684, 2011.

XU, Y-L. et al. Large scale identification of odorant-binding proteins and chemosensory proteins from expressed sequence tags in insects. **BMG Genomics,** v. 10, 2009.

ZHOU, J-J. Odorant binding proteins in insects. **Vitamins & Hormones,** v. 83, p. 241-272, 2010.

4 CHAPTER II: *STOMOXYS CALCITRANS* L. (DIPTERA: MUSCIDAE) A SMALL LITERATURE REVIEW

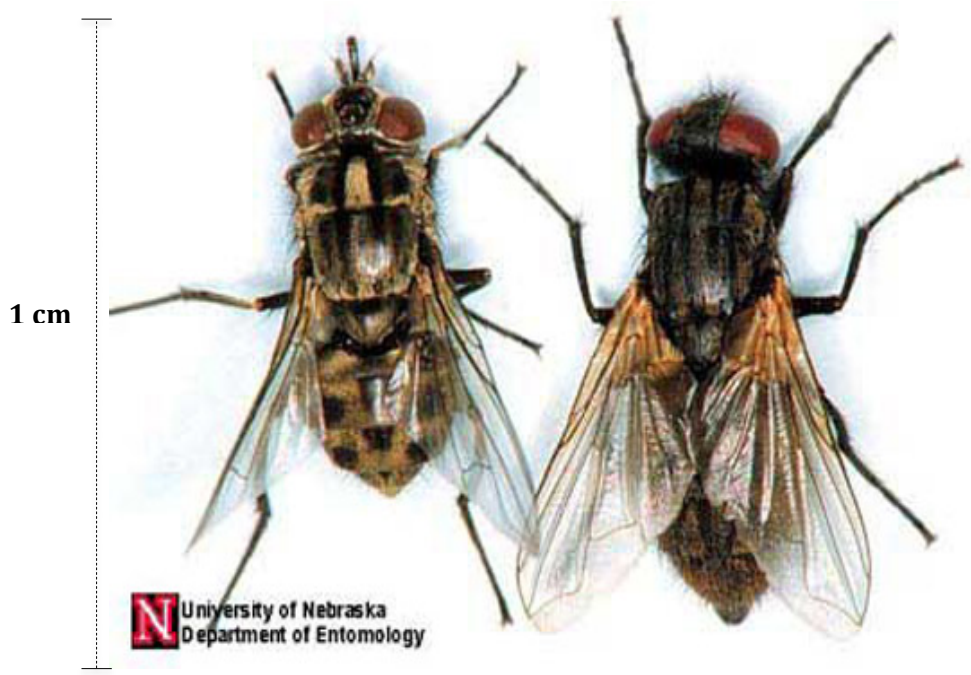
Stomoxys calcitrans L. (Diptera: Muscidae), also known as “stable fly”, “biting house fly”, “wild fly”, “dog fly”, “straw fly” and “stock fly” are classified and nomenclature as follows:

Phylum: Arthropoda
Subphylum: Mandibulata
Class: Insecta
Subclass: Pterygota
Order: Diptera
Suborder: Cyclorrhapha
Division: Schizophora
Section: Calypterate
Family: Muscidae
Subfamily: Stomoxyinae
Tribe: Stomoxini
Genus: *Stomoxys* Geoffroy 1762
Species: *calcitrans* Linnaeus 1758

4.1 Morphology

The morphology of the stable fly is similar looking to the common house fly (*Musca Domestica*) but with a broader and rounder abdomen. *Stomoxys calcitrans* are approximately 6-8 mm in length with a gray colored body and have four characteristic stripes on the thorax and dark spots on the back of the abdomen (Figure 1). There is a sexual dimorphism between males and females; females are slightly bigger and have a larger gap between its two compound eyes. The proboscis is another characteristic that identifies stable flies; it is long, thin and protrudes in front of the head. Unlike other biting insects, the labellum of the stable fly proboscis has a row of teeth hence its “saw-like” appearance. The eggs are white/yellowish with approximately one mm length (Figure 2 a). Larvae range between five to twelve mm in length depending on the instar (Figure 2 b). When mature, the larvae resemble a white yellowish maggot with a cylindrical shape. The pupae are dark brown/reddish and approximately four – seven mm long (Figure 2 c).

Figure 1 - (Left) Female stable fly, *Stomoxys calcitrans*, with its characteristic protruding proboscis and spotted abdomen. (Right) House fly, *Musca domestica.*



Source: University of Nebraska, Department of Entomology, 2015.

Figure 2 – a – c: (a) morphology of *S.calcitrans* eggs. (b) morphology of *S.calcitrans* larvae. (c) morphology of *S.calcitrans* pupae.

Source: Buss, 2012

4.2 Life cycle

Stable flies are holometabolous thus its complete life cycle includes four stages. Females can lay about 90 eggs in four – five different places (≈ 600 eggs in its lifetime) but each clutch requires a full blood meal. Temperature is a crucial factor

in its lifetime span. In higher temperatures the eggs can hatch after only 20 hours while in cooler temperatures it can take up to four days. Larval stages can range from 11 – 30 days depending on environmental suitability and availability of food. The length of pupating also depends on food abundance and environmental qualities. Once completed the adult digs its way out of the soil. The adult fly lives approximately 3-4 weeks and starts blood-feeding only 6 hours after emergence.

4.3 Reproduction

Male stable flies are able to inseminate more than one female in its lifetime while females only mate once (TANGTRAKULWANICH, 2012). Both genders are able to survive based on a sugar diet (SALEM et al., 2012) however, in order for females to successfully develop viable eggs they require several blood meals (JEANBOURQUIN 2005; TANGTRAKULWANICH, 2012). Mating occurs approximately two days after emergence. Oviposition usually occurs in decaying organic matter: rotting hay, grass clippings, manure, hay bales, and hay mixed with urine (JEANBOURQUIN et al., 2007; BROCE et al., 2005). These breeding sites provide suitable food for larval growth and development.

4.4 Feeding habits

Stomoxys calcitrans have a diurnal feeding rhythm and field studies have confirmed that the most active hours are from 10 a.m. to 4 p.m (ZHU et al., 2016). It has been reported that in warmer climates, stable flies have a feeding peak in the morning and in the afternoon (HAFEZ et al., 1959) while in colder climate there is only one afternoon peak (GIBSON et al., 1999). The male stable fly is estimated to engorge approximately 11,2 µL of blood and the female stable fly 15,1 µL and it takes approximately two – five minutes to obtain a complete blood meal (SCHOWALTER et al., 1979; COOK). Sugar feeding is common in stable flies; they obtain nectar from decaying fruit and plants (ZUMPT, 1973). Post feeding they usually rest on nearby fence or vegetation exposed to warmth to digest the blood meal which lasts approximately 14 – 21 hours (BISHOP et al., 1913).

4.5 Vision

Visual cues play an important role in understanding how stable flies find its host. Electroretinogram (ERG) recordings have shown that stable flies are sensitive to light in the range of 330 – 360 nm UV range however, gravid females have a significant ERG response around 480 nm (ZHU et al., 2016). Trapping experiments have shown that more stable flies are caught when a white colored trap is used rather than blue (ZHU et al., 2016). Shape is also an important factor; stable flies prefer a horizontal rectangular shape rather than vertical rectangular (TANGTRAKULWANICH, 2012).

4.6 Nuisance

Stable flies were introduced into North America from Europe during the 1700's. Today they are distributed worldwide but in greater abundance in warmer and tropical countries. They are considered to be a cosmopolitan pest with livestock as its primary host but it is not uncommon for humans and domestic animals to get bitten when in close proximity (HOGSETTE et al., 1987). Upon landing on its host, stable flies pierce the skin with its saw-like proboscis causing a painful bite. The saliva has a degree of toxicity that induces an immune response contributing to stress and immunosuppression (BALDACCHINO et al., 2013). Upon attack, the animals induce a defense-behavior that involves tail switching, muscle twitching, head shaking, ear flicking, and foot stomping (MOORING et al., 2007; TORR et al., 2006). This defense mechanism manages to interrupt the flies feeding but increases the number of biting wounds.

Stomoxys calcitrans commonly attack the legs and lower extremities of the animal. It is therefore common for cattle to herd in order to avoid being bitten. In addition to cattle, stable flies also attack other herding animals such as horses and sheep with the exception of poultry (FRIESEN et al., 2012; ANDERSON et al., 1970).

4.7 Economical Importance

The stress and nuisance that stable flies causes cattle has a great economical impact. A high number of stable flies reduce the productivity of the livestock

(TAYLOR et al., 2012). The induced defense behavior carried out by the animal reduces grazing time and increases heat stress (WELLMAN et al., 1973; DOUGHERTY et al., 1993; CAMPBELL et al., 1993). It is estimated that >10 flies per animal results in secondary health consequences (BALDACCHINO et al., 2013). Severe biting activity results in reduced milk and meat production. Taylor et al., estimated that in 2009 there was a total loss of US$ 2,211 million dollars in the cattle industry, even though a more recent economical evaluation has not been executed, this value is believed to have increased. This included dairy cattle, cow-calf herds, pastured cattle, and cattle on feed. In Brazil, another major meat producer estimated a potential loss of US$ 335,46 million dollars in 2012 among dairy and feedlot cattle (GRISI et al., 2014). A 7 % weight reduction on grazing yearling cattle has been estimated per stable fly (CAMPBELL et al., 2001).

Another aspect in which stable flies can cause economical losses is through transmission of pathogen. Blood loss, energy loss, weight decrease and nuisance caused by *S.calcitrans* favors pathogen transmission.

4.8 Pathogen transmission

Mechanical transmission is the process of pathogen transmission in which there is no direct association between host and vector. This process is common in biting flies like *S.calcitrans.* Upon feeding of a host the biting fly is often disturbed due to animal defense behavior, this leads to a secondary bite on a nearby host. Regurgitation of previously obtained infected blood meals increases the chances of transmitting pathogens. A variety of viruses, bacteria, rickettsia and helminthes are transmittable through the bite of stable flies (BALDACCHINO et al., 2013). Equine infectious anemia (EIAV) is a worldwide disease that stable flies are capable of transmitting through contact with infected blood (FOIL et al 1983). Wide ranges of laboratory studies have shown that stable flies have the ability to transmit various pathogens (HOCH et al., 1985; TRAVERSA et al., 2008; TURELL et al., 1987).

4.9 Olfactory system

Stable flies have their sensory organ on the antennae. The antenna is divided into three segments: a proximal scape; a medial pedicel, and the funicles (LEWIS,

1971). There are four main types of sensillas all mainly located on the funicles: basiconic, trichoid, clavate, coeloconic. Based on their structure, it has been suggested that the trichoid and coeloconic sensilla function as mechano-receptors (TANGTRAKULWANICH et al., 2011). There is no sexual dimorphism in sensillar distribution or structure only in abundance (Table 1). The structure of the stable flies olfactory system is almost identical to that of the house fly (KELLING et al., 2001). The olfactory system has been explored in order to understand host seeking behavior, oviposition choice, and ecology.

Table 1 - Abundance and distribution of sensillum types of the stable fly antenna.

Area of funicles		Basiconic	Clavate	Coeloconic	Trichoid, long	Trichoid, medium	Trichoid, short
Tip	Male	100 ± 34	6 ± 1	5 ± 1	611 ± 2	302 ± 32	587 ± 86
	Female	159 ± 36	26 ± 6	9 ± 4	542 ± 204	379 ± 79	1165 ± 306
Dorsal	Male	120 ± 40	10 ± 6	1 ± 1	371 ± 78	549 ± 76	949 ± 269
	Female	158 ± 18	31 ± 27	1 ± 1	626 ± 142	852 ± 161	1339 ± 400
Inner side	Male	263 ± 82	70 ± 29	9 ± 3	767 ± 439	2157 ± 1059	2542 ± 535
	Female	238 ± 39	41 ± 5	10 ± 4	907 ± 590	1452 ± 404	2316 ± 1389
Outer side	Male	400 ± 64	54 ± 4	26 ± 3	616 ± 64	2566 ± 273	3196 ± 470
	Female	325 ± 10	73 ± 2	26 ± 1	670 ± 7	1028 ± 4	3258 ± 400
Ventral	Male	266 ± 37	29 ± 3	11 ± 4	450 ± 117	1651 ± 267	1102 ± 375
	Female	311 ± 9	47 ± 2	11 ± 1	338 ± 7	1002 ± 4	996 ± 4
Total	**Male**	**1149 ± 110**	**169 ± 30**	**52 ± 3**	**2815 ± 462**	**7225 ± 475**	**8376 ± 1025**
	Female	**1190 ± 35**	**218 ± 40**	**57 ± 11**	**3082 ± 382**	**4713 ± 97**	**9072 ± 1641**

Source: TANGTRAKULWANICH et al., 2011.

4.10 Host-seeking behavior

Host seeking is conducted in three stages. The first stage involves appetitive-searching. Secondly, the stable fly is oriented and activated through the perception of chemical stimuli emitted by the hosts known as *kairomones.* Attraction is deemed to occur upon host location and feeding (LEHANE et al., 1991). Chemical stimuli such as semiochemicals play an important role in host –seeking behavior (GIBSON et al., 1999; LOGAN et al., 2005). 1-octen-3-ol, a compound found in ruminant breath is attractive to various biting flies including *S.calcitrans.* CO_2 a compound found in the breath of most animals is universal kairomone and shown to attract stable flies as well. Most studies have focused on cattle derived semiochemicals that attract stable flies (BIRKETT et al., 2004). Several compounds present in cattle urine and manure have also shown to be important cues for stable flies such as phenol, *m* cresol and *p*

cresol; three compounds that cause a strong electrophysiological and behavioral responses (TANGTRAKULWANICH et al., 2011; ZHU et al., 2016; BIRKETT et al., 2004). There is not doubt that stable flies are guided through a wide range of carboxylic acids, alcohols, and phenols.

4.11 Oviposition sites

Semiochemicals can also provide information about a suitable and advantageous oviposition site. It is known that female stable flies oviposit in decaying organic matter, and manure, however, what attracts them to these sites is still under research. Chemical analyses through gas chromatography has shown that manure contains a range of carboxylic acids, short chain alcohols, aldehydes, indoles, ketones, sulphides, and terpenes that are attractive to stable flies (JEANBOURQUIN et al., 2007). Indoles and phenols are known oviposition attractants for mosquitoes (DU et al., 1999). Dimethyl trisulphide an oviposition stimulant for *Culex* mosquitoes has also been confirmed as an active stimulant to gravid *S.calcitrans* (JEANBOURQUIN et al., 2007). Bacteria symbiosis is an important factor for stable fly oviposition as they produce chemical compounds that are attractive such as phenol, and *p* cresol (ROMERO et al., 2006).

4.12 Control

The most economical approach to control stable flies is to manage and eliminated potential breeding sites such as rotting hay, bedding and manure piles. This is however an approach that requires manpower and constant attention. The use of insecticides is the most common approach to target adult stable flies. There are a number of insecticides available against nuisance flies, however the most used insecticide is permethrin. In order to target stable flies the insecticide should be applied to the lower extremities where they normally feed. Insecticides applied on the legs are easily washed away when cattle are grazing in high pastures and therefore do not provide long-term protection. Another setback with the use insecticides is the possible development of insecticide resistance. In Florida, several field populations have demonstrated resistance to permethrin (PITZER et al., 2010). Stable flies have also demonstrated a natural variation to insecticide susceptibility regardless of prior exposure (MARCON et al., 1997). Resistance to organophosphates has also been

recorded in southwestern Kansas (CILEK et al., 1994). Impregnated ear tags are also available as a control method against stable flies however this approach is more efficient against horn flies as stable flies feed in the lower body parts (HARVEY et al., 1970). Ear tags impregnated with flucythrinate have shown to control stable flies for a period of maximum seven weeks (HOGSETTE et al., 1986).

4.13 Biological Control

Natural enemies can regulate a fly species when its present in low quantities but it is difficult to combat highly infested areas. Female parasitoid wasps have the ability to deposit its egg into stable fly larva or pupae (BARNARD, 2003). *Spalangia cameroni* (Hymenoptera: Pteromalidae) a parasitoid used against both horn- and stable flies (WEINZIERL et al., 1998) has shown to successfully locate stable fly larvae even when hidden deep within bedding (PITZER et al., 2011). Mites have also been suggested as a potential biological control as they have shown to prevent the dispersion of stable flies (BERESFORD et al., 2009).

Parasites are commercially available as a biological control against stable flies. However, various factors need to be implemented and taken into consideration in order to obtain good results including: management such as: manure management, effective water management and control of weeds and vegetation.

4.14 Traps / attractants

Traps can be used for both surveillance and control of stable fly populations. The first developed trap against stable flies is the William trap made of reflective fiberglass. The William trap consist of two rectangular panels of fiberglass notched halfway and fitted together in a cross configuration (FOIL et al., 1994). Nowadays, the William trap has been modified in a number of ways. A wide variety of traps including sticky traps, Broce trap, Nzi cloth traps, and Alsynite traps (Williams trap) have been tested and evaluated for stable flies (Figure 3 a-d) (TAYLOR et al., 2006). Traps are a cost-effective approach but it requires maintenance and regular cleaning in order for them to maintain its efficacy (RUGG et al., 1982). Visual stimulation plays an important role in the efficacy of traps. Black and blue colored traps have been tested against stable flies and have shown to be less effective than the white colored

traps (WILLIAMS et al., 1973; BERESFORD et al., 2006; OSA et al., 2014; GILLES et al., 2007). It has been argued that this is because white is a combination of all colors and thus create synergistically active wavelengths (ZHU et al., 2016). The use of semiochemicals is an ongoing research that has proven to efficiently increase the efficacy of trapping methods. It provides a targeted control and surveillance approach. 1-octen-3-ol is a known attractant for many heamatophagous insects including stable flies but its efficiency in trapping enhancement have been contradicting for stable flies (MIHOK et al., 1995; ALZOGARAY et al., 2000). Carbon dioxide is also a universal attractant that also is efficient against stable flies (HOY et al., 1969). Wind tunnel tests have shown that both 1-octen-3-ol and CO_2 cause an upwind anemotaxis but CO_2 significantly increases sinuosity and angular velocity (SCHOFIELD et al., 1997). An interaction between visual and olfactory stimuli would optimize trapping methods against stable fly management.

Figure 3 - a-d: Shows different types of traps used for *Stomoxys calcitrans* catches. (a) Sticky trap covered in *S.calcitrans.* (b) Broce trap covered in *S.calcitrans.* (c) Williams trap also known as Alsynite trap. (d) Nzi trap commonly used for tsetse flies.

Source: (a) Department of Agriculture and Food, Australia, 2016; (b); (c) Kaufman, University of California; (d) Torr et al., Natural Resources Institute, 2016.

REFERENCES

ALZOGARAY, R. A.; CARLSON, D. A. Evaluation of *Stomoxys calcitrans* (Diptera: Muscidae) behavioral response to human and related odors in a triple cage olfactometer with insect traps. **Journal of Medical Entomology,** v. 37, p. 308-215, 2000.

ANDERSON, J. R.; TEMPELIS, C. H. Precipitin test identification of blood meals of *Stomoxys calcitrans* (L.) caught on California poultry ranches , and observations of digestion rates of bovine and citrated human blood. **Journal of Medical Entomology,** v. 7, p. 223-229, 1970.

BALDACCHINO, F. et al. Tranmission of pathogens by *Stomoxys* flies (Diptera, Muscidae): a review. **Parasite,** v. 20, p. 26, 2013.

BARNARD, D. R. Control of fly-borne disease. **Pesticide Outlook,** v. 14, p. 222-228, 2003.

BERESFORD, D. V.; SUTCLIFFE, J. F. Studies on the effectiveness of chloroplast sticky traps for sampling stable flies (Diptera: Muscidae), including a comparison to Alsynite. **Veterinary Entomology,** v. 99, p. 1025.1035, 2006.

_____; _____. The effect of *Macrocheles muscaedomesticae* and *M. subbadius* (Acarina: Macrochelidae) phoresy on the dispersal of *Stomoxys calcitrans* (Diptera:Muscidae). **Systematic & Applied Acrology Special Publications,** v. 23, p. 1-30, 2009.

BIRKETT, M. A. et al. The role of volatile semiochemicals in mediating host location and selection by nuisance and disease-transmitting cattle flies. **Medical and Veterinary Entomology,** v. 18, p. 313-322, 2004.

BISHOP, F. C. The stable fly (Stomoxys calcitrans L.), an important live stock pest. **Journal of economical entomology,** v. 6, p. 112-126, 1913.

BROCE, A. B.; HOGSETTE, J.; PAISLEY, S. Winter feeding sites of hay in round bales of major developmental sites of *Stomoxys calcitrans* (Diptera: Muscidae) in pastures in Spring and summer. **Veterinary Entomology,** v. 98, p. 2307-2312, 2005.

CAMPBELL, J. B. et al. Effects of stable flies (Diptera: Muscidae) and heat stress on weight gain and feed conversion of feeder cattle. **Journal of Agricultural Entomology,** v. 10, p. 155-161, 1993.

CAMPBELL, J. B. et al. Effect of stable flies (Diptera:Muscidae) on weight gains of grazing yearling cattle. **Journal of Economic Entomology,** v. 94, p. 780-783, 2001.

CILEK, J.E.; GREENE, G.L. Stable fly (Diptera: Muscidae) insecticide resistance in Kansas cattle feedlots. **Journal of Economic Entomology,** v. 87, p. 275-279, 1994.

COOK, D. **Managing stable flies**. Bruce: University of Western Australia, 2012.

DOUGHERTY, C. T. et al. Stable flies (*Stomoxys calcitrans* L.) and the behavior of grazing beef cattle. **Applied Animal Behavior Science,** v. 35, p. 215-233, 1993.

DU, Y.; MILLAR, J. G. Electroantennogram and oviposition bioassay responses of *Culex quinquefasciatus* and *Culex tarsalis* (Diptera: Culicidae) to chemical odors from Bermuda Grass Infusions. **Journal of Medical Entomology,** v. 36, p. 158-166, 1999.

FOIL, L. D. et al. Mechanical transmission of equine infectious anemia virus by deer flies (*Chrysops flavidus*) and stable flies (*Stomoxys calcitrans*). **American Journal of Veterinary Research,** v. 44, p. 155-156, 1983.

_____.; HOGSETTE, J.A. Biology and control of tabanids, stable flies, and horn flies. **Revue Scientifique et Technique,** v 13, p. 1125-1158, 1994.

FRIESEN, K. M.; JOHNSON, G. D. Reproductive potential of stable flies (Diptera: Muscidae) fed cattle, chicken, or horse blood. **Journal of Medical Entomology,** v. 49, p. 461-466, 2012.

GIBSON, G.; TORR, S. J. Visual and olfactory responses of heamatophagous Diptera to host stimuli. **Medical and Veterinary Entomology,** v. 13, p. 2-23, 1999.

GILLES, J. et al. Efficiency of traps for *Stomoxys calcitrans* and *Stomoxys niger niger* on Reunion Island. **Medical and Veterinary Entomology,** v. 21, p. 65-69, 2007.

GRISI, L. et al. Reassessment of the potential economic impact of cattle parasites in Brazil. **Brazilian Journal of Veterinary Parasitology,** v. 23, p. 150-156, 2014.

HAFEZ, M.; GAMAL-EDDIN, F. M. On the feeding habits of *Stomoxys calcitrans* L. and *sitiens* Rond., with special reference to their biting cycle in nature (Diptera:Muscidae). **Journal of Bulletin Social Entomology Egypt,** v. 43, p. 291-301, 1959.

HARVEY, T. L.; BRETHOUR, J. R. Horn fly control with dichlorvos-impregnated strips. **Journal of Economical Entomology,** v. 63, p. 1688-1689, 1970.

HOCH, A. L.; GARGAN, T. P.; BAILEY, C. L. Mechanical transmission of Rift Valley fever virus by heamatophagous Diptera. **American Journal of Tropical and Medical Hygiene,** v. 34, p. 188-193, 1985.

HOGSETTE, J. A.; RUFF, J. P. Evaluation of flucythrinate and fenvalerate impregnated ear tags and permethrin ear tapes for fly (Diptera: Muscidae) control on beef and dairy cattle in Northwest Florida. **Journal of Economic Entomology,** v 79, p. 152-157, 1986.

_____; _____. Control of stable flies and horn flies (Diptera: Muscidae) with Permethrin tapes applied to tails of beef and dairy cattle. **Journal of Economic Entomology,** v. 80, p. 417-420, 1987.

HOY, J. B. Trapping the stable fly by using CO_2 or CO as attractants. **Journal of Economic Entomology,** v. 63, p. 793, 1970.

JEANBOURQUIN, P. The role of odour perception in the sensory ecology of the stable fly, *Stomoxys calcitrans* L. Dissertation for the Degree of Doctor of Philosophy, University of Neuchatel, p. 1-107, 2005.

J_____; GUERIN, P. M. Chemostimuli implicated in selection of oviposition substrates by the stable fly *Stomoxys calcitrans.* **Medical and Veterinary Entomology,** v. 21, p. 209-216, 2007.

_____; _____. Sensory and behavioral responses of the stable fly *Stomoxys calcitrans* to rumen volatiles. **Medical and Veterinary Entomology,** v. 21, p. 217-224, 2007.

KELLING-JOHANNES, F. **Olfaction in houseflies: morphology and electrophysiology**. Dissertation for the Degree of Doctor of Philosophy, University of Groningen. 2001.

LEHANE, M. J. **The biology of blood-sucking insects**. 2nd ed. Liverpool: Cambridge University Press, 1991.

LEWIS, C. T. Superficial sense organs of the antennae of the fly, *Stomoxys calcitrans.* **Journal of Insect Physiology,** v. 17, p. 449-461, 1971.

MARCON, P. C. R. G. et al. Susceptibility of stable flies (Diptera: Muscidae) from Southeastern nebraska beef cattle feedlots to selected insecticides and comparison of 3 bioassay techniques. **Journal of Economic Entomology,** v. 90, p. 293-298, 1997.

MIHOK, S.; KANGETHE, E.; KAMAU, G. K. Trials of traps and attractants for *Stomoxys* spp. (Diptera: Muscidae). **Journal of Medical Entomology,** v. 32, p. 283-289, 1995.

MOORING, M.S. et al. Insect-repelling behavior in bovids: role of mass, tail length, and group size. **Biological Journal of the Linnean Society,** v. 91, p. 383-392, 2007.

OSE, G. A.; HOGSETTE, J. A. Spatial distribution, seasonality and trap preference of stable fly, *Stomoxys calcitrans* L. (Diptera: Muscidae), adults in a 12-hectare zoological park. **Zoo biology,** v. 33, p. 228-233, 2014.

PITZER, J. B.; KAUFMAN, P. E.; TENBROECK, S. H. Assessing permethrin resistance in the stable fly (Diptera: Muscidae) in Florida by using laboratory selections and field evaluations. **Veterinary Entomology,** v. 103, p. 2258-2263, 2010.

_____. et al. Seasonal abundance of stable flies and filth fly pupal parasitoids (Hymenoptera: Pteromalidae) at Florida equine facilities. **Journal of Economic Entomology,** v. 104, p. 1108-1115, 2011.

ROMERO, A.; BROCE, A.; ZUREK, L. Role of bacteria in the oviposition behavior and larval development of stable flies. **Medical and Veterinary Entomology,** v 20, p. 115-121, 2006.

RUGG, D. Effectiveness of Williams traps in reducing the numbers of stable flies (Diptera: Muscidae). **Journal of Economic Entomology,** v. 75, p. 857-859, 1982.

SALEM, A. et al. Feeding and breeding aspects of *Stomoxys calcitrans* (Diptera: Muscidae) under laboratory conditions. **Parasite,** v. 19, p. 309-317, 2012.

SCHOFIELD, S.; BRADY, J. Effect of carbon dioxide, acetone, and 1-octen-3-ol on the flight responses of the stable fly, *Stomoxys calcitrans,* in a wind tunnel. **Physiological entomology,** v. 22, p. 380-386, 1997.

SCHOWALTER, T. D.; KLOWDEN, M. J. Blood meal size of the stable fly, *Stomoxys calcitrans,* measured by the hicn method. **Mosquito News,** v. 39, p. 110-112, 1979.

TANGTRAKULWANICH, K. et al. Characterization of olfactory sensilla of *Stomoxys calcitrans* and electrophysiological responses to odorant compounds associated with hosts and oviposition media. **Medical and Veterinary Entomology,** v. 25, p 327-336. 2011.

_____.; **Sensory morphology and chemical ecology of the stable fly, *Stomoxys calcitrans**:*** host-seeking and ovipositional selection. Dissertation for the Degree of Doctor of Philosophy, University of Nebraska, 116 p, 2012.

TAYLOR, D. B.; BERKEBILE, D. Comparative efficiency of six stable fly (Diptera: Muscidae) traps. **Journal of Economic Entomology,** v. 99, p. 1415-1419, 2006.

TAYLOR, D. B.; MOON, R. D.; MARK, D. R. Economic impact of stable flies (Diptera: Muscidae) on dairy and beef cattle production. **Journal of Medical Entomology,** v. 49, p. 198-209, 2012.

TORR, S. J.; MANGWIRO, T. N. C.; HALL, D. R. The effects of host physiology on the attraction of tsetse (Diptera: Glossinidae) and *Stomoxys* (Diptera: Muscidae) to cattle. **Bulletin of Entomological Research,** v. 96, p. 71-84, 2006.

TURRELL, M.J.; KNUDSON G.B. Mechanical transmission of *Bacillus anthracis* by stable flies (*Stomoxys calcitrans*) and mosquitoes (*Aedes aegypti* and *Aedes taeniorhynchus*). **Infection and Immunity,** v 55, p. 1859-1861, 1987.

TRAVERSA, D. et al. Identification of intermediate hosts of Habronema microstoma and Habronema muscae under field conditions. **Medical and Veterinary Entomology,** v. 22, p. 283-287, 2008.

WEINZIERL, R. A.; JONES, C. J. Release of *Spalangia nigroaenea* and *Muscidifurax zaraptor* (Hymenoptera: Pteromalidae) increase rates of parasitism and total mortality of stable fly and house fly (Diptera: Muscidae) pupae in Illinois cattle feedlots. **Journal of Economic Entomology,** v. 91, p. 1114-1121, 1998.

WILLIAMS, D. F. Sticky traps for sampling populations of *Stomoxys calcitrans.* **Journal of Economic Entomology,** v. 66, p. 1279- 1280, 1973.

ZUMPT, F. The Stomoxyine biting flies of the world. Diptera: Muscidae. Taxonomy, biology, economic importance and control measures. Stuttgart: **Fischer,** 1973.

ZHU J.J. et al. Visual and olfactory enhancement of stable fly trapping. **Pest management Science,** DOI: 10.1002/ps.4207, 2016.

