PLANT-DERIVED SEMIOCHEMICALS FOR THE ATTRACTION OF STABLE FLY, *STOMOXYS CALCITRANS* TO HOST PLANTS AND FITNESS VALUE OF NECTAR FEEDING

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Plant-derived semiochemicals for the attraction of stable fly, *Stomoxys calcitrans* to host plants and fitness value of nectar feeding

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DECLARATION

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

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DEDICATION

I dedicate this work to my parents, Mr. Hezron Kosgei and Mrs Basilisa Kosgei for their continuous support and prayers throughout my educational journey.

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ABBREVIATIONS AND ACRONYMS

AAT	African Animal Trypanosomiasis
ANOVA	Analysis of Variance
ANOSIM	Analysis of Similarities
NMDS	Non Metric Dimensional Scalling
SIMPER	Simillarity Percentage
BLAST	Basic Local Alignment Search Tool
cpDNA	Chloroplast DNA
DCM	Dichloromethane
DNA	Deoxyribonucleic Acid
EAG	Electroantennogram
EDTA	Ethylenediaminetetraacetic acid
GC	Gas Chromatography
MS	Mass spectrometry
NCBI	National Centre for Biotechnology Information
PCR	Polymerase Chain Reaction
rbcL	Ribulose Bisphosphate Carboxylase Large Chain
RH	Relative humidity
VOCs	Volatile Organic Compounds
NMDS	Non-Metric Multidimensional Scaling
MBBU	Molecular Biology and Bioinformatics Unit

Icipe	International Centre of Insect Physiology and Ecology
TAE	Tris Acetate EDTA
OBP	Odorant Binding Protein
OR	Odorant Receptor
IR	Ionotropic Receptor
DA	Discrimant Analysis
SVM	Support Vector Machine
SIT	Sterile Insect Technique
HIPVs	Herbivore induced plant volatiles

ABSTRACT

Stable fly (*Stomoxys calcitrans*) is a serious vector of many livestock diseases of medical and veterinary importance. Unlike their blood-feeding characteristics, which are widely studied, little information about *S. calcitrans* plant feeding nectar is unknown. According to this study, the plant-feeding behaviour of field-collected stable flies was determined by detecting coding (rbcL) and the non-coding (trnH-psbA) regions of the chloroplast gene. From the three study regions (*Icipe*- Duduville campus, Mpala research centre, and Amboseli), 3.67% of field-collected stable flies were positive for the following seven plant species: *Parthenium hysterophorus*, *Terminalia brownii*, *Senegalia mellifera*, *Vachellia xanthophloea*, *Allium sativum*, *Lantana camara*, and *Schinus terebinthifolia*. The fitness benefits of plant feeding in adult stable flies was investigated using flowering *Parthenium hysterophorus* as a representative plant nectar source because it is readily available and can be grown in pots. Supplementing blood-feeding with flowering *P. hysterophorus* had no significant effect on *S. calcitrans* egg-laying and survival but significantly enhanced egg hatchability compared to blood alone. This variation suggests an additional resource acquired from plant feeding, which enhances egg fertility in *S. calcitrans* compared to feeding on blood alone. The plant volatiles demonstrated a great chemodiversity between the different plant species. From both laboratory behavioural assays and field experiments, the behavioural response of stable flies to plant-derived volatile organic compounds varied from attraction, neutral to repellency. Blood feeding is very important for egg development in adult flies, and supplementing blood-feeding with plant nectar improves eggs' hatchability, which will lead to an increased vector population. Results from this work contribute largely to a better understanding of stable flies' survival, reproduction, and disease transmission and the possibility of applying plant odours in the surveillance and control of stable flies.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Stomoxys calcitrans Linnaeus, 1758 also known as stable fly, is a blood-feeding insect and a cosmopolitan species within the eighteen *Stomoxys spp*. (MAG, INTA, & USDA, 2018; Zumpt, 1973). They are serious pests of cattle on pasture and range, thus making them an important pest of medical and veterinary importance. *Stomoxys calcitrans* sucks blood from livestock and other animals with varying degrees of preference (Getahun *et al*., 2020a; Taylor et al., 2012). Their hosts range from cattle, horses, camels, small ruminants, buffalo, dogs and occasionally humans when other hosts are unavailable (Baldacchino *et al*., 2013; Tainchum *et al*., 2010; Mihok & Clausen, 1996; Getahun *et al*., 2020a). Stable fly (Figure 1.1A) greatly resembles the common housefly (Figure 1.1B), but can be differentiated by its sharp piercing proboscis projecting in front of the head, which is absent in house fly. They also have a characteric four longitudinal dark stripes on the dorsal part of the thorax. They also have a checkerboard pattern with seven spots at the back of the abdomen which also makes it unique from other *Stomoxys spp*.

Figure 1. 1: Morphology of (A) *S. calcitrans* and (B) *Musca domestica*

(Source: Getahun *et al*.,2020b).*Stomoxys calcitrans* causes a lot of annoyance to the animals resulting in reduced feeding time, weight loss, possible transmission of pathogenic microorganisms. They are mechanical vectors of many livestock diseases

such as viruses (Rift valley fever virus and West Nile fever virus), bacteria (*Bacillus anthracis* and *Pasteurella multocida*), protozoans (Trypanosomes and *Besnoitia besnoit*) and Helminths (*Habronema microstoma* and *Dirofilaria repens*) (Baldacchino *et al*., 2013; Sharif *et al*., 2017), and surra disease in camels (Njiru *et al*., 2000; Getahun *et al*., 2020a).

If not managed, *Stomoxys calcitrans* can cause serious economic losses. Previous studies in USA alone, reported the economic losses as a result of stable flies infestations to be around 2.2 billion USD per year (Taylor *et al*., 2012). These losses are suspected to be higher due to their feeding habit and their wide distribution in nature. The female stable fly lay eggs on animal dung and decomposing organic matter on farms (Steve Mihok & Clausen, 1996; Jeanbourquin & Guerin, 2007a; Baleba *et al*., 2019). An ovipositing female stable fly chooses a substrate that increases the fitness of its offspring (Baleba *et al*., 2019). It has been shown that these flies prefer to lay on donkey and sheep dung because they contain β-citronellene and carvone, respectively, which attract gravid females.

Despite blood being the main protein source for stable fly and essential to mating and ovarian development, previous studies have shown that *S. calcitrans* feeds on sucrose solution when supplied in the laboratory (Moonola & Cupp, 1978). Stable flies have been observed resting on some flowering plants in Europe and North America (Jarzen & Hogsette, 2008; Jeanbourquin & Guerin, 2007b). Recently, they were documented resting in some fruits and flowers in Mali (Müller *et al*., 2012), but the chemical ecology and physiological basis underlying this observation have not been determined yet.

Insects-plant interactions can be mutualistic, commensalistic, or antagonistic (Calatayud *et al*., 2018). Plant floral and extra-floral nectar is a source of sugars, amino acids, and other important nutritional elements essential in insect nutrition (Carter *et al*., 2019). Plant nectar has been shown to have some nutritional benefits, such as increased survivorship and autogeny when ingested by mosquitoes (Foster, 1995) and other insects such as hoverflies (Haslett, 1983). Infection of malaria vectors with Plasmodium

parasites increases its plant foraging behaviour (Nyasembe *et al*., 2014). This could be a source of self-medication and possibility of pesticides resistance and can occur in stable flies too. During photosynthesis and respiration, plants produce non-volatile and some volatile organic compounds (VOCs) from flowers, leaves, and fruits, which impacts atmospheric chemistry and interaction with the environment (Tholl *et al*., 2006).

The information on the plant species preferred by stable flies is important in understanding the chemistry of plant volatiles that are possible attractants to stable flies and used in its management. This will prompt more research on understanding the vector competence of *Stomoxyine* flies in their natural habitat, incorporating plant nectar sources in habitat manipulation, and developing effective control mechanisms through the lure and trap improvement.

1.2 Statement of the problem

Little is known about the stable fly-plant interaction, plant-derived semiochemicals produced by the preferred plants that act as attractants to the stable flies and fitness value of nectar feeding. The spread of vector-borne diseases by the *Stomoxys* spp. poses a major threat to both human and animal health globally. Vector-borne diseases transmitted by biting flies are the main cause of low animal productivity and increased livestock mortality. According to previous studies, the economic losses due to stable flies infestation in the United States alone is above 2.2 billion USD annually. These losses could be higher in other parts, such as Africa. Stable flies' rate of spreading diseases worldwide is increasing due to their diversity and ability to access alternative hosts. There have been several incidences of livestock vector-borne related diseases such as African animal trypanosomiasis (AAT) outside tsetse flies belt, most probably transmitted mechanically by biting flies such as *Stomoxys* spp.

1.3 Justification

It is postulated that despite stable flies being blood-feeders, their nectar feeding behaviour which could play a significant role in their fitness, remains unexplored. The nutrients from the ingested nectar might contain important nutrients that might be carried over to the eggs enhancing larval emergence in stable flies. The source of stable fly's blood meals has been widely studied, but its plant meal source has been overlooked over a very long period. There is a need to investigate *Stomoxys calcitrans*-plant interaction under natural environmental conditions. The knowledge of plant-derived semiochemicals from this study can revolutionise disease vector management by developing environmentally friendly trapping methods for vector control.

1.4 Research questions

(i) Do *Stomoxys calcitrans* feed on plant nectars?

(ii) Does plant nectar feeding have fitness benefits to the stable flies?

(iii) What are the plant semiochemicals utilized by stable flies when seeking for plant sugar sources?

1.5 Objectives

1.5.1 General objective

To establish the evidence of plant sugar feeding in *Stomoxys calcitrans*, and the role of sugar feeding in stable fly's physiology.

1.5.2 Specific objective

1. To establish the evidence of plant feeding in *Stomoxys calcitrans.*
2. To determine fitness value of sugar feeding in *S. calcitrans.*
3. To evaluate plant volatile compounds for their attractiveness to *S. calcitrans* under field conditions.

1.6 Scope of the study

The main focus of this study was to: (i) Determine the nectar feeding behaviour of stable fly, (ii) Physiological basis of plant nectar feeding in adult stable flies, (iii) Understand

the semiochemical basis driving plant sugar feeding, and (iv) Determine how this knowledge can be utilized for stable flies' management.

1.7 Limitations of the study

Only *Parthenium hysterophorus* plant was used while evaluating the fitness benefits of plant-feeding in stable flies. There is a need to conduct fitness experiments using the other identified plants because different plants vary in their nutritional compositions, impacting observed results.

CHAPTER TWO

LITERATURE REVIEW

2.1 Stable flies (*Stomoxys calcitrans*)

Stable flies resemble the common housefly but have long, thin black proboscis for piercing. They use their sharp proboscis to pierce the skin and suck blood from mammals. It has four unique black longitudinal stripes across the thorax and abdomen characterized by several dark spots. The females lay eggs in animal waste and other decaying matter. During plant feeding, they suck the sap from flowers as their source of sugar. Stable flies feed on diverse nectar sources and sugars when supplied in the laboratory, impacting their survival and reproduction (Tawich *et al*., 2021; Jones *et al*., 1992). Like other hematophagous arthropods, stable flies require blood for reproduction and rely on chemical and physical stimuli to locate their host (Gibson & Torr, 1999). Hematophagous insects feed on nectars to acquire sugar which is their energy source to help them seek blood hosts.

2.1.1 Taxonomy of stable flies

According to previous studies by Zumpt (1973), and Skidmore (1985), stable flies taxonomic information have been classified 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

2.2 Biology of stable fly

2.2.1 Feeding behavior of stable flies

Both male and female stable flies are blood feeders. Sharp-bayonet-like mouthparts characterize them for piercing through the host's skin to suck blood and plant surfaces for plant sap/nectar (Gerry *et al*., 2007). The stable flies lack anesthetics in their saliva (Cortinas & Jones, 2006), leading to a painful piercing on the animal skin. This causes host disturbance whereby the animal responds by tail flicking, skin shaking, and leg stamping during feeding reducing feeding time (Ashmawy *et al*., 2019).

S. calcitrans prefers feeding on the livestock ears, head, and lower extremities, such as the front legs of cattle (Campbell *et al*., 2001), because they cannot be easily disturbed in these regions by the host defense mechanisms (Dougherty *et al*., 1993). They also prefer feeding on dog ears and the ankles of humans, sometimes leading to the development of necrotic dermatitis (Geden & Hogsette, 1994). According to comparative studies on feeding habits of Stomoxys spp. and tsetse fly conducted in Zimbabwe, tsetse fly is more responsive to host defensive character as compared to stable fly. This will take the host's least time, therefore not completing its blood meal in one host (Holloway & Phelps, 1991; Schofield & Torr, 2002; Mullens *et al*., 2006).

From Schowalter (1979), a single blood meal taken by stable flies ranges between 11-15 µl volume of blood from the host. Stable fly requires one to two blood meals per day, depending on the climatic conditions, and they take about 3-5 minutes to complete the

blood-feeding on a host for their ovarian development (Hillerton *et al*., 1984). The stable fly is mainly known as a mechanical vector (Baldacchino *et al*., 2013), transmitting pathogens through regurgitation rather than contact through mouthparts during blood feeding. However, it is reported to be both a mechanical and biological vector for *Enterobacteria sakazakii* (Mramba *et al*., 2007). Other insect species, such as tsetse flies, can act as biological and mechanical vectors (Gubler, 2009).

Different types of piercing flies have different feeding strategies and are classified as either mechanical or biological vectors for pathogen transmission. The interrupted feeding in biting flies is a reason for increased pathogen transmission. The conditions in the stable fly's gut is conducive for the survival of pathogens which contributes to delayed pathogen transmission (Baldacchino *et al*., 2013). *Stomoxys calcitrans* feeding habits are of significant epidemiological impact facilitating an inter-herd transfer of pathogens (Foil & Gorham, 2000). Unlike mosquitoes and tabanids, whose females feed on blood for egg production and male solely on sugars (Baldacchino *et al*., 2014), both male and female stable flies feed on a blood meal (Taylor & Berkebile, 2014).

Stable flies feed on nectars from selected fruits and flowers (Tawich *et al*., 2021; Erasmus, 2015; Müller *et al*., 2012). Most biting flies are hematophagous in both sexes, while others, such as tabanids and mosquitoes, have males solely feeding on nectar for somatic maintenance (Baldacchino *et al*., 2014; Ma & Roitberg, 2008). Taylor & Barkebile (2014) study showed that they also feed on plant sugars for energy to enable them to fly in search of blood meals. Biology and ecology resulting from insect-plant interactions utilizing insect's feeding behavior form the basis for their control. The architecture of floral nectar can affect the accessibility of plant nectar by probing insects. Stable flies have been observed in some fruits and flowers, but there is inadequate information on what drives their nectar preference (Müller *et al*., 2012). Stable flies are opportunistic nectar feeders. A correlation between nectar feeding and blood-feeding has been reported because the flies obtain sugar from plants to provide energy for seeking blood meals (Taylor & Berkebile, 2014). The ability of biting flies to feed on sugars is

greatly influenced by environmental factors such as humidity, temperature, and photoperiod. Stable flies are diurnal feeders biting during the day (Usher *et al*., 1985), but other blood-feeders like mosquitoes are nocturnal, biting more during the night than the day (Lehane, 2005). High temperature increases the biting flies' feeding capability compared to low temperatures (Martínez-de la Puente *et al*., 2010). Studies on stable flies showed a need for increased feeding with increased temperature because of increased metabolism in hot weather than in cold weather. In less than 9℃, the flies may make no effort of finding food, thus remaining quiescent, and this condition, if prolonged, may lead to starvation (Florez-Cuadros *et al*., 2019).

2.2.2 Stable fly's life cycle

When planning control and surveillance strategies for a highly mobile insect such as a stable fly, it is crucial to understand its life cycle and related stages for fly development. The duration each piercing fly takes to complete its life cycle varies due to climatic factors such as temperature, humidity, and difference in genetic factors. Moderately higher temperatures speed up the life cycle of the flies, whereas extremely low temperatures negatively affect the development and sometimes make the fly diapause (Foil & Hogsette, 1994). The majority of the piercing flies are completely holometabolous. In contrast, a few like the tsetse fly and Hippobosca camelina have their cycle being viviparous whereby females do not lay eggs but larviposit (Attardo *et al*., 2006). *Stomoxys calcitrans* are holometabolous, developing through four stages: egg, larva, pupa, and adult (Figure 2.1).

