INDUCTION OF MOLECULAR AND PHYSIOLOGIC CHANGES IN LARVAE AND ADULT *ANOPHELES GAMBIAE* S.S. MOSQUITO BY *MURRAYA KOENIGII* LEAF PHYTOCHEMICALS

Mang'era, Clarence Maikuri (BSc, MSc.)

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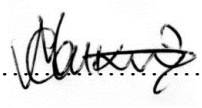
MAY, 2021

DECLARATION

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

Signature: ……  …… Date: 31st May, 2021….

Mr. Mang'era Clarence Maikuri.

31st May, 2021

SUPERVISORS

We confirm that the work reported in this thesis was carried out by the student under our supervision

Signature: ……  …… Date: 31st May, 2021……

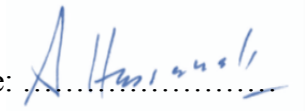
Dr. Fathiya Khamis (Ph.D.)

International Center for Insect Physiology and Ecology (ICIPE), Kenya.

Signature: ……  …… Date: 31st May, 2021….

Dr. Mireji Paul (Ph.D.)

Kenya Agricultural and Livestock Research Organization – Biotechnology Research Institute, Kenya.

Signature: ……  …… Date: 31st May, 2021……

Prof. Hassanali Ahmed (Ph.D.)

Kenyatta University, Kenya.

DEDICATION

To my cherished wife Edna and my sons (Ivan and Ethan) whose future inspires me to excellence, my parents Pr. and Mrs. Mose and my sister Lilian.

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ABBREVIATIONS AND ACRONYMS

20E	20-Hydroxyecdysone
asl	Above sea level
ANOVA	Analysis of variance
BPU	Benzoylphenyl urea
COEJHE	Carboxylesterase juvenile hormone esterase
CRISPR	Clustered regularly interspaced short palindromic repeats
cDNA	Complementary deoxyribonucleic acid
Cas9	CRISPR associated protein 9
CP	Crossing point
CPLCP	Cuticular protein of low complexity
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
DDT	Dichlorodiphenyltrichloroethane
DE	Differentially expressed
EO	Essential oil
FDR	False discovery rate
GO	Gene ontology
HCA	Hierarchical cluster analysis
hrs	Hours
IRS	Indoor residual spraying
IGD	Insect growth disrupting compound
ITN	Insecticide-treated nets
IVM	Integrated Vector Management
JH	Juvenile hormone
JHA	Juvenile hormone analogs
KEMRI	Kenya Medical Research Institute
kdr	Knock-down resistance
LC	Lethal concentration

LC-QtoF-MS	Liquid chromatography quadruple time of flight coupled with mass spectrometry
LD	Lethal dose
LLIN	Long-lasting insecticidal nets
LSM	Larval source management
mRNA	Messenger Ribonucleic acid
mins	Minutes
DEET	N,N-Diethyl-*meta*-toluamide
PC	Principal component
ROS	Reactive oxygen species
RPKM	Reads Per Kilobase of transcript, per Million mapped reads
RT-qPCR	Real-time quantitative Polymerase chain reaction
RNA	Ribonucleic acid
RNA-Seq	RNA-sequencing
SSA	Sub-Saharan Africa
VGSC	Voltage-gated Sodium channel
WHO	World Health Organization
WHOPES	World Health Organization Pesticide Evaluation Scheme

ABSTRACT

Plant-based constituents have been proposed as eco-friendly alternatives to synthetic insecticides for control of mosquito vectors of malaria. These synthetic products are increasingly becoming problematic mainly due mosquito resistance, and hence the need to search and identify alternative control agents. In this study, we first screened the effects of methanolic leaf extracts of curry tree (*Murraya koenigii*) growing in tropical (Mombasa, Malindi) and semi-arid (Kibwezi, and Makindu) ecological zones of Kenya on late third and early fourth instar (L_3/L_4) *An. gambiae* s.s. larvae. Extracts of the plant from the semi-arid region, and particularly from Kibwezi, led to high mortality of the larvae. Bioassay-guided fractionation of the methanolic extract of the leaves of the plants from Kibwezi was then undertaken and the most active fraction (20 fold more potent than the crude extract) was then analysed by Liquid chromatography quadruple time of flight coupled with mass spectrometry (LC-QtoF-MS) and a number of constituents were identified, including a major alkaloid constituent, Neplanocin A **(5)**. Exposure of the third instar larvae to a sub-lethal dose (below 4.43 ppm) of this fraction, induced gross morphogenetic abnormalities in the larvae, with reduced locomotion, a 3-fold reduction in pupation rates and protracted larval-pupa moulting by up to 15 days post-exposure relative to controls. In order to explain these hormetic responses and gain the molecular insights on the observed phenotypic effects conferred by sub-lethal doses of *M. koenigii* bioactive fractions, differential gene expression profiles were assessed by analysing RNA-Seq datasets from *An. gambiae* mosquito immature stages and validated through RT-qPCR. These deformities were accompanied by significant induction (up to 780-fold increase) of transcripts predominantly associated with hard cuticular proteins, juvenile hormone esterases, immunity and detoxification in the larvae samples exposed to the extract relative to the non-exposed control samples coupled with alteration of pathways involved in putrescine metabolism and structural constituents of the cuticle. Additionally, essential oils (EO) from *M. koenigii* leaves showed significant fumigant toxicity against adult *An. gambiae* (100% mortality in 3.30 ± 0.071 hrs, $p = .00$). These results demonstrate subtle growth-disrupting effects of the phytochemical blend from *M. koenigii* leaves on aquatic stages of *An. gambiae* mosquito and provides significant molecular evidence for differentially expressed genes linked to developmental abnormalities observed in *An. gambiae* mosquito larvae exposed to a bioactive fraction of *M. koenigii* leaf extract. Also, fumigant toxicity assays demonstrate that *M. koenigii* has a unique EO complex blend with an insecticidal potential. The study lays down some useful groundwork for the downstream development of phytochemical blends for eco-friendly control of *An. gambiae* vector population that can be swiftly deployed concurrently with existing Integrated Vector Management (IVM) technologies. Functional studies into the potential roles of the candidate genes as molecular targets that can be evaluated to shed further light on the molecular mechanisms underlying the effects of the phytochemicals.

CHAPTER ONE
INTRODUCTION

1.1 Background

Effective control of *Anopheles gambiae* using the current classes of insecticides is challenging and necessitates a search for novel insecticides with unique modes of action, different from the current ones. Current mosquitocidal classes of insecticides (pyrethroids, organochlorines, organophosphates, carbamates, and DDT) are limited to three different modes of action (little target-site diversity), with two different target sites (AchE or voltage-gated sodium channel) and a single component (central nervous system) of the vector (Meyer *et al*., 2012; Baldacchino *et al*., 2015). It is not, therefore, surprising that there is cross-resistance to the insecticides in the vector to insecticides within the classes, a phenomenon currently widespread across Sub-Saharan Africa (SSA) (Nkya *et al*., 2014; Benelli and Beier, 2017; Owusu *et al*., 2017).

Mosquito resistance to pyrethroids is an emerging challenge to the efficacy of otherwise successful insecticide-treated nets (ITN) based malaria control intervention against adult malaria vectors (Staedke *et al*., 2019). Studies in novel vector control methods are now tuned towards biological solutions such as plant-based insecticides and repellents (Bossou *et al*., 2013). Most plants produce a range of phytochemical blends as secondary metabolites, which may be structurally related, with a multiplicity of functions against different pathogens and herbivores. These phytochemical blends rarely demonstrate acute toxicity to vertebrates and are eco-friendly. Moreover, it is equally rare for insects to develop a resistance mechanism against such a range of phytochemicals in these blends. In addition, the chemical composition and yield of essential oils from leaves of plants have been observed to vary with agro-climatic and

geographical factors (Lal *et al*., 2001; Verma *et al*., 2013) hence the need to explore different ecological zones for optimum putative insecticidal effects.

Use of safer insecticides of botanical origin will offer sustainable and affordable solutions to malaria vector control. Unlike conventional insecticides, which are mainly based on single active ingredients, plant-derived insecticides comprise phytochemical blends of chemical compounds which act synergistically on both behavioral and physiological processes of mosquitoes. Moreover, such blends often have resistance-mitigating effects. Identifying bio-insecticides that are efficient, as well as being suitable and adaptive to ecological conditions is vital for continued effective vector control management.

One such plant is *Murraya koenigii* where mosquitocidal effects of relatively high doses of curry tree plant (*M. koenigii*) extracts have been demonstrated against larvae of *Anopheles stephensi* (Srivastava *et al*., 2009; Arivoli, 2015), *Aedes aegypti* (Patil *et al*., 2010; Kovendan, Arivoli, *et al*., 2012) and *Culex quinquefasciatus* (Tennyson *et al*., 2012). These studies have, however, not assessed the potential effects of longer-term exposure of larval stages to sub-lethal doses of the plant extracts and characterized the chemical basis of their activities to the *An. gambiae* mosquito. With demonstrated activity against other species of mosquito, this study was designed to assess the potential of *M. koenigii* for its mosquitocidal activity against *An. gambiae*.

Phytochemicals have subtle anti-insect properties that have evolved through endless evolutionary interactions with a range of natural pests and will have potential as new weapons in the arsenal of alternative products for fighting against the malaria vector (Maia and Moore, 2011). This study aims at determining the fumigant toxicity of major constituents of essential oils and anti-larval activity of non-volatile compounds

individually and in selected blends of *M. koenigii,* with the purpose of identifying the candidates with subtle control potential of *An. gambiae* s.s. Here, the gene expression profiles of the mosquitos exposed to *M. koenigii* phytochemicals are investigated by RNA-seq to further the understanding of insect growth disruption mechanisms. The application of this sequence-based approach allowed us to interrogate absolute changes in transcript accumulation profiles associated with resulting phenotypic changes in the mosquito.

This study reveals potential new targets in the malaria vector that can spur research and development of new insecticide formulations.

1.2 Problem Statement and justification

Malaria remains among the top killer diseases in Kenya and other countries in SSA (Gatonye and Kibet, 2014) relative to pneumonia and Acquired Immunodeficiency Syndrome (AIDS). With the increasing occurrence and spread of drug-resistant *Plasmodium* parasites in Kenya (Mang'era *et al*., 2012; Taylor and Juliano, 2014) and increasing cost of antimalarial drugs (Sicuri *et al*., 2013), there is a need for complementary methods for effective management of the disease.

Control of *An. gambiae* population reduces transmission of the malaria-causing parasite resulting in the reduction of malaria incidences. Existing techniques for the control of the malaria vector such as ITNs require a major boost by developing new control agents, such as botanicals, that are effective against the malaria vector, safe to humans and the environment and, at the same time, effective against the malaria vector. The use of synthetic products to control the mosquito population is becoming increasingly problematic mainly due to mosquito resistance to the commonly used pyrethroids, and hence the need to search and identify alternative control agents.

This study targeted subtle growth disrupting bioactive effects of major components of *M. koenigii* phytochemicals on *An. gambiae* immature stages and the mechanism(s) underlying their effects. Additionally, fumigant toxicity of essential oils *M. koenigii* growing in different locations in Kenya was compared and possible bioactive components of the essential oils identified. These phytochemicals are attractive as mosquitocidal and larvicidal candidates because of their low mammalian toxicity, short environmental persistence and multi-component blend effects that would limit the development of mosquito resistance against them.

1.3 Hypothesis

There is no significant molecular and physiologic responses in adult and larval stages of the *Anopheles* mosquito exposed to *Murraya koenigii* leaf phytochemicals.

1.4 Objectives

1.4.1 General Objective

To determine molecular and physiologic changes in larvae and adult *Anopheles gambiae* s.s. mosquito due to *Murraya koenigii* leaf phytochemicals exposure

1.4.2 Specific Objectives

(i) To determine the effect of non-volatile constituents of *M. koenigii* on growth and development of *Anopheles gambiae* s.s. immature stages

(ii) To determine differentially expressed genes associated with exposure of *An. gambiae* s.s. to sub-lethal doses of *M. koenigii* non-volatile constituents

(iii) To determine chemotypic variability and fumigant toxicity of volatiles from *Murraya koenigii* on adult *Anopheles gambiae* s.s.

CHAPTER TWO

LITERATURE REVIEW

2.1 The malaria threat in Africa

Mosquitoes cause the highest human mortality in the world as compared to other organisms since they transmit a myriad of devastating diseases, such as malaria, that kills about 450,000 million people every year (WHO, 2018). Half of the world's population is under threat from malaria, with Africa carrying a disproportionate burden of 93% of malaria deaths especially in children < 5 years of age (WHO, 2018), mainly because the transmission pattern is centred largely in the tropics. Despite these worrying statistics, a rigorous campaign against malaria dubbed Roll Back Malaria led to substantive scale-up of effective treatments across Sub-Saharan Africa (SSA) leading to a marked reduction in reported malaria-linked mortality in SSA (O'Meara *et al*., 2010) by up to 50% over the past two decades (WHO, 2016). The decrease in main malaria indicators (infection rates, disease burden, and asymptomatic carriers) is considered to be a result of advancement in health care systems, including enhanced diagnostics and proper application of efficacious anti-malarial drugs. The malaria burden has been further reduced in parts of SSA by widespread coverage of bed nets, rigorous campaigns for indoor residual spraying (IRS), and implementation of full therapeutic course of antimalarial medicine to vulnerable groups regardless of whether the recipient is infected with malaria (Intermittent preventive treatment) (Tizifa *et al*., 2018) (Fig 2.1).

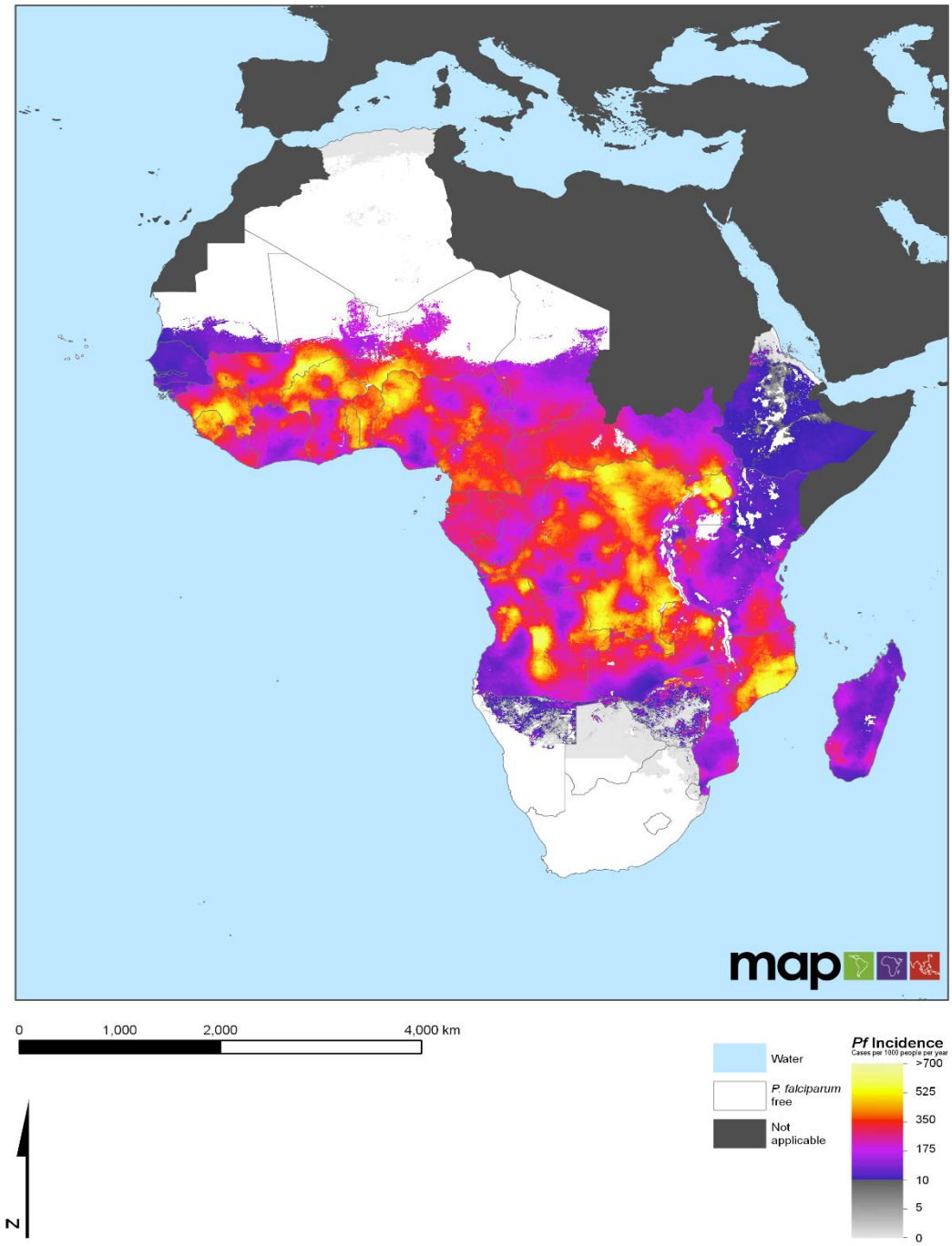

Figure 2.1: *Plasmodium falciparum* malaria incidence distribution (2017) in the WHO African Region. Source: Malaria Atlas project 2019

Notwithstanding this remarkable reduction in malaria prevalence, malaria remains a looming threat in Africa, given the projected growth of the continent's population by 2030 in countries where malaria remains a risk due to the emergence of parasites resistant to antimalarial drugs and of mosquito resistant to insecticides.

Global climatic change contributing to changes in rainfall, humidity, and temperature, impacts malaria vectors by augmenting transmission patterns in regions previously thought to have reduced disease burden. There is, therefore, a pressing need to consistently formulate an optimal combination of malaria vector control interventions suited for the continent's unique terrain and climate.

2.2 Mosquitoes as disease vectors

2.2.1 *Anopheles gambiae* mosquito and malaria

Mosquitoes have been crucial targets of biomedical research since Patrick Manson and Sir Ronald Ross first associated them with the transmission of filarial worms and malaria in the late 19th century (Cox, 2010). Transmission of malaria occurs when a female Anopheles mosquito bites a human host, injecting a protozoan parasite (*Plasmodium*) into the bloodstream.

The three most efficient malaria vectors of the malaria parasite, *P. falciparum* are mosquitoes of the *Anopheles gambiae sensu lato (s.l.*) complex (*An. gambiae sensu stricto (s.s.*) (hereafter referred to as *An. gambiae*), its sibling *An. arabiensis*, and *An. funestus* (Coluzzi *et al*., 1979; Lehmann and Diabate, 2008; Sinka *et al*., 2010). Among these, *An. gambiae* and *An. arabiensis* are more closely associated with humans and represent the major vectors of malaria (O'Loughlin *et al*., 2016; Zhou *et al*., 2016). *An. arabiensis* also feeds on other hosts such as cattle and dogs, when

available and hence reduces their efficiency in transmission of human malaria (Kent *et al*., 2007; Stone and Gross, 2018; Meza *et al*., 2019) . The power of *An. gambiae* as an efficient malaria vector was once demonstrated in the 1930s when the species expanded its range to the largely immune-naïve Brazilian population and was linked to increased (25%) malaria mortality over 9 years (Griffing *et al*., 2015).

The *Anopheles gambiae* mosquito efficiently transmits the malaria parasite due to its unique life cycle, with female *Anopheles* requiring a blood meal for ovarian development. The blood meal is then followed by maturation and oviposition of batches of eggs. Female *Anopheles* mosquitoes support sporogonic development of *Plasmodium* parasites soon after ingestion of malaria-infected blood. Within the mosquito midgut lumen, maturation of male and female gametes is followed by fertilization to form a flagellated motile zygote (ookinete) (Beier, 1998).

The life cycle of the mosquito as outlined by Service (1980), can be summarized as follows: The female Anopheles adult mosquito lays about 50-200 small (1mm long) brown or black (white when freshly laid) boat-shaped eggs on the water surface soon after mating and blood-feeding. Viable eggs hatch into larvae within 2-3 days in a warmer climate and about a week-long in cooler climates. The hatched larvae lie parallel to the surface to allow air intake and surface feeding, a distinct feature of the Anopheles species. At ambient water temperatures (25-28^{o}C), the larvae undergo four moults within 6-9 days to reach the pupal stage, which lasts 2-3 days depending on temperature.

Thus, the minimum duration for one generation may be as long as 10-11 days. When the progeny of any one egg batch emerge as adults the males emerge first. The males become ready for mating within 24 hours after emergence such that by the time the females emerge, the males are competent for mating. Most of the male mosquitoes usually die after mating (Lees *et al*., 2014).

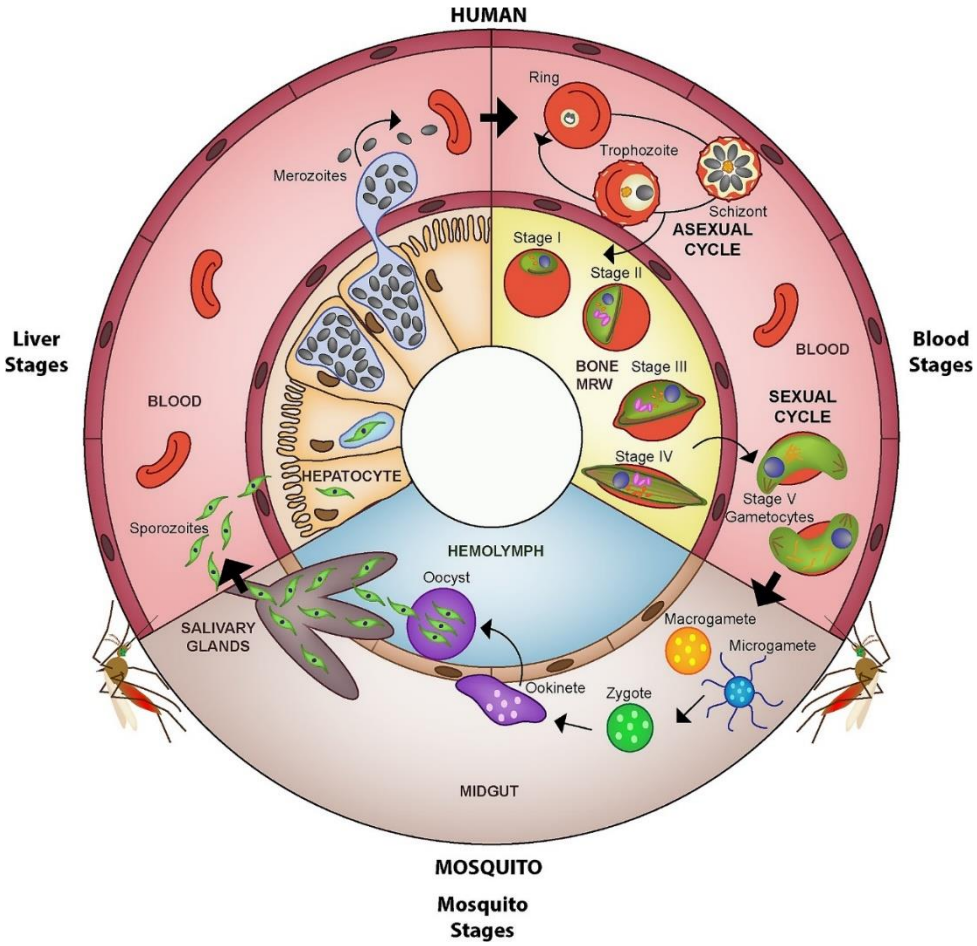

Figure 2.2: The life cycle of *Plasmodium falciparum* malaria parasite showing the exogenous (insect vector) and endogenous (human host) phase (Nilsson *et al*., 2015).

Additionally, the life cycle of human malaria parasite species is characterized by an exogenous sexual phase (sporogony) in which multiplication occurs in several species of Anopheles mosquitoes, and an endogenous asexual phase (schizogony) which takes

place in the vertebrate (human) host (Fig 2.2) who suffers the often fatal consequences of malaria disease (Antinori *et al*., 2012).

2.2.2 The mosquito threat in Kenya

In Kenya, *Anopheles* mosquito species plays a major role as malaria transmission agent. The main vectors of malaria in Kenya are *An. arabiensis* and *An. gambiae* which coexist in a sympatric (occur in the same place at the same time) manner mostly in the coastal and Nyanza regions (Okara *et al*., 2010). Additionally, secondary vectors of malaria such as *An. pharoensis* in Mwea (Central Kenya), *Anopheles nili* and *Anopheles coustani* along the Coastal and Western regions of Kenya, have been reported (Mwangangi *et al*., 2013) though their role in the transmission of malaria parasites in the country is not well established. An estimated 8.5% of Kenya's population live in areas with over 30% malaria prevalence although this represents a marked reduction over 25 years resulting from considerable interventions against the disease (Macharia *et al*., 2018).

Notwithstanding the spirited campaigns the Kenyan government has mounted in the recent past that have seen a reduction in malaria prevalence, the positive gains have not been evenly distributed across the country, hence leaving pockets of regions with high risk of malaria outbreaks (Halliday *et al*., 2014). Such vulnerable regions include arid counties such as Baringo and West Pokot, which have recently experienced malaria outbreaks leading to high incidence of malaria, patient hospitalization and associated deaths (Mulambalah, 2018).

Anopheles spp. are also associated with spread of other vector-borne diseases such as but not limited to lymphatic filariasis, and O'nyong'nyong fever (linked to *An. funestus* and *An. gambiae*).

2.3 Mosquito vector control strategies

2.3.1 Chemical insecticides in mosquito control

Mosquito vector control includes the measures directed against a disease vector, aimed at limiting its ability to transmit disease by protecting areas that are known to be prone to malaria transmission. These malaria-prone areas are determined by carrying out entomological surveys to determine the vector population size and spatial spread, conducting insecticide susceptibility tests and establishing human biting profiles of the prominent vector (Smith Gueye *et al*., 2016). Hence, mosquito control measures must match the local setting to achieve optimum efficacy since transmission parameters are strongly influenced by the local climate, ecology, and behavior of both humans and vectors (Beck-Johnson *et al*., 2013; Mwangangi *et al*., 2013). Additionally, recent successful mosquito control programmes have relied on a combination of interventions, which together have reduced vectorial capacities in malaria-prone areas. The impact of combined vector control methods in Turkey (IRS, larvivorous fish and ITNs) and in the Philippines (IRS and LLINS) was employed to reduce vector receptivity and achieve elimination (Gueye *et al*., 2016) of the malaria vector.

Current strategies are all linked to Integrated Vector Management (IVM) approach which was introduced in the year 2004 by the World Health Organization (WHO) to increase the cost efficiency of existing vector control methods and to reduce the rapid spread of drug and insecticide resistance (WHO, 2004) since most vector control methods involve the use of chemical insecticides. Integrated Vector Management focused on an array of interventions to combat the vector at different vulnerable stages of its life cycle.

For a long time, chemical insecticides (organophosphates, carbamates, and pyrethroids) have been extensively used in the control of mosquitoes. However, in recent years, malaria vectors have rapidly developed resistance against these chemicals and environmental problems (high toxicity, non-biodegradable properties and waste generated in soil, water, food, and air) have resulted from their excessive use (Fig 2.4) (Mnzava *et al*., 2015) leading to knockdown resistance (kdr) in the adult mosquito. Knockdown resistance confers resistance to the rapid knockdown action and lethal effects of DDT and pyrethrins insecticides (Bowman *et al*., 2018). Pyrethroids, the most widely used chemical class of insecticide, exert their insecticidal effect on the voltage-gated sodium channel (VGSC) located on the membrane of neurons. When pyrethroids bind an open channel, they block its closure, thus extending the action potential and resulting in the insect's rapid paralysis.

Resistance to pyrethroids, the major class of insecticide used on all long-lasting insecticidal nets, is widespread in *Anopheles* mosquitoes in SSA and India and is increasingly becoming ineffective against the malaria vector (Fig 2.3) (Kleinschmidt *et al*., 2018; WHO, 2018).

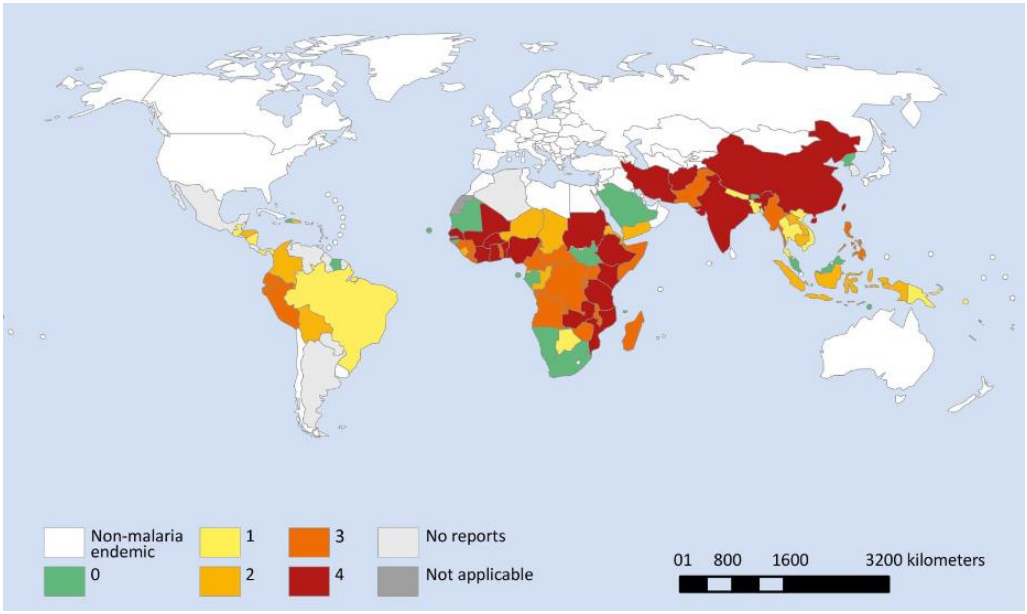

Figure 2.3: Number of insecticide classes to which resistance in malaria vectors has been reported by 2016 globally (WHO, 2018).

There are also increasing reports of resistance to other classes of chemical insecticides such as organophosphates, carbamates, and DDT (Tizifa *et al*., 2018). These reports paint a grim picture on the future of pyrethroids as the mainstay of insecticides against mosquitoes. This is why this study is exploring the utility of insecticidal botanicals (such as from curry tree leaves) in mosquito vector control.