5 CHAPTER III: BEHAVIORAL AND ELECTROPHYSIOLOGICAL STUDIES OF *STOMOXYS CALCITRANS* L. (DIPTERA: MUSCIDAE) TO TWO DIFFERENT HOSTS AND THEIR ENVIRONMENTAL OVIPOSITION STIMULANTS[1]

ABSTRACT

The role of semiochemicals in mediating the nuisance flies, *Stomoxys calcitrans* (Diptera: Muscidae) to cattle and equine and their environmental associated oviposition stimulants was investigated using coupling gas chromatography – electrophysiology (GC-EAG), coupled gas chromatography – mass spectrometry (GC-MS), and laboratory behavioral studies. Host emitted volatiles samples showed to be significantly attractive to female stable flies whilst males did not show a difference between test and control. Horse and cattle manure samples were only attractive to mated female flies whereas grass infusion sample was attractive to both mated and unmated female stable flies. Electrophysiological recordings coupled gas chromatography revealed 21 active compounds that include primary alcohols, ketones, and phenolic compounds. Active compound profile between the different hosts and their respective manure samples differed suggesting that *S.calcitrans* are attracted to a wide range of chemical stimulants that could be host specific.

Key words: Livestock pest. Biting fly. Chemical ecology.

[1] **This paper was submitted to the Journal of Economical Entomomlogy (2016)**

5.1 INTRODUCTION

Stomoxys calcitrans L. (Diptera: Muscidae) commonly known as “stable flies” are a worldwide heamatophagous pest of livestock and other warm-blooded animals. Both male and female flies are obligate aggressive blood-feeders that target the lower part of the livestock’s body. Unlike other heamatophagous insects, stable flies have a saw-like proboscis responsible for its notorious painful bite. The saliva has a degree of toxicity that induces an immune response contributing to stress and immunosuppression (BALDACCHINO et al., 2013). Sugar feeding from decaying fruits and plants is common for stable flies but in order for females to produce a viable batch of eggs, a complete blood meal is essential (SALEM et al., 2012; JEANBOURQUIN, 2005). Although stable flies are habitual pests in livestock farms including dairy and feedlot it is not uncommon to find this nuisance pest in equine farms (MACHTINGER et al., 2014). In addition to being nuisance, stable flies are mechanical vectors of pathogen including bacteria, viruses, helminthes and protozoans present in the hosts’ blood (BALDACCHINO et al., 2013). Due to the induced pain upon biting, blood feeding is often interrupted through muscle twitching, tail flicking, and foot stomping (MOORING et al., 2007). This requires finalizing its blood meal on a secondary host, which in turn increases the probability of mechanical transmission of pathogens. Blood loss, energy loss and weight decrease caused by stable flies also decreases the animals immune response thus increasing the chances of infection.

The host seeking behavior of stable flies is well understood and it has been confirmed that they are guided through olfactory cues emitted by the hosts (TANGTRAKULWANICH et al., 2011). 1-octen-3-ol, CO_2, and acetone, are three established host emitted chemical stimulants attractive to the stable fly; however, it is believed that a vast range of additional stimulants could be involved (GIBSON et al., 1999). In addition to olfactory cues, it has been suggested that biting behavior also depends on the reaction of host upon attack and host size (MOORING et al., 2007; TORR et al., 2006).

There is no direct study involving *S.calcitrans* host preference between equine and cattle. Most conducted research has been based on cattle and its derived host volatiles (BIRKETT et al. 2004; GIBSON et al., 1999). Stable flies are highly sensitive to volatile organic compounds found in rumen digesta (JEANBOURQUIN et al.,

2007[a]) and although equines are not ruminants, they are monograstic animals that also digest its diet through microbial fermentation. Many of these rumen volatiles are also found in manure, a common oviposition site for stable flies (JEANBOURQUIN et al., 2007[b]; HAFEZ et al., 1959; BROCE et al., 2005). Oviposition studies comparing horse and cattle manure have shown that gravid stable flies prefer horse manure when given a choice, however a chemical analyses of the two sources have not been conducted (JEANBOURQUIN et al., 2007[b]). The presence of hay or grass in manure has proven to play an important role in the attraction of gravid female *S.calcitrans* (MACHTINGER et al., 2014). Grass infusion has proven to be an attractive oviposition source for other heamatophagous insects (MBOERA, et al., 2000; DU, et al., 1999) but it has not been explored for stable flies.

This study explored the organic volatile compounds emitted by cattle and equines to determine their effect on stable flies. To incorporate the various chemo-stimulants present in livestock farms, three different oviposition stimulants were included: cattle manure, equine manure, and grass infusion. Determining the volatile profile of these different sources will provide knowledge and possible lures for targeted control and monitoring methods.

5.2 MATERIAL AND METHODS

5.2.1 Insects

For the behavioral assays, *Stomoxys calcitrans* pupae were obtained from a reared laboratory colony in EMBRAPA Gado de Corte, Campo Grande, MS, Brazil. All flies were sexed prior to use based on the space between the two compounds eyes. Females were divided into mated and unmated by comparison of their abdomen and separating females right after emergence. Flies were left unfed for 24 hours before conducting experiments.

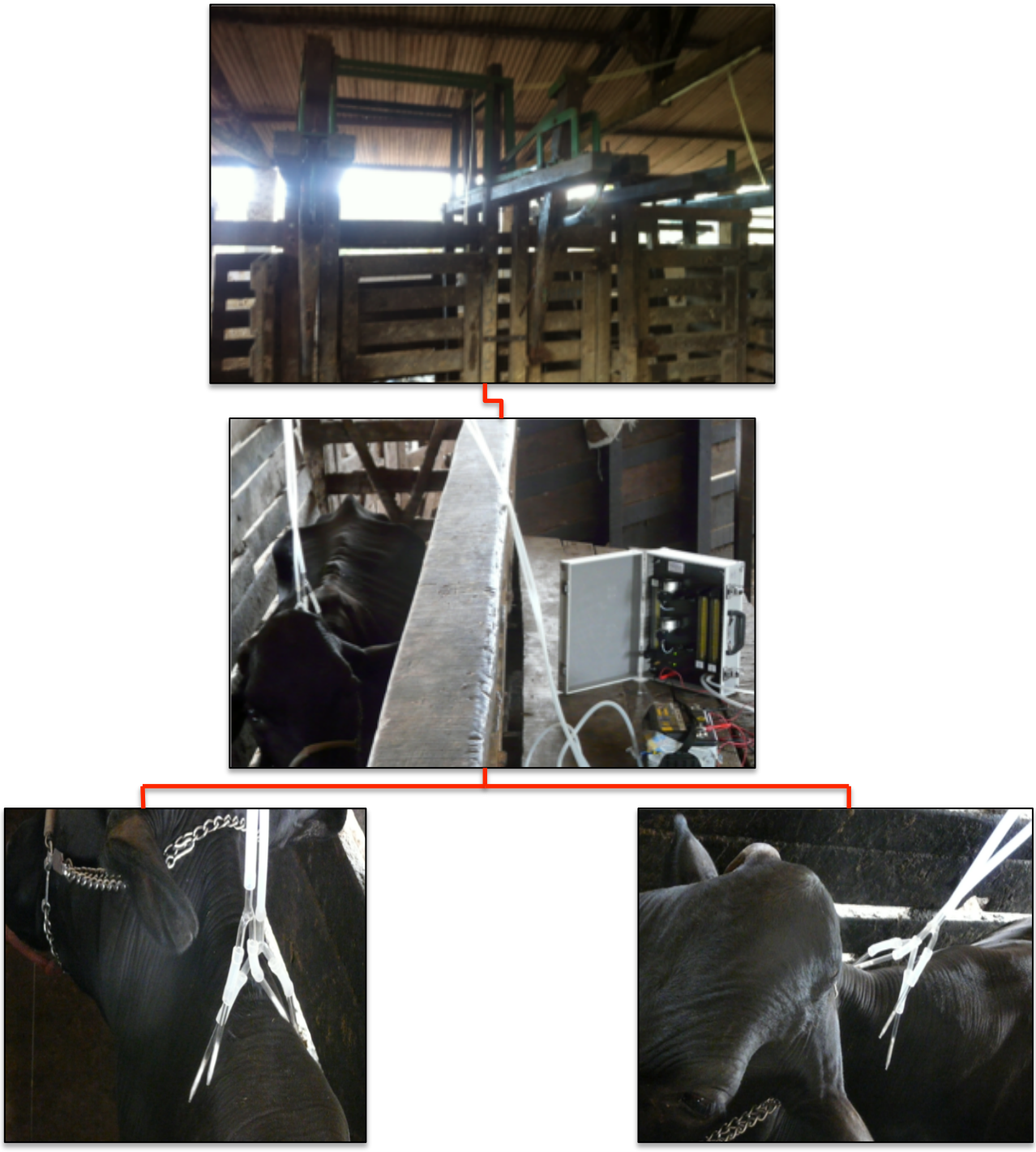
To conduct electrophysiological studies *S. calcitrans* were obtained from a cattle farm, Fazenda Sao Luis in Maceio, AL, Brazil, 9º39'57"S and longitude 35°44'6" W.

5.2.2 Host volatile collection

Host emitted volatile organic compounds (VOCs) were collected from the dorsal part of Girolando cattle (Figure 1) and Manga larga horses (Figure 2) with approximately five and six years of age from a livestock farm Alvaro Vascolenzos located in Maceio, AL, Brazil, latitude is 9º39'57"S and longitude 35°44'6" W.

Each VOC collection was conducted using air entrainment during 1 hour with Porapak Q (100 mg, 50 – 80 mesh, Supercoil, Poole, UK) as the adsorbent. All connections were made with PTFE tubing and the flow rate was set at 1400mL min^{-1}. A 750 µL aliquot of re-distilled HPLC degree hexane (Sigma-Aldrich, Brazil) was used as the extracting solvent. Samples were collected in two mL headspace vials (Sigma-Aldrich, Brazil) and stored in a freezer (-20 °C) until use.

Figure 1 - Cattle volatile collection scheme. Top: Cattle shed where the cow was maintained. Middle: Head space set up. Bottom: Close up of porapak traps located on the dorsal part of the cattle.



Source : Author, 2016.

Figure 2 - Equine volatile collection scheme. Top: area where the horse was maintained. Middle: horse with headspace set up attached to its dorsal area. Bottom: close up of porapak traps located on the dorsal part of the horse.

Source: Author, 2016.

5.2.3 Manure and grass infusion volatile collection

Volatile organic compounds were collected from three oviposition stimulants: fresh horse manure (500 g), fresh cattle manure (500 g), and grass infusion after five days of fermenting (one L of water with 500 g of grass) (Figure 3). Air entrainment was carried out in the Laboratory of Natural Resources, UFAL, during one hour with Porapak Q (100 mg, 50 – 80 mesh, Supercoil, Poole, UK) as the adsorbent. All connections were made with PTFE tubing and ferrules; flow rates were set at 1400mL min $^{-1}$ in and 600 mL min^{-1} out, creating a positive pressure. Oven roasting bags made of poly(ethylene terephthalate) (PET) were used for each headspace collection. A 750 µL aliquot of re-distilled HPLC degree hexane (Sigma-Aldrich, Brazil) was used as the extracting solvent. Samples were collected in two mL headspace vials (Sigma-Aldrich, Brazil) and stored in a freezer (-20 °C) until use.

Figure 3 - Volatile collection from three oviposition stimulants. Left: Fresh horse manure. Middle: Fresh cattle manure. Right: Grass infusion with 7 days of fermenting.

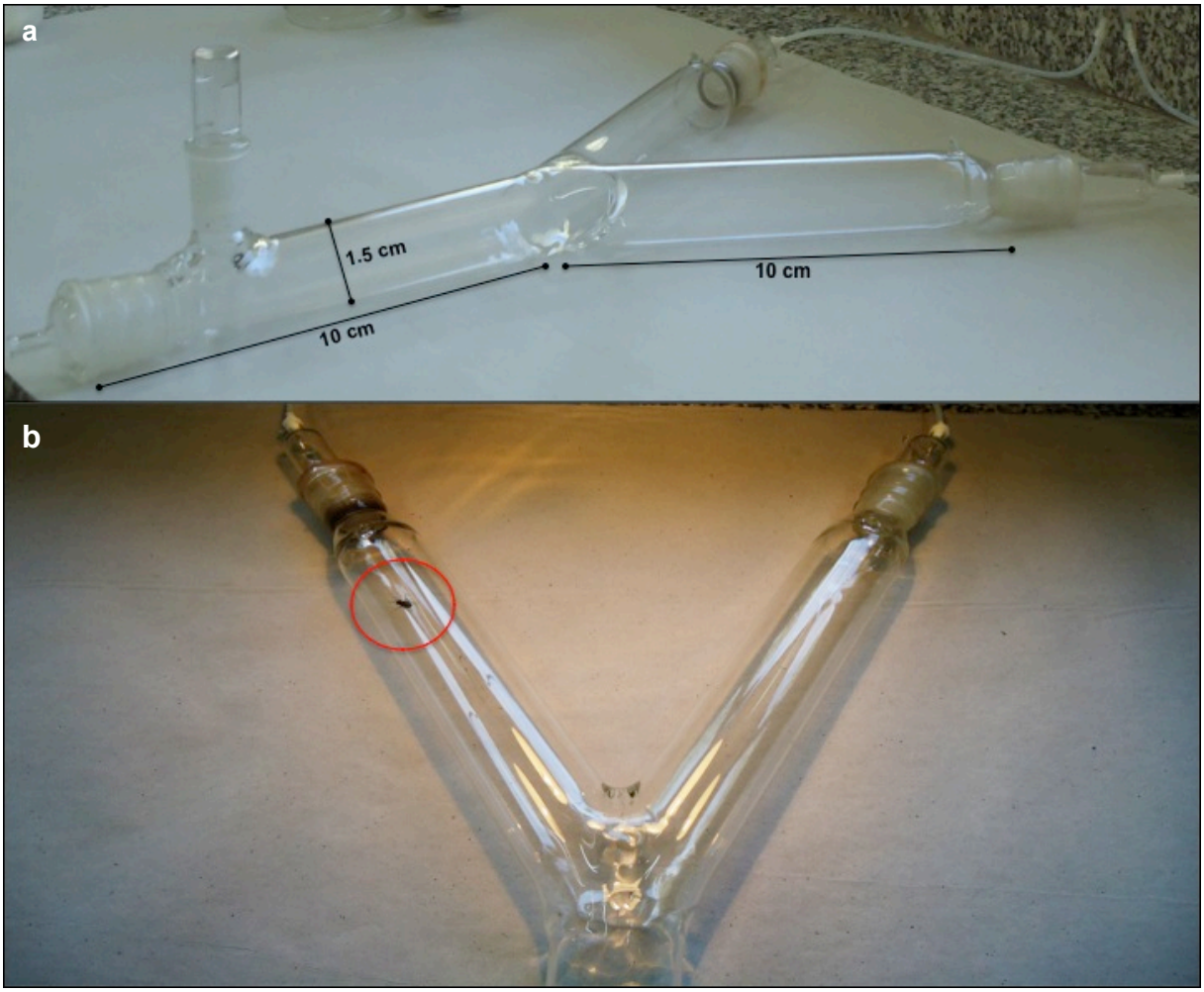
Source: Author, 2016.

5.2.4 Laboratory behavioral assays

Since both male and females *Stomoxys calcitrans* blood feed, both genders were used to test the behavioral response to host emitted stimulants (*N* = 25, respectively per sample). Mated and unmated female flies were used to test the behavioral response to oviposition stimulants (*N* = 25, respectively per sample).

A modified Y-tube olfactometer (length per arm = 10 cm; diameter = 1.5 cm) was used for all bioassays (Figure 4 a). Each assay was conducted in a controlled environment with a temperature of (24 °C) and humidity (70 %) with a photoperiod of 12:12 hours. The air flow (1000 mL min $^{-1}$) was passed through an activated charcoal filter to remove chemical contaminants and then split and passed into two glass vessels containing test material and control entering the two arms of the olfactometer. Ten microliter aliquot of each sample was loaded onto filter paper (Whatman No. 1.20 mm) and placed in the center of each end of the Y-tube olfactometer. Insects were released one by one into the stem of the olfactometer, when the fly proceeded further than one third of one of the arms it was deemed to have made a choice (three minutes were allowed per replicate, Figure 4 b).

Figure 4 - a-b: Top: Y-tube olfactometer used for all behavioural assays. Bottom: Behavioural assay showing a stable fly proceeding further than one third of one of the arms thus deemed to have made a choice.



Source: Author, 2016

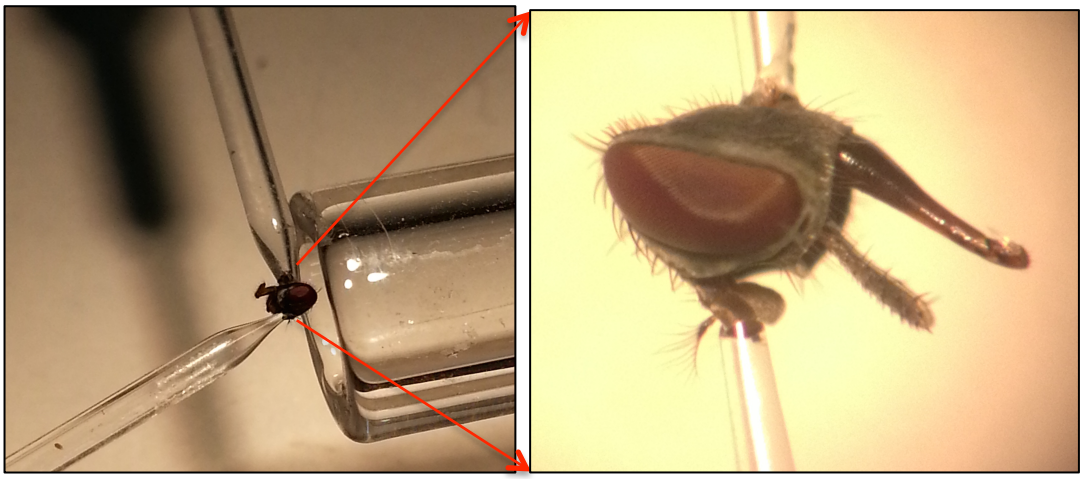
5.2.5 Electrophysiological studies

Electroantennogram (EAG) recordings were made using Ag-AgCl electrodes and glass capillaries filled with Ringer solution (8.0 g L^{-1} NaCl, 0.4 g L^{-1} $CaCl_2$) connected to silver wire, closing the electric circuit. Flies were sexed before use and chilled for one minute before excising the head. The head was placed within the indifferent electrode and the tip of one of the antenna was inserted to the recording electrode (Figure 5). The signals were passed through a high impedance amplifier (IDAC-4, Syntech 2004, Hilversum, Netherlands) and analysed using a customized software package (Syntech, NL 4.6, 2008).

Separation of the VOCs was achieved on a gas chromatography (GC) (Schimadzu, GC-2010) equipped with a flame ionization detector (FID), using an NST05 column (30 m x 0.25 mm i.d. x 25 µm film; Restek Corporation). Three microliter aliquot of each sample was injected in the splitless mode at an oven temperature of 40 °C for five minutes and then programmed at 3 °C/min to 130 °C for two minutes, then programmed at 10 °C/min to 250 °C. Electronic flow control was used to maintain a constant hydrogen carrier gas flow of 1.21 mL min^{-1}. The outputs from the EAG amplifier and the FID were monitored simultaneously and analysed using Synthech software package.

Peaks eluting from the GC column were judged to be active if they elicited an antennal depolarization in three or more runs (N= 6 per treatment).

Figure 5 - Electroantennogram using the excised head of *Stomoxys calcitrans.* Base of head was connected to one of the glass capillaries filled with Ringer solution connected to the indifferent electrode. Tip of antennae was connected to the second glass capillary filled with Ringer solution connected to the recording electrode.



Source: Author, 2016.

5.2.6 Compound identification

For chromatographic analysis, the volatile samples that elicited an electrophysiological activity were injected into a gas chromatograph coupled to a mass spectrometer (GC-MS), Schimadzu, QP2010, utilizing a non-polar NST-05 column (30 m x 0.25 mm i.d. x 0.25 µm film, Restek Corporation) with helium as the carrier gas. The temperature program stipulated 40° C for five minutes, programmed to increase at 3 °C/min to 130 °C for two minutes, then programmed at 10 °C/min to 250 °C. One microliter aliquot of each sample was injected in splitless mode. An alkane standard mixture (C_8 – C_{30} - Sigma Aldrich, Brazil) was injected to determine the retention indices of the analytes. Compounds were identified and confirmed through mass spectrometric fragmentation patterns, retention indices, and co-injection with synthetic standards. All solvents and reagent compounds were HPLC gradient and purchased from Sigma-Aldrich, Brazil/ Sweden.

5.2.7 Statistical analyses

To statistically analyze the results of each bioassay a two-way classification Chi-squared analysis was conducted. A degree of freedom of one was

used and the Yate's correction of continuity was applied. Obtained values were determined significant by consulting the chi-squared distribution table. The use of Chi- squared analysis verifies if the distribution of observed frequencies deviates significantly from the expected frequencies. Thus, the Chi-squared test is suitable for bioassays with two choices, since it is part of the principle that the insect has an equal chance to go to either side of the Y-tube olfactometer. This allows assessing whether the prevalence choice by side (treatment or control) is significant or not. Thus it is possible to know if there is significant insect attraction for the utilized treatment.

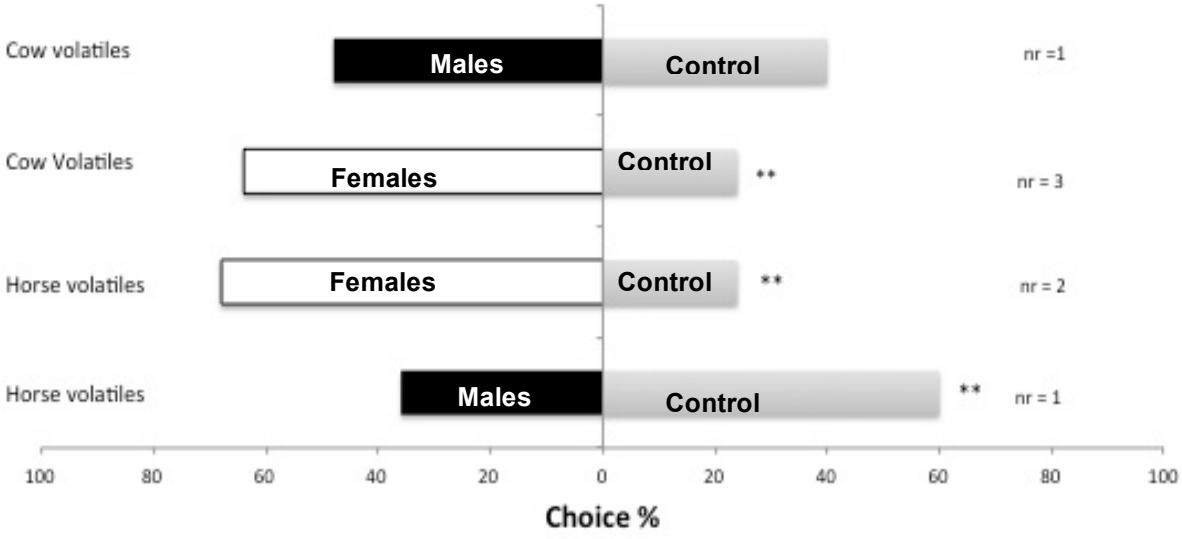
5.3 Results

5.3.1 Behavioral assays using *S.calcitrans*

Behavioural assays testing host volatiles using male *S.calcitrans* showed a significant difference between control and horse emitted volatiles, giving preference to the control (χ^2=6, d.f.= 1, p= 0,014305, Figure 6). When testing female *S. calcitrans,* results showed that they were significantly attracted to horse emitted volatiles (χ^2= 21.0434, d.f. = 1, p= 0,000004, Figure 6). Similar results were obtained when testing cattle emitted volatiles, females were significantly attracted (χ^2= 4, d.f.= 1, p= 0,0293768, Figure 6) whilst males did not show any preference between test and control (χ^2= 0,7272, d.f. = 1, p= 0,393768, Figure 6).

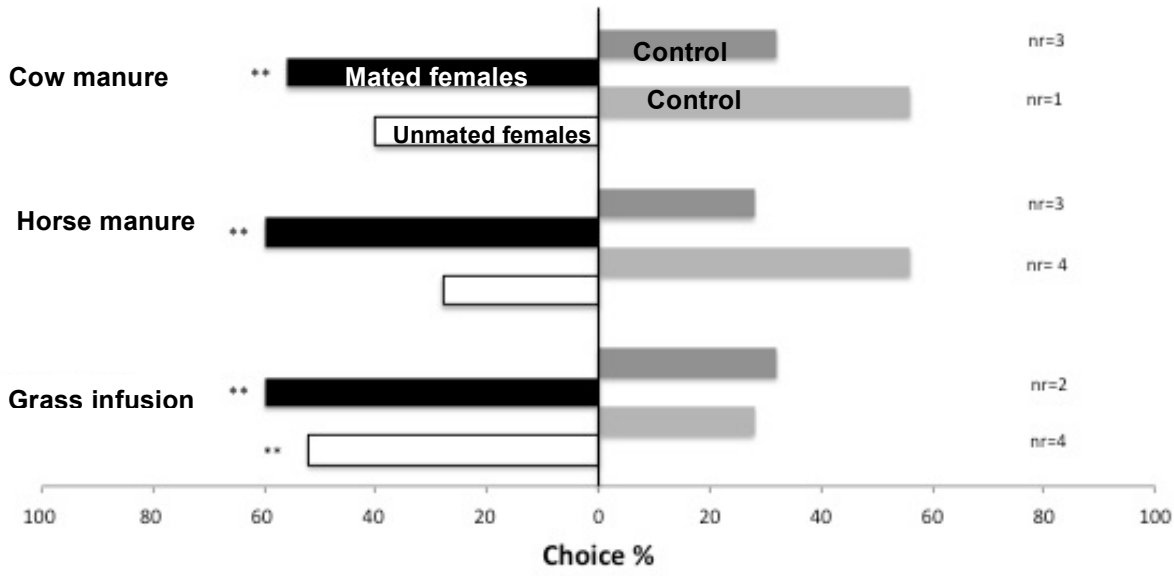
Behavioral assays of oviposition stimulants associated with cattle and equine farms showed that mated female flies were significantly attracted horse and cattle manure (χ^2= 11,6363, d.f. = 1, p= 0,000646 and χ^2= 10,45, d.f. = 1, p= 0,00543, Figure 7) unlike unmated females. Grass infusion was significantly attractive to both mated and unmated female flies (χ^2= 7.2, d.f. = 1, p= 0.0079 and χ^2= 8.5217, d.f.= 1, p= 0.003509, respectively, Figure 7).

Figure 6 - Attraction of male and female *S.calcitrans* to volatile organic compounds emitted from horses and cattle, respectively. Black bars indicate male *S.calcitrans*; White bars indicate female *S.calcitrans*. Grey bars indicate control (re-distilled hexane). *N*= 25 flies / sample; nr = no response. **= p < 0.01 in relation to control, using Chi-Squared analysis.



Source: Author, 2016.

Figure 7 - Attraction of unmated and mated female *S.calcitrans* to volatiles collected from possible oviposition stimulants present in livestock farms. Black bars indicate mated female *S.calcitrans*; White bars indicate unmated female *S.calcitrans*. Grey bars indicate control (re-distilled hexane). *N*= 25 flies / sample; nr = no response. **= p < 0.01 in relation to control, using Chi-Squared.

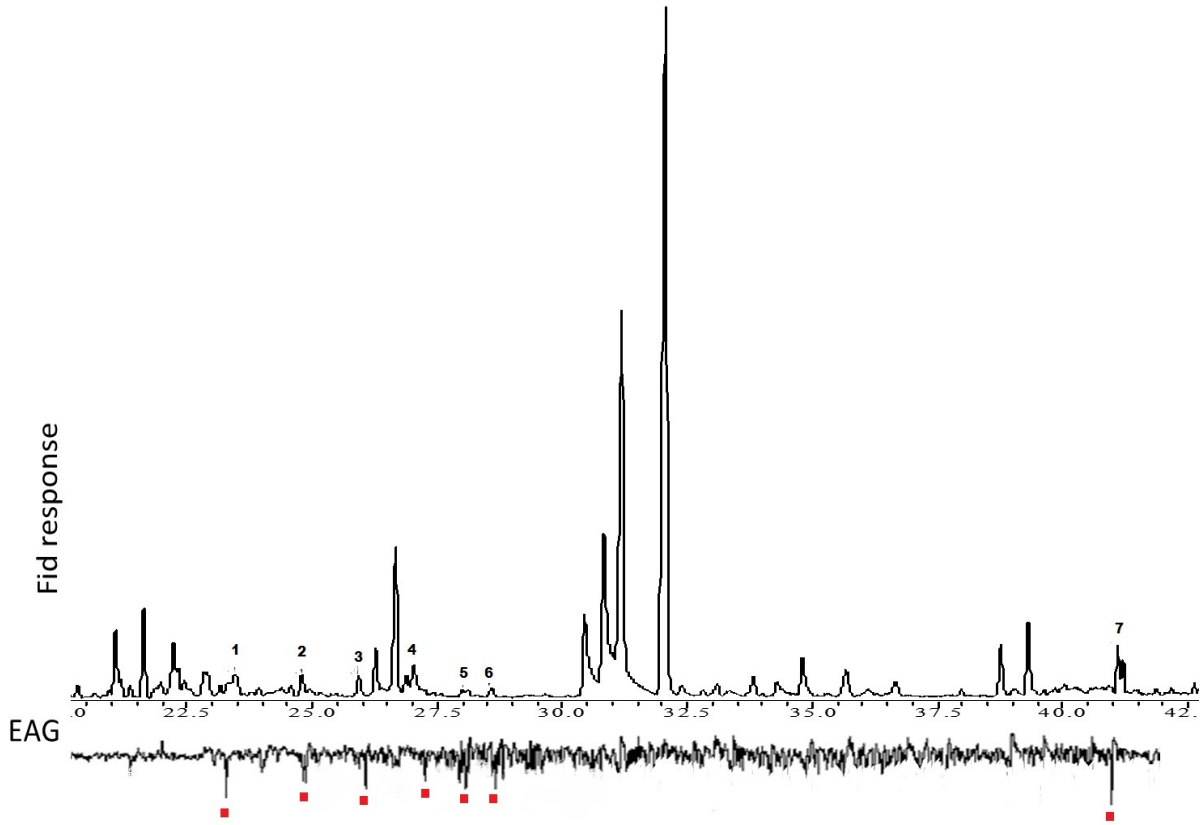


Source: Author, 2016.

5.3.2 Electrophysiological and chemical analyses

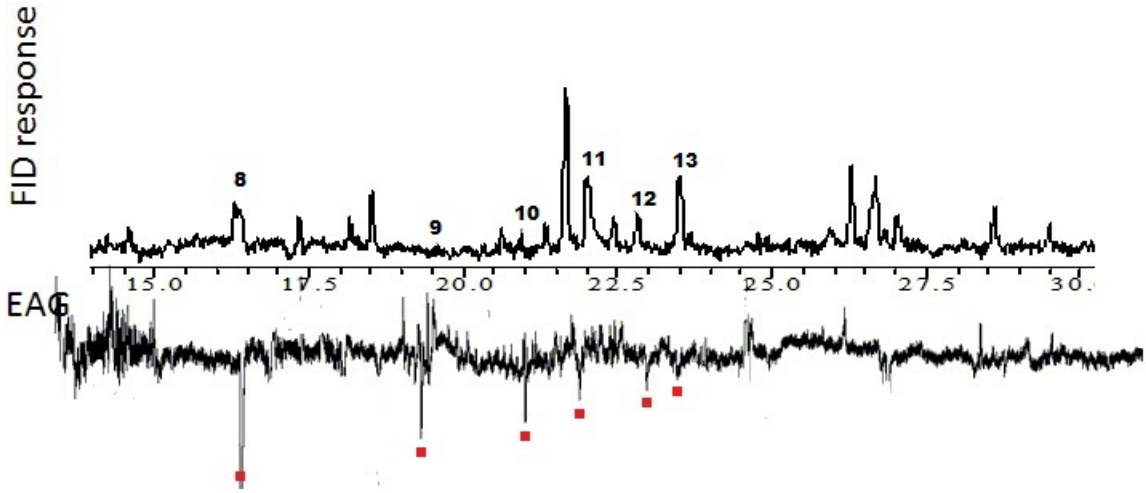
Coupled GC-EAG analyses showed a number of active compounds in host volatile samples. Cattle derived volatile sample elicited seven electrophysiological responses (Figure 8) while horse derived volatile samples only elicited six (Figure 9). Both horse and cattle manure samples elicited five electrophysiological responses each (Figure 10 and 11). Grass infusion sample only elicited four electrophysiological responses (Figure 12).