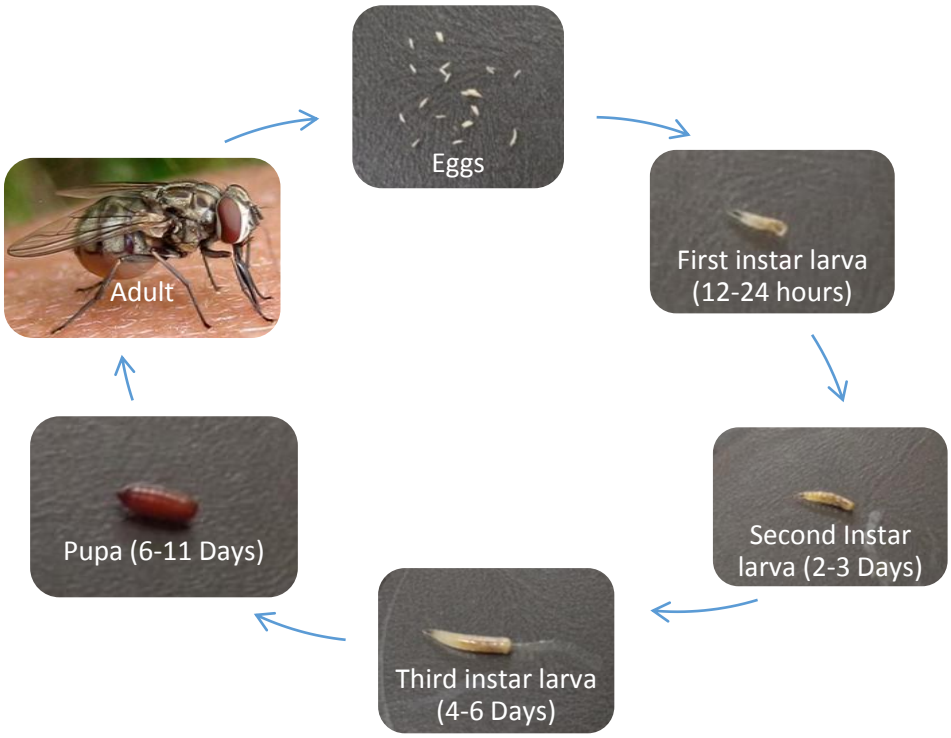


Figure 2. 1: Life cycle of *Stomoxys calcitrans* (Tawich *et al*., 2021)

Stable fly resembles houseflies but has seven circular spots on their abdomen, whereas houseflies' abdomen lacks these patterns (Birkemoe & Sverdrup-Thygeson, 2011). The stable fly has unique patterns that can be used to morphologically distinguish it from other Stomoxys spp according to Zumpt taxonomic keys (Zumpt, 1973). Stable flies lay their egg on decomposing manure or animal dung on the farms. According to the study by Jaenlike, female insects prefer to lay eggs on substrates that enhance their offspring's development host (Jaenike, 1978). According to previous studies, stable fly prefers to lay eggs on sheep and donkey waste compared to other substrates because it has increased Carvone and β- Citronellene, respectively (Baleba *et al*., 2019). When laid, the *S. calcitrans* eggs are white but turn to creamy white with time (Newstead, 1906; Akanmu, 1979). Piercing flies vary in the number of eggs laid throughout its life. Stomoxys, as well as tabanids, can lay 100-200 eggs 4-5 times in their life cycle, summing up between 800-1000 eggs throughout their life cycle (Foil & Hogsette, 1994)

as compared to tsetse flies which are larviparous producing a single larva in 10-14 days (Dean *et al*., 1969). Stable flies lay egg clutches consisting of 20-25 eggs with more than 800 eggs in a lifetime hence higher vector multiplication over a very short period (Patra *et al*., 2018). The eggs hatch into the first-instar larvae after 12-24 hours then grows to second instar larvae and third instar larvae, with all the larval stages taking 11-21 days (Malaithong *et al*., 2019) depending on the temperature, humidity of the substrate, and other biological factors like mites infestation. The emerging larvae bury deep in the substrate to avoid desiccation (Showler & Osbrink, 2015). After 12-26 days, the third instar larvae move towards the drier parts of the habitat. The grey to yellow coloured puparium hardens its cuticles forming a reddish-brown pupa, which lasts 6-20 days, then pupate, and most produce adults after 5-26 days (Patra *et al*., 2018). The newly emerged adult stable fly develops strong wings and flies in less than one hour. Fully mature adults start mating after 3-5 days, and females begin laying eggs in 5-8 days (Foil & Hogsette, 1994). The stable fly takes about 30 days to complete its life cycle (Foil & Hogsette, 1994; Patra *et al*., 2018; Showler & Osbrink, 2015), and this can vary depending on the temperature.

2.3 Odor detection and perception in stable flies

Stable flies have a wide range of sensory systems to track their host, substrate, conspecific such as sense of smell and vision. The recent fly genome study showed that stable flies got expanded gene families in olfaction and vision (Olafson *et al*., 2021). They have sensory organs called sensilla located on the antennae. The antennae of a stable fly is the primary sensory organ having sensilla on its surface, which houses several olfactory sensory neurons (OSNs) that detect volatile odours (Tangtrakulwanich *et al*., 2011, Getahun *et al*., 2020b).

A sensillum is a unit of an antenna in which the nervous system interacts with the external environment. Species and sex determine the number of OSNs and sensilla available per antenna and the number of glomeruli. Insect's sensilla can be classified into different types (placodea, trichodea, basiconica, campaniformia, chaetica, styloconica,

and coeloconica) depending on antenna pore density, length (Pellegrino, 2011; Seada, 2015), putative functions (basiconica and trichoidea), thermoreceptors (coeloconica), mechanoreceptors (e.g. chaetica), and hygroreceptors (e.g. digiformia) (Grabe & Sachse, 2018; Suliman *et al*., 2015). Stable flies' antenna has four types of sensilla: basiconica, clavate, trichoid, and coeloconica sensilla (Tangtrakulwanich *et al*., 2011, Getahun *et al*., 2020b). For example, *Drosophilla melanogaster* antenna has close to 400 sensilla houses, approximately 1200 OSNs (Shanbhag *et al* 199), which varies between species. Female *A. aegypti* antennae possess 3-4 times more sensilla than their male counterparts (McIver, 1982).

Odour perception of any relevant odour starts when an olfactory sensillum intercepts the volatile molecule. It then passes through the pores of the sensillum to reach the receptor lymph, where it is transported to an odorant-binding protein (OBP) and the dendritic membrane of an OSN for signal transduction (Tsuchihara *et al*., 2005). For successful signal transduction, the odour has to bind to its correct receptor, triggering a series of events in the dendrites, resulting in an electrical signal/action potential (Leal, 2005; Pelosi *et al*., 2006). The electrical signal reaching the glomeruli is transmitted to the mushroom body and lateral horn (Vosshall & Stocker, 2007), translating to behavioural output such as attraction or repellency (Kreheret *et al*., 2008). Glomeruli are uniquely circuited, and this shows that feeding and oviposition activate different glomeruli in the antennal lobe (Bisch-Knaden *et al*., 2018). According to previous studies on odour localization in insects, insects chemoreceptors can be regulated intracellularly to impact odour perception, and odour stimulation enhances phosphorylation of OR co-receptor. In contrast, site-directed mutation of Orco reduces the sensitivity of the OSNs and not the ionotropic receptors (IR) neurons (Getahun *et al*., 2016).

The response of an insect to odorant compounds can be easily quantified using electrophysiology entails three methods: SSR, EAG, and GC-EAD (Olsson & Hansson, 2013). Several studies on stable fly's response to odours using EAG and GC-EAD have been done; for example, 1-octen-3-ol has a higher electrical signal to *Stomoxys*

calcitrans antenna compared to other known synthetic attractants for biting flies (Schofield, cork, & brady, 1995). Stable flies have shown a varied response to host-associated odorants (Tangtrakulwanich *et al*., 2011). The antenna response of stable flies using a mixture of attractants and repellents has been evaluated (Hieuet *et al*., 2014). Those emanating from rumen extracts, horse dung and cow have also been studied (Jeanbourquin & Guerin, 2007a). According to a study on shared volatiles from camel metabolic products, VOCs that activated more OSNs elicited strong attraction in stable flies (Getahun *et al*., 2020b). Many studies have been done on the antennal response of stable flies to animal-derived odours, with little research done on plant-derived volatiles.

2.4 Plant-derived volatiles

The amount and type of volatiles released by plants are dependent on the plant species, plant growth stage, and interactions with biotic and abiotic factors (Vivaldo *et al*., 2017). Different plant species express variations in their volatilome. Much has been done on plant volatiles with phytophagous insects compared to blood-feeding insects. Plant-based volatiles has been used successfully to manage insect pests in organic farming (Shrivastava *et al*., 2010). The semiochemicals emitted by plants can serve as signals promoting or deterring insect-plant interaction, and this observation has been widely utilized in the control of insect herbivores (Paré & Tumlinson, 1999). When damaged, some green leave volatiles compounds released by plants attract predatory insects such as parasitoids or predators.

The utilization of plant volatiles through the integrated pest management system has revolutionized the control of plant-feeding insects. This has contributed positively to managing pests of economic importance through innovations such as push-pull technologies (Hassanali *et al*., 2008). Push-pull technology has proven effective in improving soil nutritional status when leguminous plants are used and controlling fall armyworm (Khan *et al*., 2018). This leads to ecologically sustainable higher yields. Plant-derived volatiles has great potential to control livestock disease vectors, reducing the possibility of insecticide resistance and disease burden. Essential oils from catnip

have proven their insecticidal effects in controlling stable flies (Noll, 2020). Botanical pesticides and repellents from previous studies have been utilized to manage hematophagous arthropods (De Boer *et al*., 2010).

2.5 *Parthenium hysterophorus*

Parthenium hysterophorus (Famine weed) belongs to the *Asteraceae* family, and native to Mexico, caribbean, south and central America but widely distributed globally. It has numerous and small-sized flower heads clustered together and when mature produces five small seed. These seeds are highly dispersed by wind, water, clothings, machinery, and contaminated agricultural produce. This weed negatively impact crop farming and dairy due to its allelopathic property causing skin irritation and respiratory probles and mutagenesis in humans (Patel 2011). Its smell and chemical composition can be carried to milk affecting milk quality. *P. hysterophorus* plant is known to be a source of different sugars which acts as energy reserve for most of the flying insects and this has been confirmed through foraging behavior of *Anopheles gambiae* mosquito (Guseman, 2014). However, the chemical composition of this plant might have some physiological effects on stable fly's reproduction and survival. *P. hysterophorus* was used in this experiment because the other plants were very large trees and difficult to grow in pots for use in laboratory assays. *P. hysterophorus* is a source of secondary metabolites with various phytochemicals, antioxidants, fatty acids, hydrocarbons and terpenes among other essential elements (Ahmad *et al*., 2018).

2.6 Stable fly as an agent of disease transmission

2.6.1 Factors determining transmission of diseases by stable flies

2.6.1.1 Host behavior

Stable flies utilize both visual and chemical stimuli for host identification. The host colour intensity and polarization properties are important parameters in determining the degree of attraction to stable flies. These disease vectors are visually attracted to animals of specific colours and thus increase feeding on them more than others (Baldacchino *et*

al., 2014). The host animals release several odours through the skin, breath and excreta, which aid the stable flies in identifying them (Getahun *et al*., 2020b). Host preference in stable flies depends on how the host responds when these flies pierce the skin surface.

When under attack, animal hosts with little reaction are mostly preferred by stable flies to those responding vigorously by tail flicking, head swings, and foot stamping (Warnes & Finlayson, 1987).

2.6.1.2 Vector anatomical characteristics

The vector's anatomical characteristics and sensitivity to host defensive mechanisms determine its feeding habits and the amount of blood imbibed to complete one blood meal. According to a study by Schowalter (1979), stable flies suck approximately 11-15 µl of blood and require atleast two blood-meal daily. The painful piercing into the animal skin is the reason for animal response leading to interrupted feeding in these flies leading to the increased number of animals visited to complete one blood meal. These flies lack anaesthetics making their piercing very painful. Tabanids, for example, is another close family of biting flies that is 3-5 times larger in size than *Stomoxys calcitrans* and thus requires a large amount of blood as dictated by body size. The fly's biting intensity and persistence to switch between animal hosts also matters in understanding the transmission of vector-borne diseases (Baldacchino *et al*., 2014).

2.6.2 Diseases transmitted by stable flies

While estimating the mechanical transmission of pathogens by a vector in the natural population, consideration must be made of prevalence and immunity in the host population. The pathogens thrive better in the host species that have reduced immunity levels. Highly virulent pathogens have an increased rate of efficient disease transmission in a population. Animals can pick up pathogens from feeding areas prone to pathogens. A disease vector with a wide range of hosts has increased chances of pathogenic transmission. *Stomoxys calcitrans* is an insect of great medical and veterinary importance spreading a wide range of diseases to humans and livestock, as shown in table 2.1. Thus, proper control methods need to be established for its management.

Table 2. 1: Diseases transmitted by *Stomoxys calcitrans* (Baldacchino *et al*., 2013)

Disease agent	Transmission	Reference
1. Viruses		
Equine infectious Anaemia (EIAV)	Mechanical	Foil *et al*., 1983
African Swine Fever Virus (ASFV)	Mechanical	Mellor *et al*., 1987
West Nile Fever Virus (WNFV)	Mechanical	Doyle *et al*., 2011
Rift Valley fever Virus (RVFV)	Mechanical	Hoch *et al*., 1985
Lumpy Skin Disease Virus (LSDV)	Mechanical	Chihola *et al*., 2003
Bovine Herpes Virus (BHV)	Mechanical	Gibbs *et al*., 1973
Bovine Leukosis Virus (BLV)	Mechanical	Buxton *et al*., 1985
Vesicular Stomatitis Virus (VSV)	Mechanical	Ferris *et al*., 1955
Poliomyelitis Virus	Mechanical	Anderson & Frost, 1912
2. Bacteria		
Bacillus anthracis	Mechanical	Schuberg & Boing, 1914
Pasteurella multocida	Mechanical	Nieschulz & Kraneveld, 1929
Francisella tulerensis	Mechanical	Olsufiev,1940
Dematophillus congolensis	Mechanical	Richard & Pier, 1966
Erysipelothrix rhusiophiae	Mechanical	Wellman, 1950
Enterobacteria sakazakii	Biological & Mechanical	Mramba *et al*., 2007
3. Rickettsia		

Anaplasma marginale	Mechanical	Oliveira *et al*., 2011
4. Protozoa		
Trypanosoma evansi	Mechanical	Bouel & Rouband, 1912, Getahun *et al*., 2020a
Trypanosoma equinum	Mechanical	Lehane, 1991
Trypanosoma vivax	Mechanical	Mihok *et al*., 1995, Getahun *et al*., 2020a
Trypanosoma brucei	Mechanical	Mihok *et al*., 1995
Trypanosoma congolense	Mechanical	Sumba *et al*., 1998
Besnoitia besnoiti	Mechanical	Bigalke, 1968
Leishmania tropica	Mechanical	Berbarian, 1938
5. Helminths		
Dirofilaria repens	(Mechanical)	Krinsky, 1976
Dirofilaria yoemeri	(Mechanical)	Krinsky, 1976
Onchocerca gibsoni	(Mechanical)	Krinsky, 1976

2.7 Effects of stable flies on livestock production

Stable fly causes a lot of significant direct and indirect losses to the livestock. In the United States alone, stable flies causes more than 2 billion USD annually (Taylor *et al*., 2012). This could even be higher in other parts of the world, such as Africa. It causes reduced outcomes from livestock keeping due to the spread of disease and animal annoyance, which reduces animal feeding, resulting in weight loss and significant milk reduction (Campbell *et al*., 1987). They induce stress on animals through sucking blood, skin lesions due to skin piercing with their sharp mouths posing a risk to other opportunistic infections and increased immunosuppressive effect (Taylor & Berkebile, 2014). The list of diseases transmitted by these vectors is long and therefore quest for a need to develop an efficient control technique to reduce the losses caused by these disease vectors. The wide range of disease agents proved to be transmitted by *S. calcitrans* are shown in table 2.1.

2.8 Control of stable flies

Stable flies are controlled through several methods such as cultural, chemical, mechanical, biological, and sterile insect techniques (SIT) (Taylor & Berkebile, 2014). No one control method has proved best in managing stable flies population, and thus integration of the various techniques are required for increased accuracy.

Proper sanitary measures are required to avoid a dumpy environment that usually acts as their breeding ground. These significantly reduce the fly population on the farm. This technique has been very effective in controlling phlebotomine sandflies (Warburg & Faiman, 2011). Removing any substance or waste that can act as a developmental site for maggots helps reduce their breeding. Organic waste drying by dispersing discourage larval development, which thrives best in a humid environment like decomposing manures.

Environmentally-friendly control methods are advised to control stable flies. Plant-derived semiochemicals and plant extracts have singly increased trap catch in stable flies (Tawich *et al*., 2021). Developing a component blend of both animal and plant-derived odours could be the best option to control this important fly because of their additive effect hence increased efficacy. The VOC's blend has no adverse effects on the environment reducing the chances of development of insecticides resistance in stable flies. Chemical control through spraying has also been proven effective in reducing stable flies populations, but the disadvantage is developing resistance to the insecticides used over time. An example of the most widely used insecticide in controlling stable flies is Permethrin. Other disadvantages of chemical use are the higher cost of buying insecticides and their toxic nature to non-target species and, therefore, non-economically selective.

Furthermore, Developing resistance to multiple insecticides in stable (Reissert-Oppermann *et al*., 2019) necessitates alternative control tools. Biologically, parasitoids and entomopathogenic fungi have been utilized to control stable flies. Recent studies conducted to determine the infection of *Metarhizium anisopliae* on stable flies have

shown that this fungus negatively affects survival, reproduction, and other fitness parameters and can be used in their management (Baleba *et al*., 2021). According to a study on parasitism of stable flies and houseflies conducted in Illinois feedlots, *Spalangia* spp could parasitize up to 93% of stable flies (Jones & Weinzierl, 1997).

Apart from chemical stimuli, visual stimuli or combinations can be applied to manage the fly population (Baldacchino *et al*., 2013). This involves using surfaces like fibreglass that reflect near UV light and blue coloured fabrics as applied in insecticide-impregnated screens and traps such as Vavoua, Nzi and white sticky traps (Solórzano *et al*., 2015).

2.9 Potential of Host-Vector Semiochemicals interaction for vector management

Semiochemicals are signal chemicals used by insects to convey a specific chemical message that modifies behaviour or physiology. They are divided into pheromones that act within a given species and allelochemicals that work between different species (Heuskin *et al*., 2011). Allelochemicals are classified into other classes that signal various biological activities in the ecosystem. These are Allomones (when the signal benefits only the emitter), kairomones (when it helps only the receiver), synomones (when the signal benefits both receiver and emitter), and finally, apneumones (when it is of non biological origin) (Bell *et al*., 2013).