2.3.2 Long Lasting Insecticide Net (LLIN)

Long-lasting insecticidal nets (LLINs) have been widely promoted and implemented as a public health intervention tool for malaria control in most malaria-endemic countries such as Kenya (Ernst *et al*., 2016) and neighbouring Uganda (Staedke *et al*., 2019) since they are cost-effective. This widespread distribution of LLIN has rapidly replaced the historical distribution of untreated bed nets or insecticide-treated bed nets (ITNs) that required frequent retreating, hence a cumbersome approach in rural

settings. However, in the face of rising cases of pyrethroid resistance, new formulations of insecticides for LLINs have been developed, with the first generation of these formulations comprising a pyrethroid and a growth regulator (Mnzava *et al*., 2015). A noteworthy step is to examine potential benefits of the combined (pyrethroid and an insect growth regulator) formulation against pyrethroid-resistant *Anopheles* mosquito.

2.3.3 Personal protectants (repellents)

Repellents have been the primary tool for regulating the biting behaviour of arthropods on humans and animals. N,N-Diethyl-*meta*-toluamide (DEET), the most effective and long-lasting repellent currently available commercially, has long been considered the gold standard in insect repellents, but with reported human health issues (Brown and Hebert, 1997). The mechanism of action of DEET, along with other repellents, is to provide an offensive odor vapour barrier that deters the insect from coming into contact with the skin. Plant-derived sources of personal protectants have recently been reported but nearly all of these essential oils have low residual activities, of up to 4 hours as compared to 10 hours of DEET (Rehman *et al*., 2014). This inadequacy can be extended up to eight hours by use of additives such as vanillin (Lupi *et al*., 2013). Plants that have essential oils with promising repellent activity include, *Cymbopogon* spp., *Ocimum* spp. and *Eucalyptus* spp., with high concentrations of limonene, α-pinene, citronellol, thymol and camphor (Nerio *et al*., 2010).

There is currently inadequate data to assess whether repellents could play a sustainable role in controlling the malaria vector. However, studies are ongoing to build evidence

for the wide-scale usage of repellents as part of national control programs (Mnzava *et al*., 2015).

2.3.4 Indoor residual Spraying (IRS)

IRS involves the application (fumigation) of insecticide products to the interior surfaces (walls and ceilings) of human or animal dwellings in malaria (or other vector-borne diseases) endemic regions. It is most effective against endophilic (indoor-resting), endophagic (indoor-biting) mosquitoes that rest on indoor surfaces. IRS has been effective in many regions, such as Southern Africa and India (Mabaso *et al*., 2004) which have incorporated the intervention under national malaria control programs. However, population coverage by IRS has declined on both regional (SSA) and global scale due to factors such as increased LLIN coverage, reduced government commitment to funding, poor community acceptance (Magaço *et al*., 2019) and the development of insecticide resistance (WHO, 2018). Two new long-lasting formulations of existing IRS insecticides but with increased longevity beyond the benchmark of 2–4 months to 6–12 months are currently being deployed (Mnzava *et al*., 2015). Also, a range of new active insecticidal ingredients of are being explored for commercial applications (Dengela *et al*., 2018; Kweka *et al*., 2018), more so in the control of pyrethroid resistant and for delaying pyrethroid resistance (Rowland *et al*., 2013).

2.3.5 Biological control methods

Biological mosquito control methods are eco-friendly alternatives that do not rely on synthetic chemical insecticides. These alternatives are being explored to combat selection pressure for insecticide resistance and involve strategies that target various

stages of the mosquito lifecycle. A major advantage of using biological control measures in vector control is that existing predators are conserved, which would prey on newly-hatched mosquito larvae, and thus boost control efforts (Enayati and Hemingway, 2010). Biological methods may involve the use of predators, parasites or pathogens in augmenting the population of mosquito antagonist using two main strategies: (i) inoculation where small number of the control agent (predators, parasites or pathogens) are released into the mosquito habitat where they slowly multiply resulting in a sustained suppression of the target population; (ii) inundation where an overwhelming number of the control agent is mass-released resulting in an immediate significant reduction of the target population (Benelli *et al*., 2016). For example, the inoculation of fish into flooded rice fields and inundation control with microbial pathogens (entomopathogenic fungi, protozoa, bacteria, and viruses), which are produced in artificial cultures of *Bacillus thuringiensis israelensis (B.t.i.)* (Imbahale *et al*., 2011).

2.3.6 Environmental management of mosquitoes

There are three basic categories of environmental management for vector control: (i) environmental modification where there is a long term transformation of the physical habitat of the vector; (ii) environmental manipulation which involves temporary changes to the vector habitat with the aim of producing unfavorable conditions for vector breeding; and (iii) changes to human habitation or behavior, which effectively reduces interaction between human and the vector (Mordue, 1995). In rural areas and homes, proofing against the entry of mosquitoes has been efficient in reducing nuisance biting and disease transmission. Houses with open eaves, grass thatch, and mud walls have been found to have a higher density of the malaria vectors in Baringo

County, Kenya (Ondiba *et al*., 2018). Hence, house modifications such as screening of eaves and using corrugated sheets as roofing material can be an effective way of environmental modification that can be incorporated in IVM strategies to complement LLITNs and IRS to reduce indoor mosquito density.

Additionally, agricultural activities in rural areas such as use of irrigation in rice farming, ponds for fish rearing and the storage of water in tanks for livestock provide ambient breeding grounds for mosquito larvae (Oladepo *et al*., 2010). Here, improved farming practices such as adjusting rice field levels to avoid uneven flooding and puddles can ease the disease vector burden in rural areas with intense farming activities.

In urban regions, construction sites have played host to a number of mosquitoes including *Aedes*, *Culex* and *Anopheles* species. Open water storage containers, water-filled pits, flooded cellars, car tyres, empty drink cans, plant pots, and temporary waste drainage systems are common breeding sites for such vectors. Destruction, recycling, disposal or regular emptying of such containers and larval sites can reduce larval indices of container-breeding mosquitoes such as *Aedes* spp. and a resultant reduction of disease transmission (Mireji *et al*., 2008). Such ideas have previously been proposed in Kenya.

2.3.7 Larval Source Management

Larval source management (LSM) offers an attractive intervention strategy to reducing malaria transmission by targeting the immature stages (larvae and pupae) of the Anopheles mosquito (Worrall and Fillinger, 2011; Tusting *et al*., 2013; WHO, 2013;) in their natural habitats. Larval control strategies have been proposed to be very

effective where larval populations are readily located (Killeen *et al*., 2002; Walker and Lynch, 2007), and as most cost-effective in well-defined habitats of the malaria vector (Fillinger and Lindsay, 2011). Mosquito population dynamics depends on factors such as larval productivity, and control of larval populations would reduce mosquito vectorial capacity and significant reduction in transmission patterns of malaria (Kweka *et al*., 2015).

2.4 Insecticidal botanicals

2.4.1 Plant sources of mosquito control compounds

Botanicals from plant sources are secondary metabolites that act as a defence mechanism of the plants against continuous selection pressure from herbivore predators, insect attack and other environmental factors (Kishore *et al*., 2014). Several groups of phytochemicals such as alkaloids, steroids, terpenoids, essential oils and phenolics from different plants have been reported previously for their insecticidal activities (Ghosh *et al*., 2012).

Efficacies of plant-based oviposition deterrents have been explored with the leaf oil of *C. leptophloeos* being one of the most bioactive reported so far, suggesting its possible application as an alternative to synthetic insecticides (Benelli, 2015; Navarro-Silva *et al*., 2009).

Larvicides of plant origin form a large collection of potent phytochemicals against immature stages of a wide range of disease vectors (Ghosh *et al*., 2012; Piplani *et al*., 2019; Samuel *et al*., 2016; Thomas *et al*., 2017). Despite the potential activity of phytochemicals, their commercial feasibility greatly depends upon its economic viability in relation to synthetic larvicides.

2.4.2 *Murraya koenigii* Plant description

Murraya koenigii is an unarmed, semi-deciduous aromatic shrub or small tree with slender but strong woody stem, six meters in height and 15 to 40 cm in diameter, and branches with a dense shady crown covered with dark grey bark. The bark of the stem can be peeled off longitudinally to expose a white wood underneath. Leaves have one terminal leaflet (9-25 leaflets), with no hair, and strongly aromatic. Flowers are small, white fragrant, with hairy surface and dotted glands (Figure 2.4).

Figure 2.4: *Murraya koenigii* plant growing in the field with leaves, unripe berries, and flowers (field sample).

Fruits occur in close clusters, small ovoid and glandular, having thin pericarps and enclosing one or two seeds. The fruits bear spinach green colour while unripe, but turn purplish-black after they have ripened and are edible (Figure 2.4).

2.4.3 Biological activity of Curry leaf tree

*M. koenigii i*s a rich source of organic compounds with an array of chemical components such as alkaloid rich leaves (Arivoli *et al*., 2012; Gahlawat *et al*., 2014) and roots (Chakrabarty *et al*., 1997; Nayak *et al*., 2010). The leaves are associated with a range of bioactivities, such as antioxidant (Tachibana *et al*., 2003), antimicrobial (Muthumani *et al*., 2010), and mosquitocidal (Das *et al*., 1996; Patil *et al*., 2010; Sukari *et al*., 2013; Mang'era *et al*., 2018;) activities.

2.5 Insect Growth Disrupting (IGD) compounds

Insect growth disrupting compounds (IGDs) refers to substances that are either analogues or antagonists of insect hormones and interfere with their development (Fig 2.6) (Schneiderman, 1972). Concerns over eco-toxicology and mammalian safety have driven the search for novel IGD chemicals that 1) target unique physiological and biochemical systems in insects; 2) target a molecular mechanism or vulnerable developmental stage; and 3) are eco-friendly and safe to non-target species. These substances may act as: 1) juvenile hormone (JH) analogues and mimics; 2) ecdysone agonists; or 3) chitin synthesis inhibitors (Dhadialla, 2012) as shown in figure 2.5.

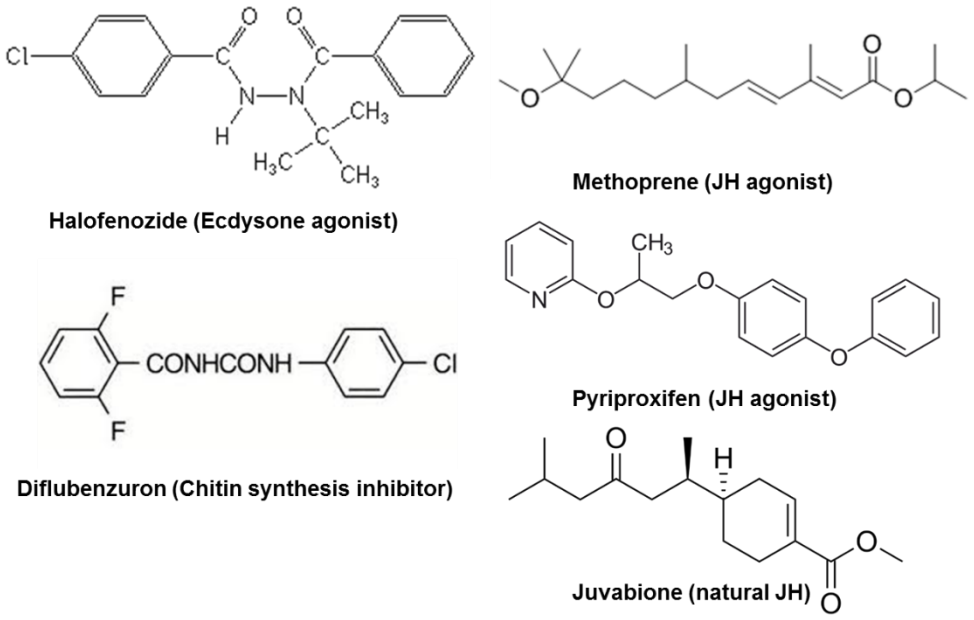

Figure 2.5: Chemical structures of IGDs insecticides with their mode of action indicated (in brackets).

Efficacy of IGDs has been demonstrated across mosquito species where inhibition of adult emergence was observed in *Aedes* (Su *et al*., 2003; Suárez *et al*., 2011) and *Anopheles* (Arredondo-Jiménez and Valdez-Delgado, 2006b; Yapabandara *et al*., 2001) species.

2.5.1 Juvenile hormone (JH) analogues

Juvenile hormone (JH) is secreted by the *Corpora allata* (Figure 2.6) prior to each insect moult and is regulated by two neuro-hormones, the allatotropins that induce secretion and the allatostatins that inhibit synthesis.

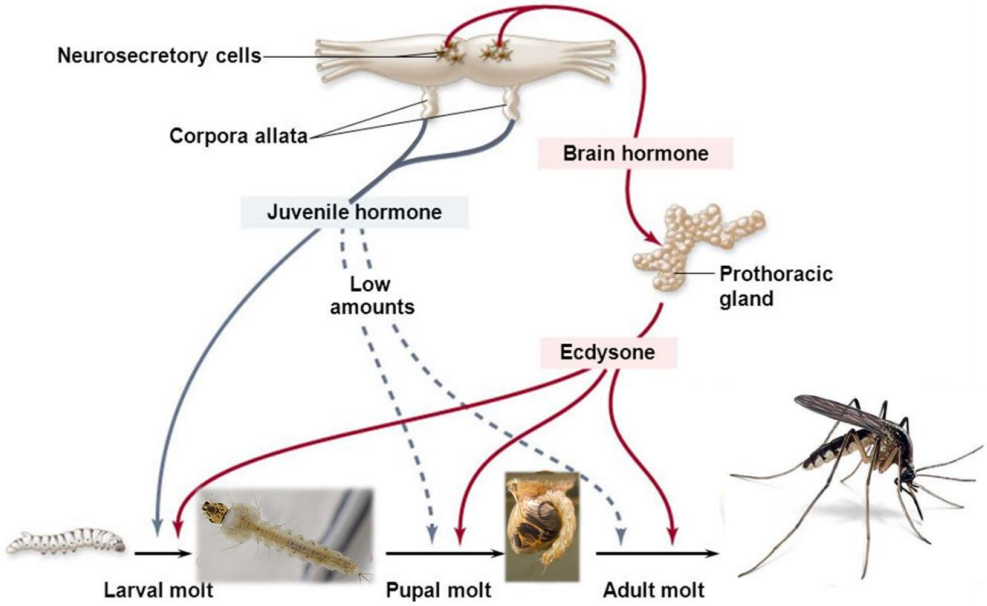

Figure 2.6: A depiction of the neuroendocrine pathway of the *Anopheles* spp. Mosquito (Singh Kaleka *et al*., 2019).

The main role of JH in immature insects is to suppress the genes that promote the development of adult features, causing the insect to remain as larvae (Huybrechts, 2018).

During the last larval instar, the *Corpora allata* becomes atrophied and stops producing juvenile hormone. This frees inhibition on the development of adult structures and leads to transformation into an adult or a pupa. Two of the most important JH analogues (JHAs) are fenoxycarb (Grenier and Grenier, 1993) and pyriproxyfen, the latter of which is regarded as the most potent JHAs available today (Miyamoto *et al*., 1993). JHAs are particularly suitable for the control of disease vectors such as mosquitoes that have larval stages that do not require fast kill because they are relatively harmless and are commonly found in concentrated populations.

JHAs can selectively target insects within an order or even at the family level; a level of selectivity that is seldom associated with the other classes of chemical insecticides.

High target selectivity is one reason that JHAs are considered exceptionally safe. This safety is echoed by the World Health Organization's approval of methoprene use level of up to 1 mg liter^{-1} (i.e., 1 ppm) as a mosquito larvicide in human drinking water (Fawell and Ong, 2012).

2.5.2 Ecdysone agonists

The insect moulting hormone, 20-hydroxyecdysone (20E) is secreted as ecdysone (also called ecdysteroid) by a pair of endocrine glands located in the prothorax of insects. Ecdysone stimulates the synthesis of a new exoskeleton and at high titers it inhibits other endocrine structures.

This moulting hormone also triggers the physical process of shedding the old exoskeleton (ecdysis) when hemolymph concentrations fall to allow secretion of eclosion hormone (Smagghe and Degheele, 1998).

Synthetic non-steroidal compounds belonging to the chemical class of bisacylhydrazines (Figure 2.7) act like the natural moulting hormone at a molecular level by inducing the expression of a group of moult-related genes.

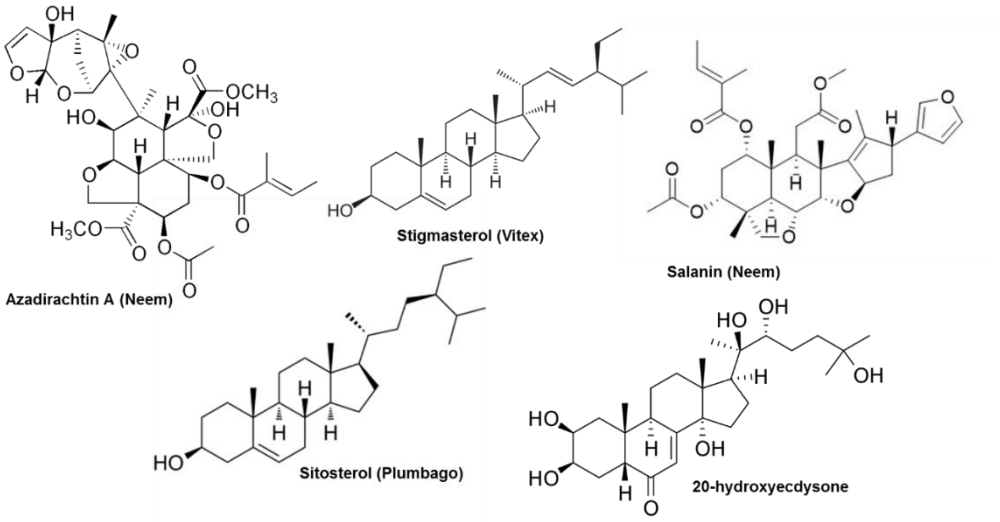

Figure 2.7: Chemical structure of plant-derived IGDs with their sources indicated (in brackets) and their similarity to 20E.

As a result, insect larvae undergo apolysis (separation of the cuticle from the epidermis) and form larva-pupae intermediates (pharate larvae) (Subramanian and Shankarganesh, 2016). However, unlike natural 20E, synthetic analogues bind strongly to the receptors and results in repression of down-regulatory genes necessary for cuticle development, sclerotization, and ecdysis. The repressed genes cause a developmental arrest in this pharate state hence the treated larva goes into a precocious incomplete moult that is often fatal (Retnakaran *et al*., 2003).

2.5.3 Chitin synthesis inhibitors

Chitin, a major constituent of the cuticle, serves as an exoskeleton and protects insects against microbial infection, dehydration, and physical injury. Chitin also acts as a permeability barrier between the food bolus and the midgut epithelium, and thus protects the gut from mechanical disruption, toxins, and pathogens. Insects consistently synthesize and degrade chitin in a highly controlled manner to allow

ecdysis and regeneration of the peritrophic matrices. Diflubenzuron, belonging to the benzoylphenyl urea (BPU) chemical class, was the first chitin synthesis inhibitor to be discovered. This discovery led to the development of several BPU derivatives, such as teflubenzuron (Merzendorfer and Zimoch, 2003), and novaluron (View, 2017). Non-benzoylphenyl urea compounds, such as cyromazine and dicyclanil, do not inhibit chitin synthesis but interfere with cuticle formation and are also considered as moult inhibitors (Subramanian and Shankarganesh, 2016).

2.5.4 Plant-derived IGDs

Plant growth steroids structurally resemble phytoecdysteroids which defend plants against attack from phytophagous insects. Once ingested by insects, these chemicals produce detrimental effects on insect development such as premature moult, weight loss, and structural and metabolic damage (Martinez and Van Emden, 2001). Many plant extracts with promising efficacies have been investigated for mosquito control targeting immature stages of the vector since mosquitoes spend a significant portion of their juvenile development stage in water, and hence can be effectively targeted.

Plant-derived compounds with diverse structural characteristics (terpenes and phytoecdysteroids) have been associated with efficacy against immature mosquito by mimicking endogenous developmental hormones (Fig 2.7).

In earlier studies, limonoids from *Azadirachta indica* (neem) and *Melia azedarach* (China berry) elicited over 95% mortality at 1 ppm against *An. stephensi* larvae (Sengottayan Senthil Nathan *et al*., 2006). In another related study, limonoids derived from *Turraea abyssinica* and *T. cornucopia* inhibited mosquito (*An. gambiae*) larvae development at a dose of LC_{50} 202–265 ppm (Owino *et al*., 2014). This dose is much

higher in relation to much lower doses of triterpenes extracted from *Melia volkensii, Dysoxylum malaricum* and *D. beddomei* that caused larval mortality through growth disruption at 5.4 and 10ppm against *An. arabiensis* and *An. stephensi* respectively (Mwangi and Mukiama, 1988; S. Senthil Nathan *et al*., 2008). Additionally, phytoecydsteroids (γ-sitosterol, stigmasterol and 20-hydroxyecdysone) derived from *Vitex schiliebenii, V. payos* and *Plumbago* spp. have shown marked toxicity (over 98% mortality) on *An. gambiae* larvae at relatively high doses (100ppm) and causing developmental aberrations at sub-lethal doses (<50ppm) (Nyamoita *et al*., 2013). Moreover, non-steroidal plant compounds (proanthocyanidins) from *Camellia sinensis* (green tea) have recently been implicated in inducing developmental defects on developing malaria mosquito larvae at sub-lethal doses of 5ppm, suggesting their potential in the control of mosquito populations (Muema *et al*., 2016).

2.5.5 Mechanism of action of plant-sourced IGDs

Structural resemblance of phytoecdysteroids to endogenous insect developmental hormones 20-E is understood to affect the moulting process through competition for the same endogenous hormone receptors in insects, causing the arrest of normal physiologic and morphologic abnormalities and consequently death (Mordue *et al*., 2005). Though the specific mode of action of phytoecdysteroids and limonoids is unclear, however, these compounds are thought to either antagonize or agonize insect ecdysteroid and juvenile hormone receptors (Bowers, 1991; Gade *et al*., 1997)

Further, limonoids extracted from neem (Azadirachtin B to K, salanins, nimbins) have been reported to inhibit mitosis by disrupting tubulin polymerization (Salehzadeh *et al*., 2003) and cell cycle arrest by inducing apoptosis signals (Priyadarsini *et al*., 2010)

in mammalian cells, suggesting a possible anti-proliferative effect on larvae growth. Similarly, on exposure to other plant-derived compounds, mosquito larvae exhibited cuticular de-melanization, extended abdominal region, impaired chitin formation, protracted larval period, tonic immobility (state of paralysis), midgut damage and deformed flight muscles in mosquito adults, indicating a possible neuroendocrine dysregulation (Fig 2.6) (Ndung'u *et al*., 2004; Sharma *et al*., 2006; Nyamoita *et al*., 2013; Procópio *et al*., 2015).

Other growth disruption effects on mosquito larvae on exposure to sub-lethal doses of these plant-derived compounds include reduced fecundity, reproductive fitness and egg viability, suggesting further downstream negative effects on the malaria vector that can limit its vectorial competence (Rattan, 2010).

2.6 Molecular approaches to developing insecticides against mosquitoes

Molecular advances over the last decade have provided a vast amount of information on the biology of mosquito vectors. Species of *Anopheles* and *Aedes* were some of the first insects after *Drosophila* to be sequenced (Holt *et al*., 2002; Nene *et al*., 2007) and currently 13 further *Anopheles* species alone are being sequenced. The genomic resources now available on sites such as *VectorBase* (Giraldo-Calderón *et al*., 2014) and elsewhere provide unparalleled insights into the cellular processes of the disease vectors.

Vast parallel sequencing of RNA molecules (RNA-seq) has also significantly augmented the utilization of genome resources by providing highly quantitative transcript abundance data, as well as a wealth of sequence, isoform, and expression information for the vast majority of encoded genes in a vector species (Martin and

Wang, 2011). Importantly, because RNA-seq largely captures only fully spliced transcripts, an informative *de novo* transcriptome assembly of RNA sequences can be generated affordably and analyzed efficiently, even in the absence of an assembled genome. Already, *de novo* assemblies of RNA-seq-derived insect transcriptomes have provided invaluable sequence information for vector insects such as the tsetse fly (*Glossina morsitans*) (IGGI, 2014), *Anopheles funestus* (Crawford *et al*., 2010) and *Aedes aegypti* (Nene *et al*., 2007) genome projects. RNASeq provides in-depth and more precise information on transcriptome characterization and quantification. Based upon availability of reference genome, transcriptome assembly can be reference-guided or de novo. Once transcripts are assembled, downstream analysis such as expression profiling, gene ontology, and pathway enrichment analyses can give more insight into gene regulation.

Next-generation sequencing (NGS) is a highly parallel or high-output sequencing method that produces data at or beyond the genomic scale. mRNA sequencing (RNA-seq) is a powerful high throughput sequencing approach that can generate concomitantly gene expression and polymorphism data over the whole transcriptome from a single experiment for comprehensive profiling of transcripts (David *et al*., 2014).

Genomic resources offer the prospect for the rational design of new insecticides and repellents, a better understanding of mosquito population structure of relevance to the design of control interventions, as well as the more radical possibility of genetic modification of mosquitoes either to render them incapable of transmitting diseases or directly to affect their mortality or fecundity (Abd-Alla *et al*., 2013).

Conventional insecticides have several molecular targets including acetylcholinesterase, nicotinic acetylcholine receptors, mitochondrial complex electron transport inhibitors, feeding and growth disruptors, voltage-gated ion channels, G-protein coupled receptors, kinases, ATPases, synthases, and carboxylesterases (Hardy, 2014). Additionally, comparative genomics has aided in the elucidation of the molecular mechanisms underlying insecticide resistance, including metabolic resistance and target-site resistance. Metabolic resistance is characterized by alterations in expression and activity of detoxification enzymes involved in the insecticide biodegradation process, including carboxyl/choline esterases (CCE), glutathione-S-transferases (GST), cytochrome P450 monooxygenases (P450), and ATP-binding cassette (ABC) transporters (Hemingway *et al*., 2004).

Techniques applied in studying these molecular targets have accelerated the quest for insecticides with novel mechanisms. One such technique is RNA interference (RNAi) that has facilitated narrowing the search to specific protein targets by aiding in the functional and behavioral characterization of genes. RNAi mediated gene silencing is often completed via microinjection or ingestion of double-stranded RNA or small interfering RNA (Zhang *et al*., 2010). The RNAi technique is often utilized to identify targets that are essential to function; by silencing the target's expression, there is potential to disrupt growth and development, causing early mortality.

Additionally, whole-genome sequencing has enabled more efficient and accurate sequencing of whole genomes, triggered by technological advancements and lower costs, and the utilization of resultant data to answer biological questions related to insect physiology and development.

Mosquito biology is a topic that requires in-depth understanding through modern, state-of-the-art molecular physiological analyses, which have the potential to provide new and environmentally safe control strategies. However, target specific alternatives are still to be advanced which can provide a sustainable way forward to the discovery of plant-based alternatives.

2.7 Mosquitocidal chemical use in Kenya

Pyrethroids for mosquito use are currently the mainstay of mosquito control in Kenya (WHO, 2018). This has not always been the case. The four classes of synthetic pesticides (pyrethroids, organophosphates, carbamates and organochlorides) have been in use in Kenya for decades (Ondeto *et al*., 2017) until the Ministry of Health in Kenya banned DDT (an organochloride pesticide) in 1986 due to its high association with harmful hormone and psychological effects on the human population (WHO, 2006). Pyrethroids are currently the only allowed pesticides for use in IRS and LLINs in Kenya (MOH, 2016). Exclusive implementation of this intervention has yielded significant success in the control of mosquito vectors populations over the years, contributing to the 68% malaria mortality reduction in Africa (Bhatt *et al*., 2015). However, extensive use of pyrethroids in mosquito control interventions has fueled development of resistance among mosquitoes, with a risk of retrogression on the gains made so far. The first case of pyrethroid resistance in malaria vector was reported in Western Kenya in the early 90s (Vulule *et al*., 1994), followed by widespread incidences of resistance to pyrethroids used in LLINs in the country over the years (Vulule *et al*., 1994; Ondeto *et al*., 2017; Kleinschmidt *et al*., 2018).

Consequently, this situation requires an urgent implementation of the Global plan for Insecticide Resistance Management outlined by WHO (WHO global malaria program,

2012). Organophosphates and carbamates have limited use in Kenya due to public health restrictions. Hence, insecticides from these classes can be considered for use in IRS as an alternative vector control tool in areas where pyrethroid-resistant malaria vectors have been reported. However, constant resistance monitoring needs to be implemented especially in the largely agricultural areas where there is use of pesticides such as fenitrothion (an organophosphate) and carbofuran (a carbamate) that may contribute to resistance against respective classes of insecticides (Ondeto *et al*., 2017).

2.8 Integration of mosquito control measures

A single method of mosquito control such as chemical larviciding may be adequate in limited number of situations. However, such single-solution approach is disposed to failure due to a number of factors which include resistance to chemical insecticides, and environmental and public health concerns, hence the need for IVM as longer term solution. IVM rationally combines all available control methods in the most effective, economical, and safe manner to maintain mosquito vector populations at acceptable levels (WHO global malaria program, 2012). The common mosquito control methods in IVM programmes are chemical, environmental, physical, genetic and biological control (Beier *et al*., 2008). An optimum combination of methods has to be rigorously tested in laboratory and semi-field conditions before being applied. This involves appropriate choice of control methods, effective dosages, biotic and abiotic conditions, appropriate choice of equipment, prevailing weather conditions and environmental persistence (Killeen, 2014). Also, ecological, epidemiological and sociological aspects need to be taken into account to enhance success of the IVM (Smith Gueye *et al*., 2016)

Successful IVM programs require well organized personnel structures, cooperative efforts between entomology and ecology researchers from local institutes and universities and support by the government. Entomological and ecological research monitoring is used to determine the species composition, abundance, and phenology (population dynamics) in relation to the local climate, as well as the spatial and temporal distribution related to human population dynamics (Matthews and Emeritus, 2011; WHO, 2018).