Figure 8 - Electroantennogram coupled gas chromatography analysis of cattle derived volatiles, using the antennae of *S.calcitrans.* Active compounds are indicated with a number. All GC analyses were conducted on an NST-05 column. *N* = 6.



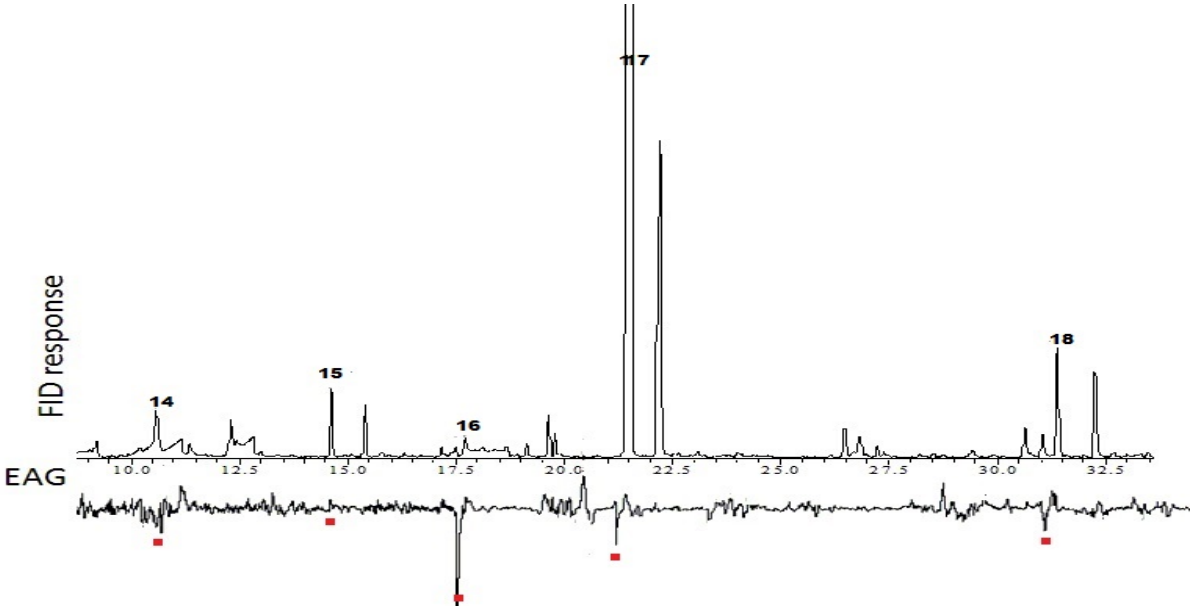
Source: Author, 2016.

Figure 9 - Electroantennogram coupled gas chromatography analysis of horse derived volatiles, using the antennae of *S.calcitrans.* Active compounds are indicated with a number. All GC analyses were conducted on an NST-05 column. *N* = 6.



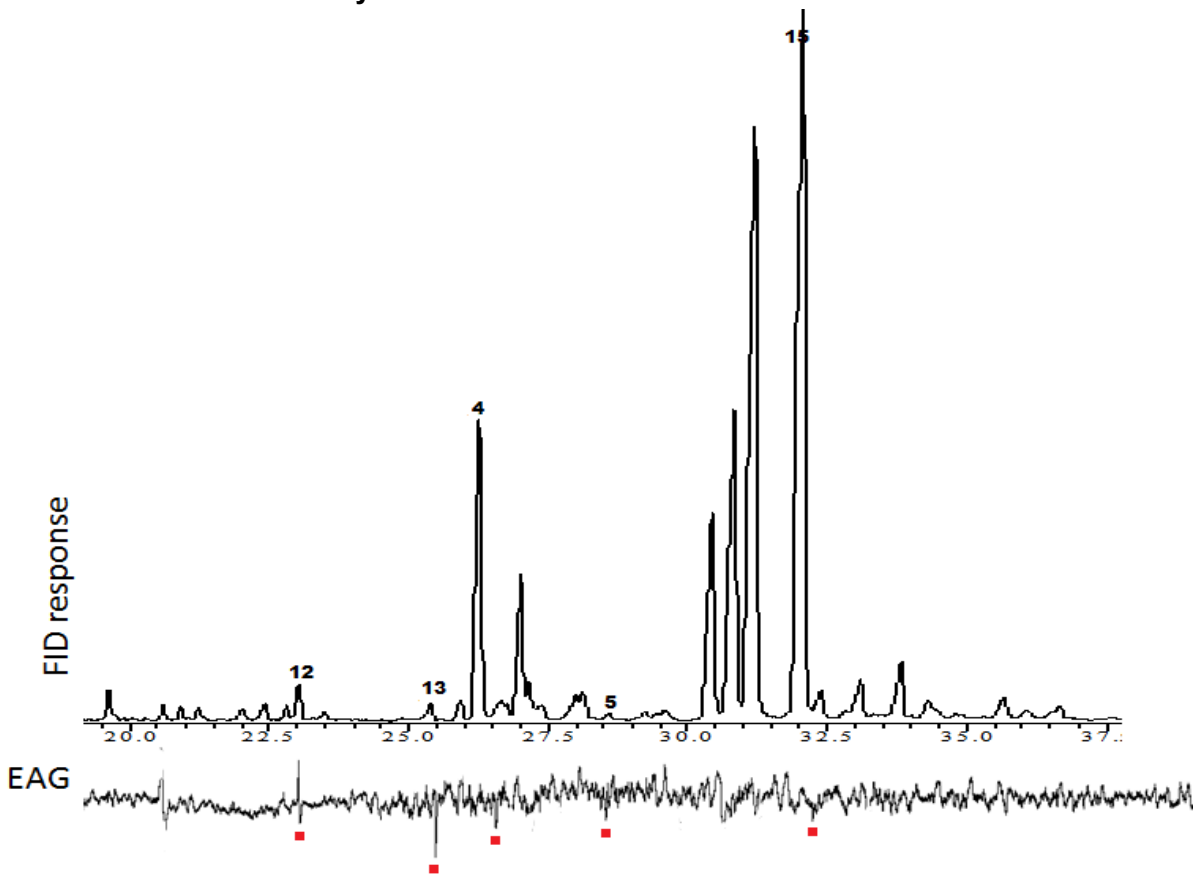
Source: Author, 2016.

Figure 10 - Electroantennogram coupled gas chromatography analysis of horse manure volatiles, using the antennae of *S.calcitrans.* Active compounds are indicated with a number. All GC analyses were conducted on an NST-05 column. *N* = 6.



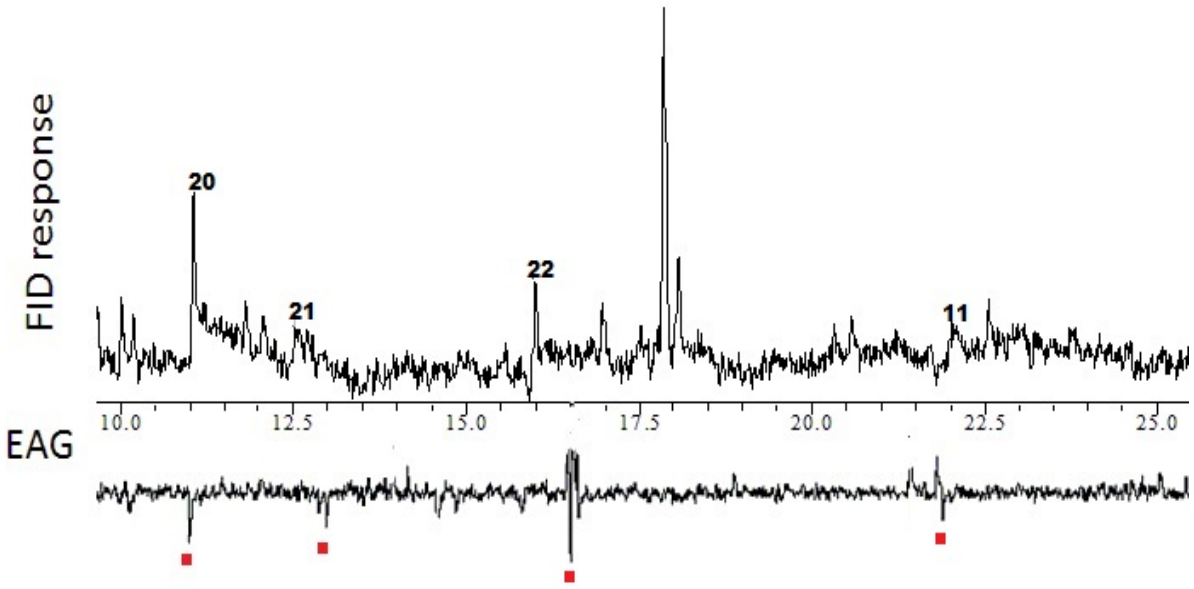
Source: Author, 2016.

Figure 11 - Electroantennogram coupled gas chromatography analysis of cattle manure volatiles, using the antennae of *S.calcitrans.* Active compounds are indicated with a number. All GC analyses were conducted on an NST-05 column. *N* = 6.



Source: Author, 2016.

Figure 12 - Electroantennogram coupled gas chromatography analysis of grass infusion volatiles, using the antennae of *S.calcitrans.* Active compounds are indicated with a number. All GC analyses were conducted on an NST-05 column. *N* = 6.



Source: Author, 2016.

Despite of *S.calcitrans* presenting different pattern of antennal responses between the two manure samples and the two host volatile samples, most of the chemical compounds occur in both substrates. The volatiles that elicited an electrophysiological response belonged to different chemical classes including, aliphatic alcohols, ketones, phenols, and aldehydes (Table 1).

Table 1 - EAG active compounds were identified using mass spectrometry and confirmed by fragmentation patterns (See appendix 1), retention indices, and co-injection with synthetic standards. Table demonstrates correlation of identified compounds in collected volatile samples. Compounds in red are those who have not previously been identified as *S.calcitrans* chemostimulants. * = confirmed with synthetic standard. RI = retention index. HF = horse manure; CM = cattle manure; GI = grass infusion; HV = horse volatiles; CV = cattle volatiles.

Peak number (Figs 8-12)	Compound name	RI	HM	CM	GI	HV	CV
1	2,6,6 – trimethyl octane	1098					*
2	Unidentified	1125					*
3	Naphthalene*	1147					*
4	4-Ethylbenzaldehyde*	1152		*			*
5	Cinnamaldehyde*	1174		*			*
6	Decanal	1200					*
7	4- Acetylacetophenone	1444					*
8	6-methyl-5-hepten-2-one	972				*	
9	Unidentified	1025				*	
10	1,2 – diethylbenzene	1051				*	
11	4- Methylphenol*	1072			*	*	
12	1-ethenyl-4-ethylbenzene	1085		*		*	
13	Nonanal*	1097		*		*	
14	*cis*-3-hexenol*	877	*				
15	3,7-dimethyl-1,6-octadiene	945	*				
16	2-octanone*	978	*				
17	Acetophenone*	1069	*				
18	3-ethylacetophenone	1262	*				
19	4-ethyl acetophenone*	1274		*			
20	1-Hexanol*	869	*		*		
21	Dimethyl trisulphide	960			*		
22	Butyl propanoate	910			*		

Source: Author, 2016.

5.4 Discussion

Behavioral assays testing host-derived volatiles demonstrated that host volatiles attracted female stable flies but males did not show a difference between test and control. Studies have shown that both male and female sugar feed but females are required to blood feed several times (TAYLOR et al., 2008) in order to oviposit a viable batch of eggs. The difference in attraction to host emitted volatiles between male and female flies noted in this study could be a suggestion that males are opportunistic feeders instead of host specific. Frequency of blood- and sugar feeding in Tabanids has shown that males feed on nectar more than females. Studies involving *Stomoxys calcitrans* and sugar feeding are contradicting as certain studies have noted that female have a higher percentage of sugar feeding than males whilst other argue that females have a decreased sugar feeding pattern as blood meals are essential for ovarian development (TAYLOR et al., 2008, KNIEPERT et al., 1980). It has been argued that the decreased sugar feeding in female is due to their increased need to blood feed (KNIEPERT, 1980), which would explain obtained results where female stable flies were attracted to host volatiles and males were not. Behavioral assays testing oviposition stimulants demonstrated that mated female stable flies were significantly attracted to both equine and cattle manure whilst both mated and unmated females were attracted to grass infusion.

The EAG data shows that *S. calcitrans* antennal receptors were sensitive to a range of compounds present in each sample. Chemical analyses of each electrophysiologically active compound revealed a wide range of aliphatic alcohols, ketones, phenols, aldehydes, hydrocarbons, esters, and benzene derivatives. The primary and aliphatic alcohols identified as chemostimulants present in horse manure included *cis*-3-hexenol and 1-hexanol which have previously been reported as constituents of cattle urine that causes an EAG response in *S.calcitrans* amongst other heamatophagous Diptera (BIRKETT et al., 2004). However, in addition to cattle urine it has not been identified as a host-derived volatile and the role of these aliphatic alcohols to Diptera is not fully clarified. Plants release certain aliphatic alcohols such as *cis*-3-hexenol as a defense component upon attack and it has been argued that certain plants have the ability to mimic manure odor in order to attract pollinators including Diptera (JEANBOURQUIN et al., 2007[b]; KITE et al., 1995;

SKUBATZ et al., 1996). The majority of the identified aldehydes have not previously been associated with *S.calcitrans.* Nonanal has previously been identified as EAG active for stable flies but whether it acts as a repellent or attractant has not been determined (JEANBOURQUIN et al., 2007[b]). However, many of the identified aldehydes in this study such as nonanal and decanal has previously been associated with human emitted odor and have proven to decrease the attraction of *Aedes aegypti* mosquitoes thus have been determined to act as a repellent (LOGAN et al., 2008). Naphthalene has also previously been associated with cattle odor (BIRKETT et al., 2004) however its behavioral affect on stable flies has not been determined. It has been found that naphthalene is a plant-derived semiochemical that functions as a repellent (KHAN et al., 1997) and it have also found to be produced by fungus in order to repel the stem sawfly, *Cephus cinctus* (DAISY et al., 2002). 6-methyl-5-hepten-2-one was amongst the identified ketones in the horse volatile sample; this chemostimulant has previously been identified as cattle emitted volatile that attracts stable flies and *Aedes aegypti* (BIRKETT et al., 2004; LOGAN et al., 2007). Acetophenone is commonly found in bovine breath (SPINHIRNE et al., 2004) but has also been previously been identified as a chemostimulant to stable flies found in rumen digesta and cow manure (JEANBOURQUIN et al., 2007[a]; JEANBOURQUIN et al., 2007[b]). In this study acetophenone, 3-ethyl acetophenone, and 4-ethyl acetophenone were found in both horse and cattle manure suggesting that these ketones are found in a wide range of sources emitted from the host and could therefore provide a strong attractant for trapping purposes. Volatiles derived from grass infusion have not been previously investigated in relation to stable flies. In this study both 4-methyl phenol and dimethyl trisulphide two well-known chemostimulants were present and determined electrophysiologically active to stable flies. Phenolic compounds including 4-methylphenol are considered to be one of the main attractants to biting flies and other heamatophagous insects commonly found in cattle urine, grass infusion, host derived volatiles etc. (BIRKETT et al., 2004; GIBSON et al., 1999; MBOERA et al., 2000; MIHOK et al., 2007; JEANBOURQUIN et al., 2007[b]; TANGTRAKULWANICH et al., 2015). In addition to being electrophysiologically active, 4-methylphenol has proven to increase the number of catches when used in the field as a lure (MIHOK et al., 2007; TANGTRAKULWANICH et al., 2015). Dimethyl trisulphide was identified in the grass infusion sample, agreeing with previous literature where it was found in Bermuda grass infusion as an oviposition

stimulant for *Culex quinquefasciatus* (DU et al., 1999). This chemostimulant have also been found in bovine breath, rumen odor, cattle and horse manure and has proven to elicit strong EAG responses and increase upwind attraction when tested in wind tunnels (JEANBOURQUIN et al., 2007[a]; JEANBOURQUIN et al., 2007[b]; TANGTRAKULWANICH et a., 2011). In addition to all the stated chemostimulants identified in this study, some of them such as 1,2 – diethylbenzene, 2,6,6 – trimethyl octane, and 1-ethenyl-4-ethylbenzene have not been reported previously as a part of the volatile of profile of host derived compounds, however, it is important to bare in mind that these compounds are do not are not synthesized in nature thus they could be artifacts of the column.

An interesting finding in this study was that there was no correlation between host emitted compounds that emitted and EAG response nor between the manure samples. This does not agree with previous studies that stated that there is no major difference between manure compound profiles from two different hosts (JEANBOURQUIN et al., 2007[b]). This study used equines and cattle from the same livestock farm and during the same period thus one would expect the compound profiles from the two hosts to not differ greatly, however, there are physiological factors that one needs to take into consideration such as: diet, physiological, and general conditions of the individuals. Nonetheless, it is interesting to see that hosts emit a combination of possible attractants and repellents opening up new tactics in which to control and/or monitor nuisance flies like stable flies. The correlation between repellents and attractants emitted by hosts has been noted in different cattle breeds, turning some more susceptible than others. This is a characteristics that been used by introducing less susceptible cattle into highly susceptible herds which results in a decreased infestation of biting flies.

5.5 Conclusion

In conclusion the current study addressed both hosts and their associated oviposition stimulants and found a wide range of chemostimulants that have previously been identified and novel compounds that have not been associated with stable flies. The significant EAG responses detected in host volatiles and oviposition stimulants indicates the use of olfactory cues to find both hosts and a suitable oviposition site. This study has provided both new and deeper knowledge regarding the olfactory use of stable flies that certainly can benefit the development of stable fly control and monitoring management.

5.6 Acknowledgment

Funding for this work was provided by the Brazilian Federal Agency for support and evaluation of graduate education, CAPES and CNPQ. The authors would like to thank EMBRAPA Gado de Corte, Brazil for providing insects.

REFERENCES

BALDACCHINO, F. et al. Transmission of pathogens by *Stomoxys* flies (Diptera, Muscidae): a review. **Parasite.** v. 20, p. 26, 2013

BIRKETT, M. A. et al. The role of volatile semiochemicals in mediating host location and selection by nuisance and disease-transmitting cattle flies. **Medical and Veterinary Entomology,** v. 18, p. 313-322, 2004.

BROCE, A. B.; HOGSETTE, J.; PAISLEY, S. Winter feeding sites of hay in round bales as major developmental sites of *Stomoxys calcitrans* (Diptera: Muscidae) in pastures in spring and summer. **Journal of Economical Entomology,** v. 98, p. 2307-2312, 2005.

DAISY, B. H., et al. Naphthalene, an insect repellent, is produced by *Muscodor vitigenus,* a novel endophytic fungus. **Microbiology,** v. 148, p. 3737-3741, 2002.

DU, Y.; MILLAR, J. G. Electroantennogram and oviposition bioassay responses of *Culex quinquefasciatus* and *Culex tarsalis* (Diptera: Culicidae) to chemical odors from Bermuda Grass Infusions. **Journal of Medical Entomology,** v. 36, p. 158-166, 1999.

GIBSON, G.; TORR, S. J. Visual and olfactory responses of heamatophagous Diptera to host stimuli. **Medical and Veterinary Entomology,** v. 13, p. 2-23, 1999.

HAFEZ, M.; GAMAL-EDDIN, F. M. On the feeding habits of *Stomoxys calcitrans* L. and *sitiens* Rond., with special reference to their biting cycle in nature (Diptera: Muscidae). **Bulletin of Social Entomology Egypt,** v. 43, p. 291-301, 1959.

JEANBOURQUIN, P. The role of odour perception in the sensory ecology of the stable fly, *Stomoxys calcitrans* L. **PhD thesis at Neuchatel University.** 2005.

——; GUERIN, P.M. Sensory and behavioral responses of the stable fly *Stomoxys calcitrans* to rumen volatiles. **Medical and Veterinary Entomology,** v. 21, p. 217-224, 2007.

——; ——. Chemostimuli implicated in selection of oviposition substrates by the stable fly *Stomoxys calcitrans.* **Medical and Veterinary Entomology,** v. 21, p. 209.216, 2007.

KITE, G. C. The floral odour of *Arum maculatum.* **Biochemical Systematics and Ecology,** v. 23, p. 343-354, 1995.

KHAN, Z. R. et al. Intercropping increases parasitism of pests. **Nature,** v. 388, p. 631-632, 1997.

KNIEPERT, F-W. Blood-feeding and nectar-feeding in adult Tabanidae (Diptera)*. **Oecologia,** v. 46, p. 125-129, 1980.

LOGAN, J. G.; BIRKETT, M. A. Review: semiochemicals for biting fly control: their identification and exploitation. **Pest Management Science,** v. 63, p. 647-657, 2007.

_____. et al. Identification of human-derived volatile chemicals that interfere with attraction of *Aedes aegypti* mosquitos. **Journal of Chemical Ecology,** v. 34, p. 308-322, 2008.

MACHTINGER, E. T., et al. Development and oviposition preference of house flies and stable flies (Diptera: Muscidae) in six substrates from Florida equine facilities. **Journal of Medical Entomology,** v. 51, p. 1144-1150, 2014

MIHOK, S.; CARLSON, D. A.; NDEGWA, P. N. Tsetse and other biting fly responses to Nzi traps baited with octenol, phenols, and acetone. **Medical and Veterinary Entomology,** v. 21, p. 70-84, 2007.

MOORING, M. S., et al. Insect-repelling behavior in bovids: role of mass, tail length, and group size. **Biological Journal of the Linnean Society,** v. 91, p. 383-392, 2007.

MBOERA, L. E. G., et al. Sampling gravid *Culex quinquefasciatus* (Diptera: Culicidae) in Tanzania with traps baited with synthetic oviposition pheromone and grass infusions. **Journal Medical Entomology,** v. 37, p. 172-176, 2000.

SALEM, A. et al. Feeding and breeding aspects of *Stomoxys calcitrans* (Diptera: Muscidae) under laboratory conditions. **Parasite,** v. 19, p. 309-317, 2012.

SKUBATZ, H. et al. The sauromattum guttatum appendix as an osmophore: excretory pathways, composition of volatiles and attractiveness to insects. **The New Phytologist,** v. 134, p. 631-640, 1996.

SPINHIRNE, J. P.; KOZIEL, J. A.; CHIRASE, N. K. Sampling and analysis of volatile organic compounds in bovine breath by solid-phase microextraction and gas chromatography – mass spectrometry. **Journal of Chromatography A,** v. 1025, p. 63-69, 2004.

TANGTRAKULWANICH, K. et al. Characterization of olfactory sensilla of *Stomoxys calcitrans* and electrophysiological responses to odorant compounds associated with hosts and oviposition media. **Journal of Medical Entomology.** v. 25, p. 327-336, 2011.

_____. et al. Behavioral responses of stable flies to cattle manure slurry associated odorants: stable fly attracted to manure slurry volatiles. **Medical and Veterinary Entomology,** v. 29, p. 82-87, 2015.

TAYLOR, D. B.; BERKEBILE, D. R. Sugar feeding in adult stable flies. **Environmental Entomology,** v. 35, p. 625-629, 2008.

TORR, S. J.; MANGWIRO, T. N. C.; HALL, D. R. The effect of host physiology on the attraction of tsetse (Diptera: Glossinadae) and *Stomoxys* (Diptera: Muscidae) to cattle. **Bulletin of Entomological Research,** v. 96, p. 71-84, 2006.

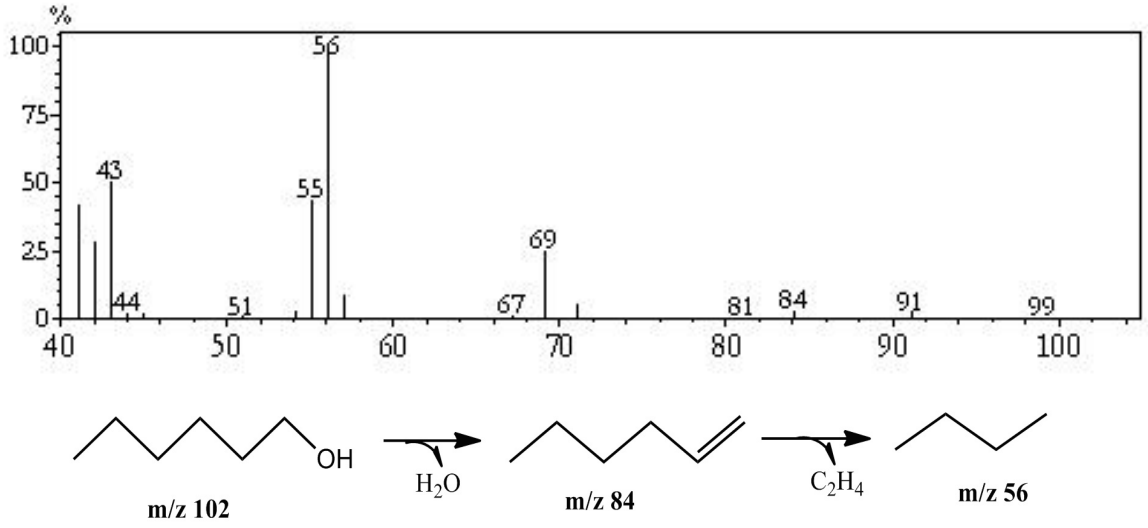
APPENDIX A

The mass spectra and fragmentation pattern of the identified compounds referred to throughout this chapter are presented.

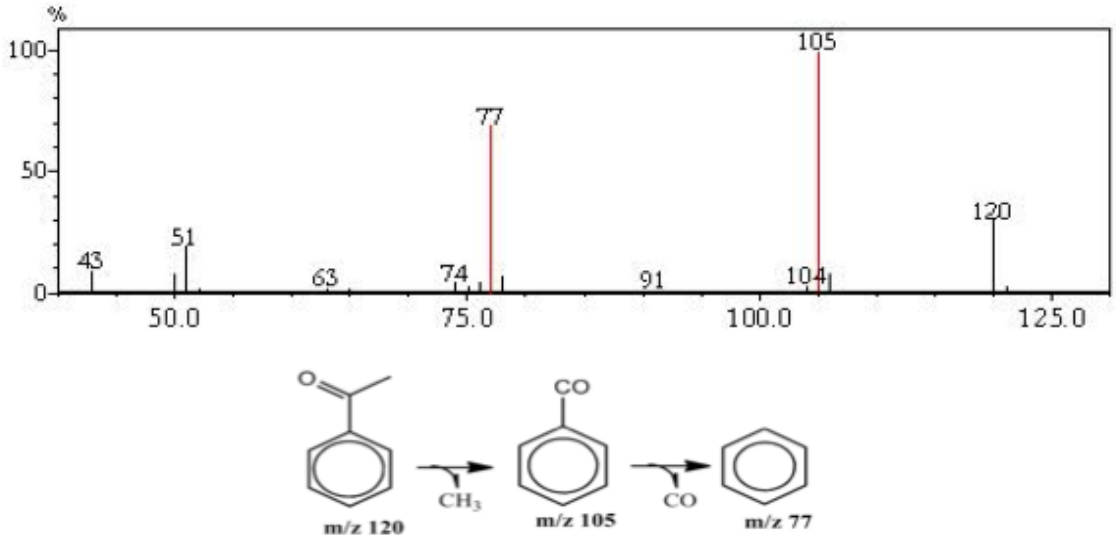
The header for each mass spectrum gives:

- The name of the compound
- Molecular weight
- m/z of each major fragment with its intensity (%)
- Fragmentation scheme

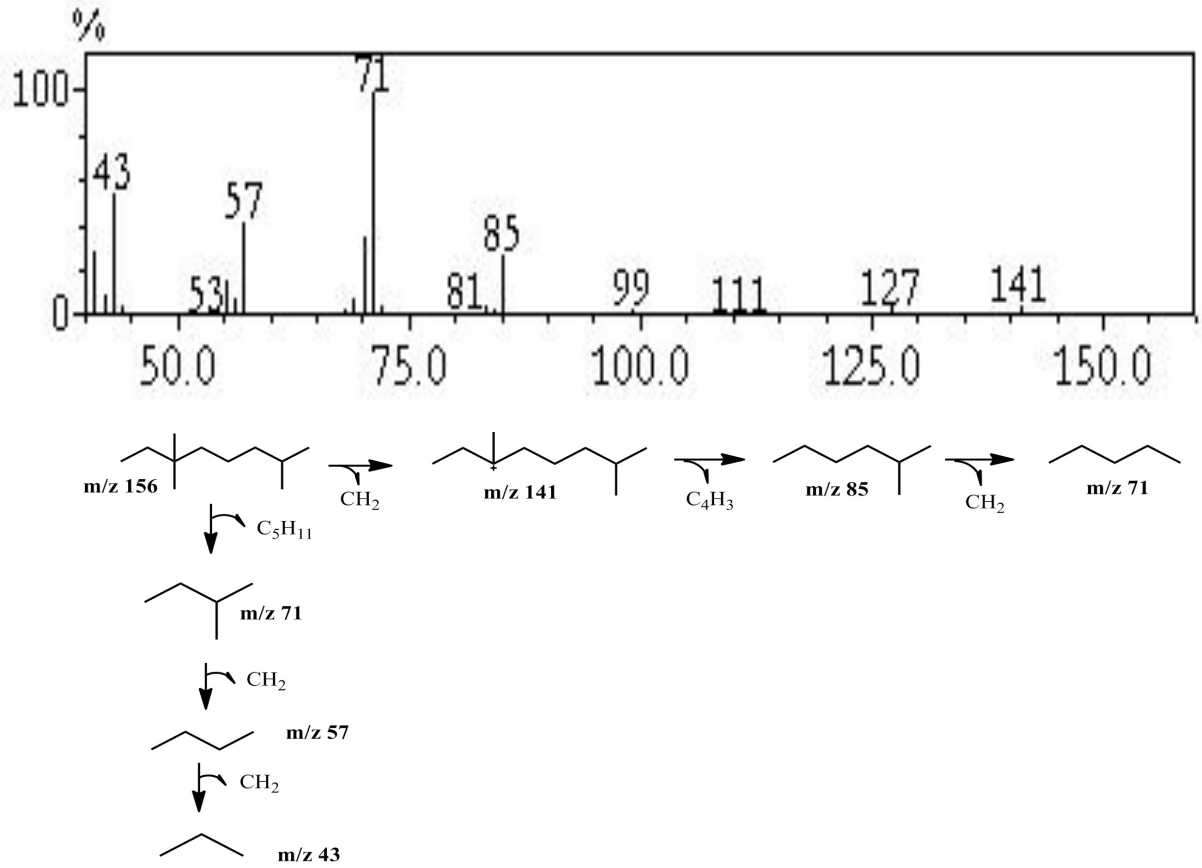
1-hexanol: MW 102, m/z 84, m/z 56 (100).