Isolation, identification, and synthesis of semiochemicals have been made easy with advancements in analytical and electrophysiological techniques, Semiochemicals have been used either singly or as a blend for population prediction by monitoring insects and mass trapping. In this study, the identified plant-derived semiochemicals can be used as potential bait enhancers in trapping stable flies. Stable flies, among other insects, rely on olfaction as the key sensory modality for sensing their external environment. The detection of specific semiochemicals by stable flies is dose-dependent. Insects' responses to semiochemicals vary and can be utilized in integrated vector management programmes to keep pests and disease vectors below damage levels. The key advantage of using semiochemicals includes: 1) They are specific and hence does not affect non-

target species 2) They are non-toxic and required in small amount and thus environmentally friendly 3) It is also very difficult for the insects to develop resistance as compared to the use of insecticides.

Stable flies respond to different semiochemicals when looking for mates, oviposition substrates, and blood or sugar meals. The other challenge of working with semiochemicals is the degree of plasticity in observed behavioural response and the development of the correct formulation that stable flies respond to. Studies done on sensory and behavioural responses of *Stomoxys calcitrans* have shown that its olfactory receptors are sensitive to rumen waste of animals and are used by these flies in locating oviposition sites (Jeanbourquin & Guerin *et al*., 2007). Their sensory behaviour has been utilized in this study to identify plant chemical lures for use in controlling *Stomoxys* spp. Livestock and wild animals excrete urine and dung, which the fly can easily detect through its olfactory receptors. The semiochemicals found in the plant, urine and livestock dung assists the flies in identifying a host to feed. The animal excreta decomposing in the animal's stables releases some volatile organic compounds that attract the flies to oviposit. These odours can be utilized alone or combined with the plant's volatile organic compounds to make efficient lures for stable fly control.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study sites

The study was conducted in three sites in Kenya: Nairobi Duduville campus (1°13′12″S, 36°52′48″E), Mpala research centre-Laikipia county (00° 23′26.98″N, 036°52′14.98″E) and Amboseli national park (2.6527° S, 37.2606°E). These regions were selected because of the previous reports indicating the presence of S*tomoxys calcitrans* in the regions and availability of diverse vegetation cover. Based on previous studies, stable flies have been reported to be common in these regions. Both Mpala Research Centre and Amboseli national park represents the rural setting with most vegetation cover consisting of shrubs and acacia bushes whereas *icipe* represented an urban set-up. The study was conducted during the rainy season, average temperature was 19±2°C, and relative humidity (60-80%).

3.2 Molecular methods

3.2.1 Sample collection and preparation

Stomoxys calcitrans were trapped using non-baited Vavoua traps (Mihok *et al*., 1995) during the rainy season of March-April 2019 in the three collection site *Icipe*-Duduville campus, mpala research center, and Amboseli national park. Abundance of these flies have previously been reported to increase during rainy seasons. The flies were then immobilized and morphologically identified and preserved in absolute ethanol before being taken to the laboratory for DNA extraction and subsequent molecular works.

Prior to fly dissection and DNA extraction, each fly was first washed to remove any exogenous plant material on the surface by dipping in 1% sodium hydroxide (NaOH) for 1 minute and then rinsed in 1× Phosphate buffered saline buffer (PBS, pH = 7.4) for another one minute. 100 flies randomly picked from the three collection sites were gut-dissected singly under a dissecting microscope to obtain gut contents for genomic DNA extraction.

3.2.2 DNA extraction and PCR detection of plant feeding in stable flies

The midgut contents of every single fly was placed in a separate 1.5 ml eppendorf tube and mechanically lysed by crushing with plastic pestles (Sigma-Aldrich, USA). DNA extraction process was then conducted using DNeasy blood and tissue kit (Qiagen, Hilden, Germany) following manufacturer's protocol. The DNA sample is first lysed using proteinase K and buffer conditions adjusted to facilitate optimal DNA binding when the lysate is loaded to mini spin columns. Each spin column containing the lysates is then placed on 2ml collection tubes and centrifuged for the sample to bind and contaminants to pass through. The spin columns are then washed twice to eliminate contaminats and enzyme inhibitors.The obtained DNA is then eluted in 50 μL elution buffer (EB) or water and the DNAis ready for use. The DNA concentration was checked using Nanodrop spectrophotometer (Thermo Scientific, Wilmongton, DE, USA) by recording the absorbance ratios at 260 nm and 280 nm. The remaining genomic DNA was stored at -20°C until use for PCR amplification.

Plant meal sources of stable flies was determined using PCR- based detection method using universal primers targeting plant-specific gene targets of chloroplast coding rbcL gene, and the non-coding trnH-psbA genes adopted from previous published studies (Shaw *et al*., 2005; Bafeel *et al*., 2012; Abbasi *et al*., 2018; Tawich *et al*., 2021). The rbcL was used as standard coding region because of its ease of amplification and universality, it has been widely adopted in previous studies in plant barcoding studies as well as phylogenetics (Vijayan & Tsou, 2010). The rbcL region is the most characterized plastid coding region in the GenBank from most other plant groups and thus forms the best baseline for comparing with other plastid genes.

PCR amplification was conducted with standard annealing temperature of each primer used. PCR amplification process was done using 96 wells Proflex thermal cycler in 10 μL reaction volume. The total reaction volume consisted of: 0.5 μL forward primer and 0.5 μL of the reverse primer (primer concentration 10 μM), 2 μL 5× HOT FIREPol Evagreen HRM mix, 6 μL Nuclease free water and 1 μL DNA of each *S. calcitrans*

sample as the template. 1µL of nuclease free water only used as template for each negative controls. DNA sample extracted from maize-fed fall armyworm (*Spodoptera frugiperda*) was routinely used as a positive control throughout the PCR process. The following are the PCR parameters used: Initial denaturation at 95°C for 15 minutes followed by 35 alternating cycles of 95°C (30 seconds for each cycle), both rbcLA and trnH-psbA primers had their annealing temperature as 53°C whereas rbcL2 annealing temperature was 50°C, elongation step was set at 72°C for 1 minute, and a final elongation step at 72°C for 10 minutes with a final hold stage at 4°C after completion of the PCR process.

The following is the base pair information of three primers used in this study:

A. Non-coding region with the target genes as Itergenic spacer region.

   TrnH-psbA primer

   Forward primer: 5’ CGCGCATGGTGGATTCACAATCC 3’

   Reverse primer: 5’ GTTATGCATGAACGTAATGCT 3’.

B. The two coding regions with target genes as the chloroplast:

   i. **rbcLA primer**

      Forward primer: 5’ ATGTCACCACAAACAGAGACTAAAGC 3’

      Reverse primer: 5’ ATGTCACCACAAACAGAGACTAAAGC 3’

   ii. **rbcL2 primer**

      Forward primer:5’ TATGTAGCTTAYCCMTTAGACCTTTTTGAAGA 3’

      Reverse primer: 5’ GCTTCGGCACAAAAKARGAARCGGTCTC 3’.

3.2.3 Confirmation of plant-meal source in stable flies by gene sequencing

All the successful PCR products were separated using a 1% agarose gel stained with 0.5µg/mL ethidium bromide and set to run at 87 volts for one hour on 1× TAE buffer.100bp ladder (Solis BioDyne) was used to determine the band sizes of the amplicons. Gel images were visualized under UV-light in a UV transilluminator with

Kodak camera (Gel Logic 200 Imaging System, Kodak, Japan). The bands of interest were cut using scarpels and purified for sequencing with QIAquick purification kit (Qiagen, Valencia, California, USA) according to manufacturer's procedures. DNA sequencing samples were outsourced for Sanger sequencing at Macrogen inc., Netherlands. The sequences were cleaned, edited and aligned using Geneious software and contigs from both the forward and reverse strands used to form consensus sequences. The plant host origin of the sequences derived was determined by aligning the obtained sequences against GenBank reference database using using bioinformatics tool BLAST search in NCBI's website with its default parameters.

3.3 Determination of the fitness value of plant nectar feeding on stable fly longevity and fecundity

3.3.1 Colony establishment of stable flies

Adult *S. calcitrans* of both sexes were trapped at the *icipe*-Duduville campus field and taken to *icipe* insect's rearing units. The flies were maintained in (75 cm × 60 cm × 45 cm) perspex cages (Astariglas®, Indonesia) and fed twice daily at 0800 hours & 1600 hours on defibrinated bovine blood obtained from local slaughter house (Choice meats). The temperature and humidity in the rearing room was kept at 25 ± 1℃ and RH 50 ± 5% respectively with a 12:12 light/dark photoperiod. The temperature as well as humidity were adjusted regularly using automated humidifiers and heaters respectively. Blood feeding was provided in a moistened cotton wool on a petri dish inside the cages. Sheep dung was used as oviposition substrate and developed pupa was picked and transferred to another cage for emergence. Blood as well as sheep substrate was provided to the newly emerged stable flies for multiplication to get enough flies for use in repeated behavioral and survival bioassays in the laboratory.

Parthenium hysterophorus was used in this experiment as a model plant because the rest of the plants identified from DNA sequences and physically in the field with the help of plant taxonomist were big trees such as A*cacia* plants which are difficult to grow in pots for laboratory experiments or get enough fresh leaves and flower cutting regularly.

Parthenium hysterophorus was an ideal experimental plant because it is readily available as weed within *icipe* research centre and can be easily grown in pots for laboratory feeding experiments.

A preliminary confirmatory experiment to ascertain whether stable flies actually feeds on *P. hysterophorus* was conducted by placing 20 stable flies in a cage (18 cm × 18 cm × 18 cm) replicated three times and providing them with potted intact *P. hysterophorus* plant for 24 hours. Each fly was then gut-dissected, extracted the DNA and detected for plant meal. *P. hysterophorus* plants were grown in plastic pots for use in subsequent laboratory experiments at flowering stage.

3.3.2 Survival assays

A group of newly emerged lab-reared male and female stable flies were kept in Perspex cage (75 cm by 60 cm by 45 cm) and supplied with the following treatments. All the treatments other than flowering *P. hysterophorus* plant were supplied in moistened cotton wool on a petri dish.

i. Blood only.
ii. Blood supplemented with 10% glucose (ratio 3:1 respectively, meaning three parts of blood to one part of 10% glucose)
iii. Intact flowering *Parthenium hysterophorus* plant and Blood.
iv. *Parthenium hysterophorus* plant only.
v. Water alone (control).
vi. Four glucose concentrations: 5%, 10%, 15%, and 20% glucose diluted in distilled water.

Survival analysis uses statistical techniques to compare the risks of death associated with given treatments over time. A total of 20 newly emerged stable flies (10 males & 10 females) were kept in smaller perspex cages (10 cm by 15 cm) per treatments and monitored for their survival throughout their life time. The feeding experiment with blood and *P. hysterophorus* were conducted in 18 cm by 18 cm by 18 cm perspex cages because the large size of the plant which can't fit in 10 cm by 10 cm by 15 cm cages. To

facilitate the survival experiment, twenty flies per treatment were placed in a cage and replicated five times. Figure below shows the feeding experimental set up with stable flies imbibing nectar when supplied with both blood and plant (Figure 3.1A) and stable flies sucking blood sample from a petri dish with a cotton wool soaked with blood (figure 3.1B).

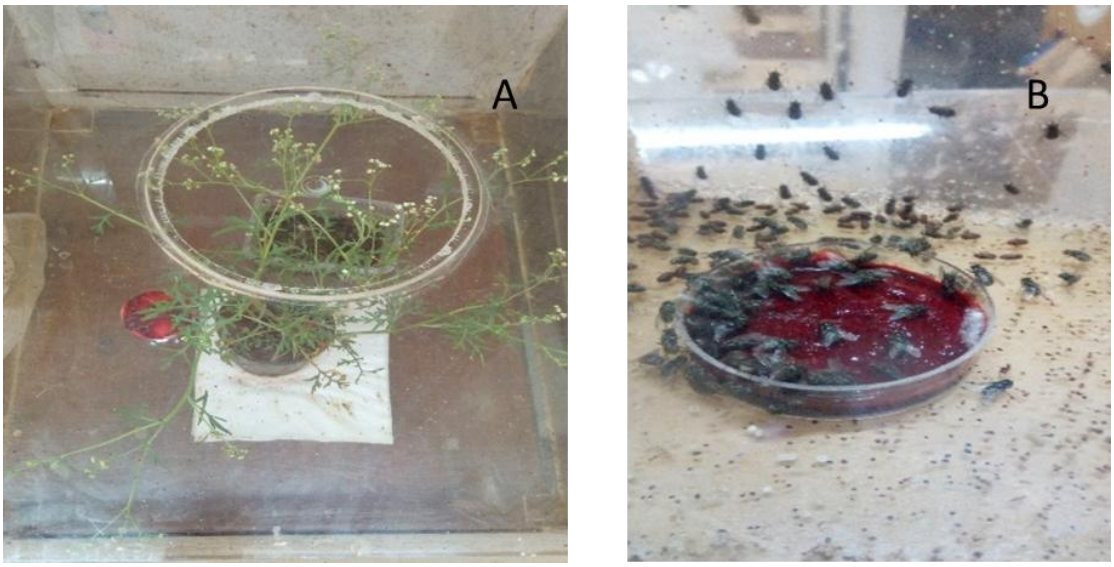


Figure 3. 1: Feeding experiment set up

(A) Shows blood supplemented with *Parthenium hysterophorus* plant (B) Stable flies sucking blood from a moistened cotton wool.

The flies under each of the various treatments were fed and monitored with daily mortality per treatment recorded. Due to the high mobility of stable flies, the survival rate per treatment was calculated by subtracting the total daily fly mortality from the initial number used in running the survival experiment daily. The difference in survival times of adult stable flies under different feeding regimes was analyzed using Kaplan-Meier method and comparison between the various treatments determined using the log-rank test.

3.3.3 Ovipositional assays

Ovipositional bioassay was used to determine the effect of sugar/nectar on the reproduction of *S. calcitrans*. The flies fed solely on distilled water, plant alone as well as four different glucose concentrations alone didn't manage to lay eggs and therefore no ovipositional data recorded. The flies from three treatments: i) Blood alone ii) Blood mixed with 10% glucose (ratio 3:1) iii) Blood supplemented with intact *P. hysterophorus* reached oviposition stage and data on number of eggs laid was recorded and analyzed.

One gravid female from each treatment was isolated and kept in an oviposition cage with 50 grams of substrate and with the correct treatment. Due to the small sizes of the oviposition cups, freshly cut *Parthenium hysterophorus* flowers were supplied on small cages (10 cm by 10 cm by 15 cm) with oviposion substrates on a petri dish (Figure 3.2A) instead of the intact plant which is large in size and could not fit in the small cages nor oviposition cups (Figure 3.2B). Cutting of flowers results in increase emission of green leave volatiles but little impact on floral nectar which was targeted in the oviposition experiment. However, volatiles emitted by cut flowers can affect the fly's feeding ability. The eggs laid per fly were recorded against the treatment throughout the fly's lifetime. The eggs were picked from the oviposition substrates using soft forceps with help of hand lense, counted and transferred to new larval developmental substrates (Figure 3.2C).

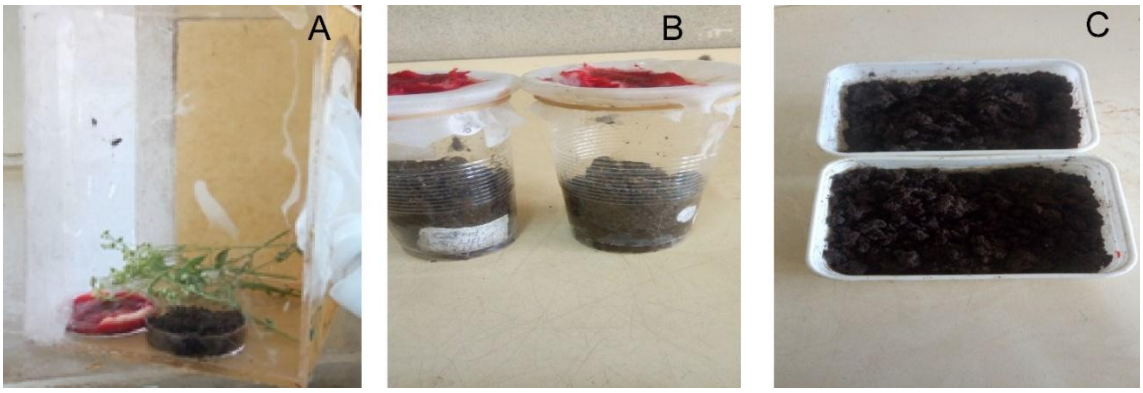


Figure 3. 2: Ovipositional experiment of stable flies

Figure 3.2A shows a stable fly oviposition experiment when supplied with blood-soaked in moist cotton wool and supplemented with *P.hysterophorus* flower cuttings. Figure 3.2B shows a stable fly oviposition experiment in oviposition cups and supplied with either blood alone or blood with sugar treatments soaked in cotton wool. Figure 3.2C shows sheep dung used in the experiments for larval development.

The data collected here is important in determining the best treatment that enhances stable fly's reproduction as this can be used in colony maintenance and understanding the physiological importance of plant/sugar feeding.

The laid eggs were incubated in a fresh substrate for a maximum of 72 hours at 18±1℃ temperature for eggs to hatch to larvae after which the eggs were considered non-viable. During this time, the percentage of larval emergence was also determined. This experiments investigated the influence of different feeding regimes on stable flies' larval emergence which is an aspect of fertility, hencecrucial in determination of sustenability of vector population in the ecosystem. The difference in hatchability of eggs from the various treatments was determined.