On the other hand, the legally mandated government needs to monitor and approve the use of mosquito control agents. Such monitoring is important in ensuring that there is use of appropriate personnel protective equipment, proper agent formulation, dosage, application technique (timing and frequency) and adequate ecological impact assessment. Also, the government enforces local community participation where the local population is sensitized and encouraged to contribute to the solution of their mosquito problem such as was observed in Coastal and Western Kenya (Mutero *et al*., 2015). This coupled with intensive public relations and educational efforts will ensure that the IVM programme is successful. IVM has now received global attention within the Global Technical Strategy for Malaria by WHO under three pillars which include: (i) Universal access to malaria prevention, diagnosis and treatment; (ii) targeting both parasite and vector by focusing on active cases of malaria, particularly in low transmission settings; and (iii) strengthening malaria surveillance as a core intervention (WHO, 2015).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Growth disrupting activity of curry tree (*M. koenigii*) leaf extracts on *An. gambiae* larvae

3.1.1 Field collections of curry tree leaf samples

Samples of *Murraya koenigii* leaves were selected on the basis of ethnobotanical information, such as their uses in traditional medicine and protection against biting insects. The leaves were collected from Malindi (35°E, 1°N), Mombasa (36°E, 1°S), Makindu (36°E, 0°) and Kibwezi (35°E, 1°N) regions in Kenya (Figure 3.1).

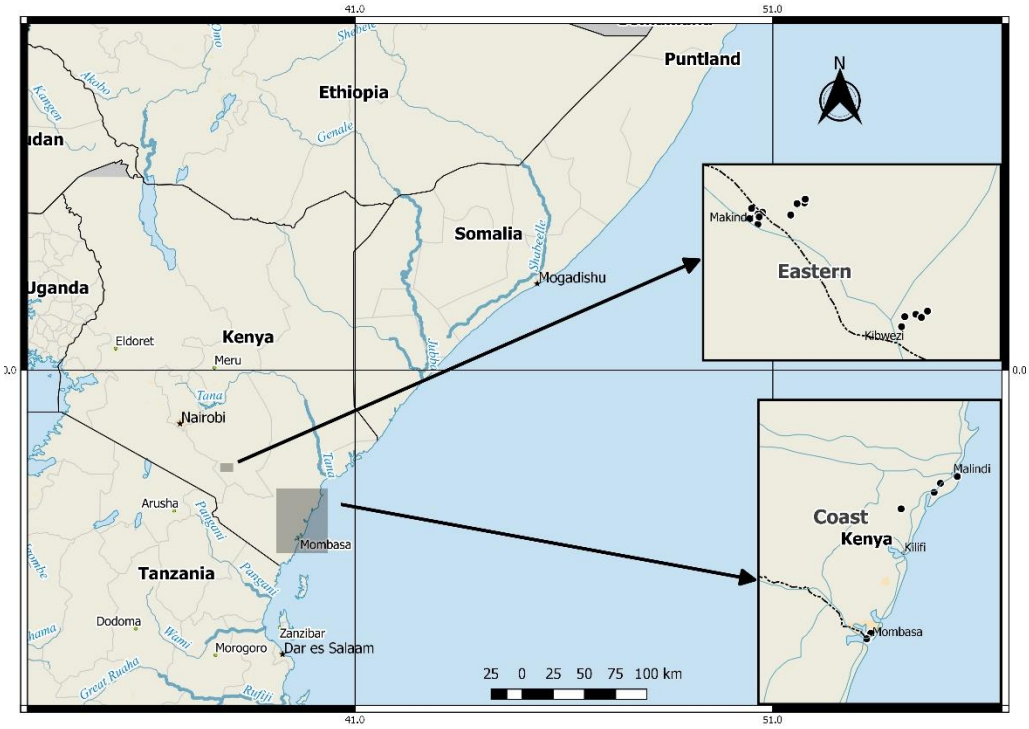

Figure 3.1: Map showing geographical sites of *M. koenigii* plant collection in Kenya. The map was created using Natural Earth Data in QGIS.

The Malindi and Mombasa regions are located in the coastal lowland semi-humid ecological regions of Kenya with an elevation of 100 – 200 m above sea level (asl),

with an average annual precipitation of 1000 – 1500 mm and mean temperatures of 25 – 27 °C. Makindu and Kibwezi, on the other hand, are located in semi-arid inland regions of Kenya at an elevation at 900 – 1000 m asl with 600 – 650 mm annual precipitation and 21 – 22 °C annual mean temperatures. The soil types across all the sites are similar and mainly consist of sandy clay loam (MoALF, 2016). The leaves were collected after the flowering period in February, placed in a separate, well-labelled gunny bag and transported to the drying site within three days. Taxonomic classification of leaf samples was done using morphological keys of Parmar (2008) and voucher specimens were subsequently deposited at the National Museums of Kenya.

3.1.2 Preparation of crude leaf extracts from curry tree (*M. koenigii*)

The leaf samples collected separately from at least three sites at each location (at Malindi, Mombasa, Makindu and Kibwezi) were separately dried under shade for 7-14 days at ambient environment temperature (27-37°C) and were subsequently powdered using commercial electrical stainless steel blender (Retsch Muhle, Haan, Germany). Each powder sample was exposed to absolute methanol (Sigma Aldrich, St. Lois, USA) at 45-50°C for eight hrs in a Soxhlet apparatus (Redfern *et al*., 2014). The extracts were then filtered using a Whatman No.1 filter paper (Whatman Inc., Haverhill, USA) for 30 min with a vacuum pump and then concentrated on a rotary evaporator (Laborota 4000 efficient, Heidolph, Germany) at 40–50°C as described by Kovendan *et al*., 2012. The residue obtained was then stored at 4°C and later solubilized in absolute ethanol until required for experimental work.

3.1.3 Rearing and maintenance of *An. gambiae*

Late third and early fourth instar (L_3/L_4) mosquito larvae of *An. gambiae* used in the experiment were obtained from mosquito colony maintained at the International Centre of Insect Physiology and Ecology (*icipe*), Nairobi, Kenya. The colony was established from *An. gambiae* s.s. mosquitoes originally collected from Mbita field station (00025'S, 34013'E), Homa Bay County in western Kenya in 2000. The mosquitoes were maintained in the standard operating procedure for rearing *Anopheles* mosquitoes where all life stages were reared at 28 ± 2 °C, 52–72 % Relative Humidity, 12 L: 12 D photoperiod. From the day of emergence, adult mosquitoes were provided with a 10% sugar solution soaked in cotton wool and at three days post-emergence, and female mosquitoes were subsequently allowed to feed on anesthetized mice. Approval for feeding mosquitoes on mice was obtained from the Kenya National Ethical Review Board (protocol number KEMRI/RES/7/3/1); the protocol was reviewed and approved by the KEMRI Animal Care and Use Committee (ACUC). Fully engorged females were allowed to lay eggs on funnel-shaped filter paper placed over oviposition cups (4cm diameter, 2cm depth) inside the cages. Oviposited eggs were dispensed into hatching trays (39cm long × 28cm wide × 14cm high) filled to a depth of 8 cm with dechlorinated water and larval densities maintained at approximately 100-150 larvae per tray. The larvae were fed on fish food Tetramin® three times daily (0.3g Tetramin/100 larvae/ day).

3.1.4 Comparison of effects of crude extracts effects on the mortality of *Anopheles gambiae* s.s larvae

The most potent *M. koenigii* leaf extract from all sampling sites was identified by larval (L_3/L_4) mortality bioassay. Briefly, five replicates were prepared from each of

the three sets of extracts. Each replicate was composed of 100ppm of the crude extract in distilled water. In each assay, 1% of ethanol in aqueous medium was used as the control. Larvae (n=20) were then placed in each replicate and control and maintained under the insectary conditions for 72hrs when the mortality of the larvae in each replicate was recorded. Larval mortalities in each set of replicates were compared with the control by one-way analysis of variance (ANOVA) at $p \leq 0.05$.

Mean larval mortalities of sets of extracts from different locations that were significantly different were identified using Tukey's HSD (Honestly Significant Difference) posthoc analysis. The results revealed that the extracts from the location with the most larvicidal potential, which was then selected for dose-response experiments to determine its median lethal concentration (LC_{50}). Briefly, different concentrations of the most potent extract (0, 25, 50, 100, 250 and 500 ppm) were prepared in distilled water with each of the three biological replicates. Larvae (n =20) were similarly placed in each replicate and control (1% of ethanol in aqueous medium), and maintained under the insectary and assessed for mortality 72hrs post-exposure (pe). Mortality data were corrected by Abbott's formula (Akçay, 2013) and then transformed to probits (Finney, 1971) for linear regression analysis. The LC_{50} values on the transformed data were determined using XLSTAT® 2017 (Addinsoft, Paris, France) and GraphPad Prism version 7.0 (GraphPad Software, San Diego, USA).

3.1.5 Bioassay guided fractionation of crude plant extracts on *An. gambiae* larvae

The most potent crude extract (location) was fractionated on a silica-packed column into its components based on their differences in the polarity of the components as described by Jandera and Churáček (1985). Briefly, the extract was placed in the column packed with Silica (200g, Kiesegel 60M [0.004-0.063 mm mesh size], Macherey-Nagel GmbH and Co. KG, Germany) in a 50 × 410 mm column and pre-conditioned with analytical grade n-hexane (Sigma Aldrich, USA) for three hrs. Components of the extract were then fractionated by gradient mobile phase of n-hexane and ethyl acetate (Sigma Aldrich, USA) of increasing polarity (100:0, 80:20, 60:40, 50:50, 40:60, 20:80 and 0:100 ratios). Fractions with similar retention factor (Rf) values (matching polarities) identified by thin-layer chromatography (Howard *et al*., 2009) were pooled into 17 major fractions. The fractions were rotor evaporated to remove the solvent and concentrate the blends of components (Kovendan, Murugan, *et al*., 2012). The acute toxicity of each concentration of the 17 fractions was then evaluated in five replicates (+ control) at 10ppm for 24hrs. The most potent fraction was subsequently separately selected for dose-response experiments to identify its median lethal concentration (LC_{50}), the effect on the regulation of larval growth and chemical components.

3.1.6 Survival assays and sub-lethal dose effect of the test compound on the development of mosquito larvae

The LC_{50} was determined as above where five concentrations of the most potent fraction (0, 1, 2, 4, 6 and 10 ppm) were prepared in distilled water in three replicates. The control group (0ppm) of larvae was treated under similar conditions for each assay

as test samples, except with 0.2% (*v/v*) ethanol diluted in distilled water. Larvae (n =20) were exposed to the treatments for 72hrs and their rates of mortality observed in 24hr intervals pe. The mortality data was corrected by Abbott's formula (Abbott, 1925), transformed to probits (Finney, 1971) for linear regression analyses and their LC_{50} values determined using XLSTAT® 2017 (Addinsoft, Paris, France) and GraphPad Prism version 7.0 (GraphPad Software, San Diego, USA).

To assess differential and temporal impacts of the alkaloid rich fraction on durations of mosquito immature stages development and survival, LC_{50} value obtained at 24 hrs in the above experiment, was used to establish incremental dose values for larvae survival time analysis. Batches of 20 L_3/L_4 mosquito larvae were placed in five replicates (n= 100) in 250ml glass beakers containing 100ml of *M. koenigii* bioactive fractions at incremental concentrations below the LC_{50} of 1 ppm, 2 ppm and 4 ppm each. The control group of larvae was treated under similar conditions for each assay as test samples, except with 0.2% (*v/v*) ethanol diluted in distilled water.

The number of affected larvae/pupa was recorded periodically every 12 h and removed from the beaker and the experiment was left to proceed until death (valid recorded event) of the last larva/pupa or upon successful emergence (censored). The proportion of surviving mosquito larvae exposed to concentrations of *M. koenigii* alkaloid rich fractions below the LC_{50} was computed relative to the control groups using the Kaplan-Meier method (Martin Bland and Altman, 1982) on SPSS statistical software (SPSS Corporation, Chicago, Illinois Statistical Package version 25.0).

Post-embryonic developmental phase time (between egg and successful emergence) was recorded and the mean larvae phase development time calculated for each of the

three replicates.

The larvae were provided with 10mg/l of Tetramin® fish food at two-day interval until the end of the observation period. Additionally, images of resulting morphological defects on mosquito developmental stages were recorded with a dissecting light microscope (Leica Corporation, Switzerland) at ×20 magnification.

3.1.7 LC-QtoF-MS Chemical analysis of bioactive fraction from *Murraya koenigii*

The chemical components of the most potent fraction were identified using Liquid chromatography quadruple time of flight coupled to a mass spectrometer (LC-QtoF-MS) as described by Cheseto *et al*. (2017). Briefly, 1.5 ml of the most potent fraction was dissolved in 1ml of 0.01% formic acid/methanol (95:5, v/v) LC−MS grade, vortexed for 30sec, and centrifuged at 14 000 rpm for 5 min, after which 0.2 µL of the supernatant was analysed by LC-QtoF-MS. Chromatographic separation of the components was achieved on a Waters Acquity UPLC (ultra-performance liquid chromatography) I-class system (Waters Corporation, Milford, MA, USA). The UPLC was fitted to a 250mm × 4.6mm i.d, 5µm particle size ACE C-18 column (Advance Chromatography Technologies, Aberdeen, Scotland) with the heater turned off and an auto-sampler thermostat at 5°C. The mobile phases used were deionized water with 0.1% of formic acid (A) and methanol with 0.1% of formic acid (B). A programmed gradient was used as follows: begin with 5% B, change from 5% to 100% B in 18 min and constant 100% B for 2 min more. A post-run of 5 min was programmed to equilibrate the column between analyses. The UPLC system was interfaced with electrospray ionization (ESI) to a Synapt G2-Si QtoF-MS (Waters) operated in full

scan MSE in positive mode. Data were acquired in resolution mode over the m/z range 100–700 with a scan time of 1 s using a capillary voltage of 0.5 kV, sampling cone voltage of 40 V, source temperature 100°C and desolvation temperature of 350°C. The nitrogen desolvation flow rate was 500 L/h. For the high-energy scan function, a collision energy ramp of 25–45 eV was applied in the T-wave collision cell using ultra-high purity argon (≥99.999%) as the collision gas. A continuous lock spray reference compound (leucine enkephalin; $[M+H]^+ = 556.2766$) was sampled at 10 s intervals for centroid data mass correction. The mass spectrometer was calibrated across the 50–1,200 Da mass range using a 0.5mM sodium formate solution prepared in 90:10 2-propanol/water (*v/v*). MassLynx version 4.1 SCN 712 (Waters Corporation, Maple Street, MA) was used for data acquisition and processing. The elemental composition was generated for every analyte. Potential assignments were calculated using mono-isotopic masses with a tolerance of 10 ppm deviation and both odd- and even-electron states possible. The number and types of expected atoms were set as follows: carbons ≤ 50; hydrogens ≤ 100; oxygens ≤ 50; nitrogens ≤ 10; chlorines ≤ 10; and sulfur ≤ 10. The empirical formulae, published literature and online database (ChemCalc, PubChem and Chemspider) were used to propose the structures (Pence and Williams, 2010; Patiny and Borel, 2013; Kim *et al*., 2016).

3.2 Molecular responses of *An. gambiae* mosquito larvae to *M. koenigii* curry tree leaves bioactive fraction

3.2.1 Preparation of *An. gambiae s.s* larvae biological samples for RNA isolation

The late third and early fourth instar (L_3/L_4) mosquito larvae of *An. gambiae* s.s. was obtained from a mosquito colony established and maintained at the International

Centre of Insect Physiology and Ecology (*icipe*), Nairobi, Kenya as described by Mang'era *et al*. (2019).

These larvae were exposed to curry tree leaf extract as described in the preceding section with relevant modifications for the current study. The extract consisted of a natural blend of alkaloid phytochemicals (neplanocin A, 3-(1-naphthyl)-L-alanine, lumiflavine, terezine C, agelaspongin and murrayazolinol) and induced morphogenetic abnormalities, reduced locomotion and delayed pupation (8-day delay) in the larvae (Mang'era *et al*., 2019). Briefly, this process was initiated by establishing the temporal range of toxicity of specific phytochemical extract concentration (2ppm) to late third-early fourth instar larvae (L_3/L_4) of the mosquito. The temporal range was critical to the determination of optimal duration of exposure of the larvae to putatively elicit molecular responses to our extract at the L_4 instar larvae just before pupation. For this purpose (establishing the temporal range of toxicity), the extract was first solubilized in absolute ethanol and diluted the resultant solution to 2ppm in a total volume of 100ml solution of distilled water (in a 250ml glass beaker). The overall ethanol concentration was 0.2% (*v/v*) absolute ethanol in the distilled water, based on the quantity of absolute ethanol initially used to solubilize the extract. A total of 20 L_3/L_4 instar larvae of the mosquito was placed into the solution, consequently exposing them to the extract and serving as the exposed treatment. Similarly, 20 L_3/L_4 instar larvae were placed in 100ml of distilled water with a similar concentration of absolute ethanol in a water beaker (in a 250ml glass beaker) that served as the control treatment. The absolute ethanol concentration equivalence between the treatments potentially assisted in contrasting the effect of the extract from that of the solubilizing agent in downstream analyses. Five replicates of each of the treatment and control was

prepared and daily mortality and pupation of these larvae monitored in both treatments under insectary conditions.

Peak pupation of the larvae was shown to be seven and 18 days post exposure to the control and exposed treatments respectively suggesting that 1) the extract appeared to delay growth in the larvae and consequently, the exposed and associated control larvae were potentially physiologically (L_3/L_4 instar larvae) similar seven- and 18-days post exposure (dpe) respectively. At this point 81 and 53% of the larvae had pupated or eclosed for control and exposed larvae populations respectively, indicating that the remaining respective proportions would potentially be appropriate L_4 instar larvae for further analyses in the definitive phase of this study. The significant extension of larval phase (delayed pupation) by the extract exposure presented unique challenges in selection of the sampling points for the RNA extraction and subsequent RNA-Seq experiments. Age-matching by sampling the larvae from either population seven days post exposure (peak pupation in control population) would constitute comparison between L_2 exposed and $L_{3/4}$ control larvae populations, hence the outcome/results would be confounded by differences in the physiological states between the populations. The alternative was to consider exposed and control larvae population seven- and 18-dpe respectively as physiologically equivalent (both at L_3/L_4 instar larvae developmental stage). The results from this approach would be confounded by the differences age differences between the larvae populations. Balancing up these options and their consequences, the latter option was selected since growth inhibition compounds typically have most profound effect on insects at L_3/L_4 instar larvae metamorphosis stage (physiologically matched) of their growth (Pener and Dhadialla, 2012). Consequently, the molecular results would reflect responses to inevitable

combination of age differences between the larvae populations and the xenobiotic (extract) challenge. The exposed will hereinafter refer to this larvae population (extract-exposed but not age-matched larvae population).

For assessment of molecular events associated with the exposure to the phytochemical extract, similar treatments (exposed and control) were repeated, but in 15 independent biological replicates of 25 larvae per treatment to enhance the odds of obtaining sufficient surviving larvae for the subsequent molecular studies, given potential 55% mortality and 59% pupation as revealed by preliminary data and the need to minimize crowding of the larvae in the beakers. Larval survivorship was monitored and recorded at 24 hrs intervals, indicating numbers of larvae alive, dead or moribund, and removing the dead larvae. Since two biological replicates each for the control or exposed treatments for our subsequent RNA-Seq molecular comparative analyses were of interest, five replicates were randomly and separately assigned from each treatment (exposed or control) into two groups of five treatments each. This collection was performed seven or 18 dpe for the control or exposed treatments respectively. From the 50 and 49 L_4 instar larvae survivors from control and exposed treatments respectively, surviving larvae in each group was pooled into two separate 1.5 ml reaction tubes, constituting two biological replicates of surviving larvae for subsequent RNA isolations and analyses. The replicates consisted of 25, 25, 25 and 24 larvae in each of the control replicates 1 and 2, and exposed replicates 1 and 2, respectively. The larvae were quickly centrifuged in the tubes at 15300 rcf for 1 min (Eppendorf AG 5417R centrifuge, Hamburg, Germany) to pellet the larvae and facilitate removal of the residual water. The larval pellet was then snap frozen in liquid nitrogen until RNA isolation.

3.2.2 Isolation and sequencing of *An. gambiae s.s* RNA

Total *An. gambiae s.s* RNA was isolated from the two biological independent replicates (from control or exposed larvae) by mechanically crushing the larvae using disposable RNAse-free plastic pestles in ISOLATE II RNA Mini Kit buffer (Bioline, Merdian Life Sciences, London, UK) following the manufacturer's protocol. The resultant total RNA was then treated with TURBO DNase™ (Ambion life technologies, TX, USA), following the manufacturer's instructions, to remove potentially contaminating DNA that could confound subsequent RNA-Seq analysis. The quality and integrity of the RNA samples was then verified using Agilent Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) according to the manufacturer's instructions. Services for cDNA library preparation from the total RNA and subsequent sequencing of the libraries was sourced from Macrogen, Korea (Geumcheon-gu, Seoul, Republic of Korea). Therein, complementary DNA libraries were prepared from 900ng (per replicate) of high-quality total RNA (RNA integrity number between 8 and 9.7) using Illumina TruSeq Stranded mRNA LT sample preparation kit (Illumina, Hayward, CA, USA) according to the manufacturer's instructions. The complementary DNA libraries were then sequenced (101bp paired-end read) on Illumina HiSeq 2500 sequencer (Illumina, Hayward, CA, USA) according to the manufacturer's instructions. Low-quality reads (<100 base pairs) and adapter sequences in the libraries were then removed using Illumina build software (Illumina, Hayward, CA, USA). Overall, four transcriptome sequences were generated from the samples (two each for control or exposed larvae). The four raw transcriptomes were then deposited at the Sequence Read Archive (SRA) of the

National Center for Biotechnology Information (NCBI), USA, under study accession number PRJNA560504.

3.2.3 Identification and validation of *M. koenigii* leaf extract responsive *An. gambiae* s.s RNA transcripts

The quality of each library was separately assessed using FastQC software (Andrews and Bittencourt, 2010) and the results used to filter and trim out low-quality sections of the reads using CLC genomic workbench version 9.0 software (CLC Bio, Aarhus, Denmark). The protein-coding gene set AgamP4.4 of *An. gambiae s.s* was then obtained from VectorBase (Giraldo-Calderón *et al*., 2014) and the filtered and trimmed reads mapped to the gene set (AgamP4.4) using RNA-Seq analysis procedures in CLC genomic workbench software version 9.0 (CLC Bio, Aarhus, Denmark) as described previously (Telleria *et al*., 2014). Briefly, the reads were mapped through settings that allowed two mismatches per read (with a maximum of 10 hits per read), with at least 80% of each read matching the gene at 95% identity. Reads per kilobase per million (RPKM) mapped reads was then used as a proxy for quantity (abundance) of transcripts (Mortazavi *et al*., 2008) for 1) assessment of baseline transcriptional processes that underpin larval developmental stage in the mosquito (in the absence of xenobiotics) and 2) comparison of relative expression of the genes between libraries from control and exposed larvae. The relative number of reads for each transcript in relation to the total read counts for each RNA-seq library was the used to calculate the *p*-value based on Baggerly's test method following Bonferroni analysis (Baggerly *et al*., 2003). Relative fold change (FC) of transcripts between the control and exposed mosquito larvae was determined as a ratio of the RPKM values and normalized based on the number of reads obtained from each

library using an inbuilt algorithm in CLC Genomics Workbench. The normalized values were used in this study. Trranscripts were considered differentially expressed (DE) between the libraries if they had a normalized value of at least 1) 1.5-fold change, 2) corrected $p < 0.05$ false discovery rate (FDR), 3) ten RPKM and 4) a support of ten unique read mappings. Fold-change was then defined as a ratio of RPKM values between those from exposed and control larvae libraries. These transcripts were thus considered and categorized as specific to control or exposed larval libraries.

Gene Ontology (GO) enrichment analyses of differentially expressed transcripts in exposed relative to control larvae libraries was conducted using gProfiler (Reimand *et al*., 2016) to establish pathways, networks and interactions associated with transcripts induced or suppressed by the exposure of larvae to the extract. For these analyses, the thresholds were set to a significance p=0.05 (to retrieve all GO terms under biological process, molecular function, and cellular component) and ordered query to identify specific functional terms associated with the most significant changes for the query (Seaman *et al*., 2015).

3.2.4 Validation of transcriptome expression profiles using RT-qPCR

DE larval transcripts were randomly selected (Appendix 2) from the RNA-seq library and compared with their relative fold changes to those obtained for the same transcripts through RT-qPCR. Briefly, five independent biological replicates (exposed and control) of the larvae were generated, and the respective total RNA libraries from our control and exposed larvae extracted and cleaned *ab initio.* The same methods and procedures used to prepare the biological samples for the RNA-seq component of this study was used for sample preparations and total RNA extraction. A volume of 1µg of

the total RNA was then reverse transcribed using the iScript™ cDNA synthesis kit (BIO-RAD, Hercules, USA) on the Arktik thermal cycler (Thermo Scientific, USA), according to the manufacturer's protocol.

Eight DE transcripts were randomly selected for validation of differential expression. These transcripts were significantly induced or suppressed in the exposed larvae library relative to the control library to encompass and validate differential expression in both directions (up and down regulated genes) of the libraries. Four transcripts were also selected that were neutral (neither induced nor suppressed in the exposed library relative to the control library) as potential internal reference neutral/loading controls (Salazar *et al*., 1993). These reference transcripts consisted of CLIP-domain Serine protease, glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) and two uncharacterized genes from VectorBase (Giraldo-Calderón *et al*., 2014). All these transcripts were abundantly expressed in the RNA-seq libraries (based on their RPKM values) to ensure that their expression levels would be within the sensitivity of the Stratagene MX3005P RT-qPCR machine (Agilent Technologies, CA, USA). DNA sequences of respective genes were obtained from VectorBase (Giraldo-Calderón *et al*., 2014) using the respective gene IDs, and primers designed from these sequences *in silico* using primer3 software (Rozen and Skaletsky, 2000). In all cases, the melting (T_m) and annealing temperatures of respective forward and reverse primers generated were similar (Appendix 2), as determined by pDRAW32 version 1.1.142 software (http://www.acaclone.com).

Reference transcripts were first interrogated for their stable expression by performing RT-qPCR in three technical replicates for each of the five biological replicates on our

Strategene MX3005P RT-qPCR machine (Agilent Technologies, California, USA) using Fast SYBR Green I Master mix (Applied Biosystems, Carlsbad, CA) according to the manufacturer's instructions. PCR was performed in reaction volumes of 10 µL for each replicate consisting of 1 µg cDNA template in three independent replicates with 5 µL of Fast SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA, USA) in the presence of 0.4 picomoles of specific primers for the respective candidate reference transcripts. The reactions were carried out in a RT-qPCR thermal cycler (Stratagene MX3005P, Agilent Technologies, CA, USA) according to the manufacturer's instructions. Thermo-cycling conditions included an initial step of 95°C for 10 mins, 40 cycles of 95°C for 30 sec, 55.0 – 63.2°C for 45 sec, 72°C for 1 min, followed by one cycle of 95°C for 1 min, 55 °C for 30 sec, and 95°C for 30 sec for all the genes. Stability (non-differential expression) of these reference transcripts was assessed using BestKeeper software (Pfaffl *et al*., 2004). From this assessment, *gapdh* and CLIP-domain serine protease transcripts were identified as less variable (with a standard deviation of crossing point (CP) of 0.56 and 0.65 respectively) among the reference transcript candidates. These two genes were thus adopted as internal housekeeping transcripts for assessment of expression of the eight randomly selected transcripts. The RT-qPCR for each of these transcripts was then separately performed under similar reaction and thermo-cycling conditions as had been previously employed in the assessment for stable expression of the reference transcripts above, but with *gapdh* and CLIP-domain serine protease as internal reference/loading controls.

3.3 Chemotypic variability of *M. koenigii* volatiles and fumigant toxicity on *An. gambiae* adult mosquito

3.3.1 *M. koenigii* leaves collection and processing

Murraya koenigii leaves were sampled from four populations associated with two bioclimatic zones. Makindu and Kibwezi are located South East inland of Kenya characterized by a semi-arid climate at an elevation at 900 – 1000 m asl with 600 – 650 mm annual precipitation and 21 – 22°C annual mean temperatures. Mombasa and Malindi are localized along the Coastal lowland strip of Kenya characterized by semi-humid climate with an average annual precipitation of 1000 – 1500 mm and mean temperatures of 25 – 27°C. The soil types across all the sites are similar and mainly consist of sandy clay loam (MoALF, 2016). The leaves were collected from at least ten healthy, disease-free plant hosts from each of the four locations after the flowering period in February, placed in a separate, well-labelled gunny bag and transported to the drying site within three days. The distance between individual plants exceeded 20 m within sites, to avoid collection from close parents. Taxonomic classification of leaf samples was done using morphological keys of Parmar (2008) and voucher specimens were subsequently deposited at the National Museums of Kenya.

This was augmented by field inspection of the plants for evidence of emission of aromatic volatiles and the absence of insects or insect attack. In the field, *M. koenigii* was found to have a strong aromatic odor when leaves were disturbed and there was no evidence of plant-feeding insects in the proximity.

3.3.2 Preparation of oil leaf extracts from curry tree (*M. koenigii*)

Leaf samples from each of the four populations were separately dried under shade for 7-14 days at ambient environment temperature (27-37°C) and were subsequently

powdered using commercial electrical stainless steel blender (Retsch Muhle, Haan, Germany).

Volatiles were obtained from the leaves by hydrodistillation as described by Clevenger (1928). Briefly, water was added to each of the powdered leaves in a round-bottomed flask and connected to a modified Clevenger apparatus which was heated for 8 hrs and oil collected in a conical flask over a condensation tube.

The collected oil was mixed with anhydrous sodium sulfate in the conical flask and further dried with a rotary evaporator to remove traces of water. The water-free oils were then placed in labeled, dark, and airtight 2mL vials and stored at −20°C for subsequent experiments.

3.3.3 GC-MS instrument conditions for *M. koenigii* oil analysis

Essential oils from the four populations were analyzed by GC/MS on a 7890A gas chromatograph (Agilent Technologies, Inc., Santa Clara, CA, USA), fitted with a HP-5MS low bleed capillary column (30 m × 0.25 mm, i.d. 0.25 μm) (J&W, Folsom, CA, USA) and linked to a 5975 C mass selective detector (Agilent Technologies, Inc., Santa Clara, CA, USA). Inlet temperature was set at 270°C, transfer line temperature oat 280°C, and column oven temperature initially maintained at 35°C for 5 min then programmed from 10°C to 280°C min^{-1}, and held at this temperature for 20.4 min. The carrier gas was helium at a flow rate of 1.25 $mL \cdot min^{-1}$. The mass selective detector was maintained at ion source temperature of 230°C and a quadrupole temperature of 180°C. Electron impact (EI) mass spectra were obtained at the acceleration energy of 70 eV. A 1.0 μl aliquot of sample was injected in the splitless mode using an autosampler 7683 (Agilent Technologies, Inc., Beijing, China). Fragment ions were

analyzed over 40–550 m/z mass range in the full scan mode. The constituents of the essential oils were identified by comparing their gas chromatographic retention indices and fragmentation patterns with those of authentic standards (Wei *et al*., 2014) as well as reference spectra published by the Library–MS databases, National Institute of Standards and Technology (NIST 05, NIST 08 and NIST 11, Adams and Chemical).