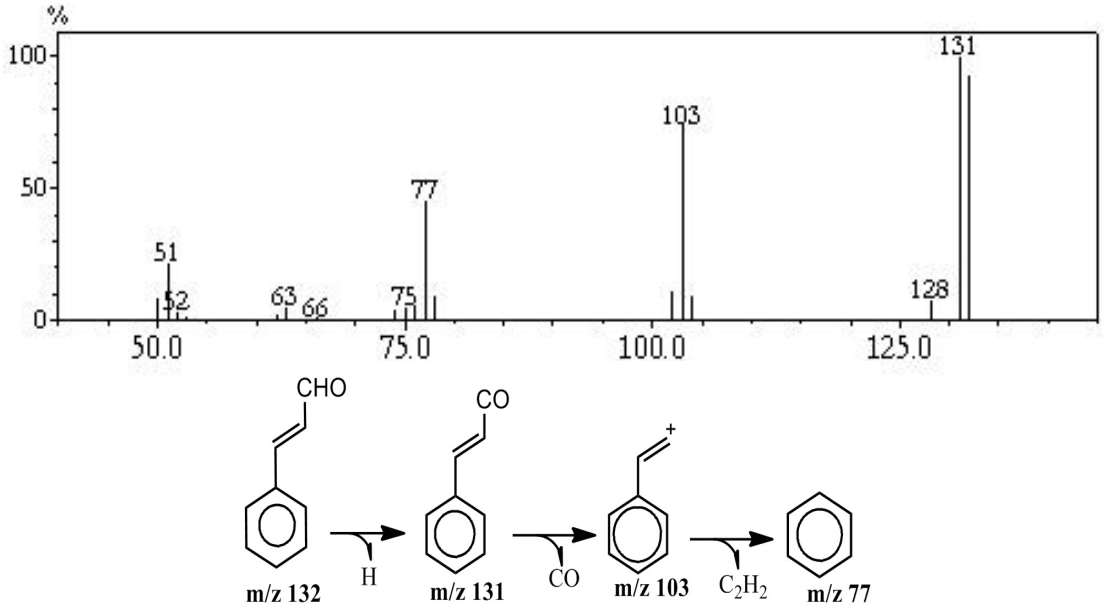
Acetophenone: MW 120 (30), m/z 105 (100), m/z 77 (69).



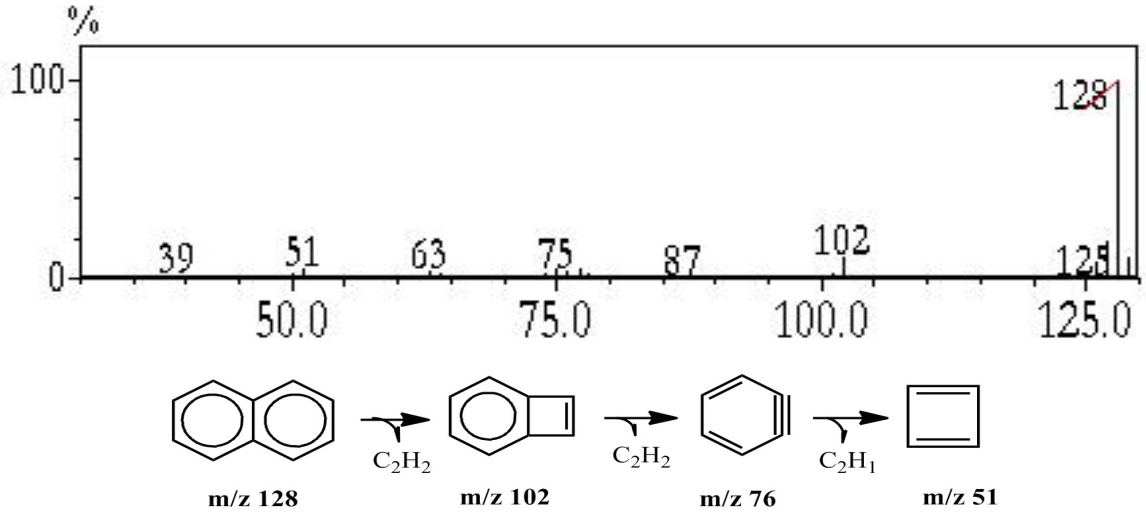
2,6,6-trimethyl octane: MW 156, m/z 85 (28), m/z 71 (100), m/z 57 (42), m/z 43 (55).



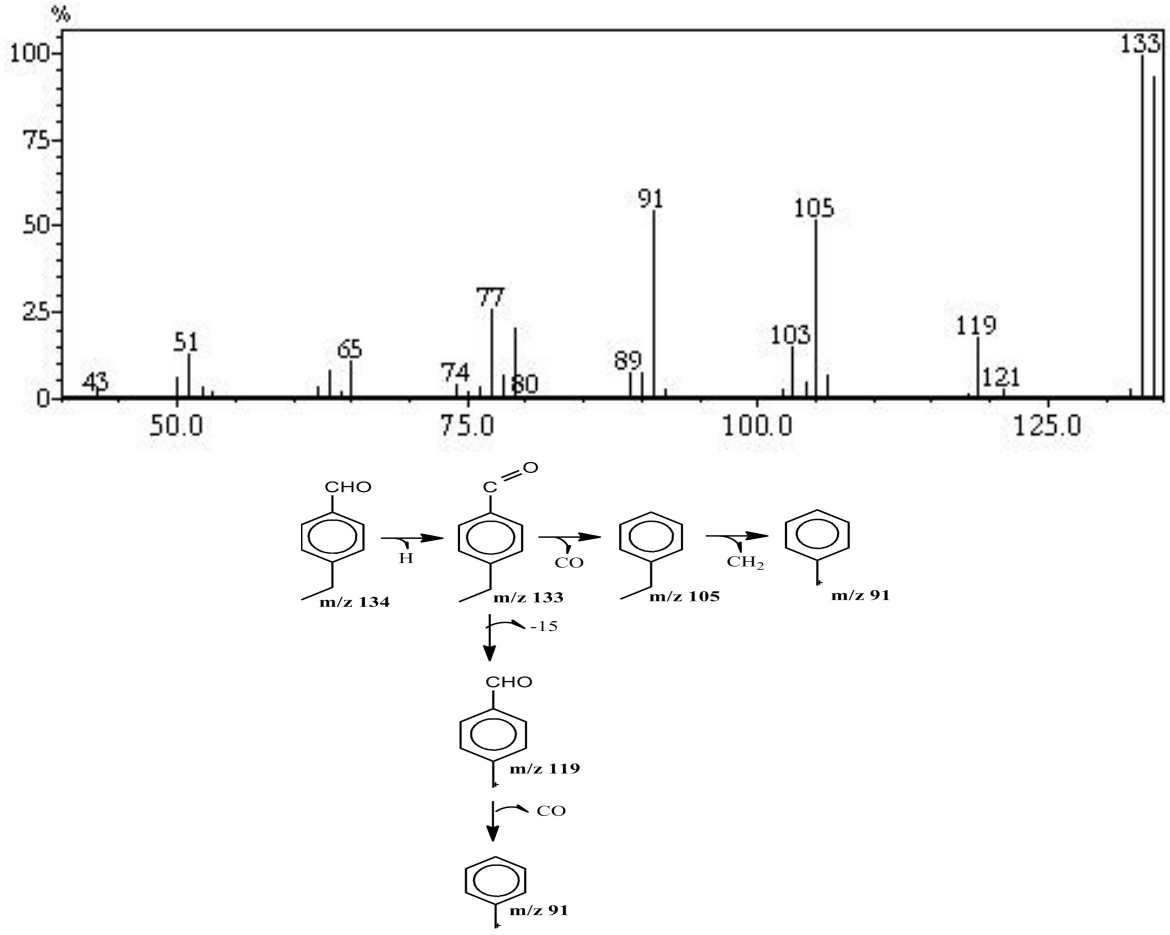
Cinnamaldehyde: MW 132 (93), m/z 131 (100), m/z 103 (74), m/z 77 (45).



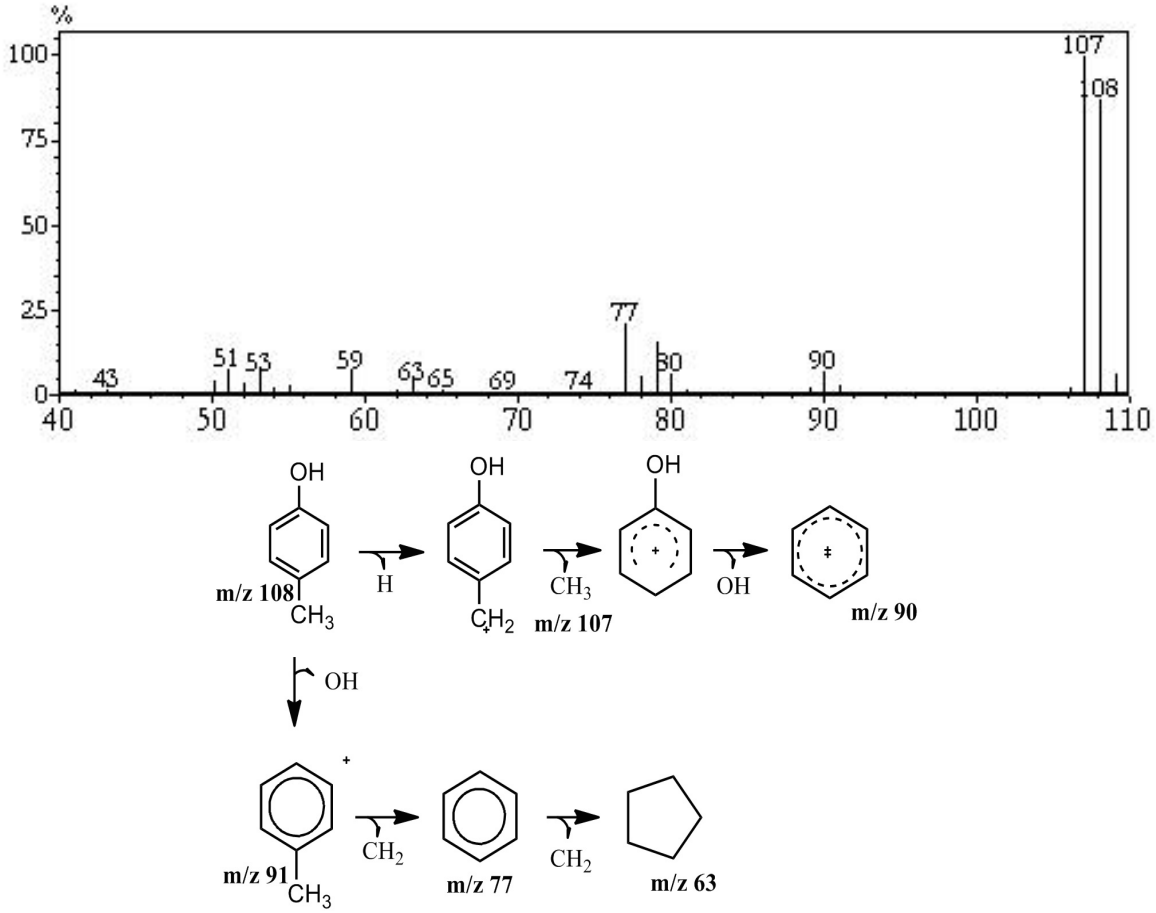
Naphthalene: MW: 128 (100), m/z 102 (11).



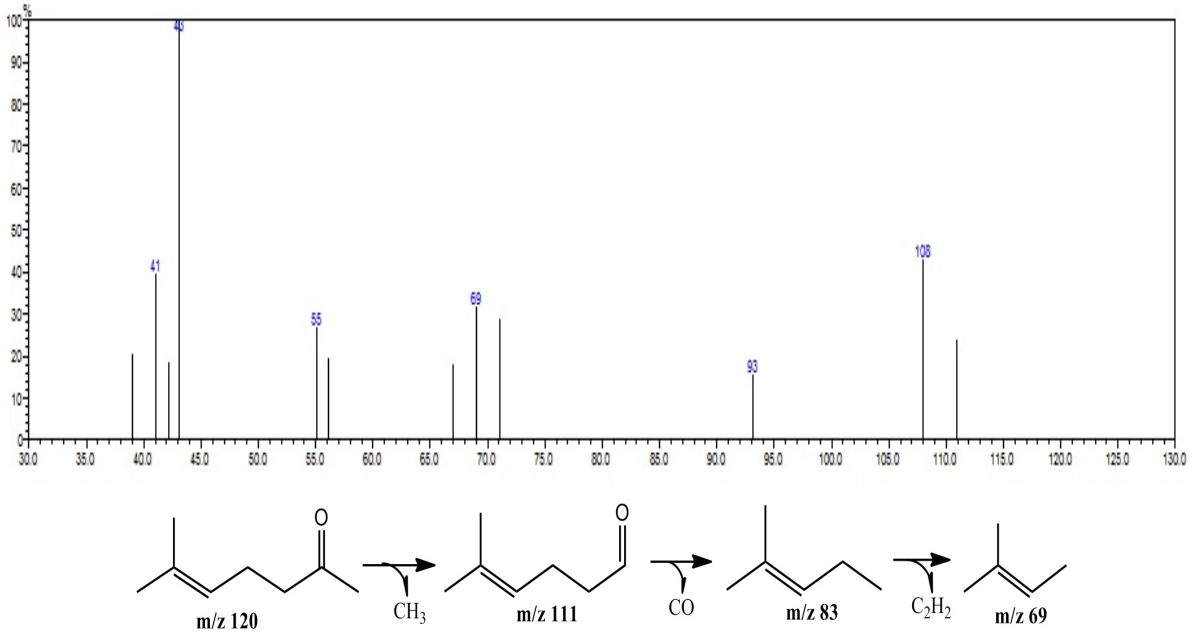
4-ethyl benzaldehyde: MW 134 (93), m/z 133 (100), m/z 105 (52), m/z 91 (55).



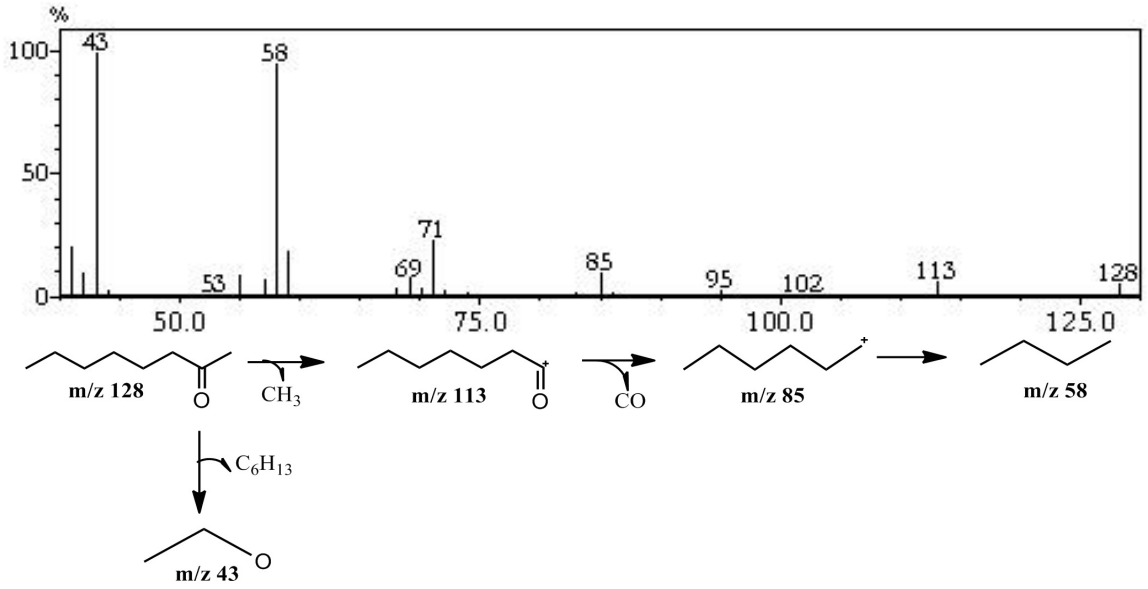
4- methylphenol: MW 108 (87), m/z 107 (100), m/z 77 (21).



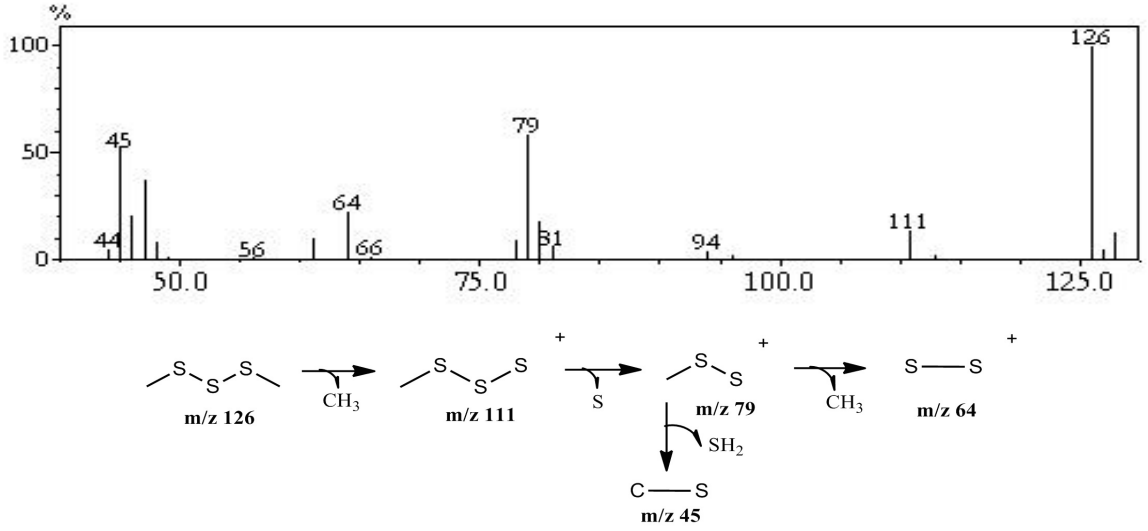
6-methyl-5-hepten-2-one: MW 126, m/z 108 (43), m/z 43 (100).



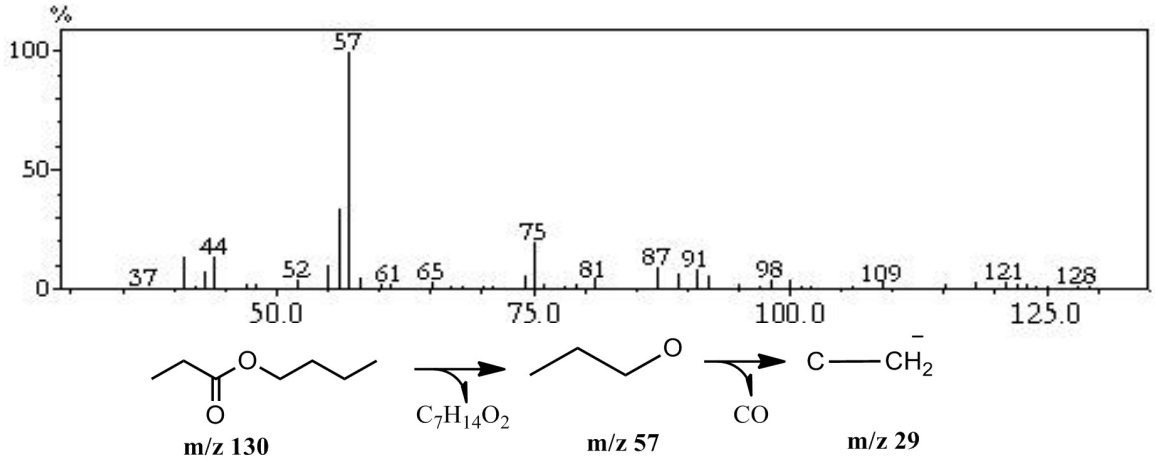
2-octanone: MW 128 (5), m/z 58 (95), m/z 43 (100)



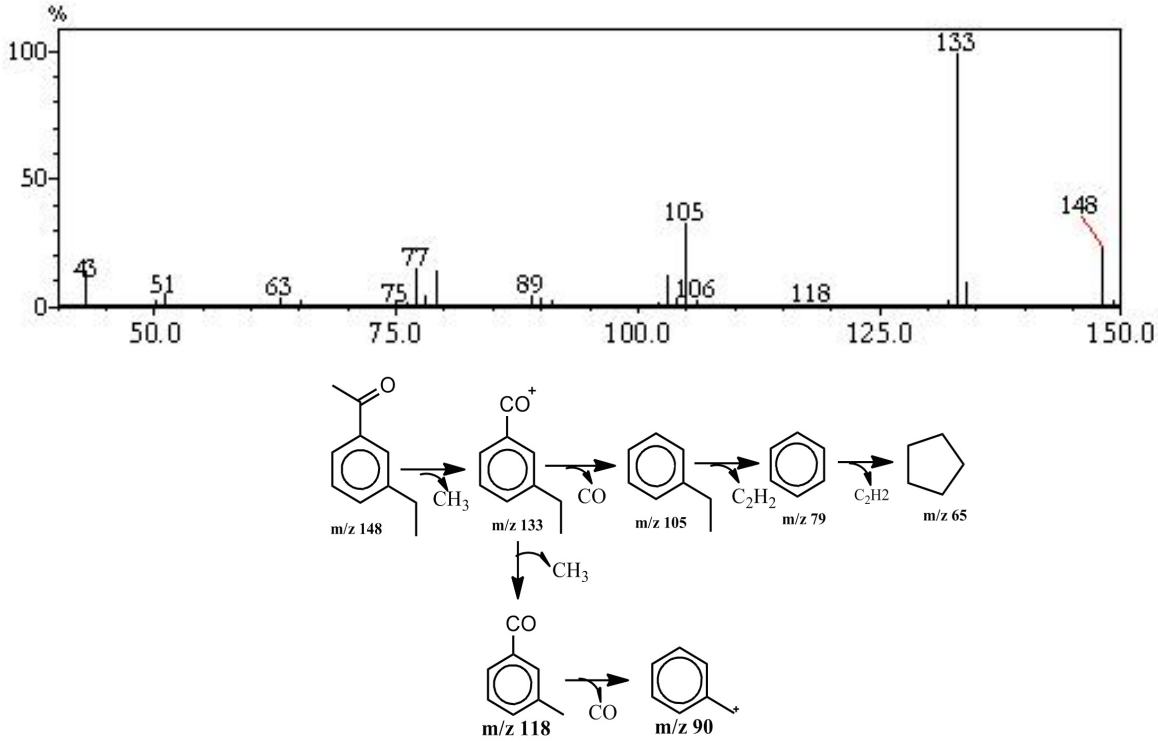
Dimethyl trisuphide: MW 126 (100), m/z 79 (58), m/z 64 (22), 45 (52).



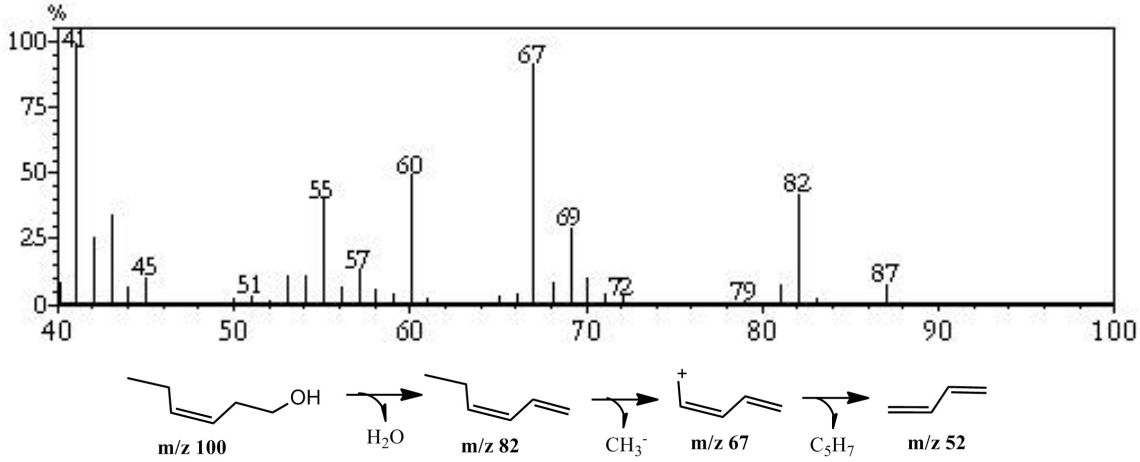
Butyl propionate: MW: 130, m/z 57 (100).



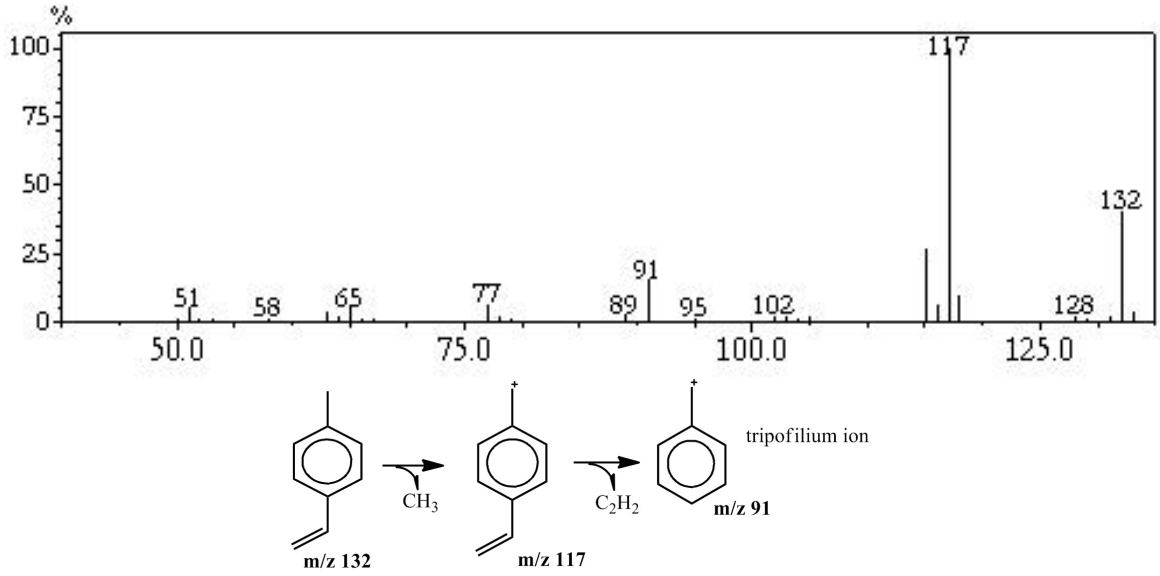
3-ethylacetophenone: MW 148 (23), m/z 133 (100), m/z 105 (33).



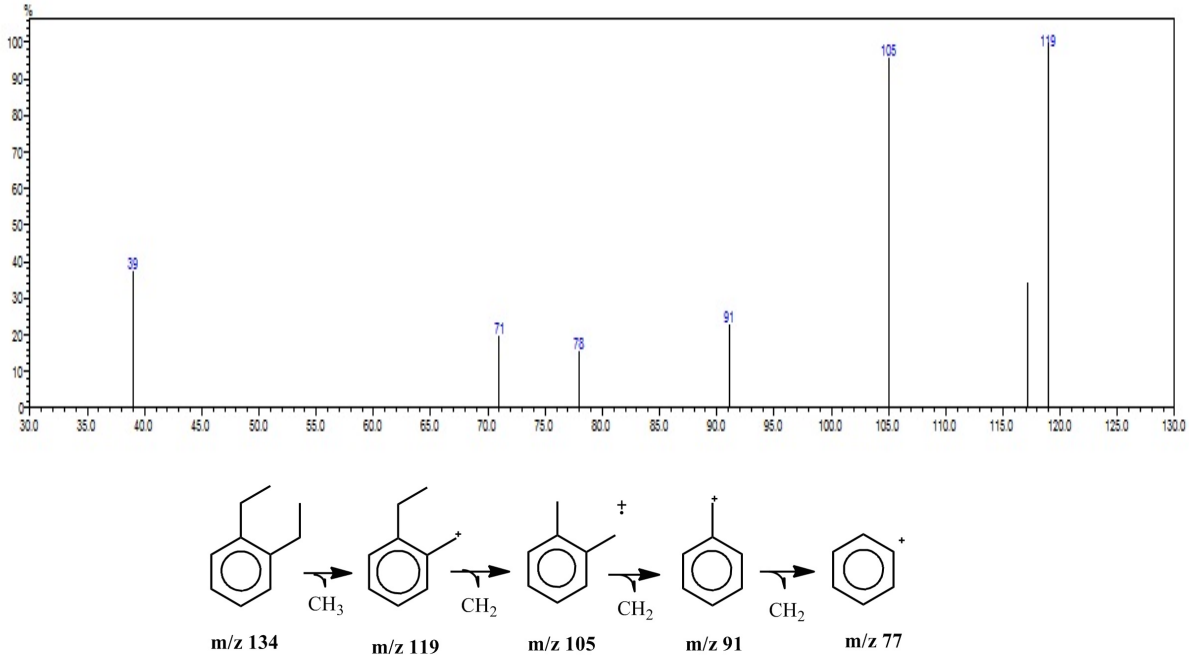
cis -3- hexenol: MW 100, m/z 82 (42), m/z (91), m/z 41 (100)



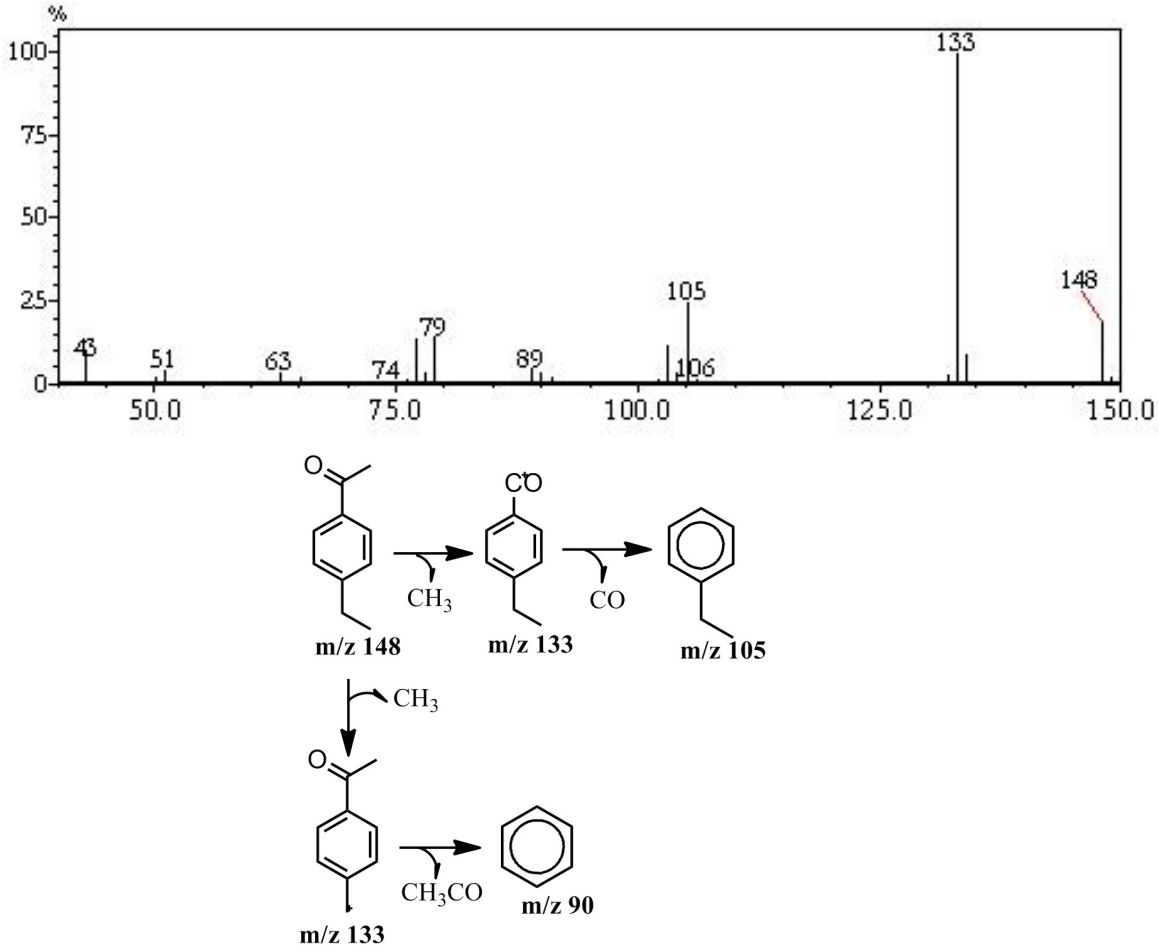
1-ethenyl-4-ethylbenzene: MW 132 (40), m/z 117 (110), m/z 91 (15).