3.4 Plant volatile chemistry

3.4.1 Plant volatile collection and extracts preparation

3.4.1.1 Plant volatile collection

Headspace volatiles were collected from plants identified to be possible nectar source for stable flies. Flies collected from mpala research center were only positive for *Senegalia mellifera* & *Senegal Senegal* and *icipe* collection were positive for *P. hysterophorus*, *T. brownii*, *S. terebinthifolia*, *V. xantophloea*. Amboseli collection were only positive for *A. sativum*. The volatiles were collected from all the identified plants except *A. sativum* which we were unable to locate in the field. Volatile collection was done in plant's natural habitats using the portable field pump (Analytical Research Systems, Gainessville, Florida, USA) (Figure 3.3). The aerial parts of the plant including flowers and leaves were enclosed gently in an air-tight oven bag (Reynolds, Richmond, VA, USA) connected with the portable field pump supplying charcoal filtered air out

through a super Q (Alltech Inc., Deerfield, IL, USA) adsorbent filters tied in the tube taking air from the back into the pump. The airflow into and out of the pump was set to be 2.5 litres per minute and 2 litres per minute respectively for 12 hours (Tawich *et al*., 2021). The analytes trapped on super Q were eluted with 300 µL GC-MS molecular grade hexane. The eluent was stored at -80℃ until analysis.

Figure 3. 3: Plant headspace volatile collection using a portable field pump

3.4.1.2 Preparation of extracts

Plant extracts in hexane were made from the identified plant species using the set up shown in figure 3.4. It consisted of 100 grams of only plant leaves and flowers. The plant material weighed and crushed using wooden mortar and pestle. 100 ml of hexane solvent was then added as an extraction solvent and covered using an alluminium foil and left for 12 hours. The extracts were then filtered using filter paper (Whatman, circles, diam. 25 mm) and kept under -80℃ until use.

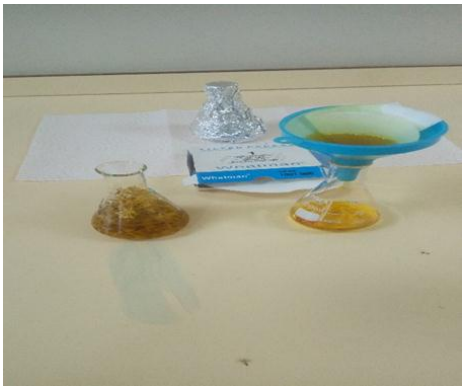


Figure 3. 4: Preparation of hexane plant extracts

3.4.2 Volatile analysis

Volatile analysis was done using the coupled Gas-Chromatography Mass Spectrometry (GC-MS). 1 μL of volatile extract was injected into the GC-MS with the auto sampler (Agilent technologies). Nonpolar capillary HP column was used to separate the compounds with helium as the carrier gas at a flow rate of 35 cm per second. The oven temperature was held at 35℃ for 5 minutes and programmed to increase at a rate of 10℃ min^{-1} to 280℃ and held at this maximum temperature for 10 minutes. The chromatograms acquired were analyzed using Agilent MSD Chemstation whereby the chemical mass spectral data was compared with the library data (Adams2. L and NIST05a.L). The absolute areas of each constituent chemical compound as calculated by the NIST05a.L software was used to estimate their percentage abundance. Dichloromethane was used as solvent blank and analyzed using the same parameters (Tawich *et al* 2021).

After the correct chemical profiles for GC-MS tested plant volatiles was determined, the individual plant volatiles were assigned to groups corresponding to species and distances between and within groups used to calculate R values which ranges between -1 and 1 for each group comparison. Higher positive R value close to 1 represents higher dissimilarity between the groups whereas lower R values imply similarity following graphical representation in NMDS by using PAST statistical software version 4.0.2. One way-ANOSIM was conducted to validate if the visual clustering patterns of similarity in NMDS was statistically different. NMDS provides unbiased insight into the patterns of chemical associations from the tested plant sources. Plants that clustered together are interpreted as to have a similar volatile composition (Majetic *et al*., 2008).

Clustering together of different plant species shows that the plants have a shared volatile profile. Identity of key chemical volatiles conserved between the plant species was determined using the similarity percentage (SIMPER). SIMPER was used to support data obtained in one-way ANOSIM to see how similar or different the variables are. The volatile chemicals data recorded in this study had high number of zeros and thus not

fulfilling the assumptions to conduct principal component analysis (PCA) hence NMDS & ANOSIM was appropriate for the analysis because it uses rank orders and therefore flexible for analyzing different data types.

3.4.3 Odor stimuli

Sixteen odors and five hexane plant extracts were tested singly for their ability to attract stable flies under field conditions. The unbaited trap was routinely used as a negative control. The odors were purchased in their synthetic standards with the highest purities. These chemicals are Linalool oxide, Mycene, Linalool, Methyl benzoate, p-cymene, ϒ-terpinene, Cis-3-Hexenyl acetate, β-Ocimene, p-cresol, Butyric acid, Sabinene, α-Phellandrene, β-Pinene, β-Caryophyllene, Toluene, and Isophorone (Sigma-Aldrich Germany).

3.4.4 Field evaluation of plant derived semiochemicals for the attraction of *Stomoxys calcitrans*

All the tested lures were released using dental cotton rolls (10 × 38 mm; Shanghai Dochem Industries Co. Ltd.) and unbaited traps used as control. 2 ml of each of thechemical and selected plant extacts in its pure undiluted standard was pipetted into a 4 ml glass tube and cotton dispenser inserted and tilted to soak the lure. The vial was covered with a perforated cap and tied 15 cm above ground on the pole of the Vavoua trap. The traps were arranged in a randomized complete block design with a distance of 500 meters between the blocks and 100 meters inter-trap distance. The field testing was done only in mpala research center in November when long rains were experienced in most regions of Kenya. This site is ideal for testing the response of stable flies to selected plant-derived VOC's and extracts because of large space to run RCBD experiment which requires a vast track of land with minimal human interference.The abundance of stable flies is high during rainy seasons than in dry seasons (Dawit *et al*., 2012; Keawrayup *et al*., 2012).

The experiment was set at the Mkenye field site around the dam on the broader Mpala conservancy because of being the central point for animals to drink water and diverse

vegetation, which is a source of background odors. Wild animals and domestic animals take water in a Mkenye dam. The area is rich in vegetation consisting of shrubs and acacia bushes. The availability of background odors ensured that the competent chemical obtained in this experiment could outshine in a natural setting. The traps were deployed at 0900hours and emptied after 24 hours, and daily trap catches per treatment were identified and recorded. Only the initial stable fly trapping experiment for plant meal analysis was done at Amboseli national park.

3.5 Sample size calculation

Proper sampling procedure need to be designed to understand the nectar feeding preference of stable flies in the wild. From previous studies, the lowest reported field positivity rate of nectar feeding prevalence under field conditions was between 3-23% (Jones *et al*., 1992) and therefore took 3% which is the minimum positivity rate in order to get maximum sample population for this study. The sample size was determined using the formula: $n = \frac{\ln(\alpha)}{\ln(1-p)}$ (Cameron & Baldock,1998). All analysis were conducted at 95% confidence limit, $\alpha = 0.05$, $p = 0.03$ (probability of nectar fed flies). Therefore, the minimum number of flies to be sampled must be more than 99 for accurate reporting of wild nectar feeding in stable flies. From the total number of flies collected from the field for plant meal analysis from each site, 100 flies were randomly sampled for DNA extraction and detection of their plant meal source. These samples were used for molecular experiment on detection of plant materials in stable fly's gut DNA content.

3.5 Data analysis

The eggs laid as well as their hatchability followed a normal distribution (Shapiro Wilk test, $P > 0.05$) and was analyzed using analysis of variance (ANOVA) and the means separated using the Student's Neuman Keuls -post hoc test (SNK test).

The chemical/volatile analysis was done by first correctly identifying GC-MS chromatograms and comparing them with th library mass spectral data available on NIST software using Chemstation. A multivariate analysis of identified key volatiles profile was done using NMDS in PAST statistical software version 4.0.2, which

uniquely clustered the identified headspace volatiles. The observed chemical clustering between plant VOCs was statistically compared using one-way ANOSIM using the Bray-Curtis dissimilarity index. Kaplan Meier curve was used to show the survival probabilities of stable flies under varying feeding regimes and differences in survival rates between the individual groups compared using the log-rank test. A generalized linear model with negative binomial error distribution and a log-link function was used to analyze field data on the response of stable flies to the test compounds. SNK post hoc test determined the significant difference between the calculated means. GLM analysis, ANOVA, and survival analysis were done using R statistical software version 3.6.2. The DNA sequences were analyzed using geneious statistical software and aligned with the pre-existing sequence data on the GenBank database for correct sequence identification using NCBI BLAST search.

CHAPTER FOUR

RESULTS

4.1 Evidence of plant-nectar feeding in stable flies

Stable flies feed on plant nectar from diverse plants. According to the laboratory feeding experiment designed to confirm *S. calcitrans* plant nectar-feeding, 85% (n=20) of the experimental flies had detectable chloroplast DNA. However, the percentage of plant-feeding in the field-collected stable flies was 3.67% (n=300) from the three study sites. Molecular analysis by extracting gut DNA from field-collected *S.calcitrans* and amplifying for cpDNA using plant barcoding genes proved that these flies were positive for cpDNA (Figure 4.1). Stable flies feed on selected plants (Figure 4.4), and this study found seven plants in the three study regions and collected volatiles from only six. We were unable to collect volatiles from *A. sativum*. Stable flies utilize their long proboscis to imbibe nectar from flowers when supplied with flowering *P. hysterophorus* provided in the laboratory (Figure 4.3). Plant-feeding preference varied between sites (Figure 4.2), and this could be due to variations in the types of vegetation cover.

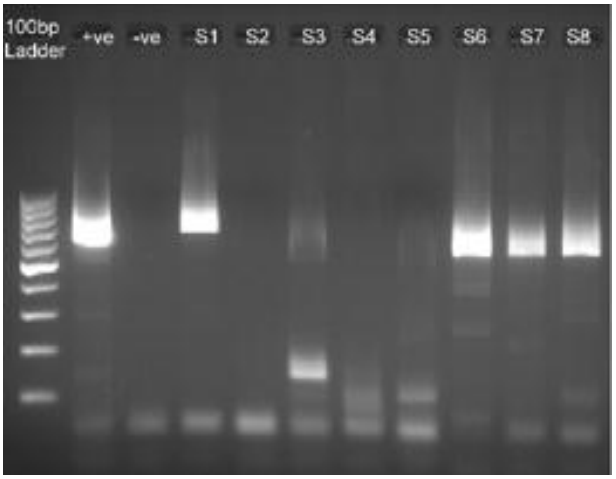


Figure 4. 1: Agarose gel electrophoresis image showing PCR amplicons of cpDNA from DNA extracted from the gut of field collected stable flies.

The PCR products were separated using a 1% agarose gel. A 100bp ladder, +ve control (gDNA of fall armyworm (*Spodoptera frugiperda*) larvae fed on maize), -ve control

(PCR Mastermix with nuclease-free water as template), *S.calcitrans* DNA samples (S1–S8 is representing *S. calcitrans*). Three of the eleven bands were faint, and re-amplification was done separately, that is why only eight samples are displayed on the gel image above.

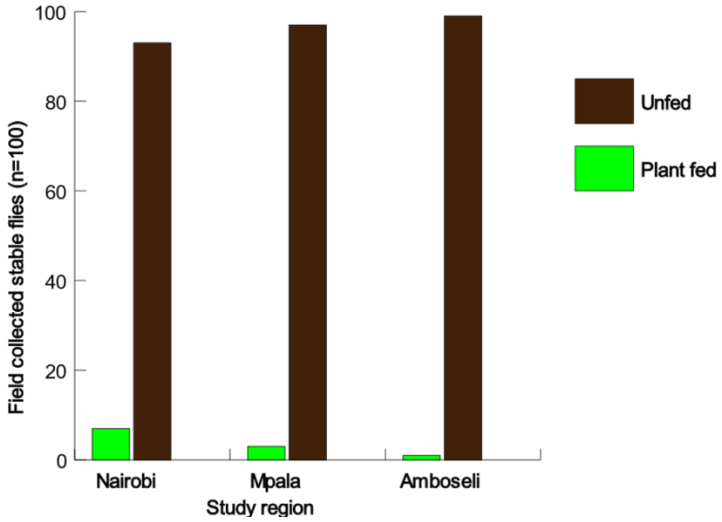


Figure 4. 2: Histogram depicting the percentage abundance of plant-fed stable flies collected from three different sampling sites in Kenya.

A high number of field-collected stable flies from *Icipe*-Duduville campus Nairobi had fed on a plant, therefore positive for cpDNA followed by Mpala and Amboseli, respectively. However, it is not clear why there were differences in the plants fed on at different sites, but the type of vegetation might be a possible factor.

Figure 4. 3: *S. calcitrans* feeding direcly on intact *Parthenium hysterophorus* nectar in the laboratory.

The image above showing stable flies feeding on intact *P. hysterophorus* in the laboratory is a physical evidence that despite being known as blood feeders, they also feed on plant nectar.

Figure 4. 4: Images of selected plants identified to be fed on by stable flies in the wild from this study.

Plant identification from this study was done by sequencing and bioinformatic analysis. We obtained guidance from a plant taxonomist in identifying the exact plants in the field before volatile collection. Some sequences like *S. terbinthifolia* and *T.brownii* amplified using primers from this study were the first to be submitted to the GenBank. According to plant taxonomic identification, they were the only *Schinus* and *Terenbinthifolia* species within the vicinity. The plants fed on by stable flies in this study belonged to only five plant families, i.e. *Fabaceae*, *Amaryllidaceae*, *Anacardiaceae*, *Asteraceae*, and *Verbenaceae*, with the majority having fed on the *Fabaceae* family. The plant identity was confirmed by BLAST analysis, and the results are shown in Table 4.1. Sequence alignments data are presented as supplementary materials in the appendix section (Appendix 2).

There is variation in the percentage of plant feeding in the field (3.67%) as compared to when stable flies are supplied with flowering plant in the laboratory and detected for plant feeding (85%). In *Icipe* trapping site, stable flies were positive for *Parthenium hysterophorus*, *Lantana camara*, *Schinus terebinthifolia*, *Vachellia xanthophloea*, and *Terminalia brownii* plants whereas Mpala collection were positive for *senegalia mellifera* (*Acacia mellifera*) and *Vachellia xanthophloea*, and finally Amboseli field collection had fed only on *Allium sativum*.

Table 4. 1: List of the identified plants with their sequence accession numbers submitted to GenBank, and the BLAST results.

Plant family	Common name	Scientific name	Study sequence Genbank accession number	Reference sequence accession number	BLAST nucleotide sequence identity
Fabaceae	Mururuku	*T.brownii*	MT993360	NC_053924	98.5%
Fabaceae	Blackthorn	*S. mellifera*	MT993363	KY100269	100%
Fabaceae	Fever tree	*V.xanthophloea*	MT993362	MF590086	99.2%
Asteraceae	Famine-weed	*P. hysterophorus*	MT951308	MH017893	99.3%
Amaryllidaceae	Garlic	*A.sativum*	MT951309	KY363332	100%
Anacardiaceae	Brazillian pepper tree	*S.terebinthifolia*	MT993361	MG946926	99.6%
Verbenaceae	Tickberry	*L. camara*	MT951307	MH837804	100%

4.2 Fitness benefits of nectar feeding

4.2.1 Stable fly oviposition and larval development

Despite the stable flies feeding on glucose alone surviving for 16 days, they did not lay eggs showing that blood is essential for egg development. There was no statistical

difference in the number of eggs laid by stable flies fed under the three treatments: experiment I (Blood alone), experiment II (Blood+intact *P. hysterophorus*), and experiment III (Blood+10% glucose) (ANOVA; df = 2; F = 1.082; P = 0.346). At a 95% confidence interval, the average number of eggs laid was as follows: Blood alone (149 ± 79), Blood supplemented with *P.hysterophorus* (167 ± 84), and 160 ± 76 eggs under both blood and 10% glucose (Figure 4.5A). The maximum number of eggs laid per female stable fly during this study was 530 eggs.

Supplementing blood-feeding with plant nectar from *P. hysterophorus* plant significantly enhanced stable flies' egg's hatchability compared to blood alone used as control (Figure 4.5B) (ANOVA; df = 2; F = 3.82; P = 0.0281).

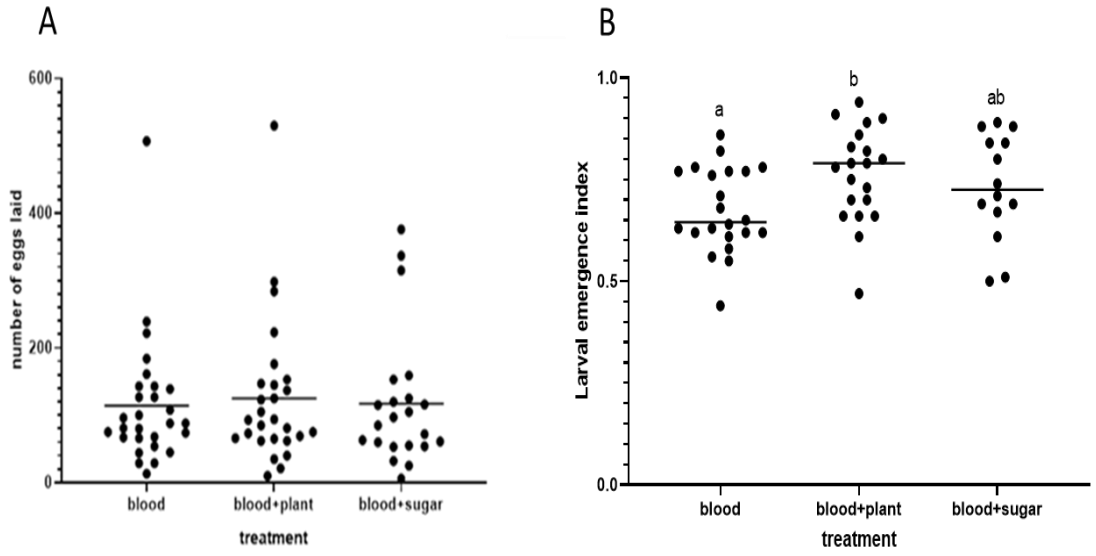


Figure 4. 5: Impact of sugar feeding in stable flies oviposition (A) and larval emergence (B), different letters show significant difference in larval emergence (Tawich *et al* 2021).