The percentage area under peak was used to tabulate the identified chemical compounds which were further grouped into their respective classes of terpenes. Non-parametric (non-Normal distributed variables) statistical test, Kruskal-Wallis test was applied to test for variability between study sites influenced by the availability of these terpenes in the essential oils. To assess possible chemical compound relationships between volatile chemical compounds from different populations, agglomerative hierarchical cluster analysis was done on major (≥2%) essential oil components in all populations of *M. koenigii* using Excel XLSTAT® 2018.6 (Addinsoft, Brooklyn, NY, USA). Pearson correlation was selected as a measure of similarity, and the unweighted pair-group method with arithmetic average (UPGMA) was used for cluster definition.

Principal component analysis (PCA) (unsupervised machine learning) was performed to partition the observations (chemical compounds) based on the four variables (study sites) for each individual observation. PCA was also used to project the variables in our study into the PC1 and PC2 axes to determine the relationship between the chemical compounds and study sites. This established the relative influence of individual compounds to fumigant bioactivity for each study site.

3.3.4 Mosquito rearing for bioassay

Kisumu strain (R70) mosquitoes were used in this experiment. This colony had never been previously exposed to any insecticide or growth regulating xenobiotics, including plant extract and were thus considered susceptible to known insecticides and other xenobiotics. The mosquitoes were maintained in the standard operating procedure for rearing *Anopheles* mosquitoes where all life stages were reared at 28 ± 2 °C, 52–72 % Relative Humidity, 12 L: 12 D photoperiod (Costa-da-Silva *et al*., 2013). The adults were freely provided with a 6% sucrose solution in a glass tube (2 × 8cm) connected to a filter paper (Whatmann®) tube as a wick. Female mosquitoes (3-4 days old) were blood-fed after being starved for 12 h, after which they were allowed to feed regularly on an anesthetized and restrained mouse for approximately 10-mins. Approval for feeding mosquitoes on mice was obtained from the Kenya National Ethical Review Board (protocol number KEMRI/RES/7/3/1) (Appendix 9). Fully engorged females were allowed to lay eggs on funnel-shaped filter paper placed over oviposition cups inside the cages. Eggs were collected and dispensed into plastic trays (25 cm long × 20 cm wide × 14 cm high) filled to a depth of 8 cm with distilled water. Upon hatching, larvae were reared in these trays at densities of 100-150/tray and fed fish food (Tetramin®) three times daily (the total amount of food provided was 0.3 g Tetramin®/100 larvae/ day). Pupae were collected from rearing trays and transferred to standard 30 × 30 × 30 cm mesh-covered cages with access to water and 6% glucose solution *ad libitum*. Newly emerged adult females intended for use in bioassays were fed on 6% glucose solution only (no blood meal) until they were 2-3 days old.

3.3.5 Fumigant toxicity assays

Fumigant toxicity assays were carried out as described by Omolo *et al*. (2005). Briefly, preliminary screening of essential oils to establish the mean duration of exposure of mosquitoes to *M. koenigii* essential oils from each of the four study sites was separately conducted on three batches of 30 female *An. gambiae* (5 days old) adult mosquitoes. Single doses of 1ml of 0.1g/ml in acetone of essential oil from each of the four sites was applied on Whatman® No. 1 filter papers (9cm diameter) in separate Petri dishes and introduced to the batches of mosquitoes in separate small transparent Perspex cages, each measuring 20×20×35.5cm. The Petri dishes were covered with wire gauzes to prevent the mosquitoes from making direct contact with the filter papers. Rolled filter papers dipping into glucose solution (6%) served as sources of food for the insects for the duration of the experiment. The cages were then monitored for 6 h to count dead insects that were immobile and not reacting to three probings with a blunt dissecting probe. The assay was replicated 6 times for essential oils from each of the four locations. Two control cages were similarly set for each replicate, with the solvent (acetone) replacing the test solution in one and Permethrin (Sigma-Aldrich, St. Louis, MO) in another. The time taken to achieve 50 (T_{i50}) and 100% (T_{i100}) mortality was recorded from each of the six replicates.

The essential oils were further assessed for their potency (LC_{50}) by assaying adult mosquito mortality over a range of doses (1 ml of 0.02, 0.04, 0.06, 0.08 and 0.1g/ml) over 6 hours as described above. Percent fumigant toxicity (mortality) of the oils (FT), was calculated according to the formula $FT = (N_T\text{-}N_C)/N_I \times 100$; where N_T and N_C represent the number of dead mosquitoes in the test and control cages, respectively, and N_I represents the initial number of mosquitoes introduced into each cage. Dose-

response data were subjected to probit analysis and LC_{50} values were obtained (Müller *et al*., 2010) from the derived regression equations (Finney, 1971). LC_{50} values (95% CI) were converted to LD_{50} by applying the formula $LD_{50}=LC_{50}/V$, where V (14,200 cm^3) is the volume of the experimental cage (Kiran and Devi, 2007).

CHAPTER FOUR

RESULTS

4.1 Growth disrupting activity of *M. koenigii* leaves against *An. gambiae* mosquito larvae

4.1.1 Larvicidal effects of crude extracts from Kibwezi, Makindu, Malindi, and Mombasa

Mean percentage mortalities of *An. gambiae* L_3 larvae exposed to 100 ppm crude extracts of *M. koenigii* leaves sampled from the four sites are summarized in Table 4.1. Crude extracts at 100 ppm from Kibwezi showed a unique trend where mean larval mortality was initially delayed (18.3%) but increased to 80.3% by 72 hours. Leaf extracts from Mombasa and Malindi had lower mortality than Kibwezi and Makindu throughout the experiment (Table 4.1).

Table 4.1. Mortality (mean % ±SE) of L_3/L_4 instars of *Anopheles gambiae* over 72 hours upon exposure to acutely toxic levels of *M. koenigii* extract.

	Concentration 100 ppm		
Time	24hrs	48hrs	72hrs
Kibwezi	18.3 ± 1.74 a	43.0 ± 2.75 a	80.3 ± 1.65 a
Makindu	26.7 ± 1.80 a	40.3 ± 1.90 a	55.7 ± 2.58 b
Malindi	15.7 ± 2.50 a	27.7 ± 3.90 ab	28.7 ± 3.43 c
Mombasa	13.0 ± 2.43 ab	18.3 ± 3.73 bc	26.7 ± 4.19 c
Control	0.0 ± 0.00 b	0.3 ± 0.00 c	1.3 ± 0.59 d

Means with the same letters within a column are not significantly different at 5% level.

Over 72hr pe (post-exposure), the two extracts from the semi-arid zone (Kibwezi and Makindu) showed higher larvicidal effects compared to those from the coastal area (Malindi and Mombasa), with that from Kibwezi being the most potent. Bioassay-

guided fractionation of this extract (into 17 fractions) identified the most potent fraction with LC_{50} of 2.43 ppm 72hrs pe (Table 4.2).

Table 4.2: Median toxicity (LC_{50}) responses of L_3/L_4 *An. gambiae* to crude and fractionated *M. koenigii* Kibwezi extract over 72 hours. LC_{50} were determined for each dose at their 95% confidence intervals.

Extract type	Time	LC_{50} (ppm)	95% CI	Slope (± SE)	χ^2
Crude	24 hrs	159.80	96.90 - 263.67	1.84 ± 0.11	0.92
	48 hrs	76.40	49.60 - 116.84	2.29 ± 0.09	0.70
	72 hrs	66.56	46.96 - 94.34	3.14 ± 0.78	0.98
Fraction	24 hrs	4.43	3.30 - 6.00	3.35 ± 0.06	0.71
	48 hrs	3.62	2.68 - 4.87	3.31 ± 0.07	0.72
	72 hrs	2.43	1.86 - 3.17	4.37 ± 0.06	0.97

All χ^2 values presented are significantly different at 0.05 levels of p and represent the goodness of fit of the regression lines in the probit analyses.

Longer-term exposure of larvae to 2ppm of this fraction induced several morphogenetic effects. It was also observed that exposure of larvae to the bioactive fraction induced morphological defects and caused protracted development of the mosquito immature stages.

Test larvae exhibited various developmental disturbances such as darkened gastric caeca (GC) and anterior midgut (AMG) (Figure 4.1a) impaired feeding, and moulting abnormalities linked to protracted development.

Mosquito larvae delayed moulting into normal pupae resulting in abnormal larval-pupal intermediates (Figure 4.1b) with deformed respiratory trumpet (RT) and bristles (BR), which proceeded to die. Mosquitoes that eclosed from the beakers with the test compound abortively flew from the surface of the water and eventually died with

deformed tarsi and wings stuck in the pupal caste (Figure 4.1c) causing impaired flight ability as compared to the control that instinctively flew form the water surface after successful eclosion.

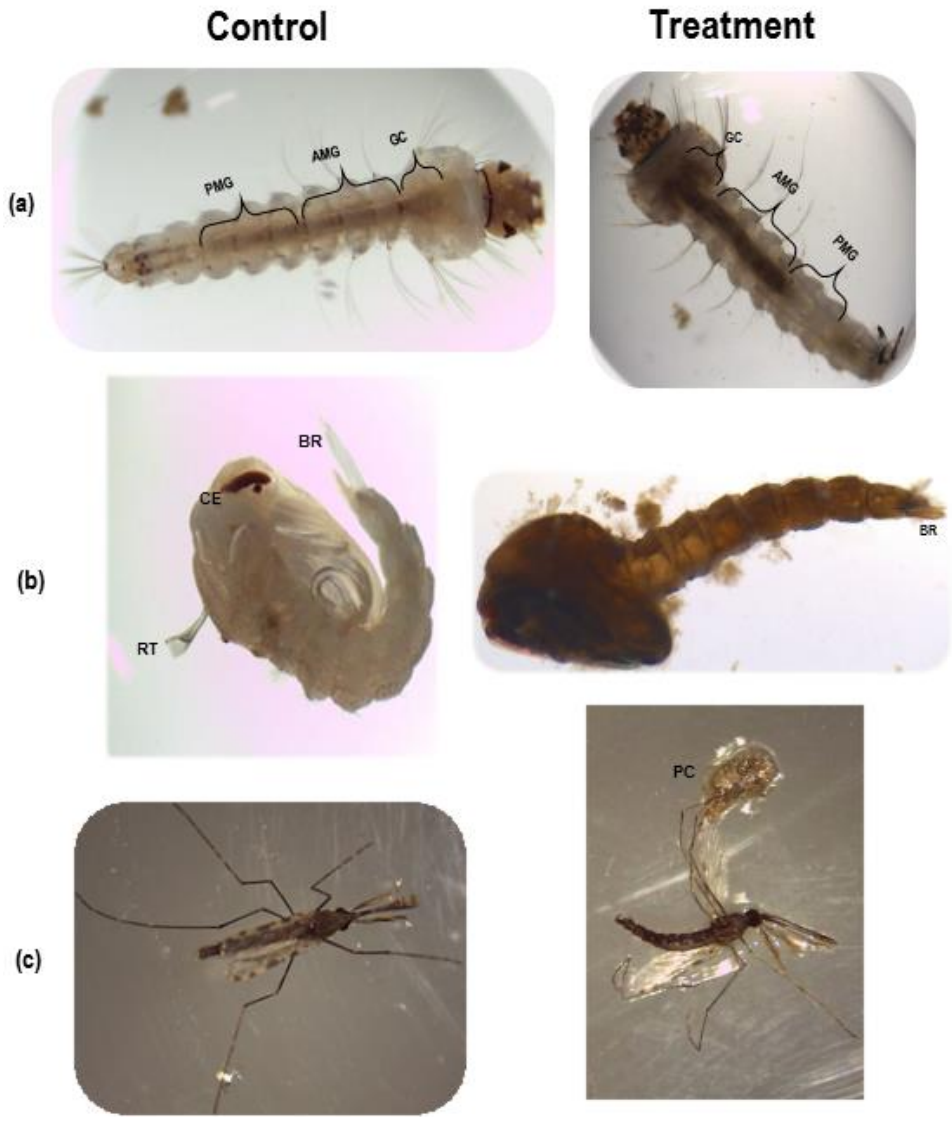

Figure 4.1: Morphologic disturbances of *An. gambiae* developmental stages resulting from exposure to the bioactive fraction of *M. koenigii* leaf extract observed at ×20 magnification. a) L_3/L_4 larvae showing PMG – Posterior Midgut, AMG – Anterior

Midgut, GC – Gastric caeca; b) BR – Bristles, RT - Respiratory trumpet, CE – Compound Eyes; c) PC – Pupal caste

4.1.2 Mosquito larval survival rates on exposure to sub-lethal doses of the bioactive fraction of *M. koenigii*

After exposing the L_3/L_4 instars to doses below LC_{50} (1, 2 and 4 ppm), the proportion of survival in larvae exposed to different concentrations of the bioactive fractions was determined and presented in Figure 4.2. The number of dead larvae was recorded periodically every 12 h and the experiment was left to proceed until death (valid event) of the last larva or upon successful emergence (censored).

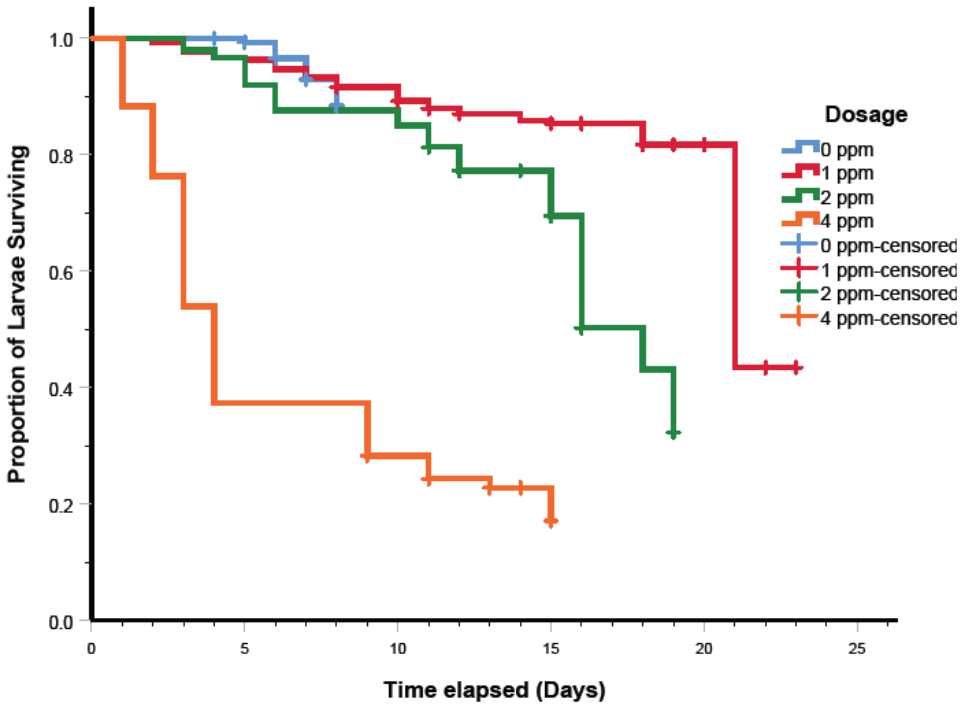

Figure 4.2: Kaplan-Meyer plot showing dose-dependent survival rates of mosquito larvae exposed to sub-lethal doses of the bioactive fraction of *M. koenigii* leaf extract

On exposure to sub-lethal doses of the bioactive fraction of *M. koenigii* leaf extract, survival rates of L_3/L_4 instars relative to controls revealed dose-dependent survival

rates with high survival rates exhibited in 1 ppm and lowest observed in 4ppm larvae. Mosquito larvae survived for 23 and 15 days post-exposure to 1ppm and 4 ppm respectively (Figure 4.2).

Pairwise comparisons (Mantel-Cox) between the mean survival times revealed significant differences among all treatments (Log Rank test, $p < .05$) with the exception of survival times between larvae in the control and 1 ppm setup whose means were not significantly different. A repeated measures ANOVA with a Greenhouse-Geisser correction determined that treatment of larvae with 1 ppm of the bioactive fraction significantly extended (at $p > 0.05$) post-embryonic mean larval development time (days) ($M = 31$, $SD = .6$) relative to the control larvae ($M = 16$, $SD = .6$), F (4.888, .452) = 119.333, $p = .001$, with a dose dependent reduction to $M = 22$, $SD = 1.0$ (Table 4.3).

Table 4.3: Mean developmental effects in *An. gambiae* larvae exposed to sub-lethal dose (2 ppm) of bioactive fraction from leaf extracts of *M. koenigii*

Dose (ppm)	Larvae		Pupae		
	Initial number†	Duration (days)^	Moulted**Δ**	Eclosed∞	Duration (days)◊
0§	100	16 ± 0.6a	97 ± 1.7a	95 ± 1.0a	2.2 ± 0.4a
1	100	31 ± 0.6b	86 ± 5.7b	80 ± 7.5b	4.3 ± 1.3b
2	100	27 ± 0.0c	71 ± 3.5c	63 ± 5.5c	6.8 ± 0.4c
4	100	22 ± 1.0d	32 ± 5.0d	23 ± 4.4d	9.0 ± 0.8d

Data are presented as the mean of three replicates (±SD) of 100 larvae each. Means with the same letters are not significantly different across columns at 5% level. § - Control; †- Three replicates per treatment at beginning of experiment; ^- Post-embryonic mosquito larvae developmental time (from egg hatching to first pupa transformation); **Δ**- Mean number of larvae that pupated post-exposure; ∞- Mean number of adult mosquitoes that successfully emerged from the experiment; ◊ -Time from pupa transformation to successful adult emergence.

Control larvae recorded shortest survival time (8 days) post-exposure (Figure 4.2) since 95% of control larvae proceeded to emerge successfully to adults as compared to less than 25% of the larvae exposed to 4ppm of the test compound (Table 4.3), indicating a significant reduction in adult emergence, F (1.7, 3.4) = 236, p = .0002.

Similar analysis (repeated measures ANOVA), determined that mean immature stage growth activity (pupation and eclosion) was significantly (p = .000) suppressed between time points (F (1.214, 13.521) = 499.090, p = .000).

Post hoc tests using the Bonferroni correction revealed a dose dependent decrease in number of larvae that pupated (M = 97, SD =1.7 to M = 32, SD = 5.0) and emerged (M = 95, SD = 1.0 to M = 23, SD = 4.4) when the dose was increased from 0ppm to 4ppm respectively (Table 4.3).

The findings further indicate significant four-fold increase in number of days taken for pupae to emerge following exposure to increasing concentrations of the bioactive fraction (Table 4.3).

4.1.3 Identification of chemical constituents of the most larvicidal fraction of Kibwezi extract

LC-QTOF-MS analysis of the fraction revealed nine prominent peaks (Fig 4.3). The major peak (retention time 4.73 min), gave a molecular ion peak $[M + H]^+$ at *m/z* 264.1021, with a molecular formula of $C_{11}H_{13}N_5O_3$ **(5)** (Table 4.4). ChemCalc, Chemspider, and PubChem (Pence and Williams, 2010; Patiny and Borel, 2013; Kim *et al*., 2016) online databases were used to reveal the identity of the peaks.

Table 4.4: Constituents of *M. koenigii* bioactive fraction.

No.	R_t (mins)	Peak Area (%)	*m/z* $[M+H]^+$	Chemical formula	Putative ID
1	0.73	0.87	216.1015	$C_{13}H_{13}NO_2$	3-(1-Naphthyl)-L-alanine
2	2.56	15.26	197.1171	$C_5H_{16}N_4O_4$	?
3	3.56	3.83	257.1048	$C_{13}H_{12}N_4O_2$	Lumiflavine
4	4.00	4.83	181.1230	$C_5H_{16}N_4O_3$	?
5	4.73	40.03	264.1021	$C_{11}H_{13}N_5O_3$	Neplanocin A
6	5.35	13.92	277.2169	$C_{14}H_{30}NO_4$	?
7	5.67	9.08	323.1522	$C_{16}H_{22}N_2O_5$	Terezine C
8	5.90	4.81	248.1079	$C_{11}H_{13}N_5O_2$	Agelaspongin
9	6.81	6.42	348.1968	$C_{23}H_{25}NO_2$	Murrayazolinol

R_t – Retention time; *m/z* - monoisotopic mass

The most prominent peak showed a fragmentation pattern associated with the alkaloid neplanocin A.

A similar comparative search identified the other constituents as 3-(1-naphthyl)-L-alanine (**1**), lumiflavine (**3**), terezine C (**7**) and agelaspongin (**8**), murrayazolinol (**9**) (Table 4.4, Figure 4.3).

Neplanocin A has been previously functionally associated with insecticidal and growth regulation in juvenile stages of mosquito (A. Sharma *et al*., 2015).

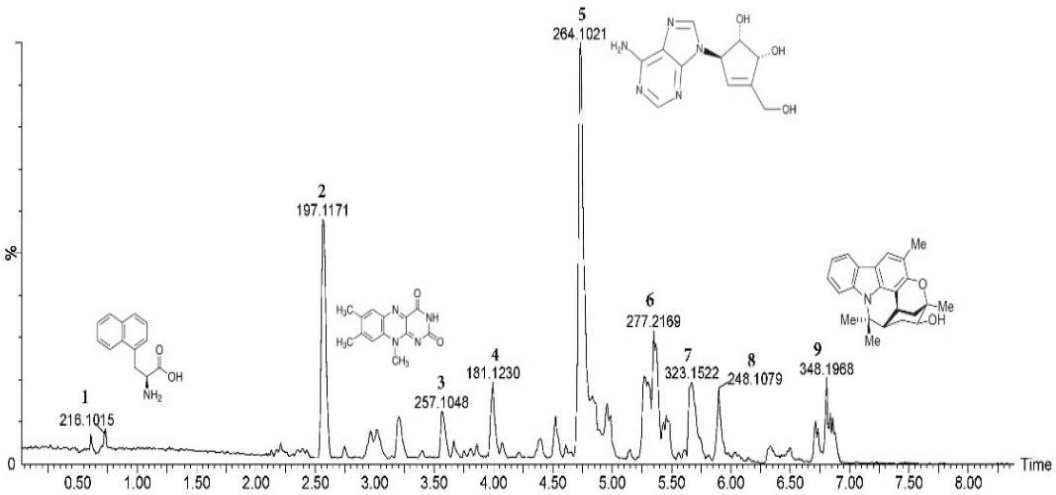

Figure 4.3: LC-QtoF-MS analysis profile of the bioactive fraction of *Murraya koenigii* leaf extract. The most prominent peak (**5**) is associated with neplanocin A

4.2 Molecular responses of *An. gambiae* mosquito larvae to *M. koenigii* curry tree leaves bioactive fraction

4.2.1 Survivorship of *An. gambiae* mosquito larvae exposed to the extract and non-exposed control

Initial pupation was observed at 4 and 11 dpe for control and exposed larvae respectively (Figure 4.4a). Most pupae (96%) from the extract-exposed larvae populations did not eclose.

At the point of sample collection, 53 and 81% of the larvae had pupated or eclosed for extract exposed and control populations respectively (Figure 4.4b). Therefore, at peak pupation for the exposed population (18 dpe) the larvae were potentially abnormal. The subsequent RNA-Seq libraries thus consisted of the normal control and the abnormal exposed larvae populations.

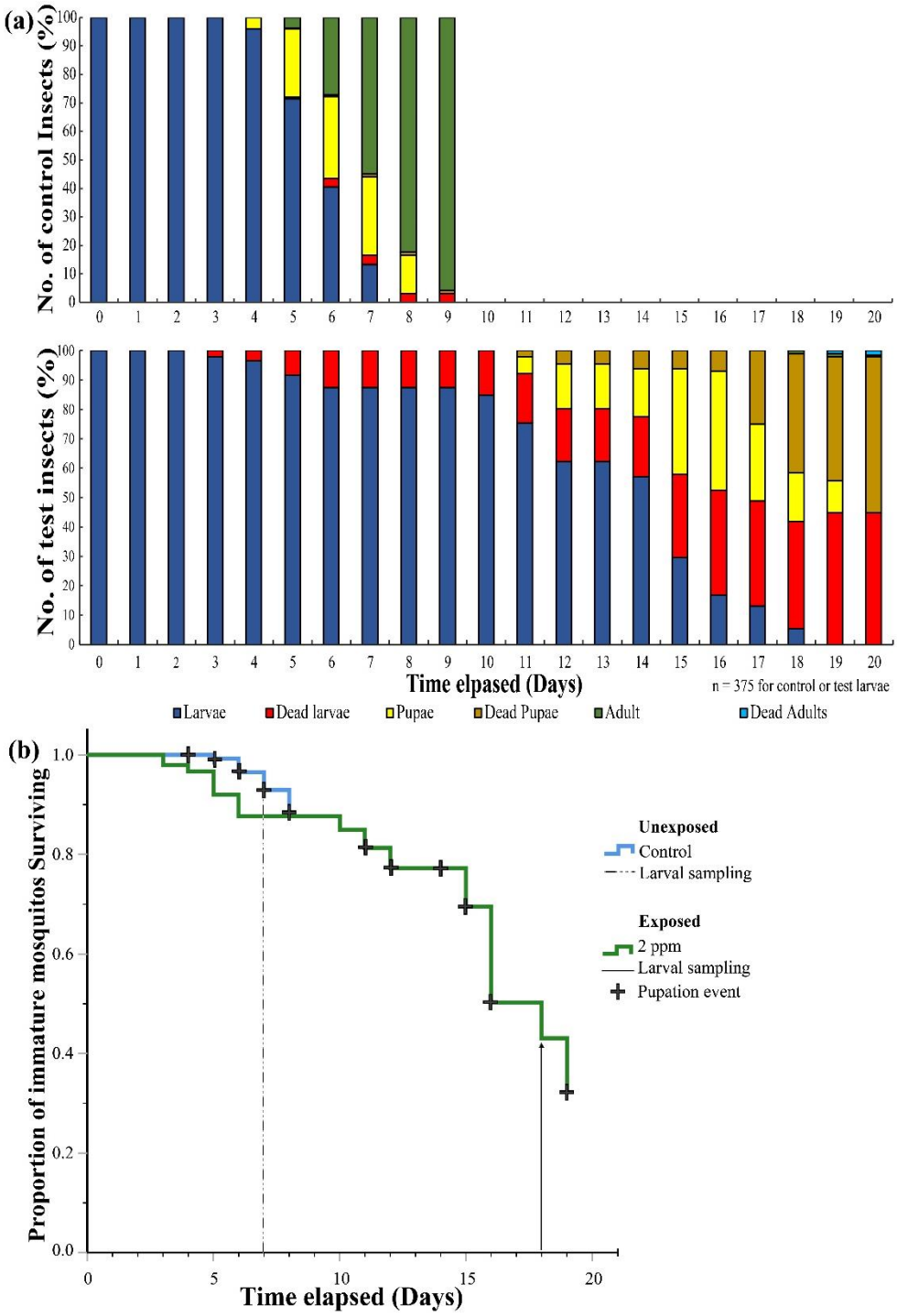

Figure 4.4: a) Bar graph showing daily pupation/mortality ratios for unexposed and exposed L_3/L_4 instar larvae throughout the experiment. b) Kaplan-Meier plot showing survival trends of unexposed and exposed (2 ppm) larvae.

4.2.2 Mapping statistics of larval RNA-seq reads on *An. gambiae s.s* gene set and validation with qRT-PCR

A Pearson correlation coefficient of 0.980 (Figure 4.5, Table 4.5) was achieved, indicating a 97.8% correlation in expression between RNA-seq and qRT-PCR results and effectively validating the transcriptomes.

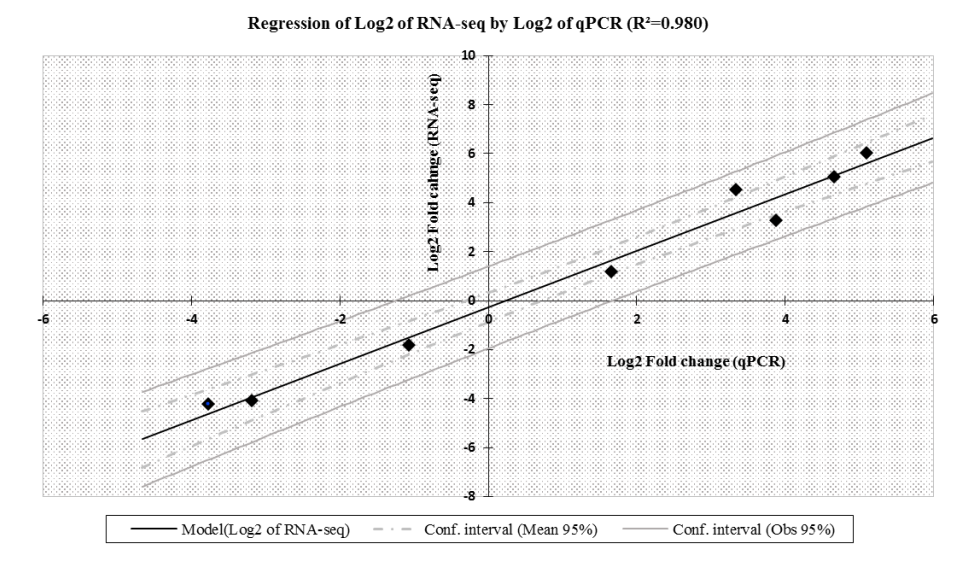

Figure 4.5: Validation of *An. gambiae* RNA-seq results with RT-qPCR using a pearson correlation between Log_2 of respective fold changes.

The expression values (log_2 ratios) for eight genes were plotted against RT-qPCR values (log_2 ratios) as shown in table 4.5 below.

Table 4.5: Gene transcripts selected for validation of mosquito larvae RNA-seq results with RT-qPCR showing their respective fold changes.

VectorBase Gene ID	Protein Name	Fold change in qPCR	Log2 of qPCR	Fold change in RNA-seq	Log2 of RNA-seq
AGAP006000-RA	CPR25: cuticular protein RR-1 family 25	-2.104290	-1.073333	-3.523449	-1.816989
AGAP010617-RA	Unknown	-13.705344	-3.776667	-18.440174	-4.204780
AGAP011277-RA	Unknown	-9.084035	-3.183333	-16.975791	-4.085407
AGAP002810-RA	45 kDa calcium	3.145596	1.653333	2.298492	1.200688
AGAP003471-RA	Osi20	34.138632	5.093333	65.807495	6.040180
AGAP005833-RA	COEJHE1E: caboxylesterase juvenile hormone esterase	14.723002	3.880000	9.817610	3.295372
AGAP008781-RA	Elongation of very long chain fatty acids protein 5	25.164767	4.653333	32.902245	5.040114
AGAP009017-RA	Cytochrome b-561 domain containing protein 2	10.056107	3.330000	23.312790	4.543050

Approximately 80 to 191 million reads were obtained from sequencing the *An. gambiae s.s* larval libraries (Figure 4.6).