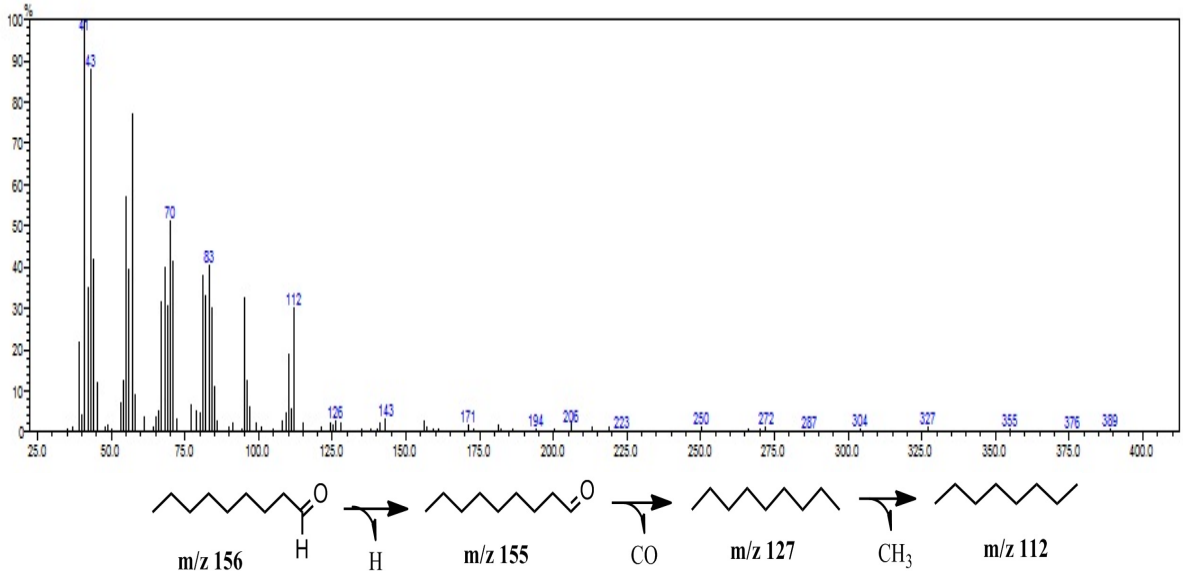
1,2-diethylbenzene: MW 134 (52), m/z 119 (100), m/z 105 (95).



4-ethylacetophenone: MW 148 (15), m/z 133 (100), m/z 105 (24).



Decanal: MW 156, m/z 112 (30), m/z 41 (100).



6 CHAPTER IV: LABORATORY AND FIELD STUDIES DETERMINING ATTRACTANTS RESPONSIBLE FOR THE SEVERE OUTBREAKS OF *STOMOXYS CALCITRANS* (DIPTERA: MUSCIDAE) IN SUGARCANE MILLS.

ABSTRACT

Stomoxys calcitrans (Diptera: Muscidae) a worldwide livestock pest have in recent years had population eruptions in sugarcane crop. This is the first time that the chemical ecology between stable flies and their occurrence in sugarcane crop is investigated. The aim is to expand current knowledge and also identify attractants that could be used as pest management tools. Both laboratory and field studies showed that sugarcane derived fertilizer, vinasse, attracts both mated and unmated female stable flies. Chemical analyses identified a wide range of carboxylic acids, alcohols, phenols, and aldehydes as chemical attractants.[2]

Key **W**ord**s**. GC-EAG. GC-MS. Integrated vector management. Agro-ecosystem. Oviposition sites.

[2] **This paper was submitted to Bulletin of Entomological Research (2026).**

6.1 INTRODUCTION

Stomoxys calcitrans L. (Diptera: Muscidae) also known, as stable flies are a major livestock pest that regardless of being heamatophagous have shown to successfully survive based on a sugar diet (SALEM et al., 2012; TAYLOR, et al., 2008). The natural habitat of stable flies is in close proximity to livestock and decaying organic matter, however, in recent years there have been outbreaks associated with different types of crop (SOLORZANO et al., 2015).

Sugarcane is the most recent crop that has had an increased number of stable fly occurrences. The expansion of sugar- and alcohol industries is believed to be one of the reasons behind the outbreaks, as pasturing land is going from cattle use to sugarcane plantations (BARROS et al., 2010; ODA et al., 2010). The economical impact that these nuisance flies have on livestock production are great, and with their expansion into agricultural crop there is a high risk of agricultural losses (TAYLOR et al., 2012; GRISI et al., 2014). The actual damage they cause on the sugarcane has not yet been determined but larvae and pupas have been found inside sugarcane stems thus showing potential threat (CANCADO et al., 2013; KASSAB et al., 2012; KOLLER et al., 2009).

The appearance of stable flies in sugarcane mills has been associated with vinasse and filter-cake application, two fertilizers derived from biomass distillation from sugarcane crop (PRADO et al., 2013). The use of vinasse in irrigation has almost completely replaced the use of chemical fertilizers. It is considered to be a more cost-effective investment and a fast application method that prevents the discharge of vinasse in rivers whilst increasing crop yield due to its high potassium content (BARROS et al., 2010; KOLLER et al., 2009). These byproducts are composed of both inorganic and organic compounds with high nutrient concentrations and are used in large scales for irrigation (ROLLIM et al., 2013). Vinasse irrigation is not always monitored leading to large puddles of stagnant vinasse which in turn creates a foul smelling decomposing environment perfectly adequate for stable flies (DOMINGHETTI et al., 2015; LEITE et al., 2013)

Pests attacking sugarcane crop are often prevented through the release of natural enemies as it manages to specifically target the vector in question or through pesticide application (POTTING et al., 1995; SILVA et al., 2012; VAN LENTEREN, et al., 2003). The parasitoid wasp *Spalangia cameroni* (Hymenoptera: Pteromalidae) is

commercially available against stable flies however they are not considered efficient in highly infested areas (WEINZIERL et al., 1998; PITZER et al., 2011; PITZER et al. 2010; MARCON et al., 1997). It is therefore crucial to understand the behavioral attraction in order to develop a targeted control approach.

Current knowledge covers the appearance of this heamatophagous pest in the agriculture but the underlying reasons remain unknown. The idea of the sugarcane derived fertilizers being responsible for attracting stable flies to sugarcane mills is a hypothesis that remains unconfirmed.

This study aims to explore this hypothesis through both behavioral and chemical analyses in order to increase current knowledge and also provide a reference for future studies involving stable flies and their occurrence in crop.

6.2 Methods and Material

6.2.1 Insects

For the behavioral assays, *Stomoxys calcitrans* pupae were obtained from a reared laboratory colony in EMBRAPA Gado de Corte, Campo Grande, MS, Brazil. Females were divided into mated and unmated by comparison of their abdomen and separating females right after emergence. Flies were left unfed for 24 hours before conducting experiments.

To conduct electrophysiological studies *S. calcitrans* were obtained from a horse stable, Mary Clark Farias, in the Municipality Coruripe, AL, Brazil. Prior to use, all flies were sexed based on the dimorphism of their compound eyes.

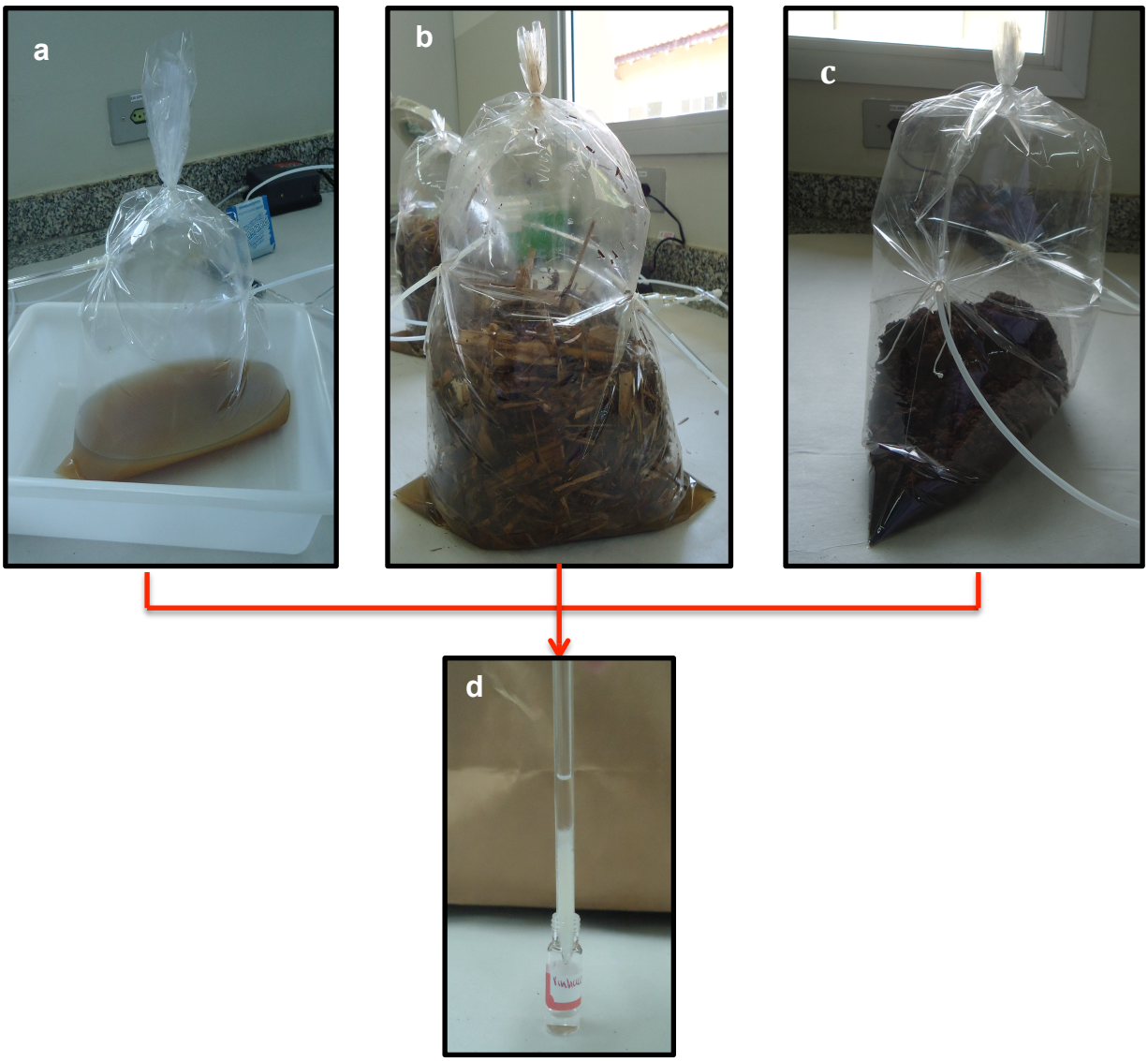
6.2.2 Volatile collection

Volatile organic compound (VOC) collection was carried out in the Laboratory of Natural Resources, UFAL, using air entrainment during two hours with Porapak Q (100 mg, 50 – 80 mesh, Supercoil, Poole, UK) as the adsorbent. All connections were made with PTFE tubing and ferrules, flow rates were set at 1400 mL min^{-1} in and 400 mL min^{-1} out, creating a positive pressure. Oven roasting bags made of poly(ethylene terephthalate) (PET) were used for each headspace collection. A 750-µL aliquot of re-distilled HPLC degree hexane (Sigma-Aldrich, Brazil) was used as

the extracting solvent. Samples were collected in two mL headspace vials (Sigma-Aldrich, Brazil) and stored in a freezer (-20 °C) until use. Volatile organic compounds were collected from three possible oviposition stimulants present in sugarcane mills: vinasse, straw mixed with vinasse, and filter cake.

The vinasse was obtained directly from a sugar-alcohol refinery factory situated in Campo Grande, MS, Brazil. One liter of vinasse (**V**) was used per headspace collection (Figure 1 a). A mixture of sugarcane straw and vinasse was made and left to ferment for two and five days under sun exposure to simulate oviposition stimulants (1 L of vinasse and 500 g of dried sugarcane straw, Figure 1 b). Filter cake (**FC**) was obtained from the same sugarcane refinery. 500 g of filter cake were left to ferment for two and five days before collecting VOCs (Figure 1 c). Volatile organic compounds were also collected from vinasse obtained from a sugar-alcohol refinery located in a different state, Boca da Mata, AL, Brazil.

Figure 1 - a-d: Volatile collection of possible oviposition stimulants present in sugarcane mills. (a) Fresh vinasse; (b) Sugarcane straw mixed with fresh vinasse; (c) Filter cake. Material was obtained from a sugar-alcohol refinery industry in Campo Grande, MS, Brazil. (d) All extractions were made with 750 µL of re-distilled hexane.



Source: Author, 2016.

6.2.3 Laboratory behavioral studies

Bioassays were conducted in order to assess the behavioral response of mated and unmated female *S.calcitrans* to the obtained VOCs samples. A modified Y-tube olfactometer (length per arm = 10 cm; diameter = 1.5 cm) was used for all bioassays (Figure 4 a, Chapter III). Each assay was conducted in a controlled environment with a temperature of (24 °C) and humidity (70 %) with a photoperiod of 12:12 hours. Air (1000 mL min^{-1}) was passed through an activated charcoal filter to remove chemical contaminants and then split and passed into two glass vessels containing test material and control entering the two arms of the olfactometer. Ten microliter aliquots of each sample was loaded onto filter paper (Whatman No. 1.20 mm) and placed in the center of each end of the Y-tube olfactometer. Insects were released one by one into the stem of the olfactometer, when the fly proceeded further than one third of one of the arms it was deemed to have made a choice (three minutes were allowed per replicate, Figure 4 b, Chapter III). Twenty-five unmated and mated female flies were used per treatment, respectively. To statistically analyze the results of each bioassay a two-way classification chi-squared analysis was conducted. A degree of freedom of one was used and the Yate's correction of continuity was applied. Obtained values were determined significant by consulting the chi-squared distribution table.

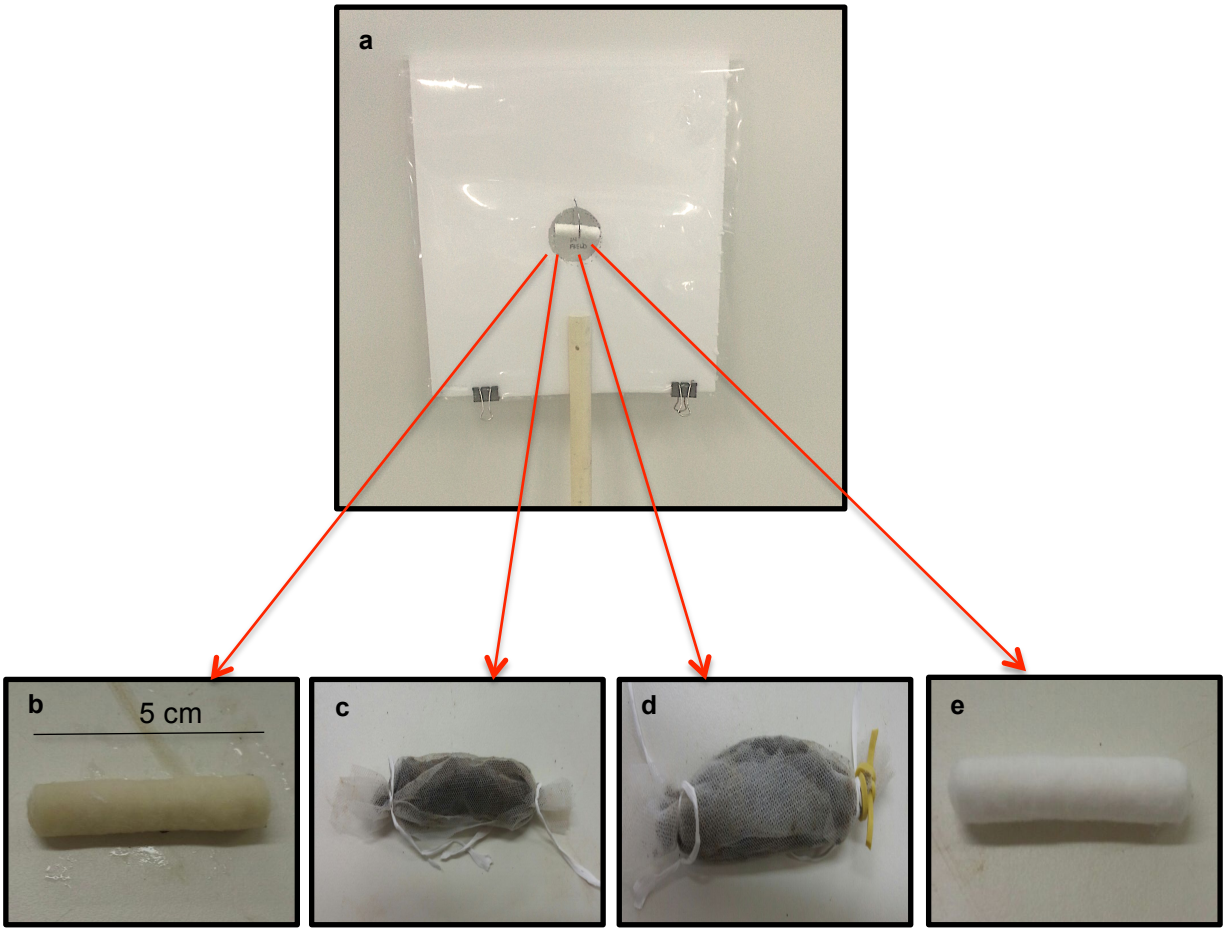
6.2.4 Field studies

In order to assess the efficiency of vinasse and filter cake in enhancing stable fly trapping field studies were conducting. White panel traps made of polysterene (28 x 28 cm and 0.3 cm thickness) were used. A five cm diameter hole was cut on the centre of the panels. A transparent plastic sleeve (58 x 58 cm) with a five cm hole was coated with BioStop adhesive glue, and attached to each panel (Figure 2 a). Traps were baited with vinasse (one mL) on a five cm long cotton roll, filter cake (50 g) suspended with thin cotton mesh, filter cake + vinasse (50 g + one mL) suspended with thin cotton mesh, and control (water) on a five cm long cotton roll (Figures 2 b, c, d, e). Lures were placed with metal wires in the centre of the white panel traps.

Trapping experiments were conducted in a horse stable, Mary Clark Farias, in Coruripe, AL, Brazil (Figure 3 a). Traps were arranged in a randomized complete

block with three replications per treatment plus control. Traps were placed inside the horse stables about two meters apart and 50 cm above ground (Figure 3 b). Trap catches were checked daily throughout three weeks (Week-days). Plastic sleeves and lures were changed daily. The Scott-Knott test of variance was used to determine statistical significance between treatments.

Figure 2 - a-e: (a) White panel trap covered with BioStop glue used to trap *Stomoxys calcitrans* . (b) lure - cotton roll soaked with vinasse. (c) lure – filter cake inside cotton mesh. (d) lure - filter cake + vinasse inside cotton mesh. (e) control – cotton roll soaked with water.



Source: Author, 2016.

Figure 3 - a-b: (a) Horse stables, Mary Clark Farias in Coruripe, Brazil, where trapping of *Stomoxys calcitrans* was conducted. (b) White panel trap baited with filter cake inside a horse stable underneath the feeding box.

Source: Author, 2016.

6.2.5 Electrophysiological studies

Electroantennogram (EAG) recordings were made using Ag-AgCl electrodes and glass capillaries filled with Ringer solution (8.0 g L^{-1} NaCl, 0.4 g L^{-1} $CaCl_2$) connected to silver wire, closing the electric circuit. Flies were sexed before use and chilled for one minute before excising the head. The head was placed within the indifferent electrode and the tip of one of the antenna was inserted to the recording electrode (Figure 5, Chapter III). The signals were passed through a high impedance amplifier (IDAC-4, Syntech 2004, Hilversum, Netherlands) and analysed using a customized software package (Syntech, NL 4.6, 2008).

Separation of the VOCs was achieved on a gas chromatography (GC) (Schimadzu, GC-2010) equipped with a flame ionization detector (FID), using an NST05 column (30 m x 0.25 mm i.d. x 25 µm film; Restek Corporation). Three microliter aliquot of each sample was injected in the splitless mode at an oven temperature of 40 °C for five minutes and then programmed at 3 °C/min to 130 °C for two minutes, then programmed at 10 °C/min to 250 °C. Electronic flow control was used to maintain a constant hydrogen carrier gas flow of 1.21 mL min $^{-1}$. The outputs from the EAG amplifier and the FID were monitored simultaneously and analysed using Synthech software package.

Peaks eluting from the GC column were judged to be active if they elicited an antennal depolarization in three or more runs (*N*= 6 per treatment).

6.2.6 Compound identification

For chromatographic analysis, the volatile samples that elicited an electrophysiological activity were injected into two GCs coupled to a mass spectrometer (GC/MS), Schimadzu, QP2010, one utilizing a non-polar NST-05 column (30 m x 0.25 mm i.d. x 0.25 µm film, Restek Corporation) and the other an polar RTX-WAX column (30 m x 0.25 mm i.d. x 0.25 µm film, Restek Corporation) with helium as the carrier gas. The same method was used for both columns, starting with an initial oven temperature of 40 °C for five minutes, programmed to increase at 3 °C/min to 130 °C for two minutes, then programmed at 10 °C/min to 250 °C.

One microliter aliquot of each sample was injected in splitless mode. An alkane standard mixture (C_7 – C_{30} - Sigma Aldrich, USA) was injected to determine the retention indices of each compound. Compounds were identified and confirmed by mass spectrometric fragmentation patterns, retention indices, and co-injection with synthetic standards. All solvents and reagent compounds were HPLC grade and purchased from Sigma-Aldrich, Brazil/ Sweden.

The chromatographic profile data of each sample analysed in the GC - MS were prepared for exploratory statistical multivariate tests using Multi Chem System® (Chemistry and Biotechnology Institute, Federal University of Alagoas). A dendogram was created based on the similarity of the chemical profile of each sample using a cluster analysis by correlation coefficients from standardized data and complete linkage from Euclidean distance method. However to determine the chemical

compounds responsible for the variation between each profile, the data was subjected on Principal Component Analysis presented in Scoreplot and Biplot. The statistical software used for these analyses was Minitab.

6.2.7 Dose-response curve using EAD

Synthetic standards of the identified compounds were used individually to conduct a dose response curve. Three doses of each synthetic analogue were tested (10 ppm, 50 ppm, and 100 ppm). The doses were determined based on an estimate of the quantity of each compound present in the collected VOCs samples.

Electroantennogram (EAG) recordings were obtained to plot a dose-response curve. The EAG system consisted of a high-impedance D.C amplifier with automatic baseline drift compensation (SYNTECH Equipment and Research, Kirchzarten, Germany). SYNTECH EAG-Pro 4.6 software was used to record and analyse the amplified EAG signals. Ten microliters aliquot of each dose were added onto a filter paper (Whatman No. 1,20mm), which was inserted into sterilized Pasteur pipettes. The stimuli was delivered over the preparation in a constant 1 $L.min^{-1}$ airstream and applied (two sec duration) at 30 sec intervals. Ten repetitions were conducted per dose. Control (hexane) was tested at the beginning and end of each repetition.

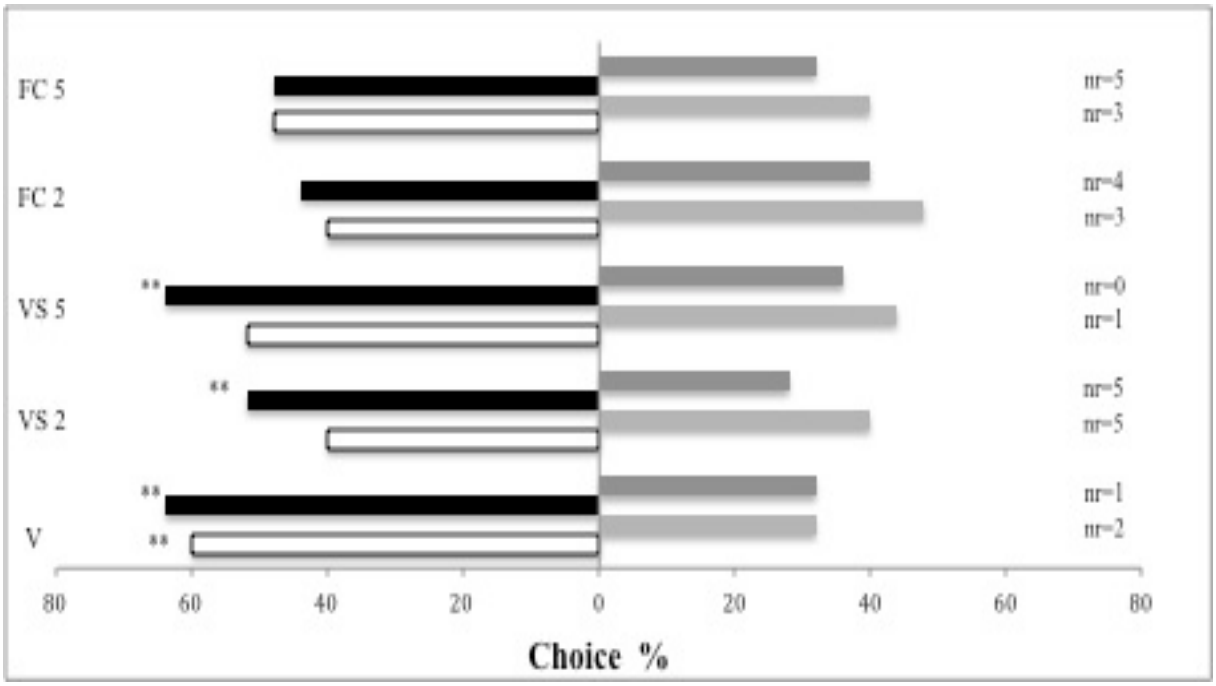
An “F” test with variance analysis to polynomial regression was used to determine the actual probability of error for each synthetic analogue. The significance equations were modelled and subjected to differential calculus to determine the right dosage for maximum effective response to treatment. Statistical Analysis and Genetics, SAEG, V.5. UFV – 1995 and Maple – Mathematics modelling and simulation system (MapleTM V.12) Maplesoft/Waterloo Maple Inc 2011, software were used.

6.3 Results

6.3.1 Behavioral assays using *S.calcitrans*

Behavioral assays using oviposition stimulants associated with sugarcane mills showed that fresh vinasse was significantly attractive to both mated and unmated female flies (Figure 4, P = 0.0035 and P = 0.001, respectively). Sugarcane straw mixed with vinasse at both fermenting stages was only significantly attractive to mated female flies (Figure 4, P = 0.0073 and P = 0.0051, respectively). Filter cake was not significantly attractive at any fermenting stage to mated (Figure 4, P = 0.66252 and P = 0,073638) or unmated female flies (Figure 4, P = 0.393768 and P = 0,393768).

Figure 4 - Behavioral assay using a Y-tube olfactometer to test volatile organic samples collected from five possible oviposition stimulants present in sugarcane mills: vinasse (V), mixture of vinasse and sugarcane straw with 2 days of fermenting (VS2), mixture of vinasse and sugarcane straw with 5 days of fermenting (VS5), filter cake with 2 days of fermenting (FC2), and filter cake with 5 days of fermenting (FC5). White bars = unmated female *S.calcitrans*. Black bars = mated female *S.calcitrans*. Grey colored bars = control. *N*=25 per treatment. **= p<0.01. nr = no response.

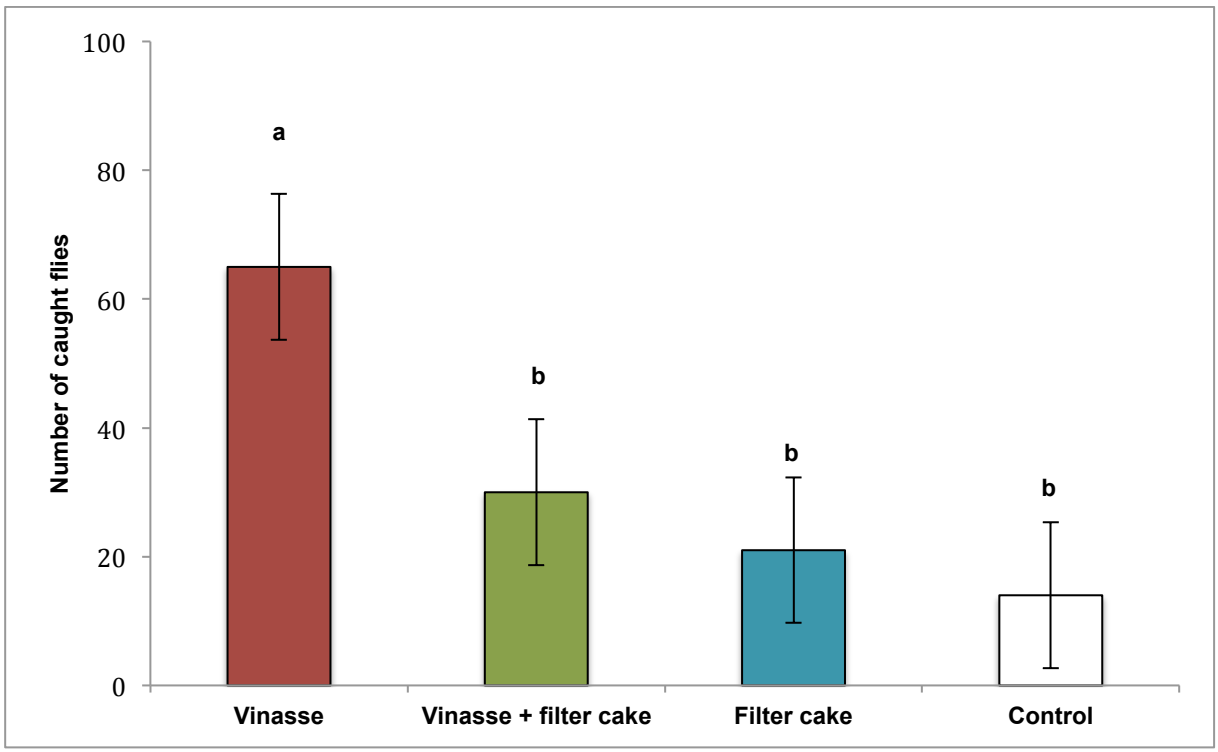


Source: Author, 2016.