4.2.2 Survival assays

From this study, there is a significant difference in the survival of stable flies fed on treatments supplemented with blood compared to the other treatments without blood. *S.*

calcitrans fed solely on blood survived for 35 days, whereas the flies provided with blood supplemented with *P.hysterophorus* lived for 34 days. Sugar alone supplied in the form of glucose enhanced the fly's survival to a maximum of 16 days which was higher than water controls and plant alone but significantly lower than blood supplemented diets as depicted in Kaplan-Meier curve (Figure 4.6). The total pooled p-value for this survival assays of the various treatments was highly significant ($p<0.0001$) showing that type of food affects the flies' survival.

A pairwise comparison was conducted to determine the difference in survival time between treatments using the log-rank test assuming a proportional hazard model. There was no significant difference in longevity between the three treatments supplemented with blood (Blood alone vs. Blood & sugar, p=0.6), (Blood vs. blood supplemented with *P. hysterophorus*, p=0.33), (Blood supplemented with *P.hysterophorus* vs blood supplemented with sugar, p=0.24). However, the pairwise survival time between the other tested treatments was significant, with the p-values less than 0.05.

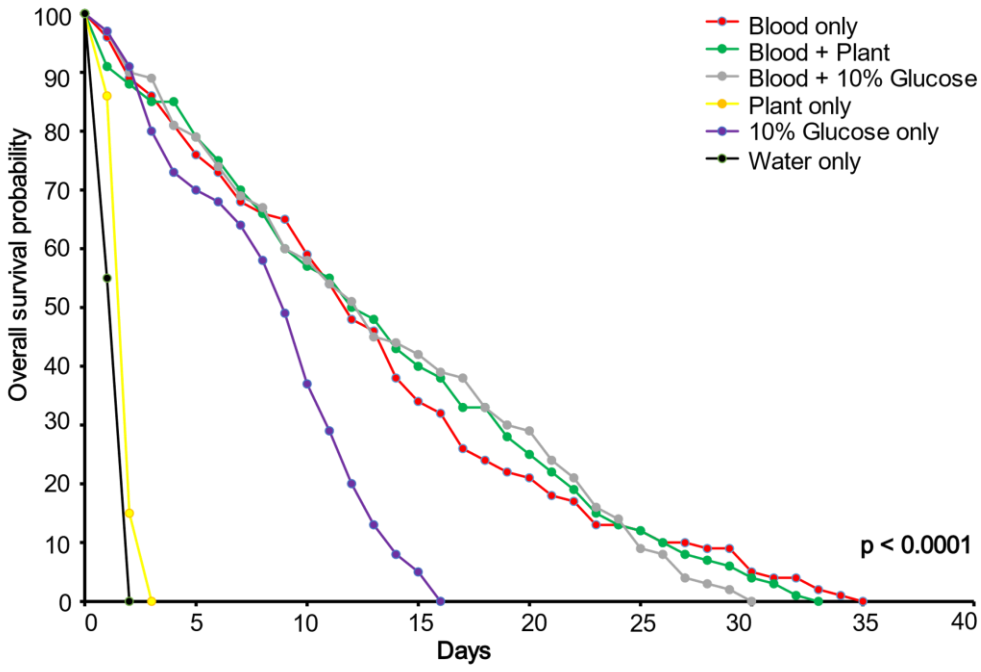


Figure 4. 6: Kaplan Meier curve showing survival of *S. calcitrans* under six different feeding regimes (Log-rank χ^2 test, n=5), $p< 0.05$ (Source: Tawich *et al*., 2021).

4.3 Chemodiversity in plant volatile chemistry

From the six plant species identified, the variation in volatile emission rates, as well as diversity, was represented in the form of chromatograms (Figure 4.7A), Heatmap (Figure 4.7B), and NMDS plot (Figure 4.7C). The odors produced by these plants consisted of various terpenes, hydrocarbons, acids, aldehydes, and alcohol. NMDS plot clustered all the identified plant volatiles into four groups with Acacia plants and *Terminalia brownii*, which are very close relatives forming one cluster. In contrast, *Lantana camara*, *Schinus terebinthifolia*, and *Parthenium hysterophorus* were clustering separately. (ANOSIM, $p<0.0001$, $R=0.7921$) meaning that the variations in chemical profile explain 79.21% of this clustering according to Bray Curtis dissimilarity index.

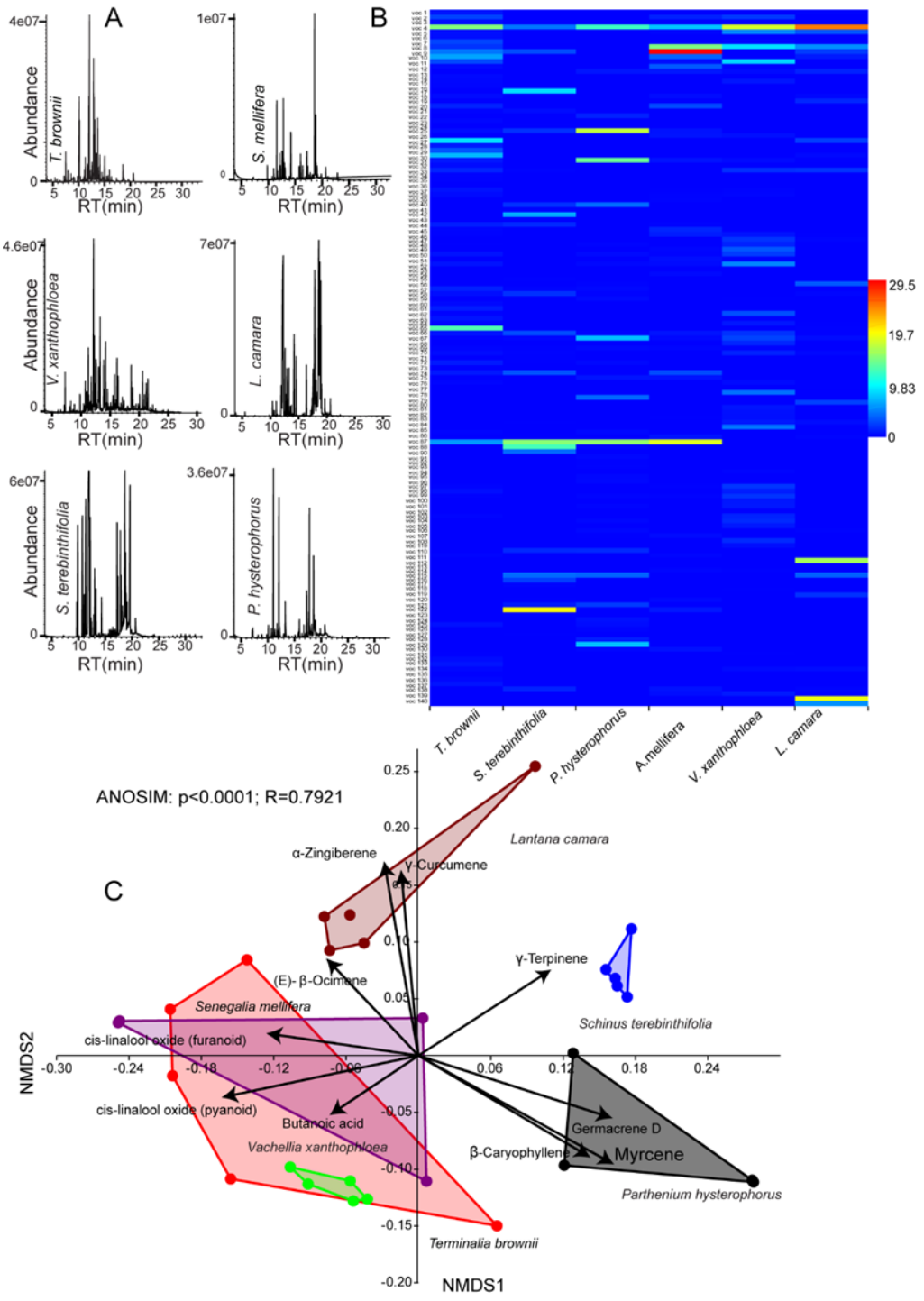
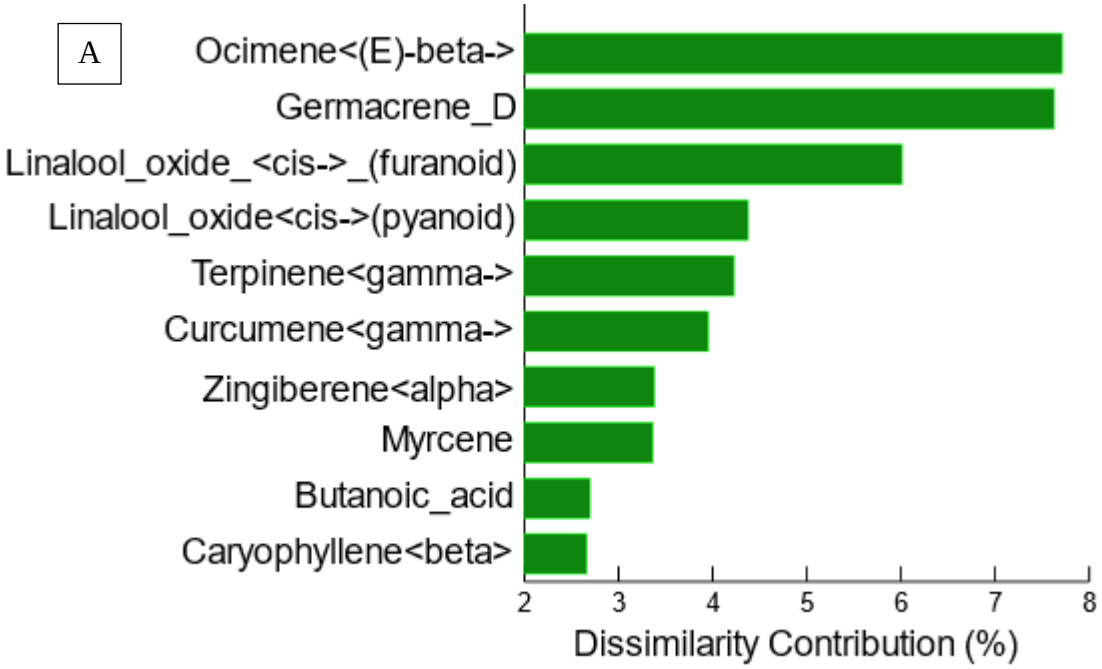


Figure 4. 7: Variations in volatiles organic compounds across six identified plant species.

(A) Chromatograms showing a representative abundance of VOCs in six plant species. (B) Heat map generated from 140 VOCs (Appendix 1) trapped from six plant species identified to be fed on by stable flies. (C) NMDS plot showing multivariate analysis of floral volatiles in six species of plant fed by stable flies (stress < 0.2). The black arrows represent overlaid bi-plot analysis of dominant compounds along each axis (Source: Tawich *et al*., 2021).

From the list of top 10 signature compounds responsible for the plant volatile chemodiversity, Linalool oxide, Beta-Ocimene, and Butanoic acid were signature compounds in the *Acacia* clustered group. *Lantana camara* was represented by Zingiberene and Curcumene compounds. β-caryophyllene, myrcene, and D-germacrene were the most prevalent volatiles in *Parthenium hysterophorus*, and only a single signature odor γ-terpinene was identified for *S. terebinthifolia* plant. The six identified plants identified about 140 chemical VOCs (Appendix 1). The volatiles released by the various plants and their floral color might play a role in the attraction of stable flies to specific plants for nectar feeding.



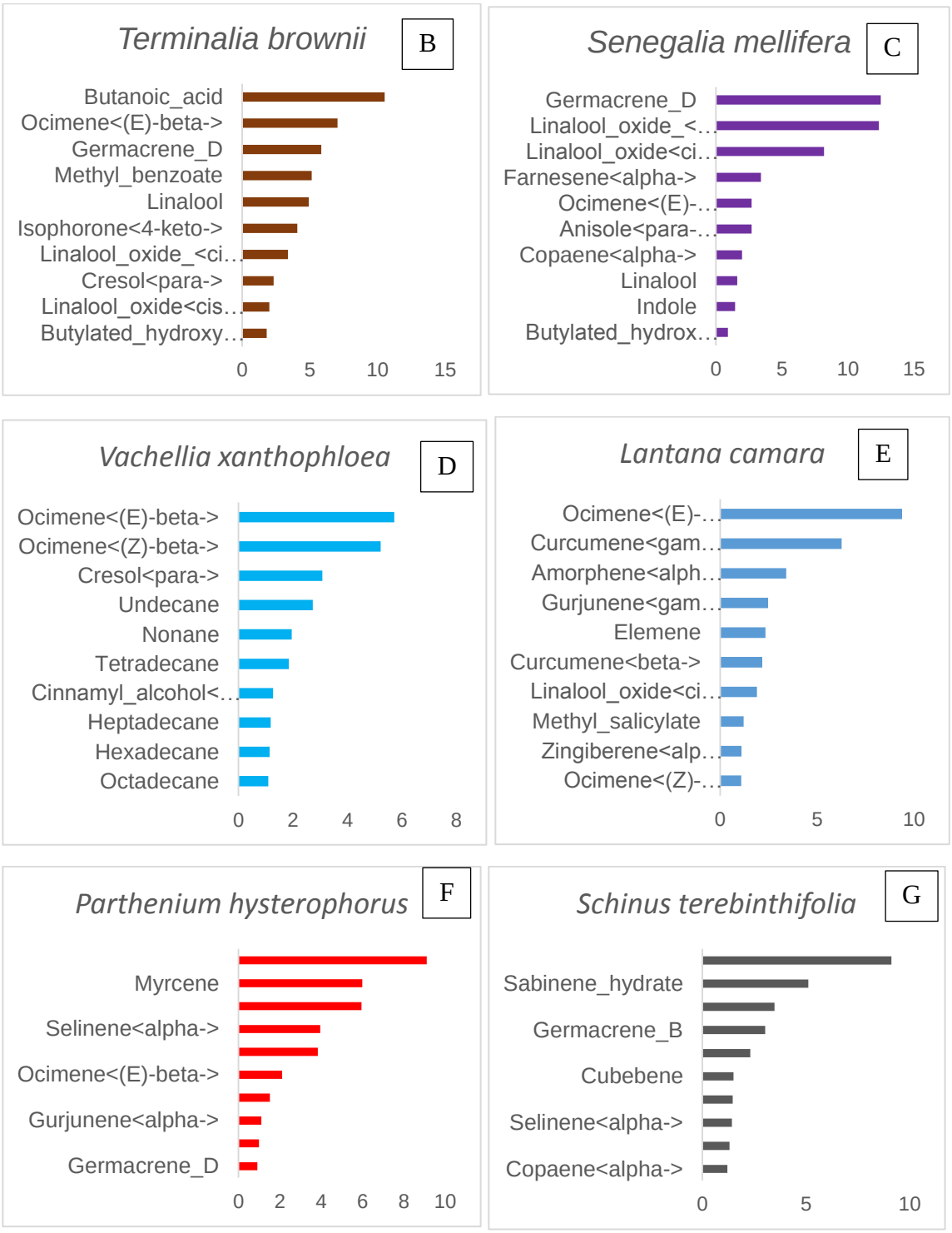


Figure 4. 8: Histograms showing ten most Indicative Volatile organic compounds within individual plant species and between the various plant species.

Analysis of each plant's volatiles confirms that each plant species has its own signature compounds (Figure 4.7C) and this compounds have varied degree of abundance (Figure 4.8 B-G). The representation of top 10 overall signature compounds across the six analysed plants is represented in figure 4.8A.

4.4 Response of stable flies' to plant derived semiochemicals under field conditions

The compounds were selected based on their SIMPER contribution. Compounds with a minimum of 1% and availability in the market were acquired and used in this study. Two compounds (p-cresol and cis-3 hexenyl acetate) were added as controls. Their response varied from neutral, attraction to repellency compared to an unbaited trap. γ-terpinene was a very attractive compound, whereas butyric acid caught 50% fewer flies than the control.

Our chosen positive controls were attractive to the stable flies. Test of attraction of the plant extracts to stable flies under field conditions confirmed that 60% of the tested plant extracts were more attractive than the control under field conditions (Figure 4.9). The ratio of female stable flies captured per trap was slightly higher than that of males. This is in line with literature where the natural occurrence of female stable flies has been reported to be higher.