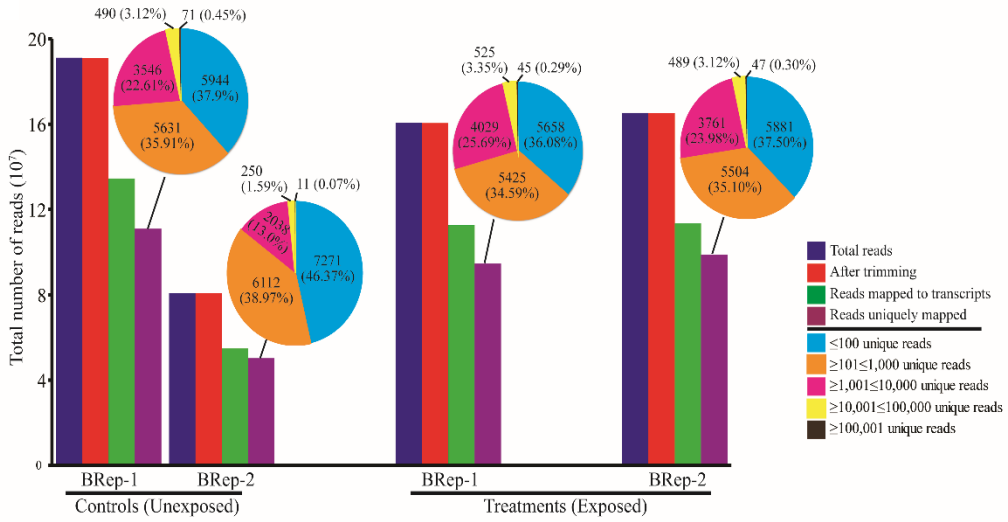

Figure 4.6: Overview of RNA-seq analysis of *Anopheles gambiae* larvae exposed to *Murraya koenigii* bioactive fraction showing mapping statistics of RNA-seq reads from *An. gambiae* larvae.

The variation in the number of reads obtained is due to the different depth we achieved in sequencing of each library. We successfully mapped 65 - 70% of these reads onto the protein-coding gene set AgamP4.4 of *An. gambiae s.s* from VectorBase (Giraldo-

Calderón *et al*., 2014), among which 58.1 – 62.3 % mapped uniquely to specific transcripts. Most of the transcripts had between 101 and 10,000 uniquely mapped reads (Figure 4.5).

The assessment of baseline transcriptional processes that underpin larval developmental stage in the mosquito (in the absence of xenobiotics) identified ribosomal, cuticular, hexamerins, cytochrome, elongation factor and muscle-related proteins as abundantly expressed transcripts (Appendix 5).

About 23.5 % (47) of 200 top-most abundantly expressed transcripts (Appendix 5) were differentially expressed between the libraries, among which expression of most (42) of the transcripts were suppressed in the exposed larvae libraries relative to those of control larvae (Figure 4.7).

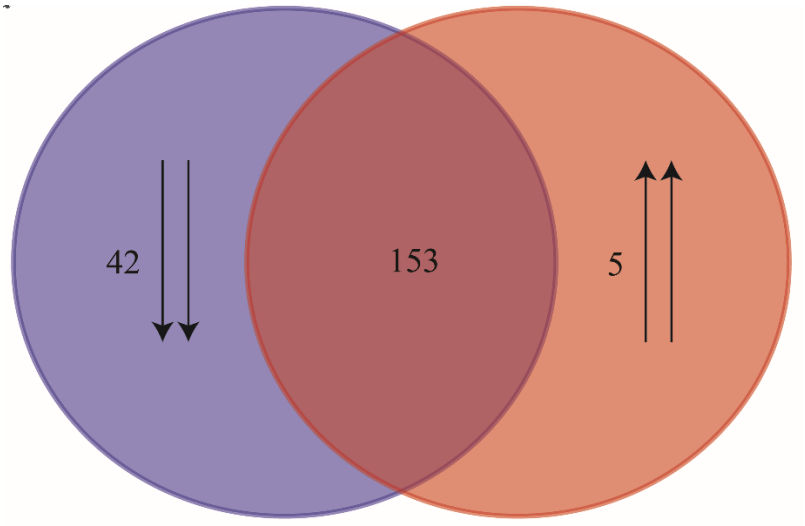

Figure 4.7: Spatial distribution of differential expression in the top 200 most abundant (RPKM) genes of the transcriptome.

4.2.3 Differentially expressed and enriched pathways between control and exposed *An. gambiae* larvae libraries

About 4.66% (730) of the transcripts were differentially expressed between the control and exposed libraries, most of which (65.75%) were induced (blue dots) by the extract in the exposed libraries (Figure 4.8).

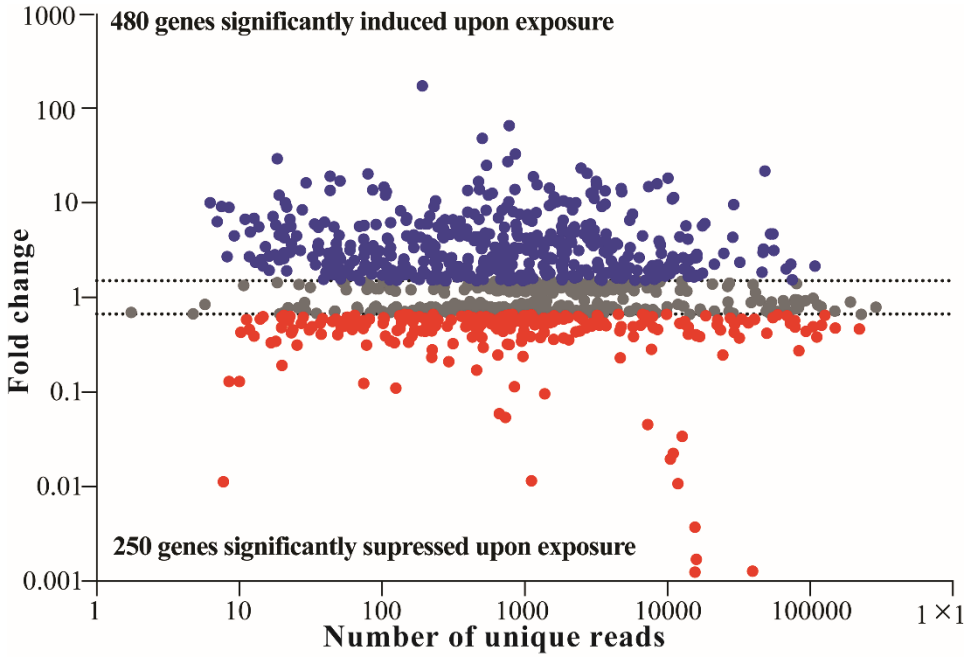

Figure 4.8: The MA plot showing differentially expressed genes between control and exposed *An. gambiae* larvae.

The differentially expressed transcripts were predominantly associated with cuticular proteins (CPs) (5.75%), cholesterol homeostasis (0.55%), osiris (0.82%), juvenile hormone metabolism (1.10%), transporters (0.82%), immunity (2.20%), redox balance and detoxification (2.88%) associated gene families among others (1.10%) (Figure 4.9).

Most (52.4%) of the CPs belonged to the CPR family, within which expression of the RR-2 subfamily was suppressed by the exposure to the extract (Figure 4.9a). Expressions of CPLC and CPF families were similarly suppressed and those of CPAP1 induced by the exposure to the extract (Figure 4.9a).

The exposure also suppressed expression of four niemann-pick type C-2 transcripts involved in cholesterol homeostasis (Figure 4.9b) while inducing expression of six osiris (Figure 4.9c), seven carboxylesterase juvenile hormone esterase (COEJHE) (Figure 4.9d), ATP-binding cassette (ABC) transporters (Figure 4.9e), ten immune-related (Figure 4.9f) and 11 detoxification associated transcripts (Figure 4.9g).

The extract exposure suppressed expression of eight C-type lysozymes (Figure 4.9f) and four glutathione S transferases (Figure 4.9g). Fatty acid elongation and chitin-binding networks were induced while putrescine and ornithine metabolism, ornithine decarboxylase activity, cell wall remodelling, cuticular structural constituents and hydrolytic activities were suppressed by the exposure as revealed by GO analyses (Table 4.6).

One term, the lysosome pathway, identified from the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database was shown to be induced. This pathway is involved in cellular detoxification (Table 4.6).

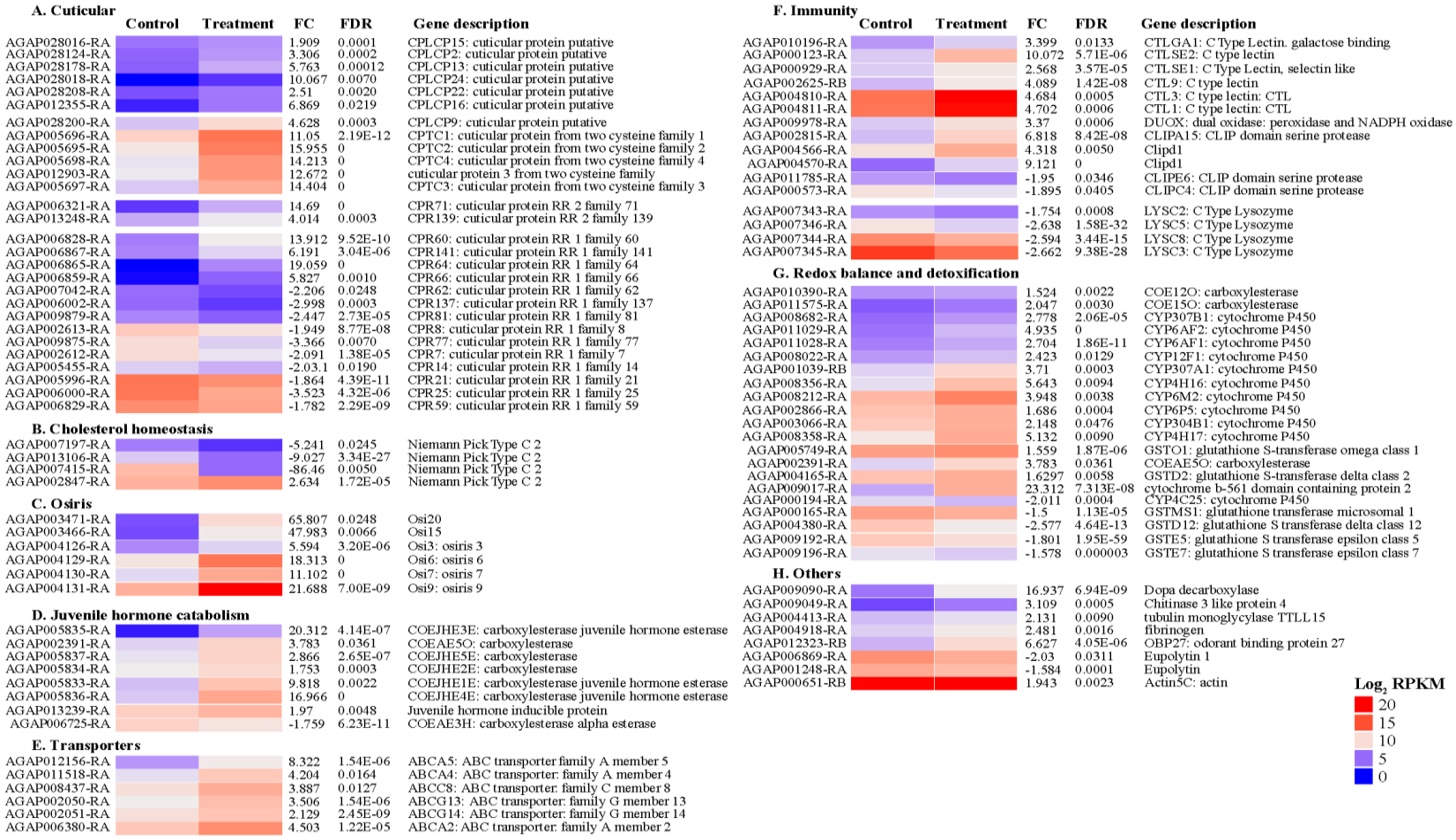

Figure 4.9: Heat map showing differentially highly expressed genes.

Table 4.6: Gene ontology analysis of induced genes in *An. gambiae*.

	GO-Category	Gene Ontology ID	Description of Pathway	General Function	Test[a] (Ref)[b]	p-value
Induced	Biological processes	GO:0006508	Proteolysis	Metamorphosis	58 (459)	0.0003
		GO:0006030	Chitin metabolic process	Insect exoskeleton	11 (210)	0.0025
		GO:1901071	Glucosamine-containing compound metabolic process	Insect exoskeleton	11 (210)	0.0034
		GO:0006040	Amino sugar metabolic process	Insect exoskeleton	11 (210)	0.0040
		GO:0019367	Fatty acid elongation, saturated fatty acid	Insect exoskeleton	2 (4)	0.0074
		GO:0019368	Fatty acid elongation, unsaturated fatty acid	Insect exoskeleton	2 (4)	0.0074
		GO:0034625	Fatty acid elongation, monounsaturated fatty acid	Insect exoskeleton	2 (4)	0.0074
		GO:0034626	Fatty acid elongation, polyunsaturated fatty acid	Insect exoskeleton	2 (4)	0.0074
		GO:0006022	Aminoglycan metabolic process	Insect exoskeleton	11 (210)	0.0090
		GO:0030497	Fatty acid elongation	Insect exoskeleton	2 (4)	0.0090
		GO:0000038	Very long-chain fatty acid metabolic process	Insect exoskeleton	2 (4)	0.0099
		GO:0042761	Very long-chain fatty acid biosynthetic process	Insect exoskeleton	2 (4)	0.0099
	Cellular component	GO:0005576	Extracellular region	Insect exoskeleton	41 (210)	0.0000
		GO:0044421	Extracellular region part	Insect exoskeleton	23 (185)	0.0000
		GO:0005615	Extracellular space	Insect exoskeleton	28 (363)	0.0000
	Molecular function	GO:0016787	Hydrolase activity	Insect exoskeleton	96 (457)	0.0000
		GO:0004175	Endopeptidase activity	Immune response	36 (404)	0.0000
		GO:0070011	Peptidase activity, acting on l-amino acid peptides	Immune response	42 (404)	0.0001
		GO:0008233	Peptidase activity	Immune response	42 (404)	0.0002
		GO:0008061	Chitin binding	Insect exoskeleton	12 (228)	0.0006
		GO:0003824	Catalytic activity	Immune response	163 (457)	0.0017
		GO:0008236	Serine-type peptidase activity	Immune response	21 (261)	0.0025
		GO:0017171	Serine hydrolase activity	Immune response	21 (261)	0.0025
		GO:0004252	Serine-type endopeptidase activity	Immune response	20 (261)	0.0030
		GO:0102338	3-oxo-lignoceronyl-coa synthase activity	Insect exoskeleton	2 (4)	0.0036
		GO:0102337	3-oxo-cerotoyl-coa synthase activity	Insect exoskeleton	2 (4)	0.0036
		GO:0102756	Very-long-chain 3-ketoacyl-coa synthase activity	Insect exoskeleton	2 (4)	0.0036
		GO:0102336	3-oxo-arachidoyl-coa synthase activity	Insect exoskeleton	2 (4)	0.0036
		GO:0009922	Fatty acid elongase activity	Insect exoskeleton	2 (4)	0.0036
		GO:0052689	Carboxylic ester hydrolase activity	Insect exoskeleton	10 (257)	0.0046
		GO:0004312	Fatty acid synthase activity	Insect exoskeleton	2 (4)	0.0056
	KEGG[c]	KEGG:04142	Lysosome	Detoxification	10 (435)	0.0027

[a]Test: Number of genes in input list with functional annotation, corresponding to the enrichment; [b]Ref: Number of genes annotated to functional term; [c]KEGG (Kyoto Encyclopedia of Genes and Genomes): A collection of databases associated with genomes, biological pathways, diseases, drugs, and chemical substance.

Table 4.6: Gene ontology analysis of supressed genes in *An. gambiae*.

	GO-Category	Gene Ontology ID	Description of Pathway	General Function	Test[a] (Ref)[b]	p-value
Suppressed	Biological Processes	GO:0016998	Cell wall macromolecule catabolic process	Detoxification	4 (132)	0.0005
		GO:0044036	Cell wall macromolecule metabolic process	Detoxification	4 (132)	0.0008
		GO:0071554	Cell wall organization or biogenesis	Detoxification	4 (132)	0.0019
		GO:0009445	Putrescine metabolic process	Cell division and growth	2 (13)	0.0041
		GO:0009446	Putrescine biosynthetic process	Cell division and growth	2 (13)	0.0041
		GO:0033387	Putrescine biosynthetic process from ornithine	Cell division and growth	2 (13)	0.0041
		GO:0006591	Ornithine metabolic process	Cell division and growth	2 (13)	0.0061
		GO:0050830	Defense response to gram-positive bacterium	Detoxification	3 (38)	0.0090
	Molecular function	GO:0042302	Structural constituent of cuticle	Insect exoskeleton	11 (124)	0.0001
		GO:0003796	Lysozyme activity	Immune response	4 (132)	0.0003
		GO:0004586	Ornithine decarboxylase activity	Cell division and growth	2 (13)	0.0018
		GO:0004553	Hydrolase activity, hydrolyzing o-glycosyl compounds	Molting	6 (80)	0.0031
		GO:0008236	Serine-type peptidase activity	Molting	13 (116)	0.0032
		GO:0017171	Serine hydrolase activity	Detoxification	13 (116)	0.0032
		GO:0070011	Peptidase activity, acting on l-amino acid peptides	Detoxification	16 (103)	0.0032
		GO:0061783	Peptidoglycan muralytic activity	Detoxification	3 (38)	0.0033
		GO:0008233	Peptidase activity	Detoxification	16 (103)	0.0039
		GO:0016798	Hydrolase activity, acting on glycosyl bonds	Detoxification	6 (80)	0.0074
		GO:0004252	Serine-type endopeptidase activity	Immune response	12 (116)	0.0075
		GO:0003824	Catalytic activity	Detoxification	82 (210)	0.0082
		GO:0016787	Hydrolase activity	Detoxification	24 (84)	0.0090

[a]Test: Number of genes in input list with functional annotation, corresponding to the enrichment; [b]Ref: Number of genes annotated to functional term;

4.3 Chemotypic variation and fumigant toxicity of volatiles from *M. koenigii* to adult *An. gambiae*

4.3.1 Chemical compositions of volatiles

Our investigation of oil samples from the four study sites using GC/MS showed 132 unique chemical compounds representing 98.30-99.98% of the total essential oils (Appendix 6). These chemical compounds were separated into five classes of terpenes, Oxygenated Diterpenes (OD), Oxygenated Monoterpenes (OM), Monoterpenes (M), Oxygenated Sesquiterpenes (OS), and Sesquiterpene Hydrocarbons (SH) based on their chemical structures (Figure 4.10).

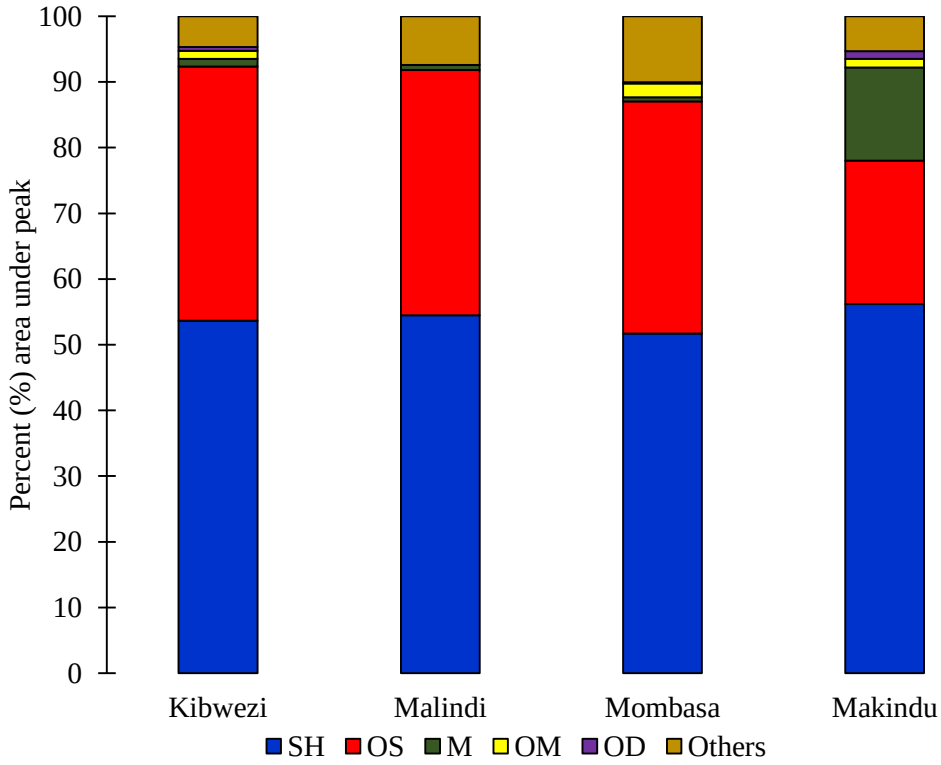

Figure 4.10: Chemical compounds in oils from the four study sites classified according to the abundance of terpenes based on their chemical structure.

The chemical composition of these essential oils revealed a high percentage of sesquiterpene hydrocarbons (51.68 – 56.18%) and oxygenated sesquiterpenes (21.82 – 38.69%) as shown in figure 4.10.

Major compounds that had an area under the peak of above 2% were extracted from the total list of compounds and displayed in table 4.7 below.

Table 4.7: Major compounds with ≥2% area under peak for oils from at least one of the geographical locations (Kibwezi, Malindi, Mombasa, or Makindu)

			Percentage area under peak‡			
Index	**Compound Name**	**Rt (min)***	**Kibwezi**	**Malindi**	**Mombasa**	**Makindu**
2	Myrcene	11.02	0.00	0.00	0.00	9.02
36	α-Cubebene	16.79	0.00	3.15	0.39	0.00
41	β-Elemene	17.38	2.09	0.73	1.19	2.13
43	(Z)-Caryophyllene	17.62	4.24	1.68	0.00	0.00
44	Caryophyllene(E-)	17.87	19.18	22.18	25.28	14.04
45	Acetonitrile, bromo-	17.94	2.34	0.00	0.00	0.00
46	Premnaspirodiene	18.05	3.00	0.00	0.00	0.00
47	γ-E-Bisabolene	18.07	0.00	3.34	0.00	0.00
48	Eudesma-6,11-diene	18.20	0.00	0.00	0.00	4.16
49	α-Humulene	18.25	8.27	8.38	7.82	5.85
51	γ-Muurolene	18.5	2.69	0.00	0.00	0.00
52	β-Selinene	18.68	8.16	7.36	6.34	16.09
53	α-Selinene	18.81	13.73	11.38	9.27	0.00
57	Amorpha-4,7(11)-diene	19.24	0.00	2.81	0.65	1.81
62	(E)-Nerolidol	19.46	1.90	1.69	1.69	3.50
63	β-Patchoulene	19.55	0.00	0.00	4.32	4.37
69	Globulol	19.86	3.23	0.81	4.38	5.69
70	Caryophyllene oxide	19.86	0.00	7.56	0.00	0.00
79	Selin-11-en-4-α-ol	20.29	0.00	0.00	0.00	10.41
80	Valerianol	20.38	2.15	0.00	2.57	0.00
83	Cadina-1(6),4-diene<cis->	20.47	1.93	0.00	2.00	0.81
84	Bicyclo[4.4.0]dec-1-ene, 2-isopropyl-5-methyl-9-methylene-	20.47	0.00	2.87	0.00	0.00
85	neo-Intermedeol	20.69	12.50	11.15	11.17	0.00
89	Spathulenol	21.39	1.50	0.33	3.62	0.00
102	Calamenene<cis->	22.68	0.16	0.00	0.23	3.29

* - Retention times in minutes. ǂ - Percentage areas under curve representing the abundance of a chemical compound.

A Kruskal-Wallis H test revealed a significant difference (χ^2 (4) = 13.70, p = 0.008) in the variability of five classes of terpenes in essential oils of leaves from Malindi relative to the other sites, where oxygenated sesquiterpenes had the most influence on variability (Mean rank 53.59) and oxygenated monoterpenes showed the least influence (Mean rank 28.83).

These findings suggest a homogenous composition of terpenoids across Kibwezi, Makindu and Mombasa. Sesquiterpenes present in all four sites were β-elemene **41**, (E)-caryophyllene **44**, α-humulene **49**, and β-selinene **52** (Table 4.11; Appendix 6).

A principal component analysis (PCA) was performed to unravel possible correlations between variables (locations) and observations (chemical components) and results shown in figure 4.11 below.

The first two principal components (PC) extracted from PCA of EO content in *M. koenigii* accounted for 61.98% of total variations, with PC1 accounting for 34.38% of this observed variation. PC1 showed a strong positive correlation with the Kibwezi and Mombasa, with Eigenvector coefficients of 0.57, 0.69 respectively.

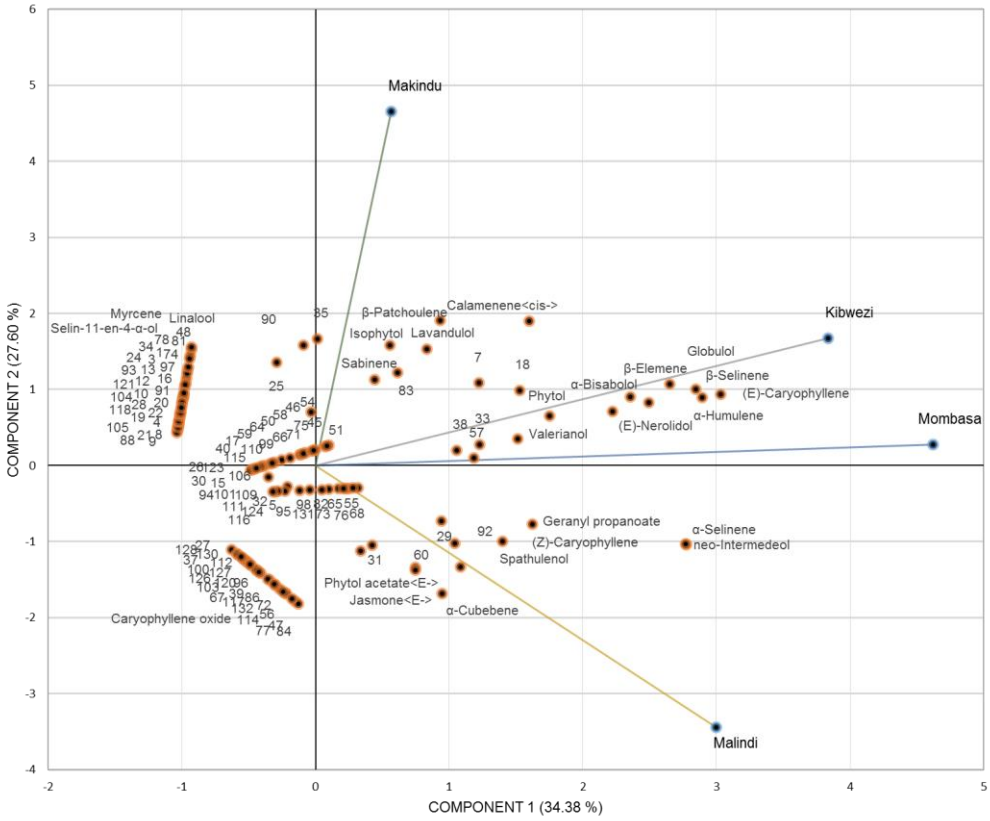

Figure 4.11: Principal component analysis of *M. koenigii* essential oil contents of leaves oil collected from four study sites. The bi-plot projects major (>2%) chemical component and geographical location based on PC1 and PC2 axes.

Projection of the study site variables on PC1 and PC2 axes confirmed that essential oil chemical compounds from Kibwezi and Mombasa are highly positively correlated, unlike the negative correlation between Makindu and Malindi (Figure 4.11).

A weak positive correlation was observed in EOs between the two (lowland semi-humid and semi-arid) climatic descriptions suggesting that additional factors could contribute to the unaccounted for variation (of about 40%).

Projection of the 132 accessions on PC1 and PC2 axes revealed a unique grouping pattern with major chemical compounds β-elemene **41**, (E)-caryophyllene **44,** α-humulene **49**, (E)-nerolidol **62**, globulol **69**, neo-intermedeol **85**, α-bisabolol **87** and phytol **119** having higher scores for PC1 strongly linked to Kibwezi and Mombasa. On the other hand, α-cubebene **36**, γ-E-bisabolene **47**, calamenene<trans-> **56**, caryophyllene oxide **70**, rosifoliol **77** and calamenene<cis-> **102** had higher PC2 scores.