6.3.2 Field studies

Traps baited with vinasse caught four times more *S.calcitrans* than non-baited traps (Figure 5). However, traps baited with both vinasse and filter cake reduced the number of caught *S.calcitrans* by half, making it not significant in comparison to the non-baited traps. Filter cake alone did not catch a significant amount of *S.calcitrans* in comparison with the non-baited traps.

Figure 5 - Number of stable flies captured in white panel traps with four different lures: vinasse, filter cake, vinasse + filter cake, and control (water). Different letters on top of column indicate significant differences (Scott-Knott analysis, p<0.01).



Source, Author, 2016

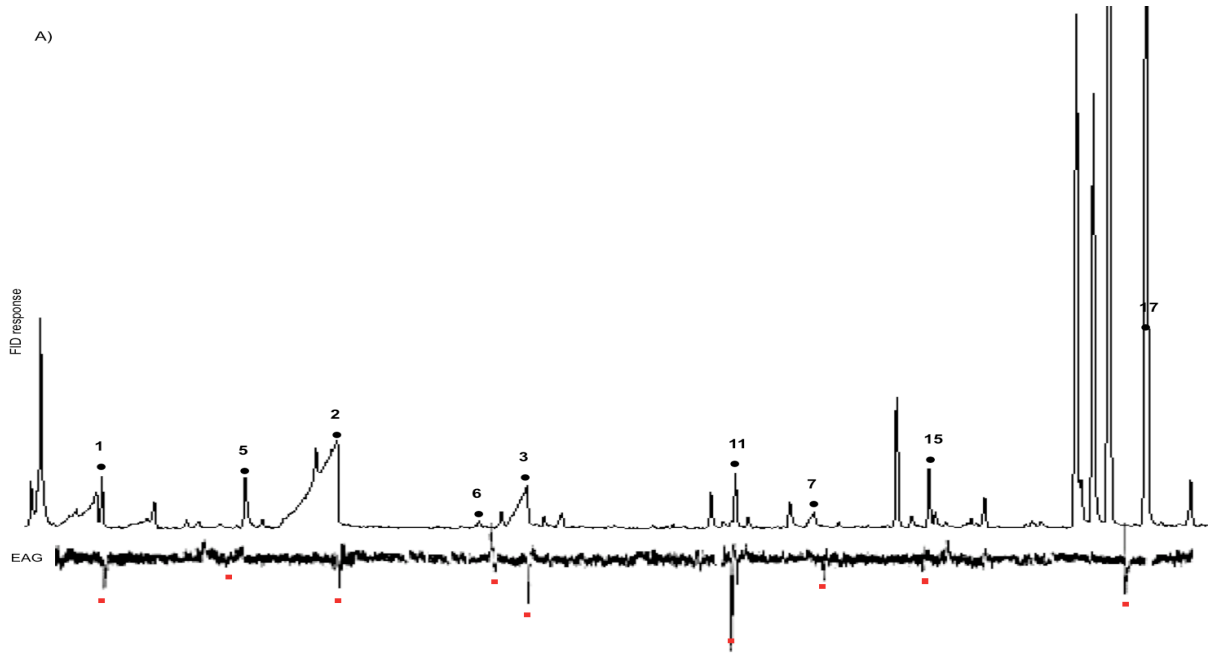
6.3.3 Chemical and electrophysiology studies

Electrophysiological studies showed that vinasse obtained from Campo Grande, MS, elicited nine EAG responses while vinasse from Boca da Mata only elicited four (Figure 6 a-b). Straw + vinasse with two days of fermenting elicited eight EAG responses (Figure. 6 c) while the mixture left to ferment for five days triggered three EAG responses on the female antenna (Figure 6 d). In total, 19 compounds present in the collected VOCs samples elicited a consistent antennal depolarization in *S.calcitrans* female (Figure 6 a-d).

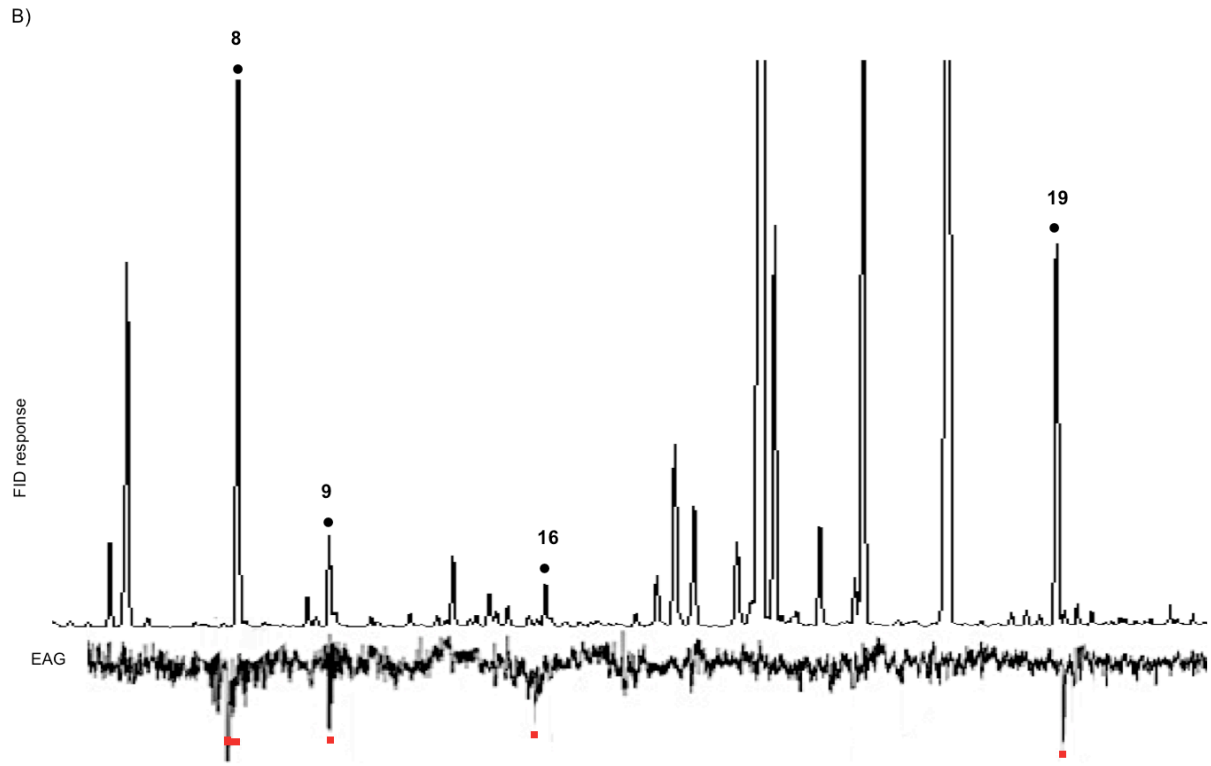
GC - MS analysis of the EAG active samples identified 18 compounds which revealed a mixture of low molecular mass compounds including: carboxylic acids, aliphatic alcohols, phenols and aldehydes (Table 1).

Chemical analyses of each sample revealed a varied compound profile. Cluster analyses of compounds present in vinasse and mixture of sugarcane and vinasse samples, demonstrated that regardless of fermentation duration there is still a significant similarity (Figure 7 a). Whilst a biplot analysis of the retention indices clarified that the compound profile between vinasse from two different states are completely different (Figure 7 b).

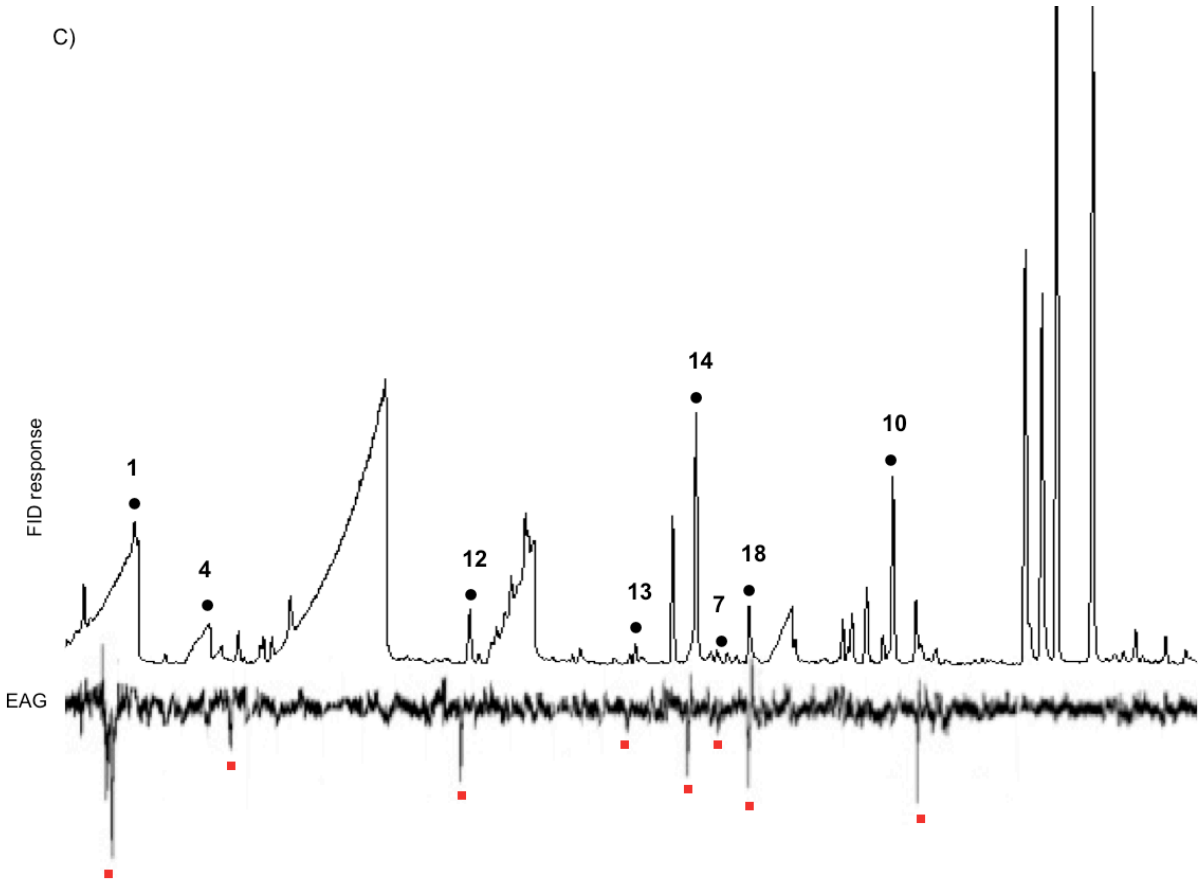
Figure 6 - a,b,c,d: GC-EAG analysis of volatile compounds using the antenna of female *S.calcitrans.* (a) GC-EAG analysis of vinasse from Campo Grande, MS. (b) GC-EAG analysis of vinasse from Boca da Mata, AL. (c) GC-EAG analysis of vinasse (Campo Grande) mixed with sugarcane straw with two days of fermenting. (d) GC-EAG analysis of vinasse mixed with sugarcane straw with five days of fermenting. *N* = 6 per treatment. Peak number correlate to compound listed in Table 1.



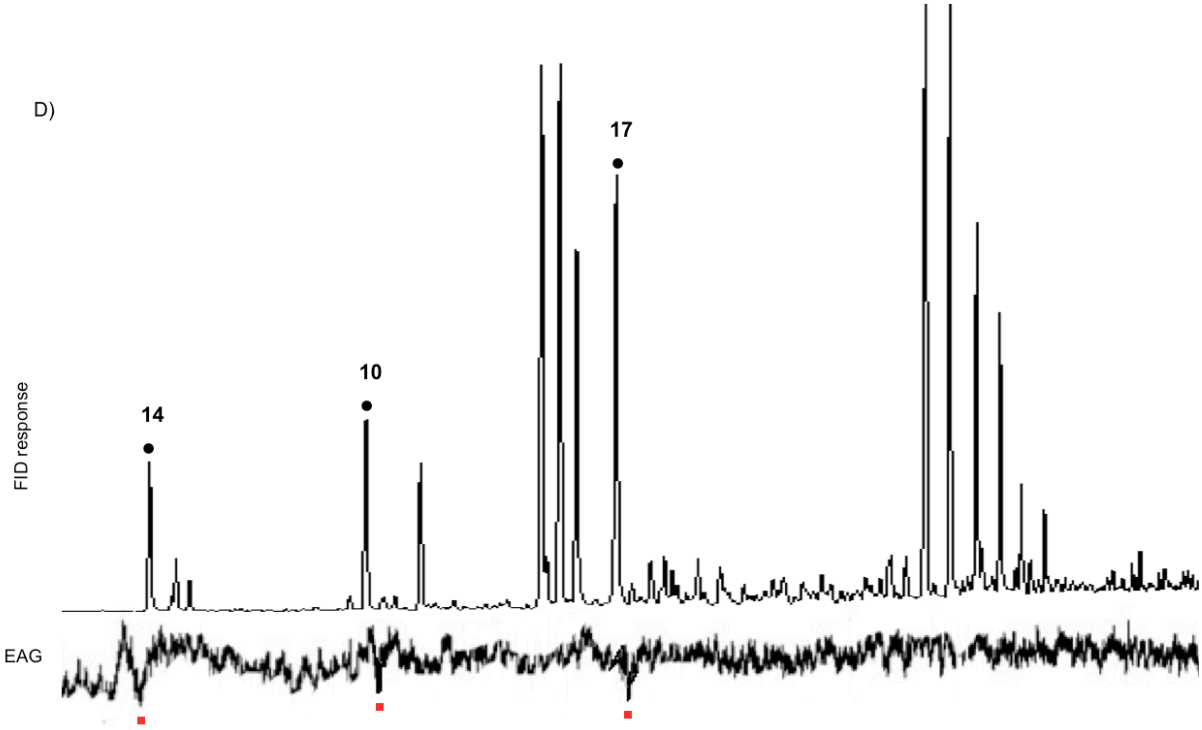
Source: Author, 2016.



Source: Author, 2016.



Source: Author, 2016.



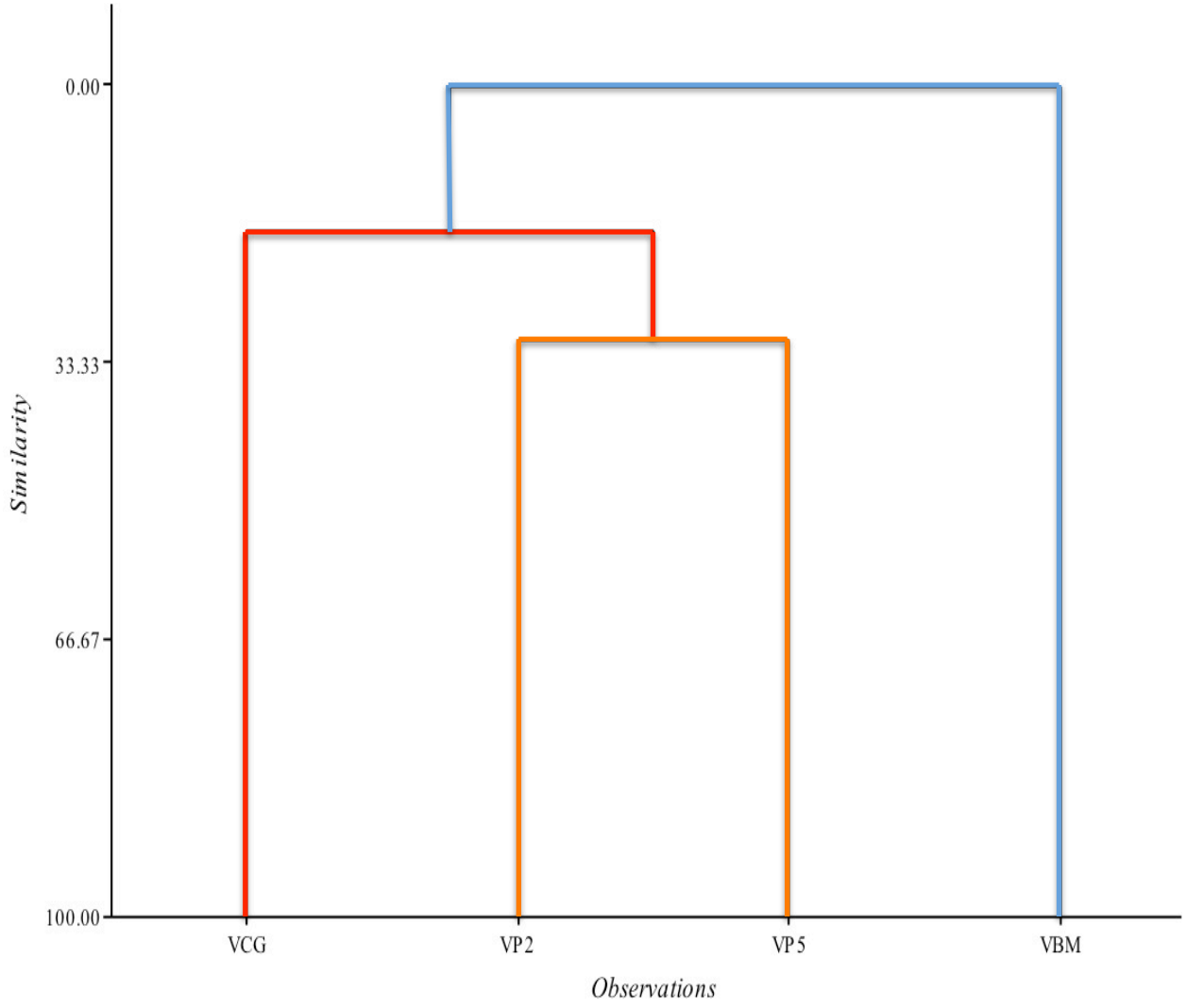
Source: Author, 2016.

Table 1 - EAG active compounds identified by coupled GC-MS and co-injection with standard synthetic compounds. 18/19 compounds considered EAG active were identified, comprising a high proportion of aliphatic alcohols, carboxylic acids, phenols, and aromatic compounds together with non-polar aromatics. RI= Retention Index; of identified compounds (1) NST-05 column; (2) RTX-WAX column. Asterisk indicates compounds confirmed through fragmentation analyses and not through co-injection of synthetic analogue (See appendix 1, Chapter IV). VCG = vinasse from Campo Grande; VBM = vinasse from Boca da Mata; VS2 = vinasse and sugarcane straw with 2 days of fermenting; VS5 = vinasse and sugarcane straw with 5 days of fermenting.

Classification	Peak n°. (Figs. 6a-d)	Compound name	RI[1]	RI[2]	VCG	VBM	VS2	VS5
Acids	1	Butanoic acid	822	1600	*		*	
	2	Pentanoic acid	908	1708	*			
	3	Hexanoic acid	1018	1845	*			
	4	3-Methylbutanoic acid	861	1600			*	
Alcohols	5	Hexan-1-ol	871	1341	*			
	6	Heptan-1-ol	969	1443	*			
	7	Phenethyl alcohol	1114	1898	*		*	
	8	2,6-dimethyl-7-octen-2-ol*	1074	1450		*		
	9	2-Phenethyl alcohol	1114	2169		*		
	10	Cymen-7- ol*	1239	1972			*	
Phenols	11	2-Methoxyphenol	1080		*			
	12	Phenol	979	1991			*	
	13	4-Methylphenol	1070	2169			*	*
	14	3-Methoxyphenol	1081	1851			*	
Aldehydes	15	4-Ethylbenzaldehyde	1175	1687	*			
	16	Cinnamic aldehyde	1179	1833		*		
Ketone	17	1- (4-Ethylphenyl)-ethanone	1280	1710	*			*
Aromatic hydrocarbon	18	Naphthalene	1174	1710			*	*
	19	Unidentified				*		

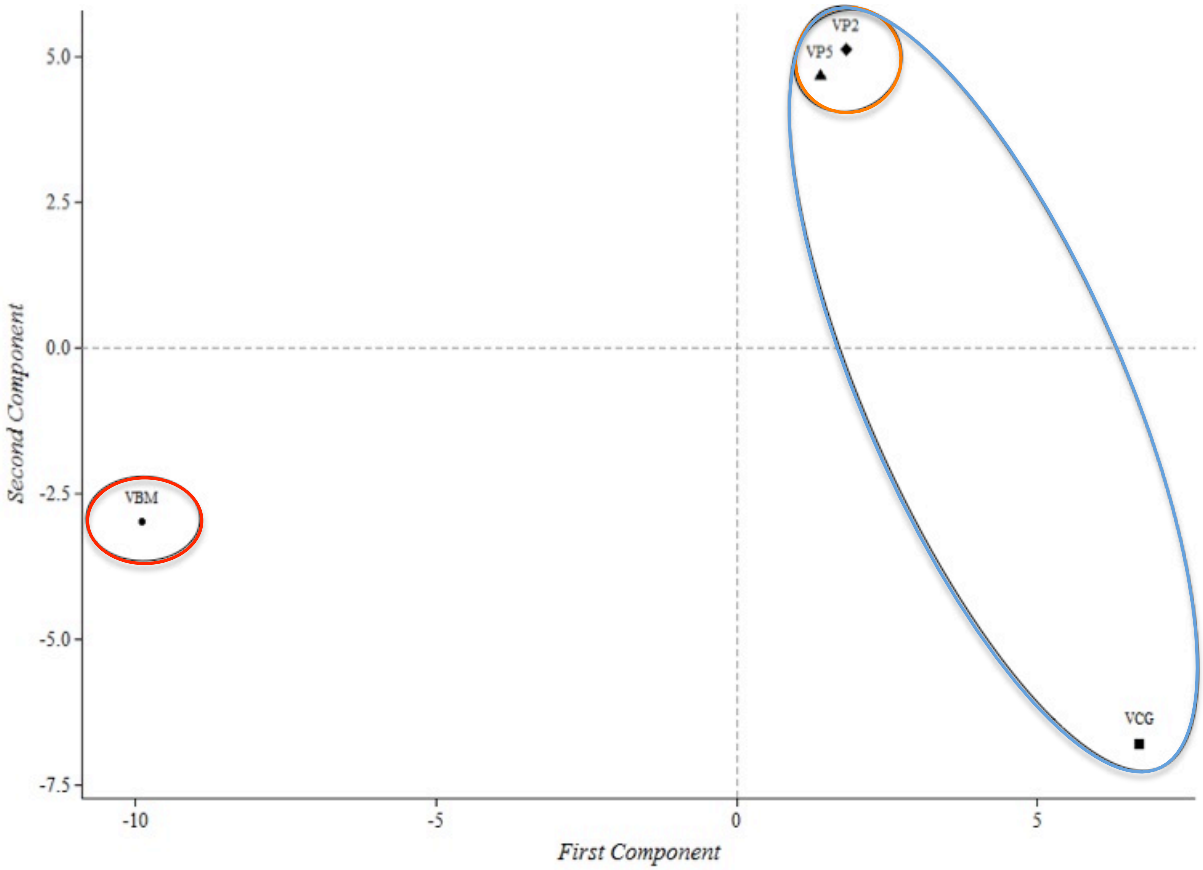
Source: Author, 2016.

Figure 7 a - Cluster analyses of the chromatographic data of the various samples containing vinasse. VCG= vinasse from Campo Grande, MS; VP2 = vinasse mixed with sugarcane straw with two days of fermenting; VP5 = vinasse mixed with sugarcane straw with five days of fermenting; VBM = vinasse obtained from Boca da Mata, AL, Brazil.



Source: Author, 2016.

Figure 7 b - Biplot analysis of retention indices of profile compound of each vinasse sample. n = vinasse from Campo Grande, MS, Brazil. w = Vinasse mixed with sugarcane straw with two days of fermenting. Δ = vinasse mixed with sugarcane straw with five days of fermenting. □ = vinasse from Boca da Mata, AL, Brazil.



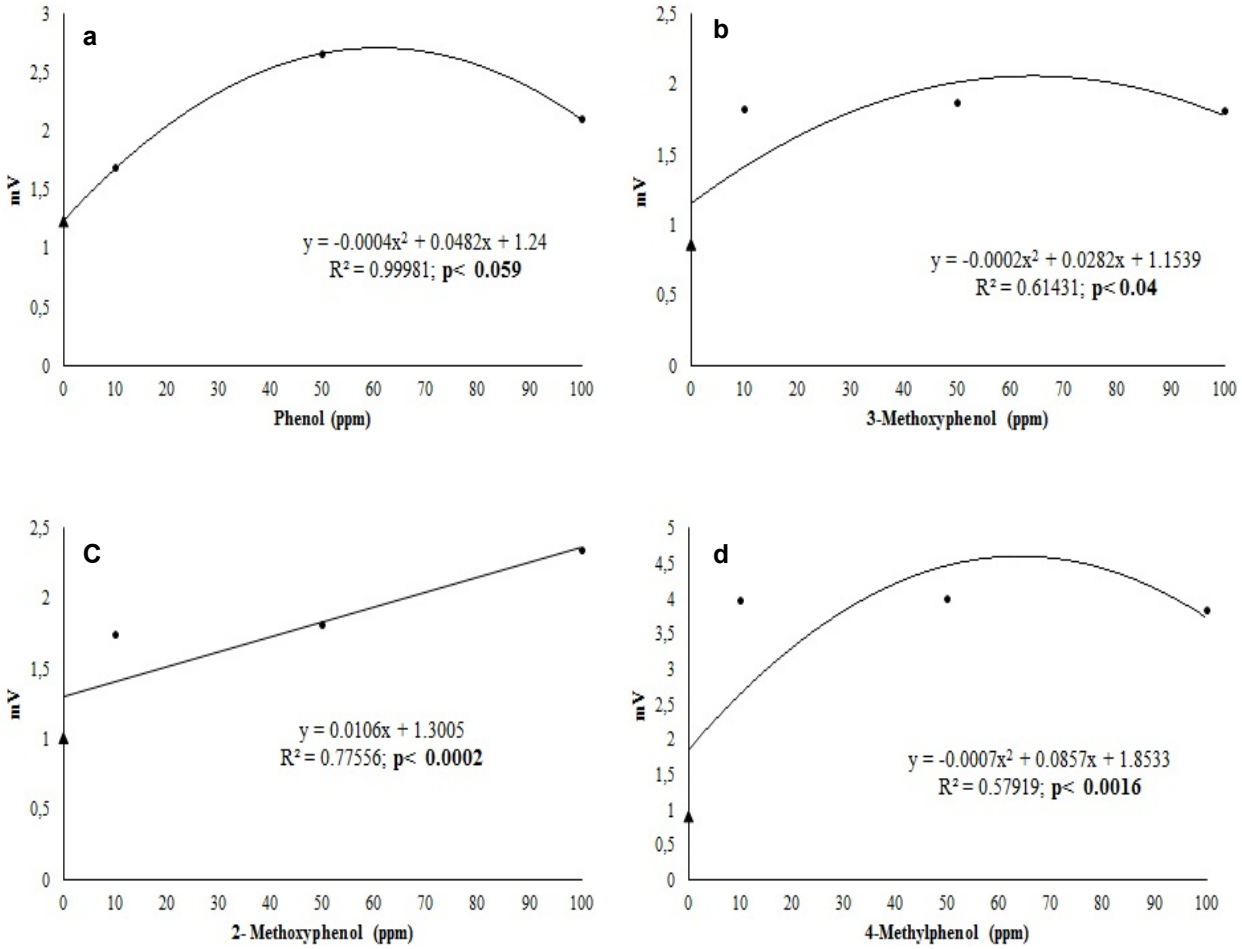
Source: Author, 2016.

6.3.4 EAG responses to synthetic compounds

Responses of *S.calcitrans* to the various doses of synthetic analogue compounds elicited a linear electrophysiological response with increasing dose (Figures. 8 a - m). Some of the synthetic compounds however elicited the highest response at lower doses and consequently decreased.

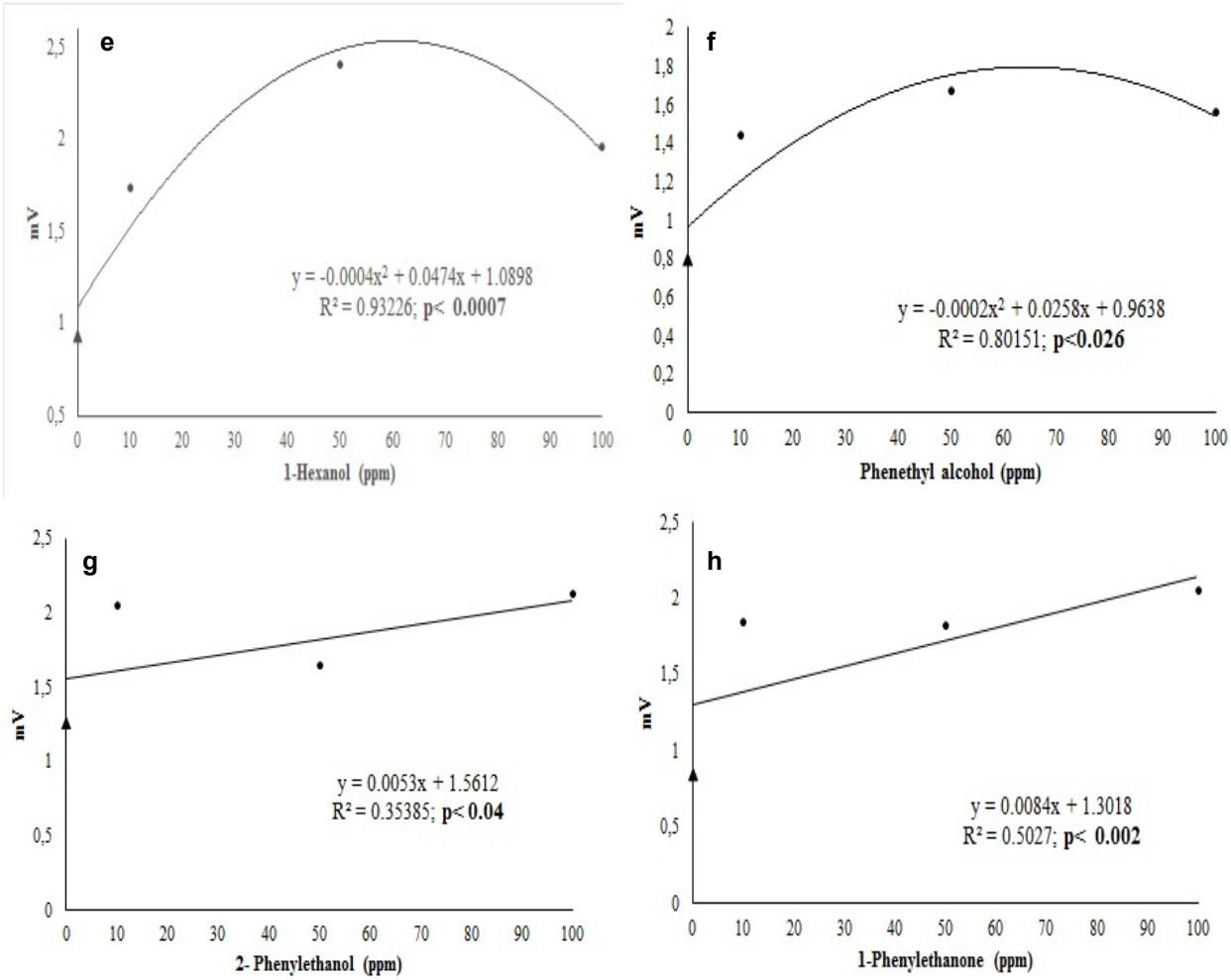
An average electrophysiological response of approximately 2.9 mV was elicited to the phenolic compounds that correspond to an average dose of 73 ppm (Figures 8 a - d). The primary alcohol groups elicited an average response of 2.1 mV but at a median dose of 74 ppm (Figures 8 e - f). 1-phenyl ethanone had a maximum electrophysiological response of 2.1 mV at 100 ppm (Figure 8 h). The carboxylic acid compounds had the highest mean dose of 83 ppm, eliciting an average response of 2.3 mV (Figures 8 I - k). The average dose for the aldehyde compounds was 77 ppm, eliciting a mean response of 2.3 mV (Figures 8 l - m).