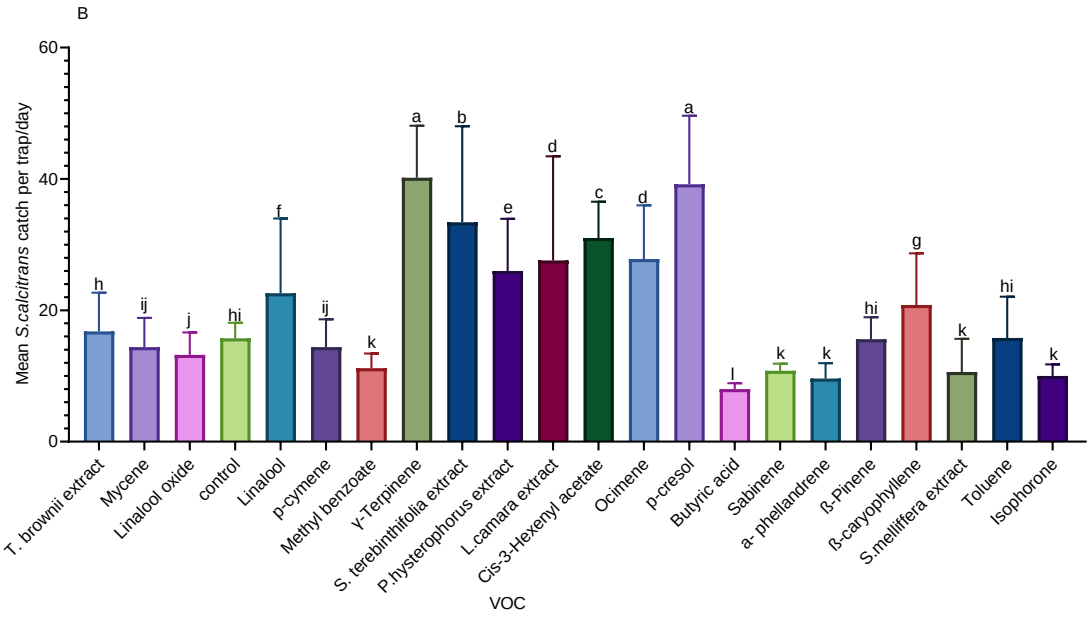


Figure 4. 9: Graph depicting the mean number of stable flies trapped using baited Vavoua traps.

The traps were baited with different plant-derived VOCs and extracts. Bars with different letters are statistically different P<0.05 (Source: Tawich *et al*., 2021).

CHAPTER FIVE

DISCUSSION

Host selection in adult stable flies is driven by visual and olfactory cues (Baleba *et* al 2020). Plants release volatile organic compounds into the air, which are detected by insects' chemoreceptors in attracting their hosts. The identified acacia plants shared some key volatile organic compounds (Linalool oxide, Ocimene, and Butanoic acid) while a few were species-specific. For instance, *S. terebinthifolia* has as signature odor as γ-terpinene, as displayed on the NMDS plot (Figure 4.7C). Volatile organic compounds vary quantitatively and qualitatively between and within plant species. Understanding these variations is crucial in *S. calcitrans*-plant communication. However, it is unclear how a given plant species' volatile composition relates to its nutritional composition. In other studies, it has been reported that the scarcity of plant nutrients hinders the growth and development of Acorn Woodpecker (Melanerpes formicivorus) (Koenig, 1982) and female parasitoids (Casas, 1999).

The use of common animal-derived chemical cues in host selection has been demonstrated in several blood-feeding flies such as mosquitoes, tsetse flies, stable flies, and culicoides (Blackwell *et al*., 1996; Kline *et al*., 1990; Naters *et al*., 1996; Getahun *et al*., 2020b). A previous study on mosquito control, the blends made by combining human/animal odors with plant-derived odors proved effective in catching malaria mosquitoes of different physiology and age (Nyasembe *et al*., 2014). The above findings show the need to develop a component blend of plant-based or animal-based lures for stable fly management. Improved efficiency of traps baited with the chemical lures confirmed the need to combine visual and olfactory cues to understand the behavior of these important vectors of medical and veterinary importance.

This study highlights the potential of utilizing plant VOCs in trapping stable flies. Stable flies feeding on plant materials are exposed to plant metabolites, which can impact their fitness and disease transmission to reduce pathogen load, as earlier reported in bumblebees and nutrient acquisition in mosquitoes (Manson *et al*., 2010; Barredo &

Degennaro, 2020). The flies can get important immune system boosting metabolites by imbibing nectar. This nectar has essential phytochemicals that can help the stable flies raise their immune system with the potential to develop resistance to most of the applied insecticides used in their control. Vector control constitutes a fundamental pillar in disease eradication or containment efforts. Understanding the volatile perception of stable flies is an environmentally friendly and species-specific method that can be applied n the management of stable flies.

Molecular detection of plant meal source of stable fly from this study confirmed their plant-feeding habits and that detection of cpDNA using either the coding or the noncoding region of the chloroplast is a very effective DNA marker for plant barcoding. Sequencing the positively detected samples and conducting a BLAST search at the NCBI online database confirmed the identity of the plants that stable flies had fed on naturally. Similar work on the DNA barcoding technique has been applied to identify plant-meal sources of wild-caught sand flies (*Lutzomyia longipalpis*) (Lima *et al*., 2016). Using the same method of DNA barcoding, a study showed that phlebotomine sand flies which are vectors of leishmania, prefer feeding on Cannabis sativa (Abbasi *et al*., 2018). Laboratory behavioral studies on the stable fly and conducting the lifetable experiments confirmed that stable flies fed on blood supplemented with flowering *P. hysterophorus* had increased larval emergence compared to the control. This observation might suggest that female stable flies acquire additional nutritional resources from nectar-feeding, such as sugars, fats, and amino acids which might be carried over in their ovaries, thus contributing to egg development and increased larval emergence.

The survival of any insect is dependent on diet, genetics, biological and environmental factors. This is due to variations in nutritional content determining an insect's growth and development. When fed on plant nectar, the increased stable fly's egg hatchability is still unclear but shows that diet is a key variable in determining the reproductive energetics of this important vector. From other published studies, pollen diffusates in water increased stable fly longevity (Jones *et al*., 1985).

Various important plant phytochemicals have also been reported to positively or negatively affect egg development (Hilker and Meiners, 2011). Supplementing blood-feeding with *P. hysterophorus* nectar-feeding in this study did not affect the number of eggs laid nor the survival of stable flies. Similar observations have been reported in other insect species. Feeding on *Lantana camara* influenced the fecundity of *Anopheles gambiae* (Manda *et al*., 2007).

Feeding on plant nectars and pollen has been reported to have numerous advantages for insects. These include antifungal, antibacterial, and antiviral, phytochemicals, nutrition as well as an energy source (Manson *et al*., 2010; Koch *et al*., 2019; Barredo and Degennaro, 2020) but can also be a source of pathogens, for example, the fungal infection reported to be affecting feeding and survival of *Anopheles gambiae* (Manson *et al*., 2010). Furthermore, nectar constituents such as carbohydrates are known to influence mosquito sugar-meal choices and longevity both positively and negatively or potentially toxic secondary metabolites such as alkaloids (Ignell *et al*., 2010: Kessler *et al*., 2013). Plant sugar feeding also has the neutralization benefit of plant defense (Malka et al., 2020). However, feeding on plants can't be ruled out as affecting the number of eggs or survival because plant species vary in the amount and quality of resources (Roulston et al., 2000; Nyasembe *et al*., 2020). Most plant nectars in *A. gambiae* habitats contain sucrose, and its hexose hydrolysis products glucose and fructose. The sugar concentration from field plants varies from 8 to 40% (Wykes, 1953) and between 3-23% (Jones *et al*., 1992). Regarding this, different glucose concentrations were tested in this study and found that higher sugar concentrations of 10% and above prolonged the longevity of stable flies to 16 days compared to the 5%, which sustained only for ten days.

The high mortality of stable flies when supplied solely with *P. hysterophorus* demonstrated the possibility of this plant having toxic metabolites that might negatively impact survival, growth, and development. Similarly, earlier studies on Anopheles gambiae discriminative plant feeding in western Kenya showed a low survival rate when

fed only on *Lantana camara* (Manda *et al*., 2007). Similarly, one night of feeding on branches of *R. communis*, *S. jasminoides* and *B. glabra* plants drastically shortened the life span of the sand flies (Schlein & Muller, 1995). However, stable flies lived longer when the same plant was presented together with blood, which might be neutralized by blood-feeding or could also be due to the amount they feed on, as toxicity is dose-dependent (Benelli and Pavela, 2018).

A confirmatory experiment of nectar-feeding conducted in this study removes the doubt that the prolonged survival might suggest that stable flies avoided feeding on the plant. The survival probability of flies fed with *P. hysterophorus* alone was higher than on water used as control but not as much as when supplied with glucose or blood. Flies fed with blood, blood + sugar, and blood in combination with intact *P. hysterophorus* plant had increased survival rate compared to the other non-blood treatments. This confirms the role of blood in enhancing stable fly's survival. These findings were in line with similar observations done by using different flower cuttings and pollen from the wild plants, which prolonged *Stomoxys* survival more than water control (Jones *et al*., 1985, 1992).

Stable flies that fed only on glucose didn't lay any amount of eggs in this study, confirming they are of the anautogenous insects (Müller *et al*., 2012), whereby blood is necessary for egg development. The autogeny has also been reported in other hematophagous Diptera, such as tabanids, chrysops, and deer flies (Rockel, 1969; Anderson, 1971; Bosler and Hansens, 1974).

Despite plant feeding being suggested in earlier studies (Taylor and Berkebile, 2014), this research work is the first evidence linking plant feeding directly to stable flies' fitness benefits. According to this study, 3.67% plant feeding was observed in wild-caught stable flies compared to 85% when fed on *P. hysterophorus* in the laboratory. This observation could result from DNA degradation over time due to enzymatic digestion. The nectar prevalence seems to be affected by the place of collection and seasonal variation. Jones et al.,1992 reported higher nectar-feeding from beach sites,

which is 23%, but 3% from diary sites and thus supporting our observation. A related study by Taylor and Berkebile reported that 8-12% of nectar-feeding depends on the season (Taylor and Berkebile, 2014). Thus, the collection region, seasons under which the flies are collected, and detection methods used might explain the disparity in the results.

The frequency of sugar feeding is determined by the abundance of sugar sources, with a positive correlation between nectar feeding and plant population in a given region (Martinez-Ibarra *et al*., 1997). Blood-feeding insects vary in their nutrient requirements for survival. Both male and female stable flies can survive on blood alone without sugar (Tawich *et al* 2021), unlike Aedes aegypti mosquitoes, which males can't survive without if deprived of sugar for a long time (Chadee *et al*., 2014). Nectar-feeding is essential for both male and female adult mosquitoes, especially males who feed exclusively from sugar and require frequent intake for survival. Nectar feeding behavior is less understood than blood-feeding, especially for stable flies as compared to mosquitoes or sand flies. Much research has been conducted on stable flies' blood-feeding behavior (Mihok and Clausen, 1996; Getahun *et al*., 2020a) compared to nectar-feeding because it is during the blood-feeding process transmission of pathogens can occur.

Various plant species emit different volatiles at different concentrations, confirmed by the chemical analysis conducted in this study. The amount of volatiles released depends on the plant's physiological state, which is determined by genetic and environmental factors. Differentiation of plants can be done using volatile chemistry about their genomic taxonomy. According to previous observations on *Pinus mugo* species, plant VOCs have been reported to have the potential to distinguish related plant species (Celiński *et al*., 2015) very closely. Each NMDS cluster in this study was determined by vital predictive compounds, confirming that plants can be characterized based on their VOCs. The behavioral response of stable flies to those odors under field conditions varied from attractive (γ-terpinene, ocimene) to neutral (β-pinene, and toluene) or even

repellent (Butanoic acid, also known as butyric acid), which caught a smaller number of flies significantly as compared to unbaited trap. The variation in the behavioral response to those odors predicted by SIMPER analysis suggests that they might have a different function.

Different odors activate different receptors in an insect and thus eliciting a different behavioral response. The absence of strong attraction from some of the identified signature compounds might also suggest the need to develop blends that have great potential to induce attraction compared to testing the compounds singly. This could be the case as observed with the tested whole-plant extracts. Three out of the five tested extracts attracted significantly more flies than the control trap, i.e. (*S. terebinthifolia*, *L. camara*, and *P. hysterophorus*) whereas *T. brownii* extract had no difference with the control. In contrast, only *S. mellifera* extract caught fewer flies than the control. *S. mellifera* was the only extract that attracted less stable flies than the unbaited trap. This could be due to the dominance of repellent linalool oxide in the composition of its signature (Tawich *et al*., 2021).

In addition to the volatiles released by the plant, other plant features such as color, floral shape, texture, as well as environmental factors such as wind speed and local CO2, might play a significant role in plant acceptance (Burkle and Runyon, 2017; Nordström et al., 2017). Understanding how insects communicate with plant and animal hosts is important in the integrated pest management of phytophagous insects. This discovery is also crucial for monitoring and controlling hematophagous insects in spreading diseases of economic importance. Most plants identified in this study have no literature on their nutritional status therefore we can’t rule out thet nectar feeding in stable flies doesn’t impact oviposition.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

Plant feeding is common in adult stable flies and has fitness benefits. The host plants were confirmed using chloroplast DNA barcoding techniques to uniquely identify the plant meal source. Stable flies feed on diverse plants and utilize the sense of smell to locate their nectar source. A combination of both animal odors and plant-derived volatiles has the potential to improve already available traps such as monoconical to enhance trap efficiency. The findings from this study have implications for diseases transmission, vector control, laboratory colony establishment, and evolutionary adaptation.

6.2 Recommendations

1. There is a need to conduct a nutrient evaluation of a wide range of plants fed on by stable flies to determine their role in stable fly reproduction. This study tested the fitness benefits of stable fly-feeding using *P. hysterophorus* plant alone.
2. There is a need to develop stable flies' surveillance and management tools using both the plant-derived volatiles alone or in combination with animal odors in making blends. These baits might better improve the attraction of stable flies and other related biting flies of importance to one health.
3. Comparative studies on stable fly fitness experiments in the laboratory should be done between different fly generations from field-collected flies to avoid the impact of their previous plant-feeding habits on the results.

REFERENCES

Abbasi, I., de Queiroz, A. T. L., Kirstein, O. D., Nasereddin, A., Horwitz, B. Z., Hailu, A., ... & Warburg, A. (2018). Plant-feeding phlebotomine sand flies, vectors of leishmaniasis, prefer Cannabis sativa. *Proceedings of the National Academy of Sciences, 115*(46), 11790-11795.

Ahmad, J., Bagheri, R., Bashir, H., Affan Baig, M., Al-Huqail, A., Ibrahim, M. M., & Irfan Qureshi, M. (2018). Organ-Specific Phytochemical Profiling and Antioxidant Analysis of Parthenium hysterophorus L. *BioMed Research International*.

Akanmu, P. O. (1979). Embryonic development of the stable fly, Stomoxys calcitrans Linnaeus (Diptera: Muscidae) : a light and electron microscopy study.

Anderson, J. F. (1971). Autogeny and mating and their relationship to biting in the saltmarsh deer fly, Chrysops atlanticus (Diptera: Tabanidae). *Journal of Economic Entomology*.

Attardo, G. M., Guz, N., Strickler-Dinglasan, P., & Aksoy, S. (2006). Molecular aspects of viviparous reproductive biology of the tsetse fly (Glossina morsitans morsitans): Regulation of yolk and milk gland protein synthesis. *Journal of Insect Physiology*.

Bafeel, S. O., Arif, I. A., Bakir, M. A., Al Homaidan, A. A., Al Farhan, A. H., & Khan, H. A. (2012). DNA barcoding of arid wild plants using rbcL gene sequences. *Genet. Mol. Res*, *11*(3), 1934-41.

Baldacchino, F., Desquesnes, M., Mihok, S., Foil, L. D., Duvallet, G., & Jittapalapong, S. (2014). Tabanids: neglected subjects of research, but important vectors of disease agents!. Infection, *Genetics and Evolution*, *28*, 596-615.

Baldacchino, F., Muenworn, V., Desquesnes, M., Desoli, F., Charoenviriyaphap, T., & Duvallet, G. (2013). Transmission of pathogens by Stomoxys flies (Diptera, Muscidae): a review. *Parasite*, *20*, 26.

Baleba, S., Agbessenou, A., Getahun, M. N., Akutse, K. S., Subramanian, S., & Masiga,

D. (2021). Infection of the Stable Fly, Stomoxys calcitrans, L. 1758 (Diptera: Muscidae) by the Entomopathogenic Fungi Metarhizium anisopliae (Hypocreales: Clavicipitaceae) Negatively Affects Its Survival, Feeding Propensity, Fecundity, Fertility, and Fitness Parameters. *Frontiers in Fungal Biology*, *2*, 4.

Baleba, S. B. S., Torto, B., Masiga, D., & Weldon, C. W. (2019). Egg-laying decisions based on olfactory cues enhance offspring fitness in *Stomoxys calcitrans* L . (Diptera : Muscidae). *Scientific Reports*, (November 2018), 1–13.

Baleba, S. B. S., Weldon, C. W., Masiga, D., Torto, B., & Getahun, M. N. (2019). Larval experience of stable fly, *Stomoxys calcitrans* Linneaus, 1758 (Diptera: Muscidae) does not influence oviposition preference in gravid females. *Ecological Entomology*, een.12748.

Barredo, E., & DeGennaro, M. (2020). Not just from blood: Mosquito nutrient acquisition from nectar sources. *Trends in Parasitology, 36*(5), 473-484.

Benelli, G., & Pavela, R. (2018). Beyond mosquitoes—Essential oil toxicity and repellency against bloodsucking insects. *Industrial crops and products, 117*, 382-392.

Birkemoe, T., & Sverdrup-Thygeson, A. (2011). Stable fly (Stomoxys calcitrans) and house fly (Musca domestica) densities: A comparison of three monitoring methods on pig farms. *Journal of Pest Science, 84*(3), 273-280.

Bisch-Knaden, S., Dahake, A., Sachse, S., Knaden, M., & Hansson, B. S. (2018). Spatial Representation of Feeding and Oviposition Odors in the Brain of a Hawkmoth. *Cell Reports*, *22*(9), 2482–2492.

Blackwell, A., Dyer, C., Wadhams, L. J., & Mordue, W. (1996). The role of 1- octen-3- ol as a host- odour attractant for the biting midge, Culicoides impunctatus Goetghebuer, and interactions of 1- octen- 3- ol with a volatile pheromone produced by parous female midges. *Physiological Entomology, 21*(1), 15-19.