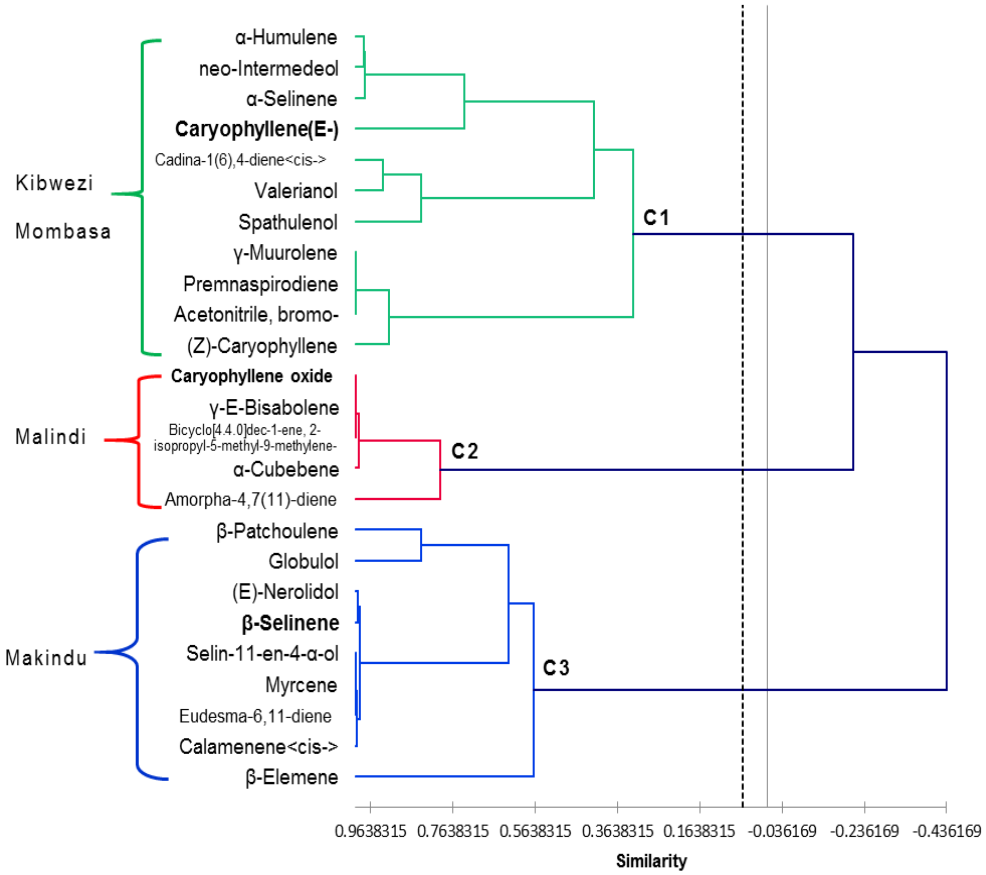

Figure 4.12: A dendrogram obtained by Hierarchical Cluster Analysis (HCA) based on the composition of the major essential oils (>2%) of the oils from Kibwezi, Mombasa, Malindi and Makindu.

Agglomerative hierarchical cluster on major (≥2%) essential oil components (Table 4.7) analysis revealed three clusters: 1) a caryophyllene (E-)-rich chemotype (C1) represented by Kibwezi and Mombasa; 2) a cluster (C2) dominated by caryophyllene oxide represented by Malindi; and 3) a β-selinene -rich chemotype (C3), represented by Makindu (Figure 4.12).

4.3.2 Fumigant toxicity of *M. koenigii* volatiles against *A. gambiae* mosquito adults

Analysis of the fumigant toxicity of essential oils of *M. koenigii* from the four locations was done against adult *An. gambiae* mosquitoes. A one-way ANOVA between the four sites and control (Permethrin) was conducted to compare the effect of the composition of essential oils of different chemotypes on fumigant toxicity to the mosquitoes.

A significant difference in EO bioactivity between the sites was observed with the exception of EOs from Kibwezi and Mombasa as determined by one-way ANOVA at 50% mortality [F (4, 25) = 247.591, p = .001], while bioactivities of EOs from Mombasa and Makindu were similar at 100% mortality [F (4, 25) = 208.065, p = .001] (Table 4.8).

Table 4.8: Mosquitocidal activity of essential oils of *M .koenigii* (from the four geographical locations) and an established pyrethroid as a control.

			LD_{50}			
	T_{i50} (h)±SE	T_{i100} (h)±SE	(mg cm^{-3})	95% CI	Slope (± SE)	χ^2
Kibwezi	1.91±0.086[a]	3.30±0.071[a]	3.6×10^{-3}	2.70 - 4.58	3.22±0.6	0.76
Mombasa	2.15±0.132[a]	4.76±0.270[b]	4.9×10^{-3}	3.59 - 5.85	2.93±0.7	0.95
Makindu	3.25±0.157[b]	5.12±0.189[b]	6.6×10^{-3}	4.86 - 7.17	2.78±0.6	0.88
Malindi	4.71±0.091[c]	7.29±0.207[c]	8.5×10^{-3}	7.46 - 9.86	3.02±0.2	0.95
Permethrin	0.13±0.013[d]	0.36±0.028[d]	0.1×10^{-3}	0.08 - 0.39	1.23±0.2	0.98

Means with the same letters in each column are not significant (p>0.05). h- Hours, SE – Standard error of mean

A Tukey post hoc test revealed a two-fold increase in mean duration to achieve 100% mortality in adult *An. gambiae* mosquitoes by EOs from Kibwezi (3.30 ± 0.071 hrs, p = .001) and Malindi (7.29 ± 0.207 hrs, p = .001). Permethrin was able to achieve 100% mortality in adult *An. gambiae* mosquitoes within 21 mins (0.36 hrs) (Table 4.8). There was no significant difference in Ti_{100} values between Mombasa and Makindu (p = .621) (fumigant toxicity means were similar in this column).

A similar trend was observed in the essential oil dose required to achieve 50% mortality (LD_{50}) in adult *An. gambiae* mosquitoes where the essential oil from Kibwezi was the most potent at the lowest dose (3.6×10^{-3} mg cm^{-3}). Essential oils from Kibwezi and Mombasa had similar LD_{50} bioactivities whereas Makindu and Malindi had unique higher LD_{50} values (Table 4.8).

CHAPTER FIVE

DISCUSSION

5.1. Growth disrupting activity of *M. koenigii* against *An. gambiae* larvae

In this study, larvicidal potential of methanolic extracts of *M. koenigii* growing in different ecological areas on *An. gambiae* larvae, as well as time-course effects of a sub-lethal dose of the most active fraction of the extract on the larvae and respective emergent pupae and adults, and potential bioactive components of the extract was explored.

There was a significant and consistently higher larvicidal potential in the extracts from Kibwezi and Makindu (semi-arid) than from those from Mombasa and Makindu (coastal zones), suggesting a significant influence of the ecology on *M. koenigii* toxicity to *An. gambiae* larvae. The differential potency of the extracts within the semi-arid zones points to micro-geographic ecological influences on the constituent profiles and toxicity of the extract. These findings suggest that further analysis of *M. koenigii* from more diverse agro-ecological zones of eastern Africa may provide more comprehensive insights on ecological factors associated with *M. koenigii* ecotypes with more potent phytochemical profiles on mosquito larvae.

Longer-term exposure of larvae to sub-lethal doses of the most potent fraction of the Kibwezi extract showed subtle time-course growth-disrupting effects with several morphogenetic consequences to the larvae, and negative effects on pupation and emergence of adults. Interestingly, this fraction was about 1.5 and 2 folds more larvicidal than those reported from green tea (*Camellia sinensis*) leaves (Muema *et al*., 2016) and Sickle Senna (*Cassia tora*) seeds (Mbatchou *et al*., 2017). The morphogenetic larvae-pupae developmental effects and decreased survival may be due

to dysregulation of juvenile hormone in the mosquito by the *M. koenigii* phytochemicals, as evidenced by the documented impact of phytochemicals in *An. gambiae* (Nyamoita *et al*., 2013; Lee *et al*., 2015) and other insects (Tusun *et al*., 2017), and/or midgut damage as observed in *Culex quinquefasciatus* larvae exposed to Garlic Vine extracts (Granados-Echegoyen *et al*., 2014; Procópio *et al*., 2015). The protracted larval and pupation duration and impaired flight in emerging adults can potentially expose the larval mosquitoes to undue predation, which together with the minimal (<2%) adult emergence would substantially reduce the population sizes of the mosquito in successive generations, and significant collapse in their populations (Athrey *et al*., 2012; O'Loughlin *et al*., 2016). In addition, this phenomenon of undue predation may minimize the risk of fueling selection toward resistance by naturally reducing the number of survivors in field conditions (Kweka *et al*., 2011).

From these findings, the alkaloid rich fraction exerted growth disrupting effects similar to those exerted by insect growth disruptors (IGDs) (Dhadialla, 2012), potentially disrupting metamorphosis in the mosquito. Curiously, the structure of chemical compounds in our bioactive fraction lack resemblance to any insect developmental hormone hence linked to other subtle molecular mechanisms leading to protracted larval development period and failed adult emergence. Semi-field assays targeting diverse larval stages derived from different co-existing broods of mosquito larvae can shed some light on the levels of survival of mosquitoes. Moreover, any possibility of risk of resistance can be evaluated by sampling mosquitoes from the site of application and monitoring incidences of any resistant strains (Swale *et al*., 2018).

LC-MS analysis of the potent fraction led to the identification of 3-(1-naphthyl)-L-alanine (**1**), lumiflavine (**3**), neplanocin A (**5**) terezine C (**7**), agelaspongin (**8**), and

murrayazolinol (**9**) as the major constituents. Compounds **1**, **3** and **5** have previously been linked to growth-disruptive effects in insects, such as darkening of gastric caeca at the anterior end of the midgut and reduced locomotion (Arrese and Soulages, 2010), delayed pupation (Haunerland, 1996), and high mortality in immature *An. gambiae* (Lu *et al*., 2013; Sharma *et al*., 2015). The phenotype observed from our growth disrupting experiments (Figures 4.1b, 4.1c) is consistent with chitin synthesis inhibition (Fontoura *et al*., 2012) similar to Novaluron, an insect growth regulator already in use against *Aedes* and *Anopheles* mosquito larvae (Su *et al*., 2003; Arredondo-Jiménez and Valdez-Delgado, 2006). The effects of inhibition of chitin synthesis could persist beyond the juvenile stages of the mosquito (Swale *et al*., 2018) suggesting possible reduced survivability and longevity of adult mosquitoes, and thus greatly reducing their vectorial capacity. Subtractive assays with blends of sub-lethal doses of the major constituents can shed some light on the contribution of each compound on the growth disruption of the larval stages of mosquitoes and on the most effective blend for potential downstream applications.

5.2 Molecular responses to growth disrupting compounds

In this study, high throughput RNA-seq expression analysis was used to determine transcriptional responses in third-fourth instar normal control and abnormal exposed larvae population exposed to sub-lethal concentrations of curry tree (*M. koenigii*) leaf extracts. The extract previously induced morphogenetic abnormalities, reduced locomotion and delayed pupation in the larvae and were predominantly composed of alkaloid phytochemicals (neplanocin A, 3-(1-naphthyl)-L-alanine, lumiflavine, terezine C, agelaspongin, and murrayazolinol compounds) (Mang'era *et al*., 2019). Control and exposed populations were age-matched due to the extract-induced delay

in pupation of the exposed relative to control larvae coupled to our need to compare L_3/L_4 populations between the treatments (exposed vs control). Thus, the molecular results would reflect responses to inevitable combination of age differences between the larvae populations and the xenobiotic (extract) challenge.

The study was initiated by establishing baseline transcriptional processes (molecular investments) that underpin larval developmental stage in the mosquito control (in the absence of xenobiotics). Evidence presented here suggests a significant investment in development and reorganization of musculature in the larvae as evidenced by preferential expression of muscle and cytoskeleton related transcripts (Vyazunova and Lan, 2004) in our study. There was a potentially enhanced investment in larval molting and pupation as shown by predominant expression of hexamerins in the larvae. The hexamerins are conserved hemolymph-proteins secreted by larval fat body (Burmester, 2015) and facilitate larvae-pupae transition (Korochkina *et al*., 1997). Hexamerins facilitate these processes through regulation of protein reserves (for amino acids and energy) (Telfer and Kunkel, 1991), transport of ecdysteroids (Enderle *et al*., 1983) and juvenile hormone (JH) (Braun and Wyatt, 1996). This study was, therefore, advanced with the aim of understanding how these and related processes were affected by the extract exposure treatment described above in relation to the associated phenotypes we observed in the larvae (Mang'era *et al*., 2019).

Analysis of processes affected by exposure to the extract revealed impairment of exoskeleton development, immunity, detoxification processes, and transport system by the exposure. Most affected processes were associated with the cuticle that typically protects insects against adverse environmental conditions and pathogens (Moussian, 2010). In that respect, the extract appeared to soften the cuticle through

reduced transcription of the RR-2 subfamily of the CPR cuticle family (Rebers and Riddiford, 1988; Willis, 2010) that encode hard cuticle proteins of the insect exoskeleton (Iconomidou *et al*., 2005) and CPLC genes implicated in increasing the thickness of the cuticle through forming rigid matrices (Huang *et al*., 2018). The reduction in hard cuticle synthesis could in turn enhance susceptibility of the larvae to insecticides (Simma *et al*., 2019), including pyrethroids (Yahouédo *et al*., 2017). The reduced synthesis is also a possible precursor to post-ecdysial molting deformities (Jan *et al*., 2017) previously observed in this mosquito (Mang'era *et al*., 2019). The suppression of the RR-1 subfamily, CPLCPs, CPFs and Niemann-Pick Type C-2 transcripts, and putrescine, ornithine and ornithine decarboxylase pathways could cause enhancement of the deformities due to the exposure. Suppression of RR-1 subfamily, CPLCPs and CPFs has been shown to potentially interrupt endocuticle development and impede ecdysis, consequently enhancing molting deformities in the mosquito (Jan *et al*., 2017; J. Zhang *et al*., 2014). Niemann-pick type C-2 controls sterol homeostasis and steroid biosynthesis precursors for ecdysteroids (X. Huang *et al*., 2007). The ecdysteroids in turn induce larval molting and metamorphosis (Niwa and Niwa, 2014) and promote pupal commitment at L_4 (Muramatsu *et al*., 2008). The putrescine, ornithine and ornithine decarboxylase pathways are critical for optimal tissue growth and development (Pegg, 2006). Suppression of their expression in our study potentially perturbs cellular processes essential for cytoskeletal structure in the larvae (Mandal *et al*., 2014). The interruption of endocuticle development and ecdysis was putatively enhanced by potentially untimely (18 dpe) induction of carboxylesterases juvenile hormone esterase (COEJHE) expression that could adversely affect pupation (Lan and Grier, 2004). The induction of COEJHE potentially

decreased JH hormone titers (Kamita *et al*., 2011) and initiated premature pupation process by changing tissue commitment from larval tissue synthesis to production of pupal tissues (Riddiford, 2012). This potentially induced the larvae-pupae-transition arrest in our previous observation (Mang'era *et al*., 2019). The extract appears to target cuticle metabolism in regulating the growth of the larvae.

The larvae putatively counteracted the exposure treatment through induction of osiris, ATP-binding cassette (ABC) transporters, cytochrome P450s (CYPs) and carboxylesterases transcripts and pathways associated with fatty acid (FA) elongation and chitin-binding. These transcripts and pathways potentially facilitated interim survival of the larvae in the xenobiotic (extract) permeated aquatic environment. Osiris facilitate phenotypic plasticity and toxicity responses essential in development, toxicology defenses and digestion in insects (Smith *et al*., 2018). The ATP-binding cassette (ABC) transporters and CYPs facilitate transformation and elimination of endogenous and exogenous compounds including insecticides (Epis *et al*., 2014) and phytochemicals (Guzov *et al*., 1998) by insects. The fatty acid (FA) elongation and chitin-binding pathways trigger intrinsic adaptation mechanisms in insects (War and Sharma, 2014; Yahouédo *et al*., 2017). However, the detoxification process appeared to be potentially counteracted by suppression of glutathione S transferases (GSTs) expression by the extract. This group of supressed GSTs are associated with the reduction of oxidative stress in mosquitoes since not all GSTs are involved in reducing oxidative stress (Ranson and Hemingway, 2005). The reduction in GSTs could have also exposed the larvae to oxidative stress (Ranson and Hemingway, 2005), suppressed their immunity to bacterial infection (Chen *et al*., 2011) and hence enhanced their susceptibility to exogenous phytochemicals (Edwin *et al*., 2016).

The immunity appears to have been further depressed by suppressed expression of C-type lysozyme in the larvae by the exposure. Lysozymes degrade macromolecules including toxic phytochemicals and have a potential role in innate immunity (Li *et al*., 2005). The exposure induced the C-type lectins (CTLs) immune transcripts (Gräf *et al*., 1994) in the larvae. The underlying physiological processes underpinning this (CTLs) immune response remains to be determined. We similarly observed induction of CPAP whose role has not been elucidated in *An. gambiae* (Zhou *et al*., 2017). Survival in the larvae in the presence of the extract seems to be dependent on opposing forces between those counteracting and those facilitating susceptibility of the larvae to xenobiotics that include our extract phytochemicals.

Overall, this study reveal significant modulation of several transcripts, some of which may not be directly related to our treatments, but are inherently perturbed, irrespective of external stimuli in the mosquito. These transcripts can potentially be identified from the rest of the differentially expressed transcripts through carefully planned and executed functional genomics studies.

These results have potential implications for integrated vector management (IVM) of *An. gambiae s.s* mosquitoes. First, the exposure treatment appears to impair the cuticular integrity in the larvae that enhances susceptibility of the larvae to insecticides. This suggests that the exposure treatment can augment efficacy of insecticide formulations to surmount resistance to insecticides in the mosquito. The current studies were limited to the L_3/L_4 instar larval stages of the mosquito, necessitating further studies to establish efficacy of the extract against younger larvae (below L_3 instar) and pupae whereas comparing their underlying molecular processes. Secondly, the larvae appear to counteract the effect of exposure by instigating adaptive

mechanisms, which can be further interrogated through generational laboratory or natural chronic exposures experiments and assessment of resultant biological cost of the adaptation to fitness in the mosquito. The findings will provide additional insight on impact of such adaptation on vectorial capacity of the mosquito following temporal exposures of the larvae. The exposure appears to perturb JH metabolism probably due to Neplanocin A constituent of the extract (Mang'era *et al*., 2019). The Neplanocin A has similar inhibition effect on JH action as 3-deazaneplanocin A in mosquitoes (Sharma *et al*., 2015; Mang'era *et al*., 2019), suggesting potential application of Neplanocin A as a potent JH antagonists. However, these putative responses extract challenge and confounded by age (exposed were older larvae) and physiological compromise (abnormal) in the larvae.

5.3 Chemotypic variability and fumigant toxicity

The chemical composition and yield of the essential oils from leaves of *M. koenigii* were observed to vary with agro-climatic and geographical factors as shown in other studies (Lal *et al*., 2001; Verma *et al*., 2013). It is not unusual for the same species sampled from different localities to have varying essential oil composition (Lakušić *et al*., 2012) since the biosynthesis of these plant-specialized essential oils (EO) is under the influence of genetic, climatic and various ecological factors (Curado *et al*., 2006; Marčetić *et al*., 2017). EO component variability was observed in this study where there was a homogeneous distribution of terpenoids across Kibwezi, Makindu and Mombasa. Mombasa belongs to the same agro-climatic regions (lowland semi-humid) as Malindi but had EO composition similar to those from semi-arid climate (Kibwezi and Makindu). The pattern of variability in this study is unique and reveals the possibility of additional underlying factors that may affect EO variability regardless

of geographical and climatic differences. One possible explanation would be that *M. koenigii* is an exotic tree grown in Kenya and is scattered in locations that are currently or had previously been inhabited by Asian communities. The Asian community's presence was influenced by the building of the Railway along the Mombasa-Makindu-Kibwezi route (Jedwab *et al*., 2017), effectively leaving Malindi out of the migratory route and hence the unique EO composition of the oil from this area observed in this study. In fact, the distribution of *M. koenigii* follows a unique pattern suggesting that the plant could be of value to the local communities such as medicinal and culinary use (Salikutty *et al*., 2012), hence its dispersal. Interestingly, the essential oil from Malindi was consistently unique in composition and bioactivity relative to EOs from the other three sites.

A relative abundance of sesquiterpenes in *M. koenigii* EO from all the four sites was further observed. Sesquiterpenes have been identified from plant sources and investigated for their mosquitocidal activities. The major sesquiterpenes (drimane and cinnamodial) in Bark extract of *Cinnamosma fragrans* was found to have insecticidal, anti-feedant and repellent activities against pyrethroid -resistant and -susceptible strains *Aedes aegypti* (Inocente *et al*., 2018). Also sesquiterpenes from *Chloroxylon swietenia* (germacrene D, pregeijerene and geijerene) showed relatively high fumigant toxicity against *Anopheles gambiae, Culex quinquefasciatus* and *A. aegypti* at low doses of LD_{50} values of up to 1.8×10^{-3} mg cm^{-3} (Kiran and Devi, 2007). Elsewhere, monoterpene constituents (perillaldehyde and perillyl alcohol) from *Conyza newii* have exhibited high fumigant toxicity against adult *An. gambiae* (1.05×10^{-4} mg cm^{-3}) (Omolo *et al*., 2005). Hence, copious amounts of sesquiterpenes in *M. koenigii* leaves show the basis underlying mosquitocidal potential of the natural

blend of these chemical compounds. There is growing evidence of plants EO with promising bioactivity of their major constituents against adult mosquitoes, which underscores the need to further explore their mosquitocidal potential.

To have a better view of the variables (sites) before clustering the data, a PCA (Azizi *et al*., 2012) was performed and revealed that agro-climatic and geographical factors accounted for only two-thirds of the total variation in the EO components between sites where Kibwezi and Mombasa were likely to have come from the same population regardless of geographical variation. Although altitude variation plays a major role in terpenoid biosynthesis (Şanli and Karadoğan, 2016), additional factors that could be responsible for the variation in EO components between sites include genotypic (Lyra *et al*., 2008) and epigenetic factors such as water availability, sun's radiation rate, soil types and temperature changes (Medina-Holguín *et al*., 2008; Gilani *et al*., 2010). However, some other factors such as the phenological stage of the plant do not have a significant effect on yield and chemotype of EOs even within the same species (Zouari *et al*., 2012). These findings, therefore, highlight the need for exploring the likely factors that may affect the oil yield of *M. koenigii* and subsequently downstream applications related to the bioactivity of the plant.

Three unique chemotype clusters were identified that showed a unique grouping pattern of sesquiterpenoid predominant chemotypes. This categorization points to the possible existence of more chemotypes (Rao *et al*., 2011) that may affect qualitative and quantitative characteristics crucial in identifying optimum blends for various applications such as culinary, anti-microbial (Muthumani *et al*., 2010), and insecticidal (Arivoli, 2015) uses.

The mosquitocidal activities of EOs from *M. koenigii* was assessed by the determination of fumigant toxicity parameters against *An. gambiae* adults, which showed varying degrees of bioactivity. The highest bioactivity observed in EO from Kibwezi and Mombasa, correlating to the clusters observed from HCA indicates that chemotypic differences may influence EO bioactivity (Gilani *et al*., 2010; Dosoky *et al*., 2016;). The overall activity of a botanical blend cannot be credited to a single major constituent (Shaalan *et al*., 2005; Isman *et al*., 2008) but a possible synergy from the less abundant or inactive compounds. These compounds might influence the rate of reactions and biological action of the active compounds (Morsy, 2017) either by exerting significant synergistic or antagonistic effect (Pandey *et al*., 2014). For example, caryophyllene oxide is often a constituent of essential oils with an effect on mosquitoes (Bossou *et al*., 2013). However, it would seem from our results that it is not one of the most abundant components of EOs with the highest potency, although synergistic effects cannot be excluded. Pure EOs can have more potent bioactivity as compared to isolated chemical components from the same oils due likely to synergistic effect of the blends of chemical components when acting together rather than separately (Kiran and Devi, 2007).

Chemotype groupings of *M. koenigii* EOs from the different locations, as revealed by HCA, was found to influence insecticidal activity patterns between the locations as demonstrated in other studies (Omolo *et al*., 2005; Kiran and Devi, 2007; Mayeku *et al*., 2014). This can be attributed to the distribution of known insecticidal compounds among the geographical locations.

The major chemical compounds associated with Kibwezi and Mombasa (most potent EO) are known to have insecticidal properties. The sesquiterpenes β-elemene **41**, (E)-

caryophyllene **44,** α-humulene **49,** and globulol **69**, have been collectively found in EOs from Benin to have insecticidal activity against *An. gambiae* (Bossou *et al*., 2013). Also, (E)-caryophyllene **44** and α-humulene **49** identified in *Commiphora leptophloeos* leaf oil have been successfully tested as oviposition deterrents against *Ae. aegypti* (Silva *et al*., 2015). (E)-Nerolidol **62,** α-humulene **49** and β-elemene **41,** EO components from *Swinglea glutinosa* (Rutaceae family) have displayed insecticidal actions against *Ae. Aegypti* mosquito larvae (Ríos *et al*., 2017). On the other hand, the above results suggest that oxygenated monoterpenes (OMs), such as linalool **11** which were less abundant (1.23 – 2.06%) in the *M. koenigii* EO blend, could contribute by exerting a synergistic effect on the other EO components, thereby influence the bioactivity of the EO blend. Linalool **11** has demonstrated significant knockdown rates in *An. gambiae* mosquitoes only when added to known repellent formulations partly due to the delayed mortality (reversible inhibitor of acetylcholinesterase) effect it has on mosquitoes (Tambwe *et al*., 2014).

Fumigant toxicity of a complex blend of EO components cannot be attributed to a particular chemical compound. In fact, the least abundant compounds may have synergistic or antagonistic effects that play a crucial role in modulating fumigant toxicity activity of the entire essential oil blend. In this way, essential oils are ultimately defined not only by its major compounds but rather by a unique blend of chemical components and their putative interactions.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

In conclusion, this exploratory study shows the presence of a blend of secondary metabolites in the leaves of *M. koenigii* growing in a semi-arid region of Kenya that has subtle growth-disrupting effects and fumigant toxicity on *An. gambiae* larvae and adult respectively. Some of these metabolites were previously shown to have negative morphogenetic effects on different disease vectors.

Anopheles gambiae s.s larvae heavily invest in cuticular development which was disrupted by the exposure treatment. This disruption (exposure) potentially induced the gross morphogenic abnormalities that we previously observed in the larvae exposed to the extract. The cuticle genes can potentially be targeted in development of more efficacious curry tree phytochemical based IGRs against *An. gambiae s.s* mosquito larvae. Survival of the larvae in the presence of the extract appears to be dependent on their ability to withstand oxidative stress associated processes induced by the extract.

Unlike conventional insecticides, constituents of phytochemical blends have different mechanisms of action, and thus act synergistically on both behavioral and physiological processes of arthropods and demonstrate resistance-mitigating effects.

This study reveals potential new targets in the malaria vector and novel plant-based chemical blends that could spur research into new insecticide formulations. We, therefore, reject the null hypothesis and conclude that there is significant induction of molecular and physiologic changes in adult and larval stages of the *Anopheles* mosquito exposed to *Murraya koenigii* leaf phytochemicals.

6.3 Recommendations for future research

i. Functional analysis of the *An. gambiae* developmental genes related to cuticular development and hormone regulation that were differentially expressed in test insects relative to controls.

ii. Investigate individual and combined effects of characterized chemicals from *M. koenigii* (and from other plant sources) on growth, development and reproduction capacity of *An. gambiae* mosquito.

iii. The effect of a broader profile of agro-ecologies in Eastern Africa on the levels of the active constituents in the plant needs to be comprehensively established to see if the active blend can be produced on a large scale.

iv. Subtractive bioassays of EOs chemical constituents of *M. koenigii* on *An. gambiae* adult mosquito to establish an optimum blend of chemical components for fumigant toxicity and repellence against mosquitoes.

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APPENDICES

Appendix 1: Front page of publications associated with this thesis

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Growth-disrupting *Murraya koenigii* leaf extracts on *Anopheles gambiae* larvae and identification of associated candidate bioactive constituents

Clarence Maikuri Mang'era[a,b,d,*], Ahmed Hassanali[c], Fathiya M. Khamis[d], Martin K. Rono[e], Wilber Lwande[d], Charles Mbogo[c], Paul O. Mireji[c,f,**]

[a] Department of Biochemistry and Molecular Biology, Egerton University, Njoro Campus, PO Box, 536 – 20115, Egerton, Kenya
[b] Department of Biochemistry and Biotechnology, School of Pure and Applied Sciences, Kenyatta University, Ruiru Campus, PO Box, 43844-00100, Nairobi, Kenya
[c] Department of Chemistry, School of Pure and Applied Sciences, Kenyatta University, Ruiru Campus, PO Box, 43844-00100, Nairobi, Kenya
[d] International Centre of Insect Physiology and Ecology (icipe), Duduville Campus, Kasarani, PO Box, 30772 00100, Nairobi, Kenya
[e] Centre for Geographic Medicine Research – Coast, Kenya Medical Research Institute, PO Box, 230 80108, Kilifi, Kenya
[f] Biotechnology Research Institute - Kenya Agricultural and Livestock Research Organization, PO Box, 362 00902, Kikuyu, Kenya

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ABSTRACT

Plant-based constituents have been proposed as eco-friendly alternatives to synthetic insecticides for control of mosquito vectors of malaria. In this study, we first screened the effects of methanolic leaf extracts of curry tree (*Murraya koenigii*) growing in tropical (Mombasa, Malindi) and semi-arid (Kibwezi, and Makindu) ecological zones of Kenya on third instar *An. gambiae* s.s. larvae. Extracts of the plant from the semi-arid region, and particularly from Kibwezi, led to high mortality of the larvae. Bioassay-guided fractionation of the methanolic extract of the leaves of the plants from Kibwezi was then undertaken and the most active fraction (20 fold more potent than the crude extract) was then analyzed by Liquid chromatography quadruple time of flight coupled with mass spectrometry (LC-QToF-MS) and a number of constituents were identified, including a major alkaloid constituent, Neplanocin A (**5**). Exposure of the third instar larvae to a sub-lethal dose (4.43 ppm) of this fraction over 7-day periods induced gross morphogenetic abnormalities in the larvae, with reduced locomotion, and delayed pupation. Moreover, the few adults that emerged from some pupae failed to fly from the water surface, unlike in the untreated control group. These results demonstrate subtle growth-disrupting effects of the phytochemical blend from *M. koenigii* leaves on aquatic stages *An. gambiae* mosquito. The study lays down some useful groundwork for the downstream development of phytochemical blends that can be evaluated for integration into eco-friendly control of *An. gambiae* vector population targeting the often overlooked but important immature stages of the malaria vector.