Figure 9 - a-d: Dose response curve with *S.calcitrans* antenna. Triangle = control (hexane). *N*= 10 per dose per compound.



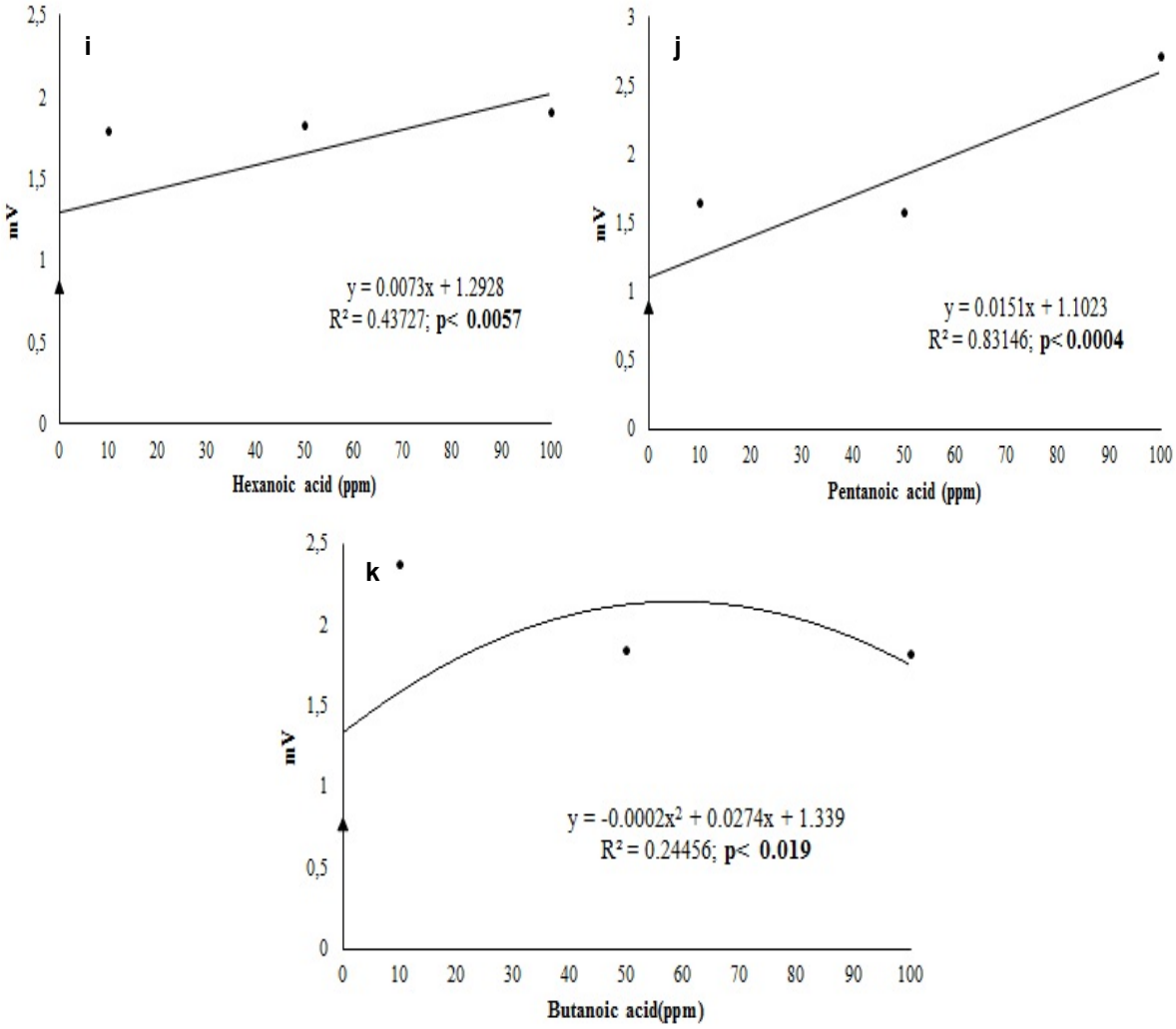
Source: Author, 2016.

Figure 8 – e – h: Dose response curve with *S.calcitrans* antenna. Triangle = control (hexane). *N*= 10 per dose per compound.



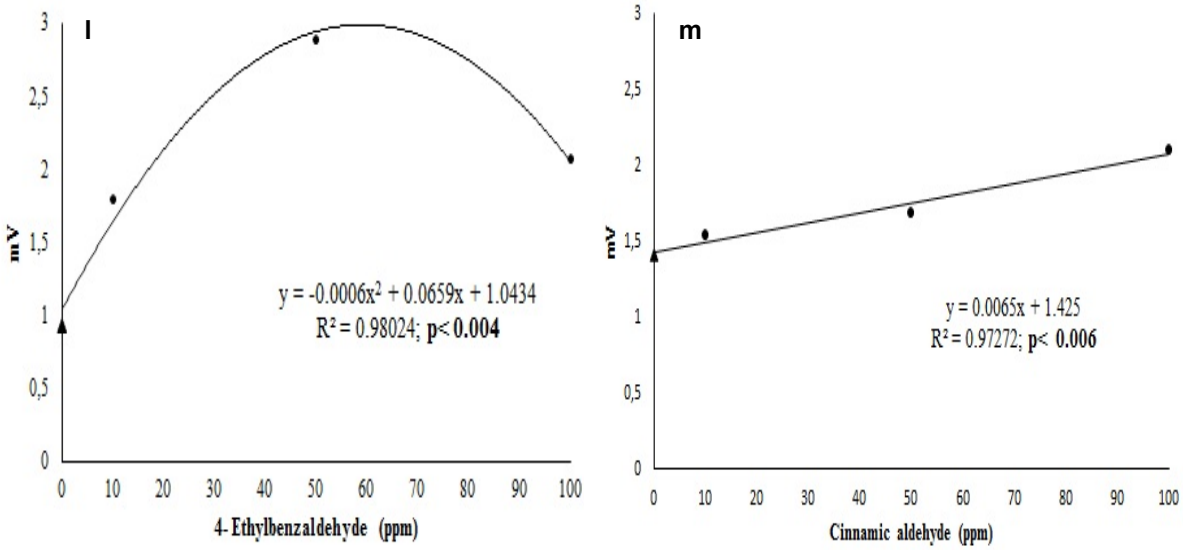
Source: Author, 2016.

Figure 8 - i-k: Dose response curve with *S.calcitrans* antenna. Triangle = control (hexane). *N*= 10 per dose per compound.



Source: Author, 2016.

Figure 8 l-m: Dose response curve with *S.calcitrans* antenna. Triangle = control (hexane). *N*= 10 per dose per compound.



Source: Author, 2016.

6.4 Discussion

This is the first time where the chemical ecology between *S.calcitrans* and sugarcane has been studied. Until recent years, biting flies has not been of agricultural concern but the recent appearance of stable flies has been noted in various crop including pineapple, vegetable and now, sugarcane (CHANG et al., 2015; SOLORZANO et al., 2015; Barros et al., 2010). This has turned stable flies into an agro-economic importance. The proximity between sugarcane mills and livestock increases the chances of survival it is therefore important to understand why these nuisance flies are seeking their way to the sugarcane crop.

This study demonstrated that both mated and unmated female flies of *S.calcitrans* were behaviorally attracted to vinasse independently of origin. Throughout the metabolic process of fermentation, sugar is converted to acids or alcohol and the leftover residues make up the vinasse. Vinasse has a very distinct smell that can be perceived from far which is mainly due to its high content of organic acids. It has been previously demonstrated that *S.calcitrans* is attracted to various organic acids JEANBOURQUIN et al., 2007; TANGTRAKULWANICH et al., 2011) and this was once again confirmed in this study, identifying butanoic acid, hexanoic acid, 3-methyl butanoic acid and pentanoic acid as EAG active.

The effect of vinasse upon *S.calcitrans* was confirmed when tested in the field. The number of caught stable flies was 6 times higher when using vinasse as a lure than the control. Mixing vinasse with filter cake, another sugarcane derived fertilizer, decreased the number of caught flies by half and was not considered significantly different from non-baited traps. This demonstrates that vinasse is the fertilizer that attracts stable flies in the field and not filter cake. These results provide vital information for sugarcane producers that use vinasse as its main fertilizer as it increases awareness and understanding of the consequences that vinasse irrigation can cause. When mixing vinasse and sugarcane straw only mated females were attracted.

As mentioned earlier, management of irrigation and crop is not always monitored which in turn creates puddles of stagnant vinasse mixed with decaying sugarcane straw. Decomposing matter is a known oviposition stimulant for stable flies since it provides suitable environment and food availability for the larvae (JEANBOURUIN et al., 2007). This could explain why only mated females were

attracted to the vinasse and sugarcane straw sample. Ovipositing in decaying matter near the base of sugarcane crop provides the possibility for larvae to migrate into the sugarcane stem for better food availability, which would explain the occurrence of stable fly larva inside green sugarcane stems.

Gas-chromatographic analyses showed that a longer fermentation period emitted lesser compounds, however, the volatile sample was still behavioral and EAG active. Multivariate statistical analysis of the chromatographic profile data of the various samples shows that independent of the vinasse being of different origin they still serve as a chemical attractant to stable flies.

When working with fermenting and decaying matter it is important to take into consideration the microbial symbiosis. Microbial development has proven to not only emit chemical cues that are perceived by adult stable flies but successful larval development strongly depends on it (ROMERO et al., 2006; ALBUQUERQUE et al., 2014). Besides yielding crop growth, vinasse has shown to also enhance microbial activity and to both benefit and protect microbial development in sugarcane crop (WEI et al., 2015).

Vinasse contains abundant organic matter that includes a wide range of phenols. Phenol is commonly associated as a host emitted compound that is attractive to stable flies, it has also been found in rumen digesta and manure (JEANBOURQUIN et al., 2007; TANGTRAKULWANICH et al., 2011). This study identified a range of phenols as EAG active for *S.calcitrans* present in the sample obtained from vinasse including phenol, 2-methoxyphenol, 3-methoxyphenol, and 4-methylphenol. 4-Methylphenol has been proven to be an effective chemical stimulant for trapping Tabanidaes, Calliphoridae, Sarcophagidae, and Muscidaes amongst other biting fly species (KRCMAR et al., 2007). In the present study, 4-methyl phenol was the compound that elicited the highest electroantennogram voltage followed by the remaining phenols.

The use of chemical cues for trapping enhancement has been researched and concluded to provide a solid approach considering that the chemical in question is both efficient and cost-effective. CO_2 a universal attractant for various heamatophagous insects including stable flies is an efficient lure however, it is not considered to be cost-effective nor environmental friendly it is therefore important to seek new chemical attractants with high efficiency. Determining a viable chemical

blend with high efficiency and cost beneficial is a crucial step; it is therefore worth exploring new attractants and their use as a tool for control of stable flies.

6.5 Conclusion

This study provides new knowledge concerning *S.calcitrans* and its increasing population in sugarcane mills from both a behavioral and chemical point of view. It demonstrates that vinasse irrigation plays an important role in the increased stable fly population in sugarcane mills. It is uncommon for heamatophagous insects to become of great concern in agriculture but stable flies are a nuisance fly with the ability to transmit various pathogens, it is therefore important to understand its distribution into crop. Domestic animals and agriculture worker are prone to the painful bite caused by stable flies and although the crop damage cause by the stable fly larvae is yet unknown it is crucial to expand current knowledge.

The identified compounds serve as groundwork for further development of monitoring and control strategies and are worth exploring further in field studies. Determining their efficiency as lures against *S.calcitrans* could provide an alternative control method beneficial from both an agricultural and farming point of view.

6.6 Acknowledgment

Funding for this work was provided by the Brazilian Federal Agency for support and evaluation of graduate education, CAPES and CNPQ. The authors would like to thank Prof. Christer Löfstedt and the Pheromone Research Group at Lund University, Sweden for providing the synthetic compounds, EMBRAPA Gado de Corte, Brazil for providing insects, and Mary Clark Farias horse farm for allowing us to conduct our field study in their property.

REFERENCES

ALBUQUERQUE, T. A.; ZUREK, L. Temporal changes in the bacterial community of animal feces, and their correlation with stable flies oviposition, larval development, and adult fitness. **Frontiers in Microbiology**, v. 5, p. 1-9, 2014.

BARROS, A. T. M. et al. *Stomoxys calcitrans* outbreaks in pastured beef cattle in the state of Mato Grosso do Sul, Brazil. **Pesquisa Veterinaria Brasileir***a*. v. 30, p. 945–952, 2010.

CANCADO, P. H. D. et al. Sugarcane stems as larval habitat for the stable fly (Stomoxys calcitrans) in sugarcane plantations. **Pesquisa Veterinaria Brasileira.** v. 33, p. 741–744, 2013.

CHANG, G. P.; BLANCO-METZLER, H.; VARGAS-GUTIERREZ, M. Use of decomposers in pineapple residues in Stomoxys calcitrans populations. **Entomologia Mexicana**. v. 2, p. 312–318, 2015.

DOMINGHETTI, T. F. S. et al. Stomoxys calcitrans (Diptera : Muscidae) outbreaks : current situation and future outlook with emphasis on Brazil. **Brazilian Journal of Veterinary Parasitology.** v. 24, p. 387–395, 2015.

GRISI, L. et al. Reassessment of the potential economic impact of cattle parasites in Brazil. **Brazilian journal of veterinary parasitology.** v. 23, p. 150–56, 2014.

JEANBOURQUIN, P.; GUERIN, P.M. Sensory and behavioural responses of the stable fly Stomoxys calcitrans to rumen volatiles. **Medical and veterinary entomology,** v. 21, p. 217–24, 2007.

KASSAB, S. O. et al. New outbreaks of the population of fly-stables, Mato Grosso do Sul: prevention and control measures. **Revista Agrarian,** v. 5, p. 84–88, 2012.

KOLLER, W. W. et al. Outbreak of stable fly, *Stomoxys calcitrans,* in Mato Grosso do Sul State: New problem for the beef and sugarcane chains? Mato Grosso, **Embrapa Gado do Corte,** p. 1–31, 2009.

KRCMAR, S. Responses of Tabanidae (Diptera) to canopy traps baited with 4-methylphenol, 3-isopropylphenol, and naphthalene. **Journal of vector ecology,** v. 32, p. 188–92, 2007.

LEITE, I. H. F.; CARVALHO, É. B.; BITTENCOURT, A. J. Influence of vinasse on the development of *Stomoxys calcitrans.* **Ciênca Rural,** v. 43, p. 326–330, 2013.

MARCON, P. C. R. G. et al. Susceptibility of stable flies (Diptera: Muscidae) from Southeastern Nebraska beef cattle feedlots to selected insecticides and comparison of 3 bioassay techniques. **Journal of Economical Entomology,** v. 90**,** p. 293-298, 1997.

ODA, F. H.; ARANTES, C. A. Populational outbreak of the stable fly *Stomoxys calcitrans, Linnaeus,* 1758 (Dipteria:Muscidae) at the municipality of Planalto, SP. **Revista em agronegocios e Meio Ambiente,** v. 3, p. 45–159, 2010.

PITZER, J.B.; KAUFMAN, P.E.; TENBROECK, S.H. Assessing permethrin resistance in the stable fly (Diptera: Muscidae) in Florida by using laboratory selections and field evaluations. **Veterinary Entomology,** v. 103, p. 2258-2263, 2010.

PITZER, J. B. et al**.** Seasonal abundance of stable flies and filt fly pupal parasitoids (Hymenoptera: Ptermalidae) at Florida equine facilities. **Journal of Economic Entomology,** v. 104, p. 1108-115, 2011.

POTTING, R. P.; VET, L. E.; DICKE, M. Host microhabitat location by stem-borer parasitoid Cotesia flavipes: The role of herbivore volatiles and locally and systematically induced plant volatiles. **Journal of Chemical Ecology,** v. 21, p. 525–539, 1995.

PRADO, R. M.; CAIONE, G.; CAMPOS, C. N. S. Filter cake and vinasse as fertilizers contributing to conservation agriculture. **Applied and Environmental Soil Science,** 2013.

ROLLIM, M. M. et al. Influence of a vinasse-distributing lake on water quality of the groundwater. **An Interdisciplinary Journal of Applied Science,** v. 8, p. 1980–993, 2013.

ROMERO, A.; BROCE, A.; ZUREK, L. Role of bacteria in the oviposition behavior and larval development of stable flies. **Medical and Veterinary Entomology,** v. 20, p, 115-121, 2006.

SALEM, A. et al. Feeding and breeding aspects of Stomoxys calcitrans (Diptera: Muscidae) under laboratory conditions. **Parasite,** v. 19, p. 309–17, 2012.

SILVA, C. C. M. et al. Preference of the parasitoid Cotesia flavipes (Cam.) (Hymenoptera: Braconidae) for Diatraea (Lepidoptera: Crambidae). **Acta Scientiarum Agronomy,** v. 34, p. 23–27, 2012.

SOLORZANO, J. A. et al. Biology and Trapping of Stable Flies (Diptera : Muscidae) Developing in Pineapple Residues (Ananas comosus) in Costa Rica. **Journal of Insect Science,** v. 15, 2015.

TANGTRAKULWANICH, K. et al. Characterization of olfactory sensilla of Stomoxys calcitrans and electrophysiological responses to odorant compounds associated with hosts and oviposition media. **Medical and veterinary entomology,** v. 25, p. 327–36, 2011.

TAYLOR, D. B.; BERKEBILE, D.R. Sugar Feeding in Adult Stable Flies. **Environmental Entomology,** v. 37, p. 625–629, 2008.

TAYLOR, D. B.; MOON, R.D.; MARK, D.R. Economic Impact of Stable Flies (Diptera: Muscidae) on Dairy and Beef Cattle Production. **Journal of Medical Entomology,** v. 49, p. 198–209, 2012.

VAN LENTEREN, J. C.; BUENO, V.H.P. Augmentative biological control of arthropods in Latin America. **BioControl,** v. 48, p. 123–139, 2003.

WEI, M. et al. Effect of vinasse application on soil in sugarcane fields**. Applied Mechanics and Materials***,* v. 700, p. 270-275, 2015.

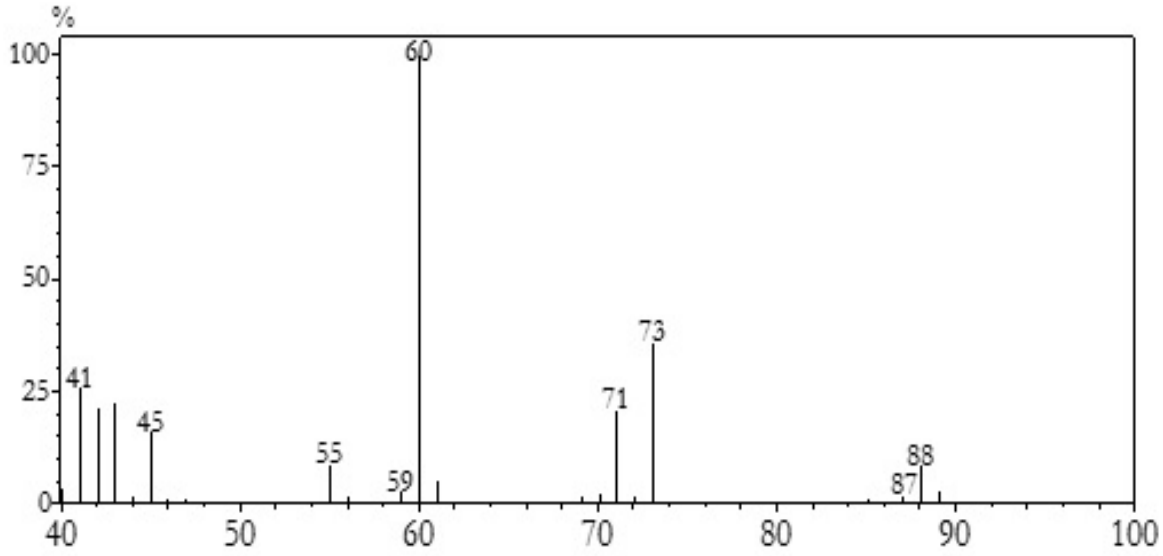
APPENDIX A

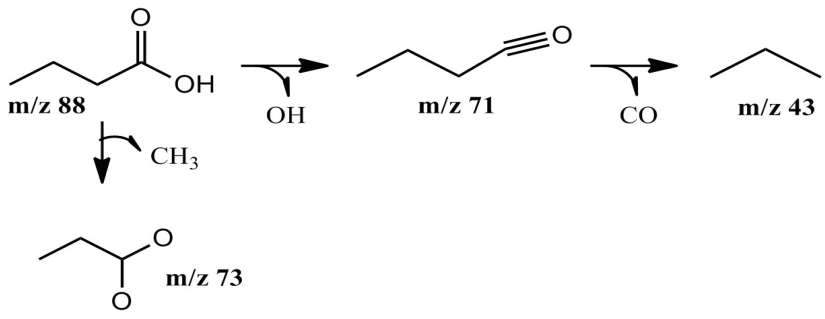
The mass spectra and fragmentation pattern of the identified compounds referred to throughout this chapter are presented.

The header for each mass spectrum gives:

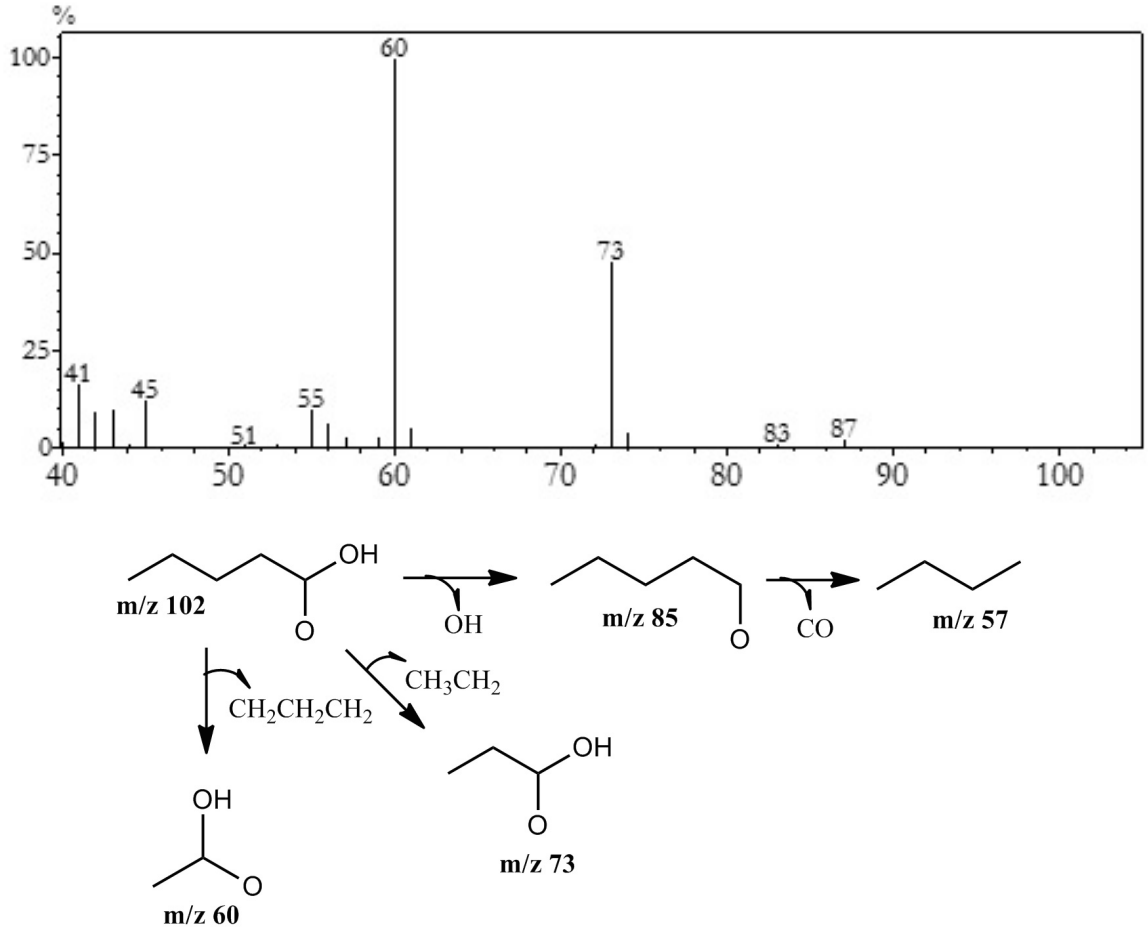
- The name of the compound
- Molecular weight
- m/z of each major fragment with its intensity (%)
- Fragmentation scheme

Butyric acid: MW 88 (8), m/z 73 (35), m/z 60 (100), m/z 41 (25).

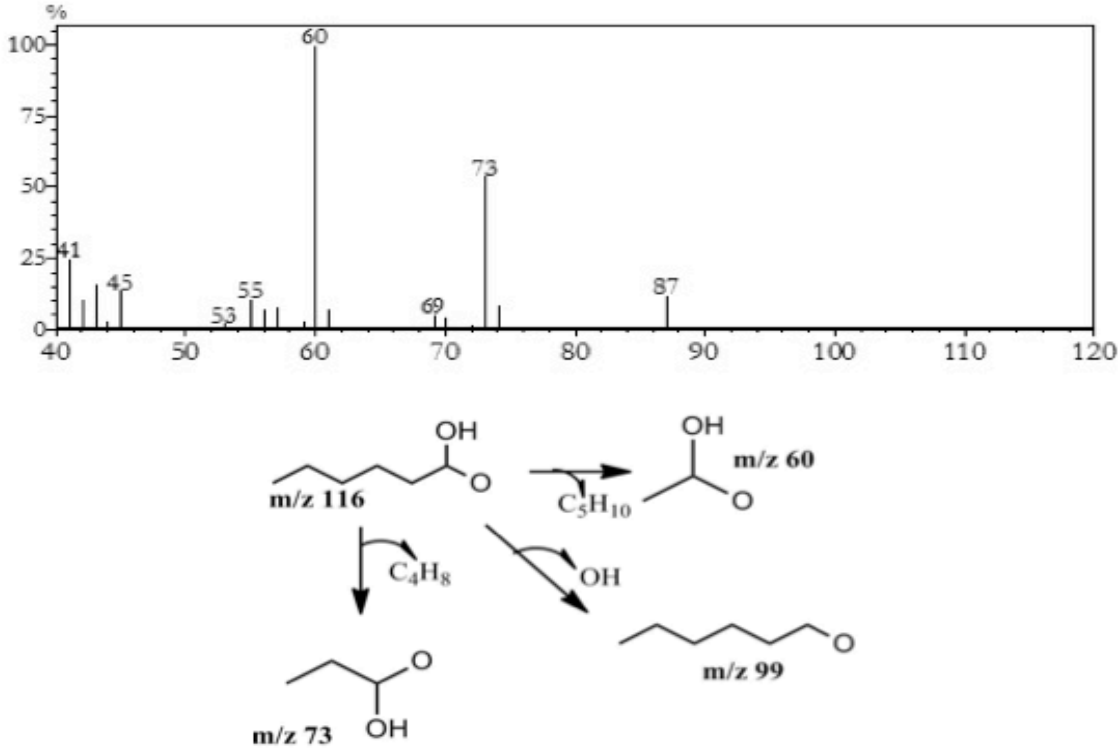




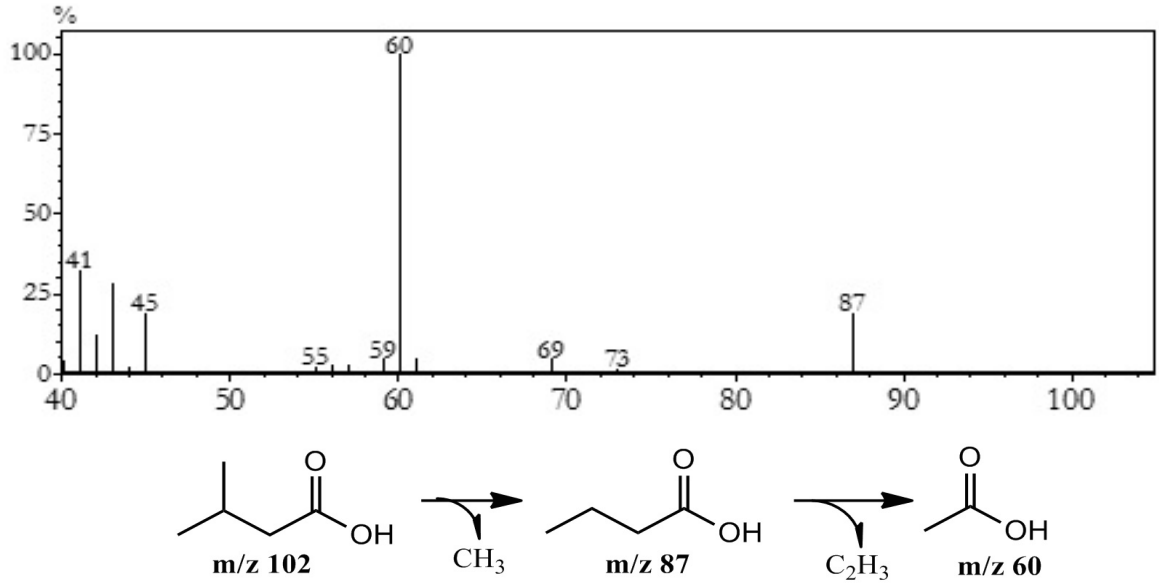
Pentanoic acid: MW 102, m/z 73 (48), m/z 60 (100).