Bosler, E. M., & Hansens, E. J. (1974). Natural feeding behavior of adult saltmarsh greenheads, and its relation to oogenesis. *Annals of the Entomological Society of America*, *67*(3), 321-324.

Burkle, L. A., & Runyon, J. B. (2017). The Smell of Environmental Change: Using Floral Scent to Explain Shifts in Pollinator Attraction. *Applications in Plant Sciences*, *5*(6), 1600123.

Calatayud, P., Sauvion, N., & Thiéry, D. (2018). Plant-Insect Interactions Plant-Insect Interactions, (January).

Campbell, J. B., Berry, I. L., Boxler, D. J., Davis, R. L., Clanton, D. C., & Deutscher, G. H. (1987). Effects of stable flies (Diptera: Muscidae) on weight gain and feed efficiency of feedlot cattle. *Journal of Economic Entomology*, *80*(1), 117–119.

Campbell, J. B., Skoda, S. R., Berkebile, D. R., Boxler, D. J., Thomas, G. D., Adams, D. C., & Davis, R. (2001). Effects of stable flies (Diptera: Muscidae) on weight gains of grazing yearling cattle. *Journal of Economic Entomology*, *94*(3), 780-783.

Carter, C., Thornburg, R. W., & Nepi, M. (Eds.). (2019). New Perspectives on the Biology of Nectaries and Nectars. *Frontiers Media SA*.

Celiński, K., Bonikowski, R., Wojnicka- Półtorak, A., Chudzińska, E., & Maliński, T. (2015). Volatiles as chemosystematic markers for distinguishing closely related species within the Pinus mugo complex. *Chemistry & Biodiversity*, *12*(8), 1208-1213.

Chadee, D. D., Sutherland, J. M., & Gilles, J. R. (2014). Diel sugar feeding and reproductive behaviours of Aedes aegypti mosquitoes in Trinidad: with implications for mass release of sterile mosquitoes. *Acta Tropica*, *132*, S86-S90.

Cortinas, R., & Jones, C. J. (2006). Ectoparasites of cattle and small ruminants. Veterinary Clinics: *Food Animal Practice*, *22*(3), 673-693.

Dawit, L., Addis, M., & Gari, G. (2012). Distribution, seasonality and relative

abundance of Stomoxys flies in selected Districts of central Ethiopia. *World Applied Sciences Journal*, *19*(7), 998–1002.

Dean, G. J. W., Paget, J., & Wilson, F. (1969). Observations on the behaviour of tsetse flies (Glossina morsitans orientalis Vanderplank and G. pallidipes Austen) during an attempt to concentrate breeding around cattle. *Journal of Applied Ecology*, 13-26.

De Boer, H., Vongsombath, C., Pålsson, K., Björk, L., & Jaenson, T. G. (2010). Botanical repellents and pesticides traditionally used against hematophagous invertebrates in Lao People's Democratic Republic: a comparative study of plants used in 66 villages. *Journal of medical entomology*, *47*(3), 400-414.

Dougherty, C. T., Knapp, F. W., Burrus, P. B., Willis, D. C., Burg, J. G., Cornelius, P. L., & Bradley, N. W. (1993). Stable flies (*Stomoxys calcitrans* L.) and the behavior of grazing beef cattle. *Applied Animal Behaviour Science*, *35*(3), 215–233.

Doyle, M. S., Swope, B. N., Hogsette, J. A., Burkhalter, K. L., Savage, H. M., & Nasci, R. S. (2011). Vector competence of the stable fly (Diptera: Muscidae) for West Nile virus. *Journal of Medical Entomology*, *48*(3), 656-668.

El Ashmawy, W. R., Williams, D. R., Gerry, A. C., Champagne, J. D., Lehenbauer, T. W., & Aly, S. S. (2019). Risk factors affecting dairy cattle protective grouping behavior, commonly known as bunching, against Stomoxys calcitrans (L.) on California dairies. *PloS one*, *14*(11).

Erasmus, A. S. (2015). The impact of Stomoxys calcitrans populations on cattle in a feedlot near Heidelberg, Gauteng, South Africa (Doctoral dissertation, North-West University (South Africa), Potchefstroom Campus).

Florez-Cuadros, M., Berkebile, D., Brewer, G., & Taylor, D. B. (2019). Effects of diet quality and temperature on stable fly (Diptera: Muscidae) development. *Insects*, *10*(7), 207.

Foil, L. D., & Hogsette, J. A. (1994a). Biology and control of tabanids, stable flies and horn flies. *Revue Scientifique et Technique (International Office of Epizootics)*, *13*(4), 1125–1158.

Foil, L. D., Meek, C. L., Adams, W. V., & Issel, C. J. (1983). Mechanical transmission of equine infectious anemia virus by deer flies (Chrysops flavidus) and stable flies (Stomoxys calcitrans). *American journal of veterinary research, 44*(1), 155-156.

Foil, L. D., & Gorham, J. R. (2000). Mechanical transmission of disease agents by arthropods. *In Medical entomology* (pp. 461-514).

Foster, W. A. (1995). Mosquito sugar feeding and reproductive energetics. *Annual Review of Entomology*, *40*, 443–474.

Geden, C. J., & Hogsette, J. a. (1994). Research and Extension Needs for Integrated Pest Management for Arthropods of Veterinary Importance - lincoln.pdf. *Proceeding of a Workshop in Lincoln, Nebraska.*, (October), 1–328.

Gerry, A. C., Peterson, N. G., & Mullens, B. A. (2007). Predicting and Controlling Stable Flies on California Dairies. Predicting and Controlling Stable Flies on California Dairies.

Getahun, M., Villinger, J., Bargul, J., Orone, A., Ngiela, J., Ahuya, P., … Masiga, D. (2020a). Molecular characterization of pathogenic African trypanosomes in biting flies and camels in surra-endemic areas outside the tsetse fly belt in Kenya. *BioRxiv*, 2020.06.18.156869.

Getahun, M. N., Thoma, M., Lavista-Llanos, S., Keesey, I., Fandino, R. A., Knaden, M., ... & Hansson, B. S. (2016). Intracellular regulation of the insect chemoreceptor complex impacts odour localization in flying insects. *Journal of Experimental Biology, 219*(21), 3428-3438.

Getahun, M. N., Ahuya, P., Ngiela, J., Orone, A., Masiga, D., & Torto, B. (2020b). Shared volatile organic compounds between camel metabolic products elicits strong

Stomoxys calcitrans attraction. *Scientific reports, 10*(1), 1-14.

Gibson, G., & Torr, S. J. (1999). Visual and olfactory responses of haematophagous Diptera to host stimuli. *Medical and veterinary entomology, 13*(1), 2-23.

Grabe, V., & Sachse, S. (2018). Fundamental principles of the olfactory code. *BioSystems*, *164*, 94–101.

Gubler, D. J. (2009). Vector-borne diseases. *OIE Revue Scientifique et Technique*, *28*(2), 583–588.

Guseman, J. (2014). Foraging Behavior of Anopheles gambiae (Diptera: Culicidae) on Parthenium hysterophorus (Compositae) and Senna occidentalis (Fabaceae) (Doctoral dissertation, The Ohio State University).

HASLETT, J. R. (1983). A photographic account of pollen digestion by adult hoverflies. *Physiological Entomology*, *8*(2), 167-171.

Hassanali, A., Herren, H., Khan, Z. R., Pickett, J. A., & Woodcock, C. M. (2008). Integrated pest management: the push–pull approach for controlling insect pests and weeds of cereals, and its potential for other agricultural systems including animal husbandry. Philosophical Transactions of the Royal Society B: *Biological Sciences, 363*(1491), 611-621.

Heuskin, S., Verheggen, F. J., Haubruge, E., Wathelet, J. P., & Lognay, G. (2011). The use of semiochemical slow-release devices in integrated pest management strategies. *Agronomy, Society and Environment*, *15*(3), 459–470.

Hieu, T. T., Jung, J., Kim, S. Il, Ahn, Y. J., & Kwon, H. W. (2014). Behavioural and electroantennogram responses of the stable fly (*Stomoxys calcitrans* L.) to plant essential oils and their mixtures with attractants. *Pest Management Science*, *70*(1), 163–172.

Hilker, M., & Meiners, T. (2011). Plants and insect eggs: How do they affect each other? *Phytochemistry*, *72*(13), 1612–1623.

Hillerton, J. E., Bramley, A. J., & Broom, D. M. (1984). The distribution of five species of flies (Diptera: Muscidae) over the bodies of dairy heifers in England. *Bulletin of entomological research*, *74(*1), 113-119.

Hoch, A. L., Gargan 2nd, T. P., & Bailey, C. L. (1985). Mechanical transmission of Rift Valley fever virus by hematophagous Diptera. *The American journal of tropical medicine and hygiene*, *34*(1), 188-193.

Holloway, M. T. P., & Phelps, R. J. (1991). The responses of stomoxys spp. (Diptera: Muscidae) to traps and artificial host odours in the field. *Bulletin of Entomological Research*, *81*(1), 51–56.

Ignell, R., Okawa, S., Englund, J. E., & Hill, S. R. (2010). Assessment of diet choice by the yellow fever mosquito Aedes aegypti. *Physiological Entomology*, *35*(3), 274–286.

Jaenike, J. (1978). On optimal oviposition behavior in phytophagous insects. *Theoretical population biology*, *14*(3), 350-356.

Jarzen, D. M., & Hogsette, J. A. (2008). Pollen from the exoskeletons of stable flies, Stomoxys calcitrans (Linnaeus 1758), in Gainesville, Florida, USA. *Palynology*, *32*(1), 77-81.

Jeanbourquin, P., & Guerin, P. M. (2007a). Chemostimuli implicated in selection of oviposition substrates by the stable fly *Stomoxys calcitrans*. *Medical and Veterinary Entomology*, *21*(3), 209–216.

Jeanbourquin, P., & Guerin, P. M. (2007b). Sensory and behavioural responses of the stable fly *Stomoxys calcitrans* to rumen volatiles. *Medical and Veterinary Entomology*, *21*(3), 217–224.

Jones, C. J., Hogsette, J. A., Patterson, R. S., & Milne, D. E. (1985). Effects of Natural Saccharide and Pollen Extract Feeding on Stable Fly (Diptera: Muscidae) Longevity. *Environmental Entomology*, *14*(3), 223–227.

Jones, C. J., Milne, D. E., Patterson, R. S., Schreiber, E. T., & Milio, J. A. (1992). Nectar feeding by *Stomoxys calcitrans* (Diptera: Muscidae): effects on reproduction and survival. *Environmental Entomology*, *21*(1), 141–147.

Jones, C. J., & Weinzierl, R. A. (1997). Geographical and temporal variation in pteromalid (Hymenoptera: Pteromalidae) parasitism of stable fly and house fly (Diptera: Muscidae) pupae collected from Illinois cattle feedlots. *Environmental Entomology*, *26*(2), 421-432.

Keawrayup, S., Duvallet, G., Sukonthabhirom, S., & Chareonviriyaphap, T. (2012). Diversity of stomoxys spp. (diptera: Muscidae) and diurnal variations of activity of stomoxys indicus and *S. calcitrans* in a farm, in wang nam khiao district, Nakhon Ratchasima Province, Thailand. *Parasite*, *19*(3), 259–265.

Kessler, S., Vlimant, M., & Guerin, P. M. (2013). The sugar meal of the African malaria mosquito Anopheles gambiae and how deterrent compounds interfere with it: a behavioural and neurophysiological study. Journal of Experimental Biology, 216(7), 1292-1306.

Kline, D. L., Takken, W., Wood, J. R., & Carlson, D. A. (1990). Field studies on the potential of butanone, carbon dioxide, honey extract, l- octen- 3- ol, L- lactic acid and phenols as attractants for mosquitoes. *Medical and veterinary entomology*, *4*(4), 383-391.

Koch, H., Woodward, J., Langat, M. K., Brown, M. J. F., & Stevenson, P. C. (2019). Flagellum Removal by a Nectar Metabolite Inhibits Infectivity of a Bumblebee Parasite. *Current Biology*, *29*(20), 3494-3500.

Koenig, W. D. (1982). Ecological and social factors affecting hatchability of eggs. *The Auk*, *99*(3), 526-536.

Kreher, S. A., Mathew, D., Kim, J., & Carlson, J. R. (2008). Translation of Sensory Input into Behavioral Output via an Olfactory System. *Neuron*, *59*(1), 110–124.

Leal, W. S. (2005). Pheromone reception. In The chemistry of pheromones and other semiochemicals II (pp. 1-36). *Springer*

Lehane, M. J., Lehane, M. M., & Lehane, M. J. (2005). The biology of blood-sucking in insects. *Cambridge University Press*.

Lima, L. H. D. M., Mesquita, M. R., Skrip, L., de Souza Freitas, M. T., Silva, V. C., Kirstein, O. D., ... & Costa, C. H. (2016). DNA barcode for the identification of the sand fly Lutzomyia longipalpis plant feeding preferences in a tropical urban environment. *Scientific reports, 6*(1), 1-6.

Ma, B. O., & Roitberg, B. D. (2008). The role of resource availability and state-dependence in the foraging strategy of blood-feeding mosquitoes. *Evolutionary Ecology Research*, *10*(8), 1111–1130.

MAG, INTA, & USDA. (2018). International Stable Fly Workshop. In Stable Fly Insecticides & chemical control assessment.

Majetic, C. J., Raguso, R. A., & Ashman, T. L. (2008). The impact of biochemistry vs. population membership on floral scent profiles in colour polymorphic Hesperis matronalis. *Annals of Botany*, *102*(6), 911–922.

Malaithong, N., Duvallet, G., Ngoen-Klan, R., Bangs, M. J., & Chareonviriyaphap, T. (2019). Stomoxyinae Flies in Thailand: A Précis, with Abridged Taxonomic Key to the Adult Species. *Vector-Borne and Zoonotic Diseases*, *19*(6), 385–394.

Malka, O., Easson, M. L., Paetz, C., Götz, M., Reichelt, M., Stein, B., ... & Vassão, D. G. (2020). Glucosylation prevents plant defense activation in phloem-feeding insects. *Nature Chemical Biology, 16*(12), 1420-1426.

Manda, H., Gouagna, L. C., Nyandat, E., Kabiru, E. W., Jackson, R. R., Foster, W. A., … Hassanali, A. (2007). Discriminative feeding behaviour of Anopheles gambiae s.s. on endemic plants in western Kenya. *Medical and Veterinary Entomology*, *21*(1), 103–111.

Manda, Hortance, Gouagna, L. C., Foster, W. A., Jackson, R. R., Beier, J. C., Githure, J. I., & Hassanali, A. (2007). Effect of discriminative plant-sugar feeding on the survival and fecundity of Anopheles gambiae. *Malaria Journal*, *6*, 1–11.

Manson, J. S., Otterstatter, M. C., & Thomson, J. D. (2010). Consumption of a nectar alkaloid reduces pathogen load in bumble bees. *Oecologia*, *162*(1), 81–89.

Martínez-de la Puente, J., Merino, S., Lobato, E., Rivero-de Aguilar, J., del Cerro, S., Ruiz-de-Castañeda, R., & Moreno, J. (2010). Nest-climatic factors affect the abundance of biting flies and their effects on nestling condition. *Acta Oecologica*, *36*(6), 543-547.

Martinez-Ibarra, J. A., Rodriguez, M. H., Arredondo-Jimenez, J. I., & Yuval, B. (1997). Influence of plant abundance on nectar feeding by Aedes aegypti (Diptera: Culicidae) in southern Mexico. *Journal of medical entomology*, *34*(6), 589-593.

Masmeatathip, R., Ketavan, C., & Duvallet, G. (2006). Morphological studies of Stomoxys spp. (Diptera: Muscidae) in Central Thailand. *Kasetsart Journal - Natural Science*, *40*(4), 872–881.

McIver, S. B. (1982). Sensilla mosquitoes (Diptera: Culicidae). *Journal of Medical Entomology*, *19*(5), 489–535.

Mellor, P. S., Kitching, R. P., & Wilkinson, P. J. (1987). Mechanical transmission of capripox virus and African swine fever virus by Stomoxys calcitrans. *Research in veterinary science*, *43*(1), 109-112.

Mihok, S., Kang'ethe, E. K., & Kamau, G. K. (1995). Trials of traps and attractants for *Stomoxys* spp. (Diptera: Muscidae). *Journal of Medical Entomology*, *32*(3), 283–289.

Mihok, Steve, & Clausen, P. H. (1996). Feeding habits of Stomoxys spp. stable flies in a Kenyan forest. *Medical and Veterinary Entomology*, *10*(4), 392–394.

Moobola, S. M., & Cupp, E. W. (1978). Ovarian development in the stable fly,

Stomoxys calcitrans, in relation to diet and juvenile hormone control. *Physiological Entomology*, *3*(4), 317-321.

Mramba, F., Broce, A. B., & Zurek, L. (2007). Vector competence of stable flies, *Stomoxys calcitrans* L. (Diptera: Muscidae), for Enterobacter sakazakii. *Journal of Vector Ecology*, *32*(1), 134–139.

Mullens, B. A., Lii, K. S., Mao, Y., Meyer, J. A., Peterson, N. G., & Szijj, C. E. (2006). Behavioural responses of dairy cattle to the stable fly, *Stomoxys calcitrans*, in an open field environment. *Medical and Veterinary Entomology*, *20*(1), 122–137.

Müller, G. C., Hogsette, J. A., Beier, J. C., Traore, S. F., Toure, M. B., Traore, M. M., ... & Schlein, Y. (2012). Attraction of Stomoxys sp. to various fruits and flowers in Mali. *Medical and Veterinary Entomology*, *26*(2), 178-187.