1. Introduction

Anopheles gambiae mosquito is a major vector of malaria and is responsible for about 216 million infections and about 446,000 deaths and in humans, mainly in sub-Sahara Africa (WHO, 2017) despite significant progress in the fight against the disease (Slutsker and Kachur, 2013). Effective management of the vector by current mosquitocides has been challenging due to widespread resistance to current insecticides, especially pyrethroids, in the vector across Sub-Sahara Africa (Gnankiné et al., 2013; Namountougou et al., 2013; Yunta et al., 2016). The resistance to pyrethroids presents a real and immediate challenge to efficacy of otherwise successful insecticide treated nets (ITN) based malaria control intervention against adult vector (Hightower et al., 2010). Resistance development has also hampered larvae control, a viable alternative control of the vector populations (Bisset et al., 2014). Recent phytochemical research has begun to reveal a variety of blend effects in the bioactivities of plant natural products. Two principal blend effects have been demonstrated: (1) enhanced biological activity resulting from synergistic or other additive effects of moderately active or individually inactive compounds to give mixtures that are more active than a linear summation of individual activities (Bekele and Hassanali, 2001), and (2) mitigating effects of structurally related or unrelated compounds against rapid resistance development that characterizes most single-component bioactive compounds (Abu Hasan

* Corresponding author at: Department of Biochemistry and Molecular Biology, Egerton University, Njoro Campus, PO Box, 536 – 20115, Egerton, Kenya.
** Corresponding author at: Biotechnology Research Institute - Kenya Agricultural and Livestock Research Organization, PO Box, 362-00902, Kikuyu, Kenya.
E-mail addresses: clarence.mangera@egerton.ac.ke (C.M. Mang'era), ahmedhassanali786@gmail.com (A. Hassanali), fkhamis@icipe.org (F.M. Khamis), MRono@kemri-wellcome.org (M.K. Rono), wlwande@yahoo.com (W. Lwande), cmmbogo@gmail.com (C. Mbogo), mireji.paul@gmail.com (P.O. Mireji).

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Parasites & Vectors

RESEARCH Open Access

Transcriptomic response of *Anopheles gambiae* sensu stricto mosquito larvae to Curry tree (*Murraya koenigii*) phytochemicals

Clarence M. Mang'era[1,2*], Fathiya M. Khamis[3], Erick O. Awuoche[4], Ahmed Hassanali[5], Fidelis Levi Odhiambo Ombura[3] and Paul O. Mireji[6*]

Abstract

Background: Insect growth regulators (IGRs) can control insect vector populations by disrupting growth and development in juvenile stages of the vectors. We previously identified and described the curry tree (*Murraya koenigii* (L.) Spreng) phytochemical leaf extract composition (neplanocin A, 3-(1-naphthyl)-L-alanine, lumiflavine, terezine C, agelaspongin and murrayazolinol), which disrupted growth and development in *Anopheles gambiae* sensu stricto mosquito larvae by inducing morphogenetic abnormalities, reducing locomotion and delaying pupation in the mosquito. Here, we attempted to establish the transcriptional process in the larvae that underpins these phenotypes in the mosquito.

Methods: We first exposed third-fourth instar larvae of the mosquito to the leaf extract and consequently the inherent phytochemicals (and corresponding non-exposed controls) in two independent biological replicates. We collected the larvae for our experiments sampled 24 h before peak pupation, which was 7 and 18 days post-exposure for controls and exposed larvae, respectively. The differences in duration to peak pupation were due to extract-induced growth delay in the larvae. The two study groups (exposed *vs* control) were consequently not age-matched. We then sequentially (i) isolated RNA (whole larvae) from each replicate treatment, (ii) sequenced the RNA on Illumina HiSeq platform, (iii) performed differential bioinformatics analyses between libraries (exposed *vs* control) and (iv) independently validated the transcriptome expression profiles through RT-qPCR.

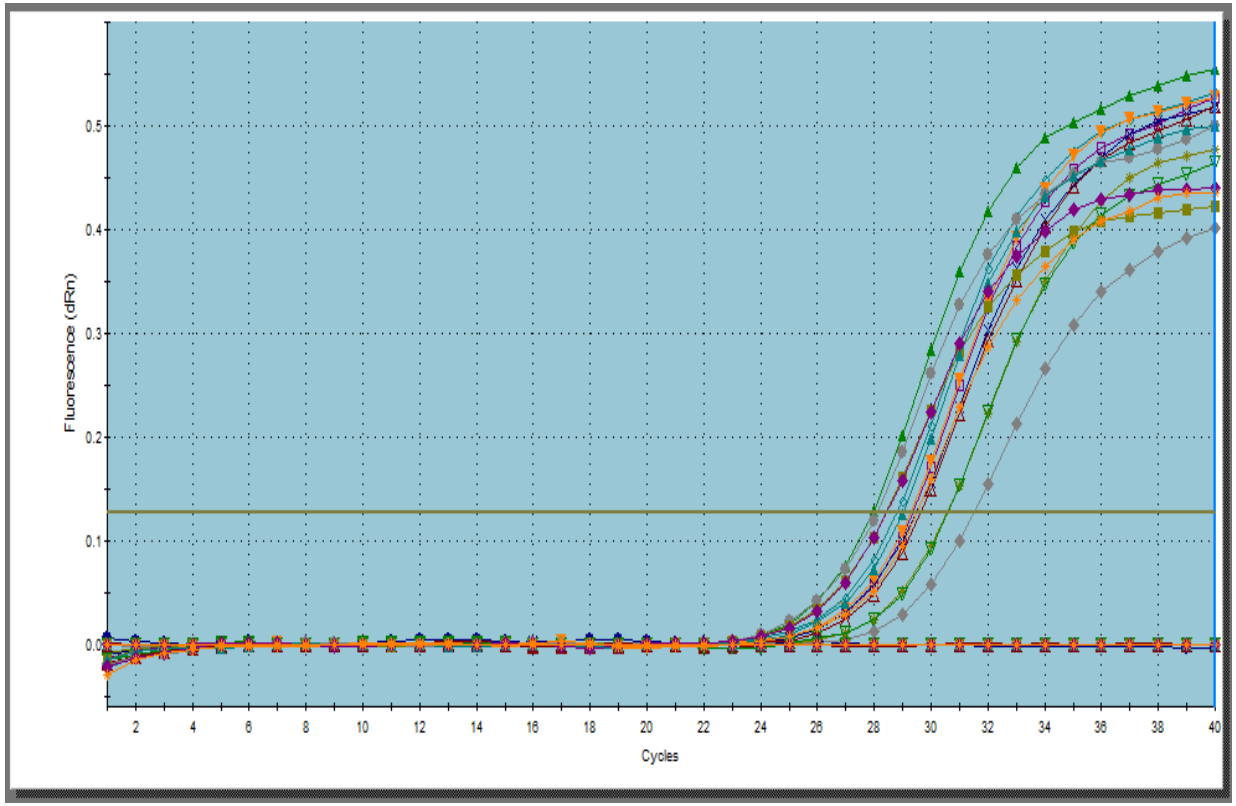
Results: Our analyses revealed significant induction of transcripts predominantly associated with hard cuticular proteins, juvenile hormone esterases, immunity and detoxification in the larvae samples exposed to the extract relative to the non-exposed control samples. Our analysis also revealed alteration of pathways functionally associated with putrescine metabolism and structural constituents of the cuticle in the extract-exposed larvae relative to the non-exposed control, putatively linked to the exoskeleton and immune response in the larvae. The extract-exposed larvae also appeared to have suppressed pathways functionally associated with molting, cell division and growth in the larvae. However, given the age mismatch between the extract-exposed and non-exposed larvae, we can attribute the modulation of innate immune, detoxification, cuticular and associated transcripts and pathways we observed to effects of age differences among the larvae samples (exposed *vs* control) and to exposures of the larvae to the extract.

*Correspondence: cmmangera@gmail.com; mirejl.paul@gmail.com
[1] Department of Biochemistry and Molecular Biology, Egerton University, Njoro Campus, PO Box 536-20115, Egerton, Kenya
[6] Biotechnology Research Institute-Kenya Agricultural and Livestock Research Organization, PO Box 362-00902, Kikuyu, Kenya
Full list of author information is available at the end of the article

Appendix 2: A list of primers used for *An. gambiae* larvae transcriptome validation

VectorBase Gene ID	VectorBase Description	Forward Primer	Reverse primer	Annealing Temperature (ºC)
AGAP006000-RA	Cuticular protein RR-1 family 25	TGTGCATTGATCTGTGTGGC	GATGCCGTTGCTAGTCTCGT	56.8
AGAP010617-RA	Unknown	CACTTGCATCAGCCGGAGTA	GCACAAGTAGCCTCCGATGT	56.8
AGAP011277-RA	Unknown	GATGGAGAGTTCTGGCTCGG	ACAGCTTCACCTCACTGTCG	63.2
AGAP002810-RA	45 kDa calcium	AAGCCGGAAGTGGTGTATCG	CTTGTCGCCATTTGTGTCGG	63.2
AGAP003471-RA	Osi20	ACACCAAAGCGAAAACGCAA	TTGGACGGTACCACTGTTCG	55.9
AGAP005833-RA	Carboxylesterase juvenile hormone esterase	GACCACTTCGTTTCGCCAAC	CCTCCTTACCCTCGACTCCA	63.2
AGAP008781-RA	Elongation of very long chain fatty acids protein 5	TATGTCGATGTCCTGCGGTG	TTCTGTTTCTTGCGCAGCAC	64.1
AGAP009017-RA	Cytochrome b-561 domain containing protein 2	GAACCGGTACTCGACTGCAA	CCCGTTATGGCATGATCCGA	63.2
AGAP009623-RA	GAPDH	GTTCATCGGCGTCGACTACA	CTCCTGGAACACGGCAATCT	56.8
AGAP010362-RA	Unknown	CAACGGGGCTTCAGACGATA	TACCACAAGTGATGCCACGG	55.0
AGAP011790-RB	CLIPA2: CLIP-domain serine protease	TCACCCAGTGCGACAGTAAC	GAAACTCCTCCGGAAAGGCA	55.0
AGAP028065-RA	Unknown	TGTACGATCATGGCAGCGAA	GAAGCCACTTGACCTTTGCG	62.0

Appendix 3: Amplification plot for reverse transcription quantitative real-time PCR (RT-qPCR)



Appendix 4: RNA-seq analysis comparing exposed and unexposed *An. gambiae* larvae from the complete transcriptome.

Feature ID	Description	Fold change (norm)	Baggerley's test: FDR p-value (norm)	Control RPKM (norm)	Treatment RPKM (norm)
AGAP004373-RB	Protein coding	172.68432	0.046686391	1.63351655	282.0826946
AGAP003471-RA	Osi20	65.807495	0.024789496	16.5991123	1092.345995
AGAP003466-RA	Osi15	47.982969	0.006596163	14.8397697	712.0562053
AGAP008781-RA	Elongation of very long chain fatty acids protein 5	32.902245	0	37.20054	1223.981292
AGAP004132-RA	Protein coding	29.288803	0	2.38961148	69.98885972
AGAP013007-RA	Protein coding	27.243013	0.00264487	38.5902181	1051.313811
AGAP003664-RA	Envelysin	25.000307	0	30.2989594	757.4832941
AGAP009017-RA	cytochrome b-561 domain containing protein 2	23.31279	7.31256E-08	152.400354	3552.87738
AGAP004131-RA	Osi9: osiris 9	21.687857	7.00038E-09	3433.49489	74465.14504
AGAP001118-RA	Protein coding	20.440965	2.76144E-08	189.410519	3871.733782
AGAP005835-RA	COEJHE3E: carboxylesterase juvenile hormone esterase	20.311989	4.14151E-07	5.41015435	109.8909981
AGAP006865-RA	CPR64: cuticular protein RR-1 family 64	19.059144	0	3.14533057	59.94730726
AGAP012498-RA	Protein coding	18.97865	0.02683556	83.5229821	1585.153464
AGAP004129-RA	Osi6: osiris 6	18.313242	0	818.97035	14998.0021
AGAP013345-RA	Protein coding	17.178679	0.011563653	7.67464853	131.8403216
AGAP005836-RA	COEJHE4E: carboxylesterase juvenile hormone esterase	16.965832	0	264.112	4480.879841
AGAP009090-RA	Dopa decarboxylase	16.936719	6.94373E-09	38.4666054	651.4980875
AGAP011553-RA	Protein coding	16.249398	2.8964E-12	2.51422066	40.85457126
AGAP005695-RA	CPTC2: cuticular protein from two-cysteine family 2	15.955152	0	776.171816	12383.93939
AGAP006468-RA	Protein coding	15.617046	5.8154E-05	106.336642	1660.664184
AGAP010168-RA	Protein coding	15.504498	3.17914E-06	288.667396	4475.643041
AGAP003465-RA	Protein coding	14.950124	6.31255E-12	724.971889	10838.41963
AGAP006321-RA	CPR71: cuticular protein RR-2 family 71	14.689639	0	9.68389076	142.2528582
AGAP005697-RA	CPTC3: cuticular protein from two-cysteine family 3	14.404002	0	264.542235	3810.466791

Feature ID	Description	Fold change (norm)	Baggerley's test: FDR p-value (norm)	Control RPKM (norm)	Treatment RPKM (norm)
AGAP005698-RA	CPTC4: cuticular protein from two-cysteine family 4	14.212867	0	473.402924	6728.412668
AGAP006828-RA	CPR60: cuticular protein RR-1 family 60	13.911639	9.52295E-10	47.1107273	655.3874102
AGAP007866-RA	SPZ4: spaetzle-like cytokine 4	13.858909	0	84.1176387	1165.778735
AGAP003049-RA	Protein coding	13.707577	0	8.55647646	117.2885607
AGAP012644-RA	Protein coding	13.629065	0	59.7735268	814.657285
AGAP005688-RA	chymotrypsin-like protease (Precursor)	13.532081	3.45631E-08	5.03295905	68.10641074
AGAP003211-RA	Protein coding	13.483219	1.53206E-06	377.726	5092.962454
AGAP002005-RA	Protein coding	13.174247	0	11.0643889	145.7649951
AGAP005686-RA	chymotrypsin-like protease	13.165192	2.77188E-10	514.232786	6769.973352
AGAP008995-RA	Protein coding	12.910981	1.82473E-06	325.870322	4207.305545
AGAP012903-RA	cuticular protein 3 from two-cysteine family	12.671541	0	534.491948	6772.836865
AGAP008997-RA	Protein coding	12.591035	6.98788E-06	63.3358172	797.4634606
AGAP012736-RA	Protein coding	12.091398	0.001747396	3.64886541	44.1198851
AGAP007164-RA	Protein coding	12.083563	1.02486E-06	61.4186743	742.1564048
AGAP003420-RA	Protein coding	12.044694	2.44421E-11	11.8206752	142.3764133
AGAP007358-RA	Cellular retinaldehyde binding protein	11.541292	6.04139E-13	591.933543	6831.67789
AGAP013234-RA	Protein coding	11.470788	6.1768E-12	1386.07791	15899.40525
AGAP004130-RA	Osi7: osiris 7	11.101968	0	380.330149	4222.413117
AGAP005696-RA	CPTC1: cuticular protein from two-cysteine family 1	11.049893	2.19275E-12	1401.35818	15484.85851
AGAP010004-RA	Protein coding	10.571793	0	52.2771764	552.6634833
AGAP001625-RA	Protein coding	10.546131	0	239.266459	2523.335456
AGAP003468-RA	Protein coding	10.282573	2.12791E-06	218.557478	2247.333289
AGAP006060-RA	Protein coding	10.257044	0	106.492406	1092.297279
AGAP007422-RB	Protein coding	10.083807	0.002347064	2.76795447	27.91151965
AGAP000123-RA	CTLSE2: C-type lectin (CTL)	10.072335	5.71379E-06	273.233981	2752.104121
AGAP028018-RA	CPLCP24: cuticular protein (putative) CPLCP24	10.06717	0.006959102	0.875	8.808773884

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AGAP005586-RA	Protein coding	10.012964	0	308.351814	3087.51557
AGAP005833-RA	COEJHE1E: carboxylesterase juvenile hormone esterase	9.8176098	0.002217101	209.660578	2058.365737
AGAP007645-RA	Protein coding	9.7381671	0.047974916	495.145491	4821.809547
AGAP003714-RA	HPX3: heme peroxidase 3	9.5523045	8.46828E-05	4454.71456	42552.78998
AGAP028610-RA	Protein coding	9.4273006	0.030967463	213.859911	2016.121674
AGAP013081-RA	Protein coding	9.2220005	0.000223873	507.867148	4683.551084
AGAP004559-RI	Ras-related protein	9.1282313	0.006634809	1.13016181	10.31637843
AGAP004570-RA	Clipd1	9.1211932	0	34.4458122	314.1869061
AGAP002743-RA	Protein coding	9.0819674	6.53789E-05	107.581198	977.0489341
AGAP007790-RD	Protein coding	9.0335533	0.001974193	3.14545607	28.41464498
AGAP006112-RA	Protein coding	8.916697	0.004921707	1.25571359	11.19681756
AGAP001269-RA	Protein coding	8.4687387	1.15854E-10	62.549701	529.7170747
AGAP000788-RA	Protein coding	8.4334535	0.000517235	338.543938	2855.09455
AGAP007094-RA	Protein coding	8.4086811	0.002650936	107.230931	901.6707035
AGAP012156-RA	ABCA5: ATP-binding cassette transporter (ABC transporter) family A member 5	8.3221586	1.53645E-06	83.7398392	696.8962268
AGAP006739-RA	Protein coding	8.2447504	3.97579E-11	21.6191184	178.2442342
AGAP000303-RA	Protein coding	8.0655613	0	131.95844	1064.318885
AGAP008123-RA	Protein coding	8.0317439	0	65.7190588	527.8386518
AGAP004124-RA	Protein coding	7.9036861	0	202.325463	1599.116954
AGAP001425-RA	Sulfotransferase (Sult)	7.666973	1.63286E-09	156.405036	1199.153194
AGAP000160-RA	Protein coding	7.6338934	4.42752E-09	1006.97516	7687.140959
AGAP004498-RA	Sulfotransferase (Sult)	7.632292	0	76.1089864	580.8860107
AGAP010349-RA	Protein coding	7.5793164	6.48849E-10	179.995073	1364.239607
AGAP009102-RA	Protein coding	7.3147659	2.00157E-07	39.0957266	285.9760887
AGAP001597-RB	Protein coding	7.1839755	4.6645E-08	35.8272486	257.3820758
AGAP005589-RA	Protein coding	6.9824342	4.60061E-08	120.641239	842.3695137

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AGAP009162-RA	Protein coding	6.9395966	0.000477045	27.7817681	192.794262
AGAP012355-RA	CPLCP16: cuticular protein (putative) CPLCP16	6.8693175	0.021875634	6.03887939	41.48298002
AGAP002815-RA	CLIPA15: CLIP-domain serine protease	6.8179122	8.42107E-08	201.641656	1374.775103
AGAP007358-RB	Cellular retinaldehyde binding protein	6.7415814	3.01226E-05	9.93467376	66.97541205
AGAP007034-RA	LRIM11: leucine-rich immune protein (Short)	6.7407483	6.56954E-08	4.40205233	29.67312666
AGAP013410-RA	Protein coding	6.7332574	0.011201111	90.780532	611.248693
AGAP013009-RA	Protein coding	6.6886716	0	197.173839	1318.831056
AGAP028657-RA	Protein coding	6.6800097	0.002004751	52.8906025	353.3097355
AGAP012323-RB	OBP27: odorant binding protein 27	6.6267577	4.04847E-06	161.744688	1071.842848
AGAP013403-RA	Sodium-coupled monocarboxylate transporter 2	6.5605309	0	29.0427668	190.5359696
AGAP028473-RA	Protein coding	6.5526641	0	363.697468	2383.187351
AGAP003750-RA	Glucose dehydrogenase (acceptor)	6.5404725	0.024472116	1100.95881	7200.790806
AGAP007613-RA	Protein coding	6.423169	0.003315438	70.3481243	451.8578888
AGAP005687-RA	chymotrypsin-like protease (Precursor)	6.412754	1.76251E-05	352.242038	2258.841546
AGAP007006-RC	Filamin	6.3682841	0.002420097	8.42692365	53.66504384
AGAP028085-RA	Protein coding	6.2584258	0.000149554	8.1739134	51.15583056
AGAP028656-RA	Protein coding	6.2168248	3.55994E-10	24.3947089	151.65763
AGAP006867-RA	CPR141: cuticular protein RR-1 family 141	6.1914901	3.03951E-06	56.0308816	346.9146503
AGAP004367-RA	Protein coding	6.0706672	0.000251224	4126.84945	25052.72974
AGAP006176-RA	Protein coding	6.0121576	0.009000627	83.8551696	504.1504968
AGAP007916-RA	Protein coding	5.9971298	2.77E-09	43.7333978	262.2748651
AGAP009011-RA	Protein coding	5.9794283	2.19528E-09	43.2338055	258.5134407
AGAP001419-RA	Protein coding	5.9775623	6.85005E-10	7.04439499	42.10830994
AGAP011178-RA	zinc/iron regulated transporter-related protein 1	5.9581534	1.74681E-08	647.11194	3855.592227
AGAP006483-RA	SPZ2: spaetzle-like cytokine 2	5.931478	9.40809E-14	186.395061	1105.598201
AGAP000596-RC	Protein coding	5.9046214	0.010331942	69.3059134	409.2251814

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AGAP028419-RA	Protein coding	5.8938172	0	20.4964238	120.8021745
AGAP006859-RA	CPR66: cuticular protein RR-1 family 66	5.826906	0.000973125	4.40194214	25.64970318
AGAP004333-RA	Protein coding	5.8036641	1.07053E-08	646.750097	3753.520344
AGAP028178-RA	CPLCP13: cuticular protein (putative) CPLCP13	5.7626173	0.000112323	23.8797673	137.6099592
AGAP010294-RA	Protein coding	5.7036639	1.29898E-09	3224.98806	18394.248
AGAP011619-RA	Protein coding	5.7030013	7.68907E-05	4228.64967	24115.99462
AGAP004552-RA	Protein coding	5.6723532	9.39122E-06	15.5913703	88.43975913
AGAP008356-RA	CYP4H16: cytochrome P450	5.6430291	0.009391723	418.505166	2361.63682
AGAP013109-RA	Protein coding	5.631881	0.020127956	5.1570665	29.04398467
AGAP003602-RA	Methyltransferase-like protein 9	5.6109198	0.006169088	30.5378374	171.3453567
AGAP003513-RA	Protein coding	5.6005494	4.80519E-05	625.91803	3505.484875
AGAP004126-RA	Osi3: osiris 3	5.5937573	3.20335E-06	60.2922587	337.2602606
AGAP002711-RA	Protein coding	5.5824186	0.007762619	10.310917	57.55985486
AGAP008199-RA	Protein coding	5.5473538	9.94806E-11	166.205666	922.0016239
AGAP002199-RA	Protein coding	5.5150907	8.76365E-08	38.2089747	210.725963
AGAP000163-RB	Protein coding	5.3629491	0.000438898	102.585859	550.1627334
AGAP000247-RB	Protein coding	5.322115	9.40809E-14	16.4759449	87.68687363
AGAP013254-RA	Protein coding	5.3165404	0.000869496	513.37565	2729.382387
AGAP013249-RA	Protein coding	5.3075702	2.3116E-12	89.8117981	476.6824197
AGAP010394-RA	Patched-related	5.2082515	0.000524638	203.948251	1062.21379
AGAP008358-RA	CYP4H17: cytochrome P450	5.1318537	0.008960186	762.917264	3915.179811
AGAP004568-RA	Serine protease like protein	4.9955877	0.000200819	86.3862224	431.5499533
AGAP011357-RB	All-trans/9-cis/11-cis	4.9594614	0.00034832	647.375423	3210.633446
AGAP011029-RA	CYP6AF2: cytochrome P450	4.9346145	0	36.9603111	182.3848866
AGAP005126-RA	SPZ6: spaetzle-like cytokine 6	4.9213041	2.91406E-08	576.394141	2836.610849
AGAP006488-RA	Protein coding	4.8259845	4.20284E-07	136.085834	656.7481296

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AGAP002993-RA	Protein coding	4.7435155	0.034031897	525.653869	2493.447263
AGAP006069-RA	Protein coding	4.7090499	2.35476E-08	145.749953	686.3438026
AGAP004811-RA	CTL1: C-type lectin (CTL)	4.7024155	0.000564897	15119.6553	71098.90171
AGAP003012-RA	SP71	4.6935652	0.000161433	398.805303	1871.818708
AGAP013525-RA	Protein coding	4.6921967	0.000273182	684.759239	3213.025011
AGAP004693-RA	nuclear receptor subfamily 6 group A	4.6896353	0.000136497	993.767977	4660.409378
AGAP004810-RA	CTL3: C-type lectin (CTL)	4.6836806	0.000490452	15795.5594	73981.35583
AGAP028200-RA	CPLCP9: cuticular protein (putative) CPLCP9	4.6276665	0.000266368	235.898616	1091.660123
AGAP013281-RA	Protein coding	4.6266663	0	79.0215414	365.6063017
AGAP002891-RE	Protein coding	4.620321	4.28664E-05	43.3550265	200.3141413
AGAP001599-RA	Protein coding	4.607746	0.000572031	5.1578513	23.76606861
AGAP011278-RA	GALE4: galectin 4	4.5702965	3.4056E-06	13.5836132	62.08113919
AGAP010930-RA	Protein coding	4.5655208	2.11887E-13	334.859987	1528.810227
AGAP008230-RA	Protein coding	4.5121503	0.002022546	108.117888	487.8441649
AGAP006380-RA	ABCA2: ATP-binding cassette transporter (ABC transporter) family A member 2	4.5032765	1.22405E-05	1844.37466	8305.729122
AGAP028213-RA	Protein coding	4.4820803	0.029606652	2.63883346	11.8274635
AGAP002485-RA	Protein coding	4.4810522	0.001150944	40.337233	180.7532482
AGAP005079-RF	Protein coding	4.4784997	0.04046698	6.79379581	30.42601219
AGAP000756-RA	Carboxypeptidase M	4.4655879	0.00278984	535.614669	2391.834358
AGAP004166-RA	Protein coding	4.449776	0.022934558	238.879104	1062.958503
AGAP005600-RA	sugar transporter ERD6-like 6	4.443963	0.000653981	96.177812	427.4106377
AGAP001375-RA	Protein coding	4.4108683	0.002004751	3524.53049	15546.23983
AGAP008080-RA	Protein coding	4.3774707	0.007475096	122.756992	537.3651365
AGAP013472-RA	Protein coding	4.3653512	0	138.237273	603.4542499
AGAP005381-RA	Hexosaminidase	4.3384594	4.0599E-05	518.576852	2249.82464
AGAP028474-RA	Protein coding	4.3255593	0.000469251	8752.22697	37858.27705

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AGAP004566-RA	Clipd1	4.3184045	0.005034778	875.950487	3782.708506
AGAP003394-RA	Protein coding	4.3054541	7.70308E-09	77.1400758	332.1230551
AGAP005588-RA	Protein coding	4.3019586	0	203.328906	874.7125318
AGAP008537-RA	Protein coding	4.2768631	0.046305197	312.823067	1337.901435
AGAP013445-RA	Protein coding	4.2725497	0.000271043	6.41574057	27.41157063
AGAP028210-RA	SRPN13: serine protease inhibitor (serpin) 13	4.2562746	0.013565572	289.522128	1232.285679
AGAP000621-RA	carboxypeptidase A1	4.2522857	0.005110625	788.501541	3352.933844
AGAP003783-RB	glucose dehydrogenase (acceptor)	4.2312823	0.009538152	120.659128	510.5428305
AGAP011518-RA	ABCA4: ATP-binding cassette transporter (ABC transporter) family A member 4	4.2038473	0.016446668	421.988406	1773.974842
AGAP008996-RA	Protein coding	4.1903881	0.006523816	839.79291	3519.058217
AGAP008005-RA	Protein coding	4.1659763	0	481.584067	2006.267786
AGAP002879-RA	Protein coding	4.1484384	0.003862479	20.7438738	86.05468278
AGAP002739-RA	Protein coding	4.1378487	0.004324639	4110.4792	17008.54109
AGAP000262-RA	Protein coding	4.1020184	0.010712174	357.658094	1467.120085
AGAP002168-RA	Protein coding	4.0919102	1.84832E-13	643.469398	2633.019003
AGAP002625-RB	CTL9: C-type lectin (CTL)	4.0892503	1.42391E-08	183.514986	750.4387216
AGAP009003-RB	Protein coding	4.0726493	0.00324937	70.8341911	288.4828213
AGAP000899-RA	Protein coding	4.0406941	0	65.2184831	263.5279373
AGAP013248-RA	CPR139: cuticular protein RR-2 family 139	4.0136278	0.000254122	133.071695	534.1002494
AGAP011205-RA	Protein coding	4.0123598	0.01797474	453.85048	1821.011418
AGAP003788-RA	glucose dehydrogenase (acceptor)	3.9998975	0.004632529	135.315941	541.2498963
AGAP010825-RA	Protein coding	3.9774074	0.008628529	1151.37946	4579.505178
AGAP008212-RA	CYP6M2: cytochrome P450	3.9476261	0.003813578	2769.3447	10932.3373
AGAP008125-RA	all-trans-retinol dehydrogenase (NAD+)	3.9173362	0	567.59441	2223.458149
AGAP008437-RA	ABCC8: ATP-binding cassette transporter (ABC transporter) family C member 8	3.8866024	0.012717676	940.951341	3657.103774
AGAP007601-RA	Protein coding	3.852802	0.003270809	1032.61487	3978.460655

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AGAP004426-RB	Protein coding	3.8265751	0.03045854	990.47828	3790.139501
AGAP000786-RA	Protein coding	3.8137353	0.000286198	414.991611	1582.668165
AGAP009282-RA	Hyphantrin	3.8032087	0	718.464062	2732.468791
AGAP005513-RA	elongation of very long chain fatty acids protein 2	3.7910423	0.000462056	541.650587	2053.420299
AGAP006489-RA	Protein coding	3.7900082	0.001580226	1422.96245	5393.039413
AGAP002391-RA	COEAE5O: carboxylesterase	3.783034	0.036082713	339.7698	1285.360697
AGAP010597-RA	Protein coding	3.7624351	0.013947652	343.625687	1292.869362
AGAP002559-RA	alpha-tocopherol transfer protein-like protein	3.7489347	9.50539E-08	64.70819	242.5867819
AGAP009274-RA	Protein coding	3.7325906	0.030988177	11.3153158	42.23544115
AGAP001597-RA	Protein coding	3.729701	0	167.581058	625.0272388
AGAP007560-RA	Protein coding	3.7293991	9.14137E-05	923.552225	3444.29482
AGAP001084-RA	fatty acyl-CoA reductase	3.7151055	0.03045854	992.982719	3689.035519
AGAP001039-RB	CYP307A1: cytochrome P450	3.7104481	0.000261365	328.832874	1220.117311
AGAP010695-RA	elongation of very long chain fatty acids protein 4	3.7008819	0.012846822	365.946866	1354.326141
AGAP000051-RA	HPX5: heme peroxidase 5	3.6874511	0.02988134	1041.79839	3841.580565
AGAP008732-RA	Protein coding	3.6754382	0.002058786	7.42342635	27.28434491
AGAP007616-RA	Protein coding	3.6572293	0.000557033	1462.36474	5348.203186
AGAP028560-RA	Protein coding	3.6248901	0.008366456	3009.38356	10908.68474
AGAP009479-RA	Protein coding	3.5813173	8.06027E-08	234.932198	841.3667334
AGAP002411-RC	regulator of G-protein signaling	3.5781057	0.022676681	64.8176594	231.9244395
AGAP012316-RA	Protein coding	3.5196786	0.000127683	29.7963746	104.8736622
AGAP005450-RA	Protein coding	3.5142713	1.03089E-07	1046.18849	3676.590201
AGAP002050-RA	ABCG13: ATP-binding cassette transporter (ABC transporter) family G member 13	3.5056003	1.53645E-06	657.277724	2304.153004
AGAP013024-RA	Protein coding	3.4727213	0.001606521	12.958191	45.00018604
AGAP010860-RA	Sodium-dependent nutrient amino acid transporter 2	3.4691712	0.005840851	79.7583023	276.6952014
AGAP005846-RA	alpha-galactosidase	3.4384196	0.027519662	5.15735944	17.73316596