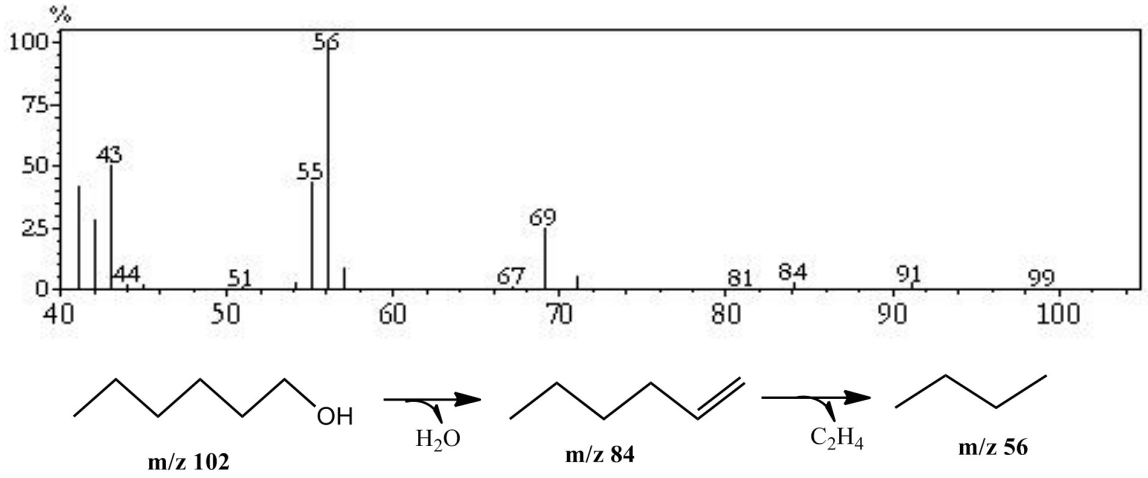
Hexanoic acid: MW 116, m/z 87 (12), m/z 73 (54), m/z 60(100).



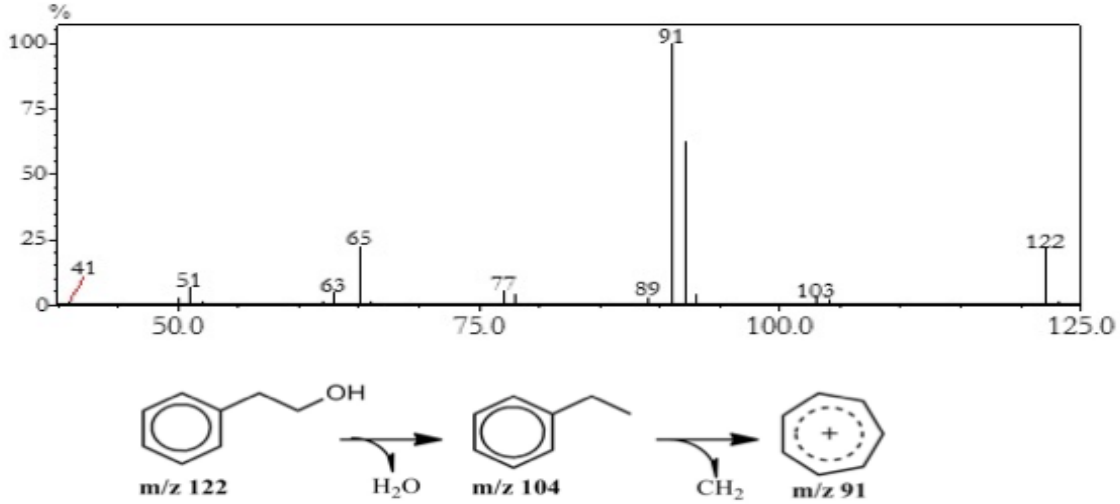
3-methyl butanoic acid: MW 102, m/z 87 (19), m/z 60 (100).



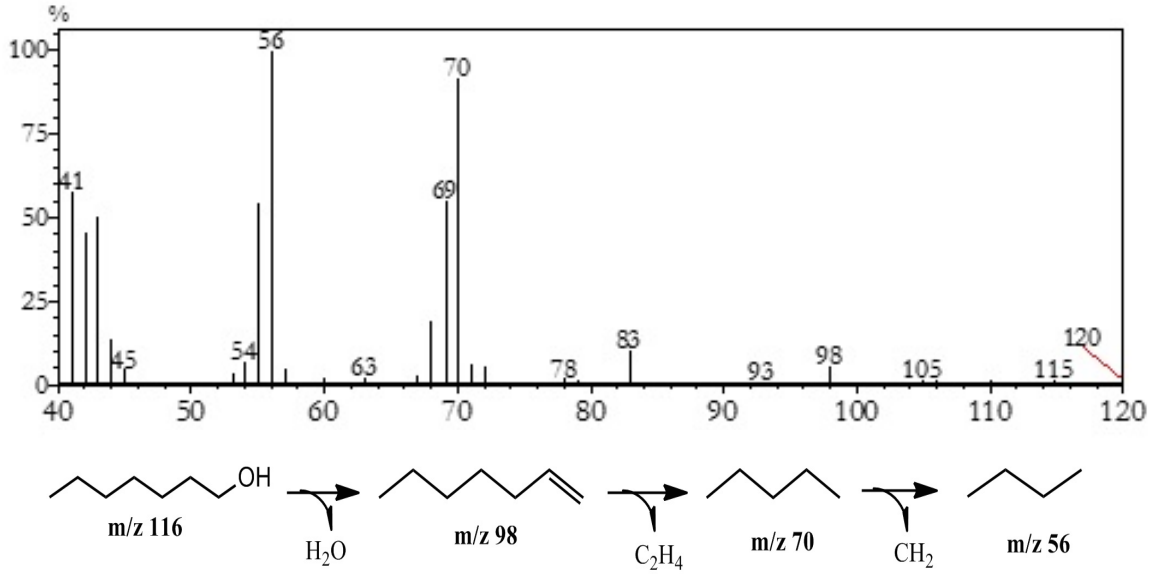
1-hexanol: MW 102, m/z 84, m/z 56 (100).



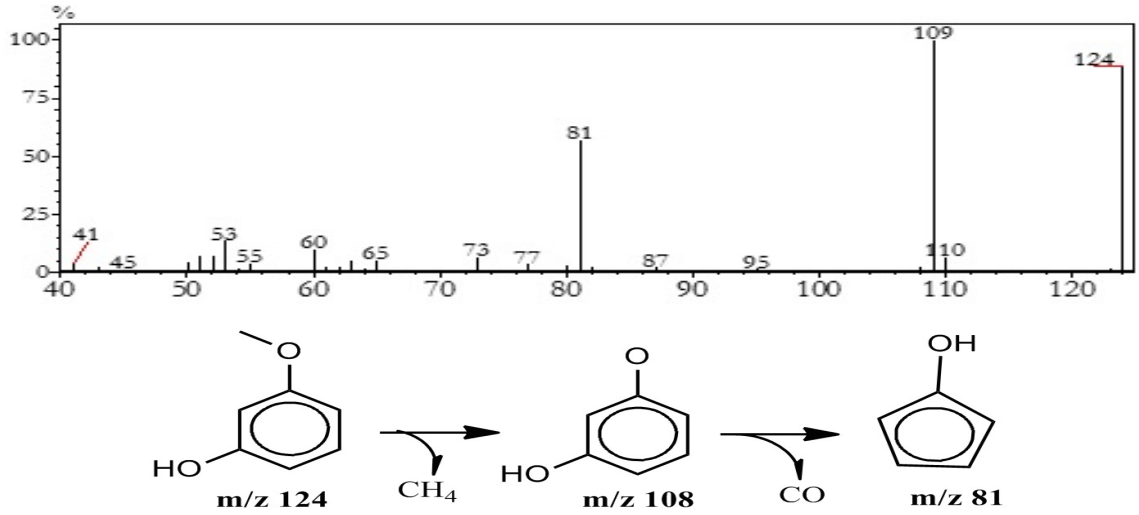
Phenylethyl alcohol: MW 122 (28), m/z 91(100).



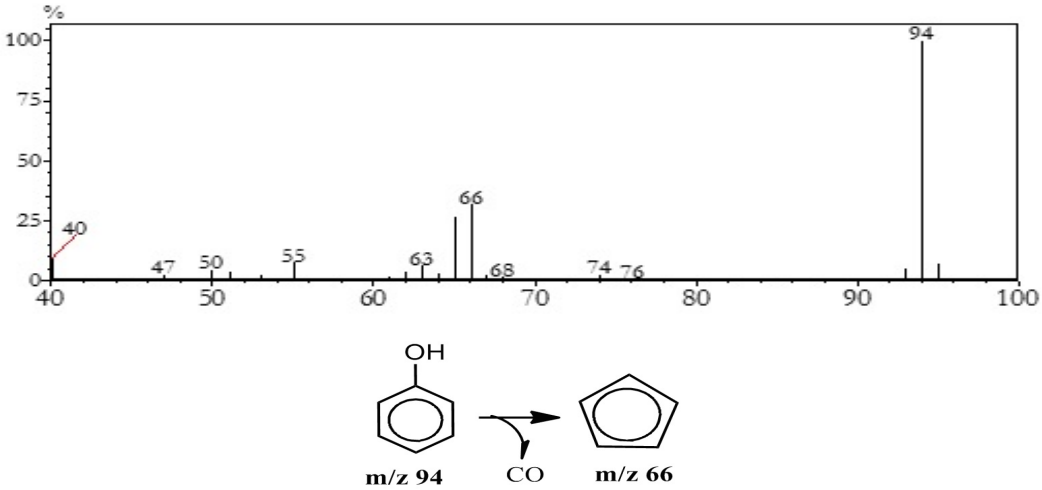
1-Heptanol: MW 116, m/z 70 (91), m/z 56 (100).



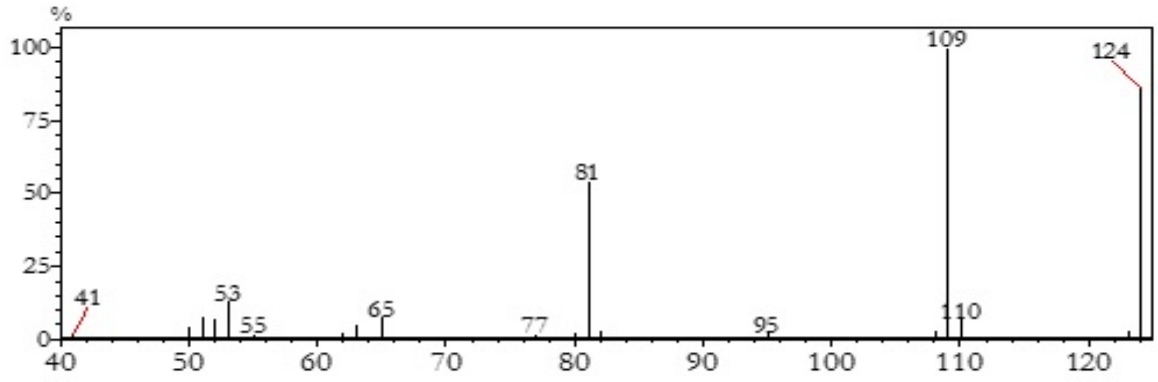
3-methoxyphenol: MW 124 (89), m/z 109(100), m/z 81 (57),

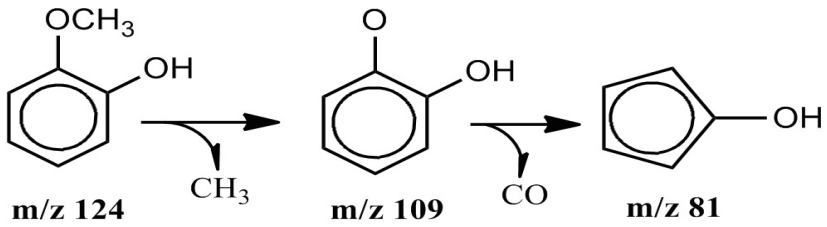


Phenol: MW 94 (100), m/z 66 (31).

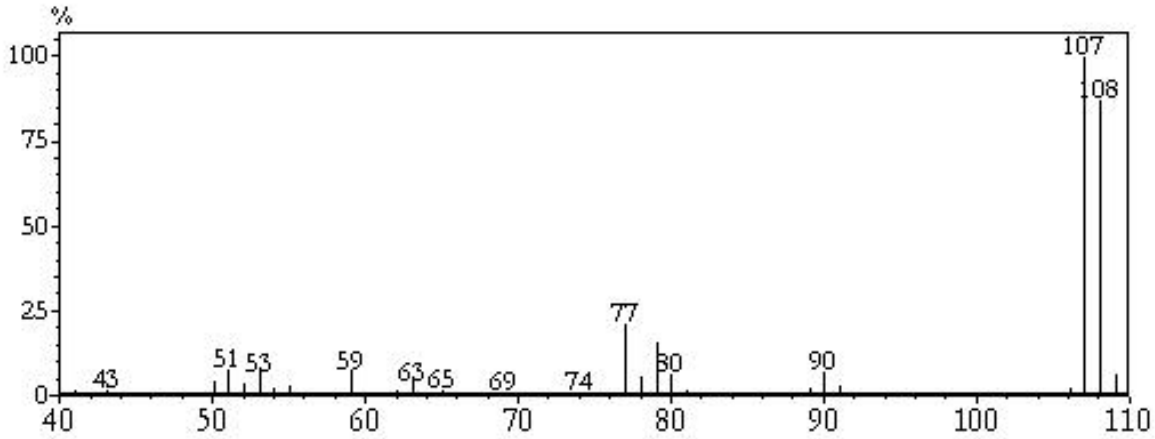


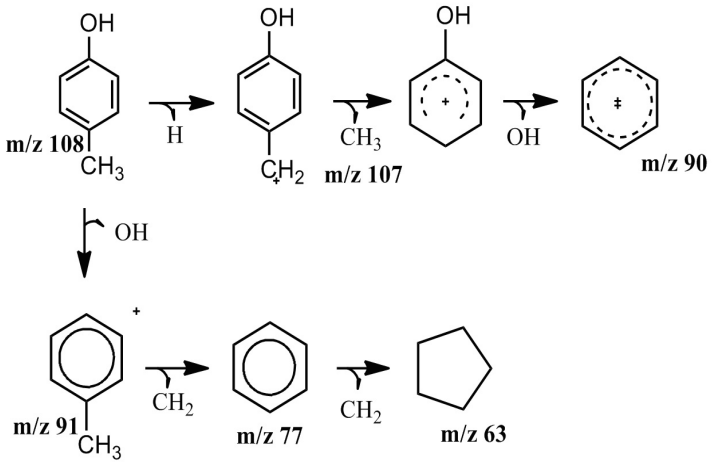
2-methoxyphenol: MW 124 (86), m/z 109 (100), m/z 81 (54).



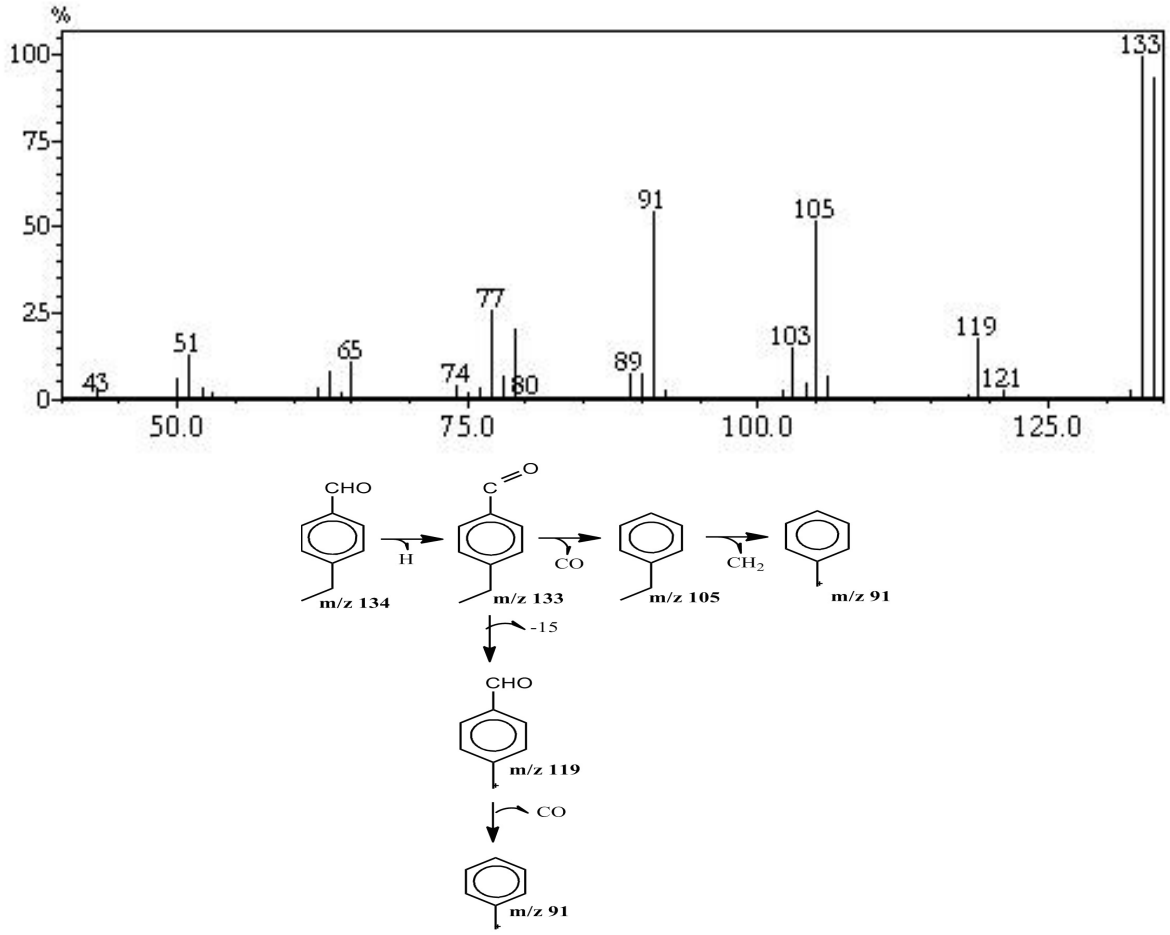


4- methylphenol: MW 108 (87), m/z 107 (100), m/z 77 (21).

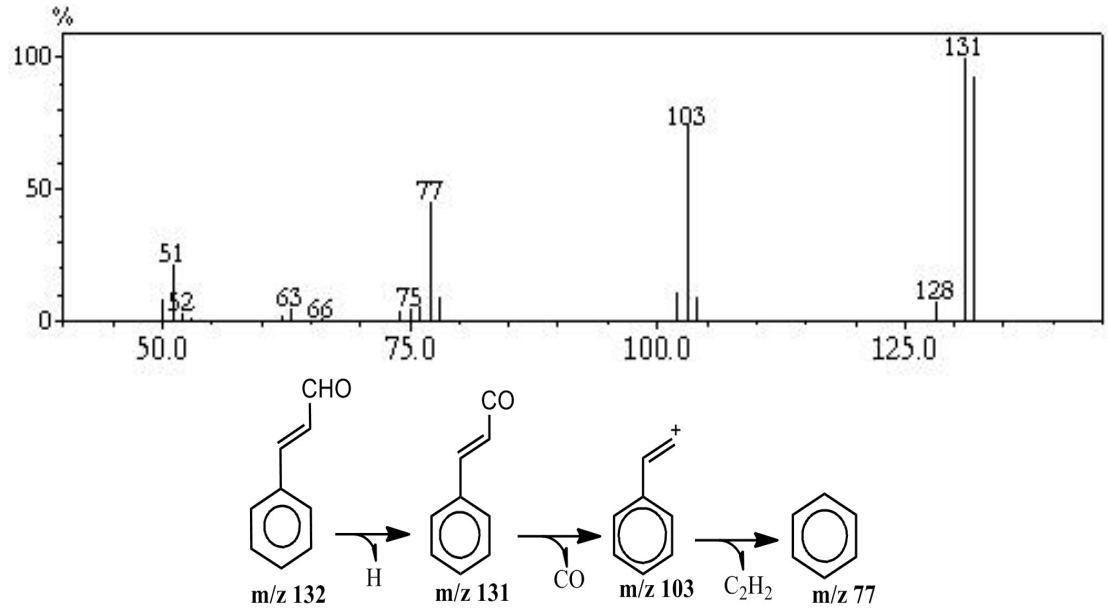




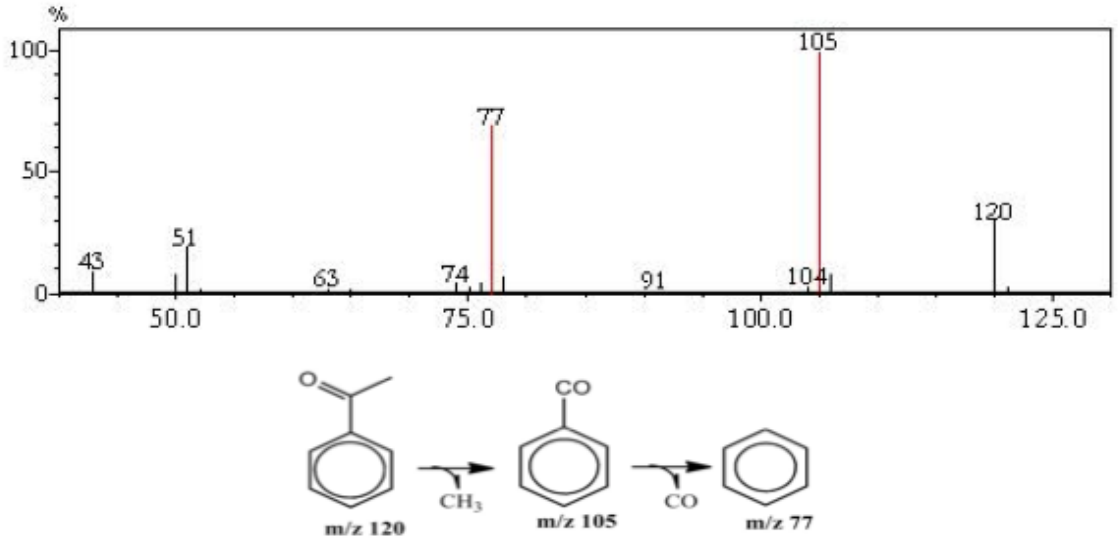
4-ethyl benzaldehyde: MW 134 (93), m/z 133 (100), m/z 105 (52), m/z 91 (55).



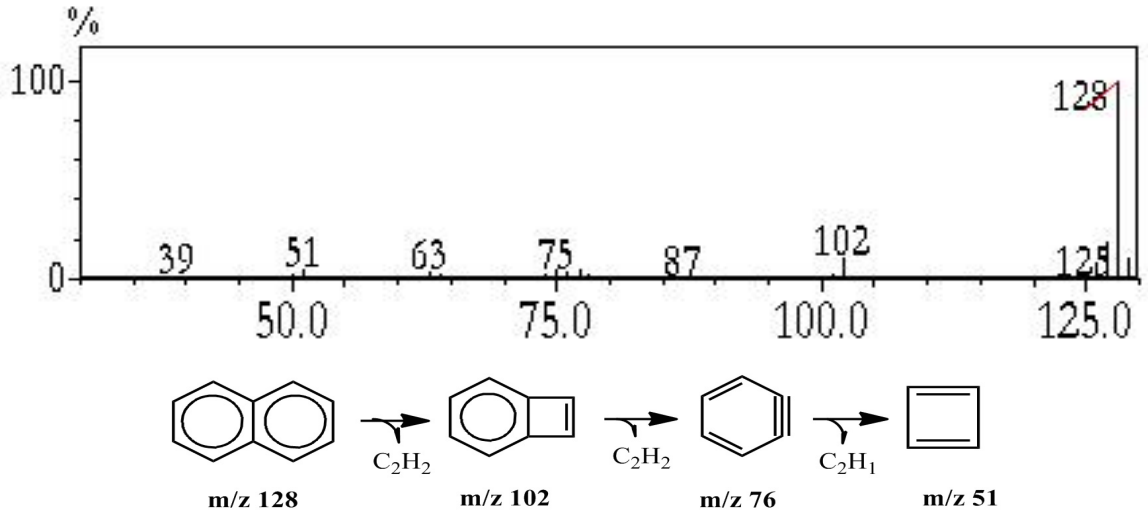
Cinnamaldehyde: MW 132 (93), m/z 131 (100), m/z 103 (74), m/z 77 (45).



Acetophenone: MW 120 (30), m/z 105 (100), m/z 77 (69).



Naphthalene: MW: 128 (100), m/z 102 (11).



7 CONCLUSION AND FUTURE ASPECTS

This study provided further knowledge regarding *Stomoxys calcitrans* and their host-seeking behavior and it also provided novel information on a rather new point of view, which is their association to sugarcane mills. Understanding the relation between chemical cues and how they affect the behavior of stable flies is a key point in the development of targeted control methods. Twenty chemostimulants associated with hosts and their environmental oviposition stimulants were identified throughout this study, providing both new and recognized chemical compounds. The key discovery in this study was the association between stable flies and sugarcane mills. No previous study has investigated the chemical cues involved in the outbreaks of stable flies in sugarcane mills thus making the results obtained both novel and innovative. Determining that vinasse irrigation attracts stable flies is a significant finding as it helps to understand the distribution of this nuisance pest into crop and the identification of chemical compounds emitted by vinasse provides vital means that can be used to control and monitor stable fly populations.

The use of insecticides is a matter of concern due to environmental consequences, cost-effectiveness, health risks, and development of resistance. The quantity and frequency of application is not always monitored nor carefully conducted. From these concerns, initiatives such as integrated vector management, use of semiochemicals, push-pull strategies, pest management have developed in order to reduce the use of insecticides and use methods that are more cost-effective and “friendlier” to both the environment and people in close proximity. Exploring the chemical ecology between vectors and their sources of interest

The identified compounds serve as groundwork for further development of monitoring and control strategies and are worth exploring further in field studies to understand the behavioral response of stable flies. Determining their efficiency as lures against *S.calcitrans* could provide an alternative control method beneficial to both livestock farming and agriculture.

ADDITIONAL COLLABORATIONS THROUGHOUT DOCTORATE DEGREE

ADDITIONAL COLLABORATIONS AND ACCOMPLISHMENTS DURING DOCTORATE DEGREE

Presentations at International Congresses

- Behavioral responses of *Stomoxys calcitrans* to possible oviposition sites found in sugarcane mills. **ALAEQ 2014.** Awarded price for best poster presentation.
- Host-seeking behavior of *Stomoxys calcitrans* in privately owned farms. **ALAEQ 2014.** Awarded price for best poster presentation.
- Intraspecific variation of cuticular hydrocarbon profiles in the *Anastrepha fraterculus* (Diptera: Tephritidae) species complex. **ISCE 2015.** Poster presentation.
- Sex attractant of the *Annona* fruit borer, *Cerconota anonella* Sepp. (Lepidoptera: Oecophoridae). **ISCE 2015.** Poster presentation.

Projects

- Attractant identification for the fruit borer *Cerconota anonella.* Responsible for conducting all electrophysiological studies in order to determine possible kairomones.
  PI: Prof. Ruth Rufino do Nascimento, UFAL, Brazil.
- Extraction and identification of hydrocarbons from the Brazilian fruit-fly populations, *Anastrepha fraterculus* (Diptera: Tephritidae): chemical and behavioral studies. Responsible for conducting all electrophysiological studies in order to determine EAG active hydrocarbons.
  PI: Prof. Ruth Rufino do Nascimento, UFAL, Brazil.
- Screwworm project. Responsible for conducting field studies testing different attractant lures for the screw-worm transmitting flies.
  PI: Dr. Junwei J. Zhu, USDA Lincoln Nebraska, USA.

- Responsible for the electrophysiological studies in determining the attraction of horn flies, *Haematobia irritans,* to different cattle breeds. Federal University of Alagoas, Brazil.
  PI: Dr. Cenira Monteiro de Carvalho, Institute of Chemistry and Biotechnology.

Exchange periods

- (May- August, 2015) Visiting student at Lund University, Sweden.
  Collaborated with the Pheromone group. Participated in a project involving odor receptor neurons of *Drosophila melangasto* perception to β-carotene. Conducted oviposition assays and used confocal microscopy.
  PI: Associate Professor Marcus Stensmyr.
- (December, 2015) Visiting student at the USDA Lincoln, University of Nebraska, USA. Participated in behavioral assays using *Stomoxys calcitrans* (Diptera: Muscidae).
  PI: Dr. Jerry J. Zhu.
- (August, 2014) Visiting student at EMBRAPA Gado de Corte, Campo Grande, MS, Brazil. Conduected behavioral assays using *Stomoxys calcitrans* (Diptera: Muscidae) and collected volatile organic compounds through headspace entrainment.
  PI: Dr. Paulo Cancado and Antonio Thadeu Barros.
- (September, 2013) Visiting student at the Entomology department, UFV, MG, Brazil. Undertook a course of electrophysiological studies and how to use gas chromatography coupled to electroantennogram (GC-EAG).
  PI: Prof. Eraldo Rodriquez Lima.

Publications

E. V. Pires, A. de L. Mendoca, L. Vanickoca, **N. S. J. Serra**, R. de C. C. da Silva, D. C. dos Santos, R. da S. Campos, A. E. G. Santana, R. R. do Nascimento. (2015). Identification and field and laboratory tests of the sex pheromone of *Cerconota anonella* Sepp. (Lepidoptera: Oecophoridae). **J. Appl. Entomol, 140,** 72-80.

E.F. da Silva Junior., D.L. da Silva., T.M. Aquino., E.A.N. Ribeiro., **N.S.J. Serra.,** J.X.A. Junior. (2014) The $GABA_A$ receptor and the neuropharmacological properties of 1,5 – benzodiazepines of medicinal interest: a review. **Pinnacle Medicine & Medical Sciences, 1**.

N. S. J. Serra, H. F. Goulart, S. S. Tavares, J. G. Costa., C. I. M. Almeida., A. E. G. Santana. (Submitted to Bulletin of Entomological Research). Laboratory and field studies determining attractants responsible for the severe outbreaks of Stomoxys calcitrans (Diptera: Muscidae) in sugarcane mills.

C. M. de Carvalho, H. F. Goulart, E. V. Pires , J. G. da Costa , **N. S. J. Serra** , M. C. C. de Andrade, M. O. F. Goulart , A. B. Fraga, M. A. Birkett, A. E. G. Santana. (2015). Differential attraction of horn flies, *Haematobia irritans,* to European and Zebu Breeds of Cattle. (Under submission).

C. K. B. Perreira , C. M. de Carvalho , T. Souza, **N. S. J. Serra** , J. G. Costa , A. E. G. Santana. (2015). Potential allelopathic effect of *Cecropia pachystachya* Trec in *Lactuca sativa.* (Under submission).