Van Der Goes Van Naters, W. M., Bootsma, L., Den Otter, C. J., & Belemtougri, R. G. (1996). Search for tsetse attractants: A structure-activity study on 1-octen-3-ol in Glossina fuscipes fuscipes (Diptera: Glossinidae). *Journal of chemical ecology*, *22*(2), 343-355.

Newstead, R. (1906). Insects and Ticks in Relation to Animal Diseases. *The Veterinary Journal (1900)*, *62*(8), 428–430.

Njiru, Z. K., Ole-Mapeny, I. M., Ouma, J. O., Ndung'u, J. M., & Olaho Mukani, W. (2000). Prevalence of trypanosomosis in camel calves: a pilot study in Laikipia District of Kenya.

Noll, M. E. (2020). The Control of *Stomoxys Calcitrans* (Stable Flies) with Essential Oils (Doctoral dissertation, University of Bristol).

Nordström, K., Dahlbom, J., Pragadheesh, V. S., Ghosh, S., Olsson, A., Dyakova, O., … Olsson, S. B. (2017). In situ modeling of multimodal floral cues attracting wild pollinators across environments. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(50), 13218–13223.

Nyasembe, Vincent O, Tchouassi, D. P., Kirwa, H. K., Foster, W. A., Teal, P. E. A., Borgemeister, C., & Torto, B. (2014). Development and Assessment of Plant-Based Synthetic Odor Baits for Surveillance and Control of Malaria Vectors, *9*(2), 1–10.

Nyasembe, V. O., Tchouassi, D. P., Muturi, M. N., Pirk, C. W., Sole, C. L., & Torto, B. (2020). Plant nutrient quality promotes survival and reproductive fitness of the dengue vector Aedes aegypti.

Olafson, P. U., Aksoy, S., Attardo, G. M., Buckmeier, G., Chen, X., Coates, C. J., … Benoit, J. B. (2021). The genome of the stable fly, *Stomoxys calcitrans*, reveals potential mechanisms underlying reproduction, host interactions, and novel targets for pest control. *BMC Biology*, *19*(1), 1–31.

Olsson, S. B., & Hansson, B. S. (2013). Electroantennogram and single sensillum recording in insect antennae. *Methods in Molecular Biology*.

Patel, S. (2011). Harmful and beneficial aspects of Parthenium hysterophorus: an update. *3 Biotech*, 1(1), 1-9.

Patra, G., Behera, P., Das, S. K., & Saikia, B. (2018). Stomoxys calcitrans and its importance in livestock.

Pellegrino, M. (2011). Structure-Function Analysis of Insect Olfactory Receptors. *The Rockefeller University*, 185.

Pelosi, P., Zhou, J. J., Ban, L. P., & Calvello, M. (2006). Soluble proteins in insect chemical communication. *Cellular and Molecular Life Sciences CMLS*, *63*(14), 1658-1676.

Paré, P. W., & Tumlinson, J. H. (1999). Plant volatiles as a defense against insect herbivores. *Plant physiology*, *121*(2), 325-332.

Reissert-Oppermann, S., Bauer, B., Steuber, S., & Clausen, P. H. (2019). Insecticide resistance in stable flies (*Stomoxys calcitrans*) on dairy farms in Germany. *Parasitology Research*, *118*(9), 2499–2507.

Rivero, A., & Casas, J. (1999). Incorporating physiology into parasitoid behavioral ecology: the allocation of nutritional resources. *Researches on Population Ecology, 41*(1), 39-45.

Rockel, E. G. (1969). Autogeny in the deerfly, Chrysops fuliginosus (Diptera: Tabanidae). *Journal of medical entomology, 6*(2), 140-142.

Roulston, T. A. H., Cane, J. H., & Buchmann, S. L. (2000). What governs protein content of pollen: pollinator preferences, pollen–pistil interactions, or phylogeny?. *Ecological monographs, 70*(4), 617-643.

Salem, A., Franc, M., Jacquiet, P., Bouhsira, E., & Liénard, E. (2012). Feeding and breeding aspects of *Stomoxys calcitrans* (Diptera: Muscidae) under laboratory conditions. *Parasite, 19*(4), 309–317.

Schlein, Y., & Muller, G. (1995). Assessment of plant tissue feeding by sand flies (Diptera: Psychodidae) and mosquitoes (Diptera: Culicidae). *Journal of Medical Entomology, 32*(6), 882–887.

Schofield, S., Cork, A., & Brady, J. (1995). Electroantennogram responses of the stable fly, Stomoxys calcitrans, to components of host odour. *Physiological Entomology, 20*(3), 273-280.

Schofeld, S., & Torr, S. J. (2002). A comparison of the feeding behaviour of tsetse and stable flies. *Med. Vet. Entomol, 16*(2), 177-185.

Seada, M. A. (2015). Antennal morphology and sensillum distribution of female cotton leaf worm Spodoptera littoralis (Lepidoptera: Noctuidae). *The Journal of Basic & Applied Zoology, 68*, 10-18.

Sharif, S., Jacquiet, P., Prévot, F., Grisez, C., Bouhsira, E., Franc, M., & Liénard, E. (2017). Assessment of persistence of Besnoitia besnoiti (Henry, 1913) bradyzoites in *Stomoxys calcitrans* (Diptera: Muscidae). *Revue de Medecine Veterinaire, 168*(7–9), 197–203.

Shaw, J., Lickey, E. B., Beck, J. T., Farmer, S. B., Liu, W., Miller, J., … Small, R. L. (2005). The tortoise and the hare II: Relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. *American Journal of Botany*, *92*(1), 142–166.

Showler, A. T., & Osbrink, W. L. A. (2015). Stable Fly, *Stomoxys calcitrans* (L.), Dispersal and Governing Factors . *International Journal of Insect Science*, *7*.

Shrivastava, G., Rogers, M., Wszelaki, A., Panthee, D. R., & Chen, F. (2010). Plant volatiles-based insect pest management in organic farming. *Critical Reviews in Plant Sciences*, *29*(2), 123-133.

Skidmore, P. (1985). The biology of the Muscidae of the world (Vol. 29). Springer Science & Business Media.

Solórzano, J. A., Gilles, J., Bravo, O., Vargas, C., Gomez-Bonilla, Y., Bingham, G. V., … Rodriguez-Saona, C. (2015). Biology and trapping of Stable Flies (Diptera: Muscidae) developing in pineapple residues (Ananas comosus) in Costa Rica. *Journal of Insect Science*, *15*(1).

Suliman, A. I. A., Mory, M. D., Saqib, A., & Man-Qun, W. (2015). Understanding Insect Behaviors and Olfactory Signal Transduction. *Enliven: Genetics, Molecular, and Cellular Biology*, *2*(2), 1–10.

Tainchum, K., Sukonthabhirom, S., Duvallet, G., Akratanakul, P., Muenworn, V., & Chareonviriyaphap, T. (2010). Population structure of *Stomoxys calcitrans* (Diptera: Muscidae) from nine regions of Thailand. *Journal of economic entomology*, *103*(3), 1012-1018.

Tangtrakulwanich, K., Chen, H., Baxendale, F., Brewer, G., & Zhu, J. J. (2011). Characterization of olfactory sensilla of *Stomoxys calcitrans* and electrophysiological responses to odorant compounds associated with hosts and oviposition media. *Medical and Veterinary Entomology*, *25*(3), 327–336.

Tawich, S. K., Bargul, J. L., Masiga, D., Getahun, M. N. (2021). Supplementing Blood Diet With Plant Nectar Enhances Egg Fertility in *Stomoxys calcitrans*, *12*(March), 1–12.

Taylor, D. B., & Berkebile, D. R. (2014). Sugar feeding in adult stable flies. *Environmental entomology, 37*(3), 625-629.

Taylor, D. B., Moon, R. D., & Mark, D. R. (2012). Economic Impact of Stable Flies (Diptera: Muscidae) on Dairy and Beef Cattle Production. *Journal of Medical Entomology*, *49*(1), 198–209.

Tholl, D., Boland, W., Hansel, A., Loreto, F., Röse, U. S. R., & Schnitzler, J. P. (2006). Practical approaches to plant volatile analysis. *Plant Journal*, *45*(4), 540–560.

Tsuchihara, K., Fujikawa, K., Ishiguro, M., Yamada, T., Tada, C., Ozaki, K., & Ozaki, M. (2005). An odorant-binding protein facilitates odorant transfer from air to hydrophilic surroundings in the blowfly. *Chemical Senses*, *30*(7), 559–564.

Usher, M. B., Coulson, R. N., & Witter, J. A. (1985). Forest Entomology: Ecology and Management. *The Journal of Applied Ecology*.

Vijayan, K., & Tsou, C. H. (2010). DNA barcoding in plants: taxonomy in a new perspective. Current science, 1530-1541.

Vivaldo, G., Masi, E., Taiti, C., Caldarelli, G., & Mancuso, S. (2017). The network of plants volatile organic compounds. *Scientific reports, 7*(1), 1-18.

Vosshall, L. B., & Stocker, R. F. (2007). Molecular architecture of smell and taste in Drosophila. *Annual Review of Neuroscience*, *30*, 505–533.

Warburg, A., & Faiman, R. (2011). Research priorities for the control of phlebotomine sand flies. *Journal of Vector Ecology*, *36*, 10–16.

Warnes, M. L., & Finlayson, L. H. (1987). Effect of host behaviour on host preference in *Stomoxys calcitrans*. *Medical and Veterinary Entomology, 1*(1), 53-57.

Wykes, G. R. (1953). The sugar content of nectars. *The Biochemical Journal*, *53*(2), 294–296.

Zumpt, F. (1973). The Stomoxyine biting flies of the world. Diptera: Muscidae. Taxonomy, biology, economic importance and control measures.

APPENDICES

Appendix I: List of 140 plant volatile organic compounds identified in this study as represented in the heatmap.

VOC name	Chemical Family	VOC ID
E-Ocimenol	Alcohol	Voc6
Linalool	Alcohol	Voc10
6-Camphenol_	Alcohol	Voc23
Phenyl_ethyl_alcohol	Alcohol	Voc29
Cinnamyl alcohol<E->	Alcohol	Voc77
Isovalencenol<(E)->	Alcohol	Voc91
3-Phenylpropanol	Alcohol	Voc99
Benzyl alcohol	Alcohol	Voc107
Terpinen-_4-ol	Alcohol	Voc120
Benzene acetaldehyde	Aldehyde	Voc62
Benzene acetaldehyde	Aldehyde	Voc108
Benzaldehyde	Aldehyde	Voc133
3-Hexanone	Ketone	Voc14
3-Penten-2-one	Ketone	Voc35
3-Heptanone,_2-methyl-	Ketone	Voc60
Acetophenone	Ketone	Voc63
Hepten-2-one<6-methyl-5-	Ketone	Voc67
Hexanoic_acid<2-methyl	Carboxylic Acid	Voc16
Pentanoic_acid	Carboxylic Acid	Voc32
Succinic_acid	Carboxylic Acid	Voc124
Citronellic_acid	Fatty acid	Voc42
Butanoic_acid	Fatty Acid	Voc64
Sulfurous_acid	Inorganic acid	Voc134
3-Hexanone	Ketone	Voc14
3-Penten-2-one	Ketone	Voc35
3-Heptanone,_2-methyl-	Ketone	Voc60
Acetophenone	Ketone	Voc63
Hepten-2-one<6-methyl-5-	Ketone	Voc67
Anisole<para-methyl->	Methoxide	Voc20
Tricosane	Alkane	Voc26

Decane	Alkane	Voc36
Dodecane	Alkane	Voc37
Nonane	Alkane	Voc46
Hexadecane	Alkane	Voc47
Tetradecane	Alkane	Voc48
Heptadecane	Alkane	Voc49
Pentadecane	Alkane	Voc50
Undecane	Alkane	Voc51
Tritriacontane	Alkane	Voc52
Docosane	Alkane	Voc53
Eicosane	Alkane	Voc68
Tridecane	Alkane	Voc69
Octadecane	Alkane	Voc96
Nonadecane	Alkane	Voc97
Heneicosane	Alkane	Voc98
Octane	Alkane	Voc100
Tetratriacontane	Alkane	Voc126
Tetracosane	Alkane	Voc136
Hexadecane_	Alkane	Voc138
Trimethyl-tridecatetraene	Alkene	Voc59
Decene	Alkene	Voc92
Hexadecene	Alkene	Voc93
Dauca-5,8-diene	Alkene	Voc129
Cumacrene	Alkene	Voc131
Indole	Aromatic compound	Voc44
Toluene	Aromatic Hydrocarbon	Voc1
Trimethyl_benzene	Aromatic Hydrocarbon	Voc61
O-xylene	Aromatic Hydrocarbon	Voc101
Mesitylene	Aromatic hydrocarbon	Voc102
Styrene	Aromatic Hydrocarbon	Voc104
Sylvestrene	Aromatic Hydrocarbon	Voc105
Naphthalene	Aromatic Hydrocarbon	Voc106
Cymene<ortho>	Aromatic Hydrocarbon	Voc113
Cymene<para>	Aromatic Hydrocarbon	Voc117
Butylated_hydroxytoluene	Aromatic Hydrocarbon	Voc2
Ethyl_isovalerate	Ester	Voc15
Methyl_benzoate	Ester	Voc27
Ethyl_benzoate	Ester	Voc28
Methyl_salicylate	Ester	Voc33
Dibutyl_phthalate	Ester	Voc58

Hexenyl valerate<3Z->	Ester	Voc71
Hexenyl butanoate<3Z->	Ester	Voc72
Methyl jasmonate	Ester	Voc81
Methyl_epi_jasmonate	Ester	Voc82
Hexenyl acetate<3Z->	Ester	Voc103
(Z)-3-hexenyl_propanoate	Ester	Voc125
cis-3-Hexenyl_isovalerate	Ester	Voc132
Menthatriene<1,3,8-para->	Monoterepene	Voc7
Phellandrene<beta->	Monoterepene	Voc18
Tricyclene	Monoterpene	Voc3
Ocimene<(E)-beta->	Monoterpene	Voc4
Ocimene<(Z)-beta->	Monoterpene	Voc5
Farnesene<alpha->	Monoterpene	Voc12
Farnesene<beta->	Monoterpene	Voc13
Phellandrene<alpha->	Monoterpene	Voc17
Terpinolene	Monoterpene	Voc21
Camphene	Monoterpene	Voc22
Myrcene	Monoterpene	Voc25
Sabinene	Monoterpene	Voc38
Sabinene_hydrate	Monoterpene	Voc41
Carene	Monoterpene	Voc43
Amorphene<alpha->	Monoterpene	Voc55
Amorphene<delta->	Monoterpene	Voc56
Pinene<alpha->	Monoterpene	Voc65
Pinene<beta->	Monoterpene	Voc66
Methyl citronellate	Monoterpene	Voc70
Limonene	Monoterpene	Voc85
Terpinene<gamma->	Monoterpene	Voc121
Valencene	Monoterpene	Voc122
a-_Thujene	Monoterpene	Voc130
Linalool_oxide<cis->(pyanoid)	Monoterpenoid	Voc8
Linalool_oxide_<trans(furanoid)	Monoterpenoid	Voc9
Dendrolasin	Monoterpenoid	Voc34
Terpineol<alpha->	Monoterpenoid	Voc119
Cresol<para->	Phenolic	Voc11
Germacrene_D	Sesquiterepene	Voc87
Nerolidol<Z->	Sesquiterepenoid	Voc45
Caryophyllene<beta>	sesquiterpene	Voc31
Sesquiphellandrene	Sesquiterpene	Voc19

Camphor	Sesquiterpene	Voc24
Selinene<alpha->	Sesquiterpene	Voc39
Selinene<beta->	Sesquiterpene	Voc40
Cubebene	Sesquiterpene	Voc57
Copaene<alpha->	Sesquiterpene	Voc73
Copaene<beta->	Sesquiterpene	Voc74
Cedrene<alpha->	Sesquiterpene	Voc75
Cedrene<beta->	Sesquiterpene	Voc76
Gurjunene<alpha->	Sesquiterpene	Voc78
Cadina-1(6),4-diene<cis->	Sesquiterpene	Voc86
Germacrene_B	Sesquiterpene	Voc88
allo-Aromadendrene	Sesquiterpene	Voc90
Apofarnesal<(E)-dihydro	Sesquiterpene	Voc94
Silphinene	Sesquiterpene	Voc95
Humulene	Sesquiterpene	Voc109
Humulene<alpha->	Sesquiterpene	Voc110
Zingiberene<alpha>	Sesquiterpene	Voc111
Y_langene	Sesquiterpene	Voc112
Elemene	Sesquiterpene	Voc114
Elemene<gamma->	Sesquiterpene	Voc115
Elemene<delta->	Sesquiterpene	Voc116
Guaiene<alpha>	Sesquiterpene	Voc118
Funebrene<alpha->	Sesquiterpene	Voc123
Cogeijerene	Sesquiterpene	Voc127
Bourbonene<beta->	Sesquiterpene	Voc128
Cadinene<delta->	Sesquiterpene	Voc137
Curcumene<gamma->	Sesquiterpene	Voc139
Curcumene<beta->	Sesquiterpene	Voc140
Eudesmol<10-epi-gamma	Sesquiterpenoid	Voc54
Nerolidol<E->	Sesquiterpenoid	Voc80
Farnesol	Sesquiterpenoid	Voc83
Farnesal	Sesquiterpenoid	Voc84
Bicyclogermacrene	Sesquiterpnene	Voc89
Gurjunene<gamma->	Sesquterpene	Voc79

Appendix II: Sequence alignments of the identified plant (A) trnH-psbA (B) rbcL PCR products used to differentiate plant species.