Feature ID	Description	Fold change (norm)	Baggerley's test: FDR p-value (norm)	Control RPKM (norm)	Treatment RPKM (norm)
AGAP004571-RA	Oviductin	3.4185218	0.000447115	481.091846	1644.622954
AGAP002316-RA	Protein coding	3.4028883	0.029774998	615.011004	2092.813744
AGAP010196-RA	CTLGA1: C-Type Lectin (CTL) - galactose binding	3.3989431	0.013256221	86.9026242	295.3770721
AGAP010940-RA	Protein coding	3.390853	0.00324159	218.872757	742.1653484
AGAP028049-RA	Protein coding	3.3871824	0.03045854	2016.40251	6829.923071
AGAP006191-RA	hitinase-3-like protein 4	3.3844188	0.003895716	39.8448743	134.8517411
AGAP009978-RA	DUOX: dual oxidase: peroxidase and NADPH-oxidase domains	3.3700077	0.000594696	275.561602	928.6447312
AGAP006690-RA	BTB (POZ) domain containing 9	3.3536083	0.009299411	58.0498491	194.6764565
AGAP002622-RA	sodium-coupled monocarboxylate transporter 1	3.3226909	0.009510406	476.321125	1582.667878
AGAP028476-RA	Protein coding	3.3205006	0.002377391	330.463029	1097.302681
AGAP013354-RA	Protein coding	3.3190439	0.015083659	29.2930477	97.22491217
AGAP008584-RA	lysosomal alpha-mannosidase	3.3160849	0.001260359	1438.47848	4770.116837
AGAP028124-RA	CPLCP2: cuticular protein (putative) CPLCP2	3.30638	0.000174849	26.0268577	86.05468143
AGAP010926-RA	Protein coding	3.2986144	0.001347167	177.618962	585.8964608
AGAP013755-RB	Protein coding	3.2827479	0.000124823	511.304647	1678.484277
AGAP028400-RA	Protein coding	3.2435557	0.000256076	17346.6089	56264.69238
AGAP001626-RA	Protein coding	3.2061182	0.000811459	32.4355123	103.9920864
AGAP003783-RA	glucose dehydrogenase (acceptor)	3.191873	0.000962343	136.104699	434.4289153
AGAP003092-RC	Protein coding	3.1821245	0.025327023	17.1020394	54.42081893
AGAP009617-RA	fatty-acid amide hydrolase 2	3.1737921	8.55842E-05	380.87662	1208.823215
AGAP006613-RA	Protein coding	3.1412418	0.001032259	9.68614617	30.42652728
AGAP005020-RA	Protein coding	3.1412072	1.81322E-06	876.599211	2753.579731
AGAP002464-RA	secreted ferritin G subunit	3.1315487	0	22804.6538	71413.88355
AGAP012950-RA	Protein coding	3.1142136	0.016214681	290.462445	904.5620821
AGAP009049-RA	Chitinase-3-like protein 4	3.1091428	0.00049535	13.5841936	42.23519768
AGAP008649-RA	Enterokinase light chain	3.0935403	0.003362886	37.7123079	116.6645431

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AGAP002970-RB	Importin-11	3.0931703	8.1398E-11	312.060979	965.2577651
AGAP007138-RA	Wurst	3.0805865	0.00056175	743.679101	2290.967766
AGAP003350-RB	phosphoenolpyruvate carboxykinase (GTP)	3.064652	1.05975E-09	10242.26	31388.9624
AGAP001623-RA	Protein coding	3.0605545	0.003247491	409.47616	1253.224099
AGAP001930-RB	Protein coding	3.0576528	1.03503E-05	16.8531763	51.53116193
AGAP000795-RC	Protein coding	3.0139276	0.009317429	76.3692349	230.171345
AGAP004427-RA	tubulin monoglycylase TTLL15	3.0116331	0.01072719	51.148691	154.0410901
AGAP006547-RA	Protein coding	3.0093585	0.008755289	273.292794	822.4359867
AGAP002465-RA	ferritin heavy chain	3.0031688	0	19983.5535	60013.98434
AGAP001269-RB	Protein coding	2.9841649	0	442.326625	1319.975586
AGAP007784-RB	enoyl-CoA hydratase / long-chain 3-hydroxyacyl-CoA dehydrogenase	2.9619008	0.02549733	110.013824	325.8500337
AGAP000247-RA	Protein coding	2.9596868	1.51149E-05	123.185051	364.5891662
AGAP028532-RA	Protein coding	2.9492847	0.002201286	1523.13546	4492.160163
AGAP008198-RA	Protein coding	2.9380245	0.000169444	708.056357	2080.286955
AGAP004497-RA	Protein coding	2.9346957	0.000834689	3619.89369	10623.28648
AGAP012949-RA	Protein coding	2.9308705	9.28764E-07	16.8547803	49.39917853
AGAP004192-RA	heat shock 70kDa protein 5	2.9249726	0.001237003	10098.7552	29538.58246
AGAP000143-RA	beta-mannosidase	2.867589	0.007101786	520.976214	1493.945661
AGAP001791-RA	Nep2: neprilysin, neutral endopeptidase 2	2.8664128	0.003999927	6516.93219	18680.21809
AGAP005837-RA	COEJHE5E: carboxylesterase	2.8658103	2.65119E-07	467.15536	1338.778635
AGAP028025-RA	Protein coding	2.858793	0.01961653	706.308718	2019.190399
AGAP000309-RA	Protein coding	2.8334171	0.016858618	260.252244	737.4031657
AGAP028067-RA	Protein coding	2.8208007	0.000623835	104.492081	294.7513332
AGAP008682-RA	CYP307B1: cytochrome P450	2.7776863	2.06101E-05	30.8004184	85.55390038
AGAP010642-RA	Protein coding	2.7722568	1.46929E-06	381.518152	1057.66629
AGAP005548-RA	Alpha-crystallin B chain	2.7685934	4.32458E-06	100.34935	277.8265467

Feature ID	Description	Fold change (norm)	Baggerley's test: FDR p-value (norm)	Control RPKM (norm)	Treatment RPKM (norm)
AGAP005064-RB	Protein coding	2.7654929	0.000815131	16.7263587	46.2566256
AGAP013161-RA	Fatty-acid amide hydrolase 2	2.7542887	0.003862479	2230.25193	6142.757644
AGAP007265-RA	Protein coding	2.7499818	0.023032056	20.7486784	57.05848864
AGAP010328-RA	Protein coding	2.7298905	0.02226518	119.408639	325.9725102
AGAP007633-RC	solute carrier family 36 (proton-coupled amino acid transporter)	2.7252218	1.43489E-07	1407.81092	3836.596974
AGAP004256-RB	Protein coding	2.7225515	8.1557E-10	173.979469	473.6680593
AGAP004567-RA	Transmembrane protease serine 11B	2.7166961	0	257.762145	700.2614243
AGAP006487-RA	Trypsin-alpha	2.7116015	0.003260675	4869.51606	13204.18708
AGAP011028-RA	CYP6AF1: cytochrome P450	2.7036532	1.85992E-11	48.3926831	130.8370343
AGAP008152-RA	Protein coding	2.6995114	5.90571E-09	292.994936	790.9431818
AGAP004805-RB	Protein coding	2.6790277	0.011934751	50.8976308	136.3561616
AGAP002733-RA	Protein coding	2.6515658	0.000876293	134.473938	356.5664876
AGAP008548-RA	Protein coding	2.6475138	0.038155035	5539.65724	14666.31903
AGAP001983-RA	Protein coding	2.6404935	0	684.858455	1808.36427
AGAP007747-RA	Protein coding	2.6360618	2.40069E-13	7624.40254	20098.39627
AGAP002847-RA	Niemann-Pick Type C-2	2.6344508	1.71685E-05	3112.99194	8201.024052
AGAP010473-RB	Protein coding	2.634393	0.032536838	34.5719811	91.07618504
AGAP004428-RA	Midline fasciclin	2.618722	0.010853063	7113.86713	18629.24058
AGAP005390-RA	Protein coding	2.5919385	0.001452844	345.652507	895.9100552
AGAP028028-RA	LRIM16A: leucine-rich immune protein (TM)	2.5832323	0.005418519	9.68703213	25.02385468
AGAP000929-RA	CTLSE1: C-Type Lectin (CTL) - selectin like	2.5675416	3.57233E-05	296.381602	760.972105
AGAP007076-RA	Protein coding	2.5486985	0.04278609	61.8169201	157.5526886
AGAP012477-RA	gamma-butyrobetaine dioxygenase	2.5464626	0.024963926	23.6396365	60.19745119
AGAP004761-RA	Protein coding	2.5461201	8.90041E-05	668.546466	1702.199625
AGAP006256-RA	Cad74A	2.5440229	0.002954005	763.226289	1941.665119
AGAP008387-RA	Protein coding	2.5358558	0	5234.67471	13274.38018

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AGAP000184-RB	malate dehydrogenase (oxaloacetate-decarboxylating)(NADP+)	2.5267032	0.036240304	2540.19266	6418.31293
AGAP004205-RA	N-sulfoglucosamine sulfohydrolase	2.5190712	0.000475924	644.85801	1624.443225
AGAP028208-RA	CPLCP22: cuticular protein (putative) CPLCP22	2.5097013	0.001997342	16.979365	42.61313416
AGAP007836-RB	Protein coding	2.5046849	0.03045854	37.7127727	94.45861262
AGAP012324-RA	Protein coding	2.4984536	9.2254E-05	186.274494	465.398182
AGAP012577-RA	Protein coding	2.4969041	0.03120574	9185.74125	22935.91517
AGAP004918-RA	Fibrinogen	2.4807189	0.001606521	294.371771	730.2536231
AGAP004846-RA	SCRB9: Class B Scavenger Receptor (CD36 domain).	2.4724396	0.004272672	336.383611	831.6881615
AGAP002070-RA	Protein coding	2.4654836	0.009299411	239.317651	590.0337359
AGAP008227-RA	trehalose 6-phosphate synthase/phosphatase	2.443767	0.017621131	2211.5224	5404.44555
AGAP028557-RA	Protein coding	2.4330305	0.043373602	1900.20784	4623.263602
AGAP008022-RA	CYP12F1: cytochrome P450	2.4231348	0.012865645	88.0515154	213.360689
AGAP004256-RA	Protein coding	2.3849998	0.001120622	4868.60599	11611.62429
AGAP007744-RA	Protein coding	2.3792369	0.006642489	5230.08128	12443.60247
AGAP010398-RA	Flavin-containing monooxygenase FMO GS-OX-like 1	2.3705842	0.000153288	362.219126	858.6709365
AGAP005213-RB	myosin XV	2.3678375	0.010331942	263.277921	623.3993248
AGAP004530-RA	Protein coding	2.360741	0.000419698	1477.5349	3488.07725
AGAP009182-RA	protein transport protein SEC61 subunit alpha	2.3547746	0.016194266	14711.229	34641.62789
AGAP009123-RA	Protein coding	2.3451022	0	227.912258	534.4775418
AGAP009763-RA	Protein coding	2.3439701	0.00021109	62.4546796	146.3919035
AGAP010395-RA	Calpain-C	2.3369512	0.028968365	836.599347	1955.091878
AGAP004426-RA	Protein coding	2.328894	0.037562858	626.679175	1459.469343
AGAP007006-RB	Filamin	2.3195082	0.001304609	4248.80243	9855.132288
AGAP003277-RA	cathepsin D	2.316789	0.024225395	9593.1714	22225.35399
AGAP000795-RB	Protein coding	2.314124	0.01138149	146.011024	337.8876156
AGAP002810-RA	45 kDa calcium-binding protein	2.2984921	0.039545965	385.145867	885.2547353

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AGAP006893-RA	Protein coding	2.2771963	1.46934E-06	58.0616841	132.217851
AGAP005637-RA	aldehyde oxidase	2.2748976	0.001520168	16.8536006	38.34021629
AGAP005630-RA	transitional endoplasmic reticulum ATPase	2.2637029	0.000209543	10131.3483	22934.36255
AGAP002955-RA	leishmanolysin-like peptidase	2.2512825	0.023320726	242.70277	546.3924891
AGAP002725-RA	Protein coding	2.2442632	0	866.571444	1944.814386
AGAP001510-RA	Protein coding	2.2439975	3.53504E-10	38252.5763	85838.68687
AGAP002361-RA	Protein coding	2.2383405	0.004341947	1110.16536	2484.928109
AGAP010237-RA	Protein coding	2.2367701	1.52719E-07	40.1006327	89.69589661
AGAP013368-RA	Lysosomal acid lipase	2.2336985	0.007568633	16.60241	37.08477821
AGAP001424-RA	heat shock protein 90kDa beta	2.2332142	0.001942782	4206.26129	9393.48237
AGAP000235-RA	Thymosin	2.2318499	0	8551.2434	19085.09132
AGAP007215-RA	phosphoacetylglucosamine mutase	2.2281401	0	918.61168	2046.795527
AGAP011025-RA	dsRNase	2.2196335	1.04232E-05	29.0432419	64.46535211
AGAP012161-RA	Guanylate cyclase	2.21277	5.00197E-10	298.385794	660.2591215
AGAP008347-RA	GPRRK: putative glycoprotein hormone rk-like receptor	2.202983	4.036E-06	138.862875	305.9125557
AGAP004920-RA	CASPS6: short caspase 6	2.1968414	4.38528E-09	82.7919595	181.8808057
AGAP004883-RA	Protein coding	2.1743334	0.001187144	729.618028	1586.43287
AGAP000426-RB	benzodiazapine receptor	2.1700731	2.65349E-07	877.354061	1903.922489
AGAP006485-RA	Trypsin-alpha	2.1684762	2.06011E-09	4249.64684	9215.25796
AGAP005449-RB	E3 ubiquitin-protein ligase CBL	2.1601528	0.00715988	153.284708	331.1183874
AGAP009002-RA	Protein coding	2.15919	0.019905844	381.13261	822.9377119
AGAP003066-RA	CYP304B1: cytochrome P450	2.1484975	0.047594413	1653.9762	3553.563668
AGAP011197-RA	Protein coding	2.1479741	0.009154781	125.315358	269.1741406
AGAP000278-RA	OBP9: odorant binding protein 9	2.1450235	0.00168065	60021.7599	128748.0841
AGAP001745-RA	26S proteasome regulatory subunit RPN2	2.1405753	6.38714E-14	5034.71205	10777.18029
AGAP007889-RA	UDP-N-acetylglucosamine pyrophosphorylase	2.1404342	0	2141.75274	4584.280897

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AGAP004413-RA	tubulin monoglycylase TTLL15	2.1314982	0.009027085	196.934022	419.7645036
AGAP002051-RA	ABCG14: ATP-binding cassette transporter (ABC transporter) family G member 14	2.1285297	2.44888E-09	930.652133	1980.920721
AGAP007886-RB	Protein coding	2.1133168	0.019172119	183.26086	387.2882552
AGAP010165-RA	dynein light intermediate chain 2, cytosolic	2.0959202	1.44363E-05	57.9358393	121.4288974
AGAP001026-RB	Protein coding	2.0829695	0	126.697119	263.90623
AGAP003623-RC	long-chain acyl-CoA synthetase	2.0806683	0.000549972	1397.98008	2908.732813
AGAP002689-RA	Protein coding	2.0730455	0.005552993	264.900718	549.1512492
AGAP000885-RA	aminopeptidase N	2.065197	0.014111256	2450.77687	5061.33696
AGAP007970-RA	APG18A: autophagy related gene	2.0624905	3.61046E-08	1687.78686	3481.044324
AGAP005404-RA	lectin, mannose-binding 1	2.0590716	0.025173979	2849.79913	5867.940329
AGAP006486-RA	Prss3	2.0516056	2.64705E-06	4360.39519	8945.811067
AGAP011575-RA	COE15O: carboxylesterase	2.0473721	0.002930031	20.8749214	42.73873191
AGAP005496-RA	LRIM12: leucine-rich immune protein (Short)	2.0463924	1.95912E-06	42.2360048	86.4314376
AGAP002463-RB	ubiquitin associated and SH3 domain-containing protein B	2.0404459	0.007118532	696.142472	1420.441087
AGAP002481-RA	26S proteasome regulatory subunit N1	2.0354478	1.24321E-13	5141.33154	10464.91184
AGAP010175-RB	Adenylyl cyclase-associated protein 1	2.03303	0.001779624	4895.75838	9953.223714
AGAP004212-RA	Crc: calreticulin	2.016788	0.000108414	35071.5627	70731.90704
AGAP001341-RA	bleomycin hydrolase	2.0055481	5.48481E-10	5994.55781	12022.3743
AGAP000889-RA	Coactosin-like protein	1.997681	0.001706006	6891.17707	13766.37325
AGAP004200-RA	Alpha-tocopherol transfer protein-like	1.9859033	0.01309262	28.4149179	56.42927961
AGAP002836-RA	Protein coding	1.978533	0.032419164	18.2349529	36.07845562
AGAP005167-RA	Short chain dehydrogenase/3-oxoacyl-(acyl-carrier protein) reductase	1.9772628	0.041042378	77.0181066	152.2850341
AGAP003418-RA	stearoyl-CoA desaturase (delta-9 desaturase)	1.9717471	5.00634E-06	2810.06067	5540.728857
AGAP013239-RA	Juvenile hormone-inducible protein	1.969773	0.004805836	1504.26141	2963.053579
AGAP002579-RA	Protein coding	1.9536533	0.041462341	942.695748	1841.700653
AGAP008100-RA	Spire	1.9484016	0.04211295	85.174961	165.9550318

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AGAP003886-RA	Protein coding	1.9450019	0.033005933	960.759414	1868.678898
AGAP000651-RB	Actin5C: actin	1.9425316	0.00228004	73180.2627	142154.9713
AGAP009016-RA	LIX1-like protein	1.9358998	0.001177663	815.394111	1578.521328
AGAP001256-RC	3'-phosphoadenosine 5'-phosphosulfate synthase	1.9358832	0.006642489	827.058369	1601.088366
AGAP002329-RA	carnitine O-octanoyltransferase	1.9348913	0.03579443	1362.16166	2635.63477
AGAP001635-RA	sodium-coupled monocarboxylate transporter 2	1.9294025	0.011239922	61.7011669	119.0463852
AGAP000376-RA	Protein coding	1.9278986	0.010975386	4156.25729	8012.842783
AGAP008830-RA	non-lysosomal glucosylceramidase	1.9218275	0.014827478	406.086621	780.42842
AGAP006745-RA	Protein coding	1.9197431	0.032794313	841.857765	1616.150649
AGAP012317-RA	Protein coding	1.9185335	0.000231145	367.352086	704.7772952
AGAP028016-RA	CPLCP15: cuticular protein (putative) CPLCP15	1.9091569	0.000136331	37.7121415	71.99839689
AGAP012544-RA	CASPS14: short caspase 14	1.9076214	0.002641738	176.86457	337.3906469
AGAP007895-RA	A-kinase anchor protein 10	1.9074118	0.030574158	298.887097	570.1007882
AGAP004890-RA	ribose-phosphate pyrophosphokinase	1.9030252	0.047177636	841.604011	1601.593618
AGAP011828-RA	cathepsin L	1.8944148	0.035590412	8346.23719	15811.23543
AGAP000795-RA	Protein coding	1.8908986	5.87659E-11	124.313151	235.0635622
AGAP012769-RA	Protein coding	1.8880692	0.027766538	107.755572	203.4499817
AGAP007099-RA	Protein coding	1.8758599	9.60142E-06	227.783428	427.2897872
AGAP009255-RA	Sorting nexin-2	1.8606727	0.041903839	768.112908	1429.206715
AGAP002888-RC	GPRNNA3: putative GPCR class a orphan receptor 3	1.8544849	0.004049185	27.788529	51.53340698
AGAP010929-RA	tubulin beta	1.8484098	1.52719E-07	26524.1354	49027.47113
AGAP001557-RA	solute carrier family 12 (sodium/potassium/chloride transporter), member 2	1.8479691	0.009692094	568.21324	1050.040531
AGAP028533-RA	Protein coding	1.8447393	0.007972016	2491.74407	4596.618187
AGAP006088-RA	Protein coding	1.8299841	3.88345E-05	179.50124	328.4844124
AGAP010870-RA	solute carrier family 27 (fatty acid transporter), member 1/4	1.82972	0.027995297	2061.15668	3771.339624
AGAP011917-RA	serine-type enodpeptidase	1.8214308	0	5462.47336	9949.517316

Feature ID	Description	Fold change (norm)	Baggerley's test: FDR p-value (norm)	Control RPKM (norm)	Treatment RPKM (norm)
AGAP003487-RA	Mesenchymal stem cell protein DSCD75	1.810215	1.77443E-08	941.928223	1705.092614
AGAP004510-RA	Innexin inx2	1.8075331	0.047594413	9805.31399	17723.42995
AGAP009076-RA	carnitine O-acetyltransferase	1.8040303	0.000602467	2230.38294	4023.678296
AGAP001323-RA	Protein coding	1.7893757	0.024613812	6707.73241	12002.65341
AGAP028140-RA	Protein coding	1.7860442	0.045287556	48.3927352	86.4315639
AGAP009466-RG	Protein coding	1.7857145	0.000144231	110.141425	196.681141
AGAP001999-RB	protein neutralized	1.7718635	0.019984378	1168.46078	2070.353039
AGAP009701-RA	alanyl-tRNA synthetase	1.7671199	0.000251224	4835.63552	8545.14797
AGAP000235-RC	Thymosin	1.7629139	2.21846E-05	862.303918	1520.167587
AGAP001681-RA	ubiquitin conjugation factor E4 A	1.7577399	4.65479E-07	1558.4819	2739.405818
AGAP002711-RB	stardust, isoform B	1.7576547	0.005458223	263.142512	462.513683
AGAP004394-RA	dipeptidyl-peptidase III	1.7540501	9.49086E-06	3197.80294	5609.106698
AGAP005834-RA	COEJHE2E: carboxylesterase	1.7525756	0.000272052	634.693925	1112.349082
AGAP006216-RA	GPRMTH2: methuselah receptor 2	1.7486028	0.000159216	280.706261	490.8437463
AGAP004961-RA	Protein coding	1.742005	0.024879673	25.3991128	44.24538116
AGAP006576-RB	malate/L-lactate dehydrogenase	1.7381908	0.000169313	2352.58882	4089.248273
AGAP012167-RA	Pgi: phosphoglucose isomerase (glucose-6-phosphate isomerase)	1.7309742	0.001123855	9864.93804	17075.95367
AGAP012166-RA	Protein coding	1.728389	0.006631923	610.856772	1055.798116
AGAP007169-RA	Sestrin	1.7269595	1.82584E-06	636.057374	1098.445295
AGAP007736-RA	Protein coding	1.7268924	0.014516057	923.874188	1595.431324
AGAP008228-RA	dual specificity phosphatase	1.7168746	0.002930031	504.151337	865.5645999
AGAP003220-RB	Protein coding	1.7128091	0.048351955	58.8131263	100.7356572
AGAP002403-RA	Nucleoside diphosphate-linked moiety X motif 18	1.7114009	0	425.531277	728.2546204
AGAP008864-RA	Protein coding	1.7085807	0.033872286	242.964433	415.1243511
AGAP001721-RA	translocon-associated protein subunit alpha	1.7074435	0.024613812	10038.6194	17140.37555
AGAP003300-RA	solute carrier family 39 (zinc transporter)	1.7068458	3.14679E-05	1350.81383	2305.630953

Feature ID	Description	Fold change (norm)	Baggerley's test: FDR p-value (norm)	Control RPKM (norm)	Treatment RPKM (norm)
AGAP003929-RA	MMP3: matrix metallopeptidase 3	1.7064179	8.06259E-12	241.583946	412.2431649
AGAP028133-RA	Protein coding	1.706078	5.4597E-05	1206.56179	2058.488512
AGAP011363-RA	Ras-related protein Rab-6A	1.7039191	3.27949E-06	2862.9253	4878.19311
AGAP005716-RA	SCRB16: Class B Scavenger Receptor (CD36 domain)	1.7022488	0.002131239	232.67503	396.0707898
AGAP000204-RA	Protein coding	1.6947179	0.004618267	220.763166	374.1312948
AGAP001961-RA	GPRVPR2: GPCR vasopressin family receptor 2	1.6938213	3.50234E-06	83.1678024	140.8713926
AGAP010139-RD	GMP synthase (glutamine-hydrolysing)	1.6932122	0	453.363795	767.6411076
AGAP001634-RA	Protein coding	1.6888314	2.43261E-09	204.081232	344.6587986
AGAP011603-RA	long-chain acyl-CoA synthetase	1.6884103	9.46941E-07	1219.46607	2058.959048
AGAP002866-RA	CYP6P5: cytochrome P450	1.6861961	0.000355269	2147.19598	3620.593507
AGAP010331-RA	Protein coding	1.6801303	0.003384733	5307.80559	8917.804943
AGAP008274-RA	snail, invertebrate	1.6703908	6.80442E-05	172.476622	288.1033604
AGAP010973-RA	Sodium/potassium/calcium exchanger 3	1.6703581	0.001276275	505.65431	844.6237654
AGAP001313-RA	Muscular protein 20	1.6657966	4.04379E-10	8914.27755	14849.37315
AGAP009666-RA	Protein coding	1.661257	0.003081114	141.874222	235.6895402
AGAP001372-RC	Protein coding	1.6572334	0.000791874	1457.96967	2416.19605
AGAP011679-RA	Protein coding	1.6540792	0	8064.49125	13339.30757
AGAP006606-RA	Protein coding	1.6526092	0.000349667	201.952118	333.7479332
AGAP003215-RA	26S proteasome regulatory subunit T2	1.6440154	2.33324E-05	2985.97518	4908.989176
AGAP009649-RA	solute carrier family 17, member 5	1.6397299	0	5107.2752	8374.551846
AGAP009668-RA	lysosomal-associated membrane protein 1/2	1.6376791	0.046955117	5344.1021	8751.924063
AGAP006254-RA	polypeptide N-acetylglucosaminyltransferase	1.6318055	0.035826302	2893.20656	4721.150428
AGAP002017-RA	Hexosyltransferase	1.6309772	9.37673E-06	102.366211	166.9569533
AGAP001890-RA	Protein coding	1.6299801	0.008866615	5438.34524	8864.394745
AGAP004165-RA	GSTD2: glutathione S-transferase delta class 2	1.6297003	0.005778446	2302.38503	3752.197525
AGAP008705-RA	Protein coding	1.6290052	0.002002321	160.311172	261.1477389

Feature ID	Description	Fold change (norm)	Baggerley's test: FDR p-value (norm)	Control RPKM (norm)	Treatment RPKM (norm)
AGAP008397-RA	Protein coding	1.6159237	4.30426E-05	1095.94629	1770.965615
AGAP007784-RA	enoyl-CoA hydratase / long-chain 3-hydroxyacyl-CoA dehydrogenase	1.6158265	0.008097425	4622.64925	7469.399121
AGAP008034-RA	solute carrier family 39 (zinc transporter), member 7	1.6130482	0.037425038	2703.94676	4361.596342
AGAP013001-RA	aminopeptidase N	1.6110513	0.025173979	1319.83845	2126.327427
AGAP006084-RA	Protein coding	1.609281	0.034417083	255.621475	411.3667761
AGAP010158-RA	Protein coding	1.6062248	4.43326E-06	512.028805	822.43337
AGAP010972-RA	Protein coding	1.6016194	1.54604E-06	749.812898	1200.914859
AGAP001267-RA	Adaptor-related protein complex 2, beta 1 subunit	1.5961011	0.030988177	2860.79629	4566.119977
AGAP000553-RA	w: protein white	1.5927471	0.040784101	154.04004	245.346821
AGAP004227-RA	ADP-ribosylation factor-like protein	1.5907238	0.02109035	2962.03813	4711.784481
AGAP001391-RA	Tetracycline transporter	1.5899539	0	599.441534	953.0844248
AGAP009466-RE	Protein coding	1.5845354	1.10452E-05	583.767294	924.9999158
AGAP008515-RA	Protein coding	1.5839811	3.09724E-07	289.857855	459.1293748
AGAP008494-RB	signal transducing adaptor molecule	1.5704679	2.92986E-07	1061.68687	1667.345182
AGAP010441-RA	Protein coding	1.565911	0.003944582	122.558903	191.9163331
AGAP009258-RA	Protein coding	1.5641455	5.58689E-10	330.239601	516.5428011
AGAP010420-RA	valyl-tRNA synthetase	1.5637036	4.60177E-05	2034.67224	3181.624342
AGAP003623-RA	long-chain acyl-CoA synthetase	1.5633423	5.68746E-06	1430.84686	2236.903457
AGAP004450-RA	Protein coding	1.5591697	4.84799E-10	442.454827	689.8621671
AGAP005749-RA	GSTO1: glutathione S-transferase omega class 1	1.5587487	1.86698E-06	5589.65893	8712.873806
AGAP011357-RA	All-trans/9-cis/11-cis	1.5507702	2.62413E-12	937.159786	1453.319485
AGAP004118-RA	SCRAL1: Class A Scavenger Receptor (SRCR domain) with Lysyl Oxidase domain.	1.5504695	0.006106851	440.570106	683.090505
AGAP013206-RA	Protein coding	1.5496966	2.54011E-06	166.332349	257.7646812
AGAP009851-RA	Protein coding	1.5488093	0.012294634	159.057148	246.3491937
AGAP007827-RA	Protein coding	1.5465986	6.40185E-09	47881.6257	74053.65721
AGAP001697-RB	20S proteasome subunit beta 3	1.5374503	0.00025159	820.920831	1262.124988

Feature ID	Description	Fold change (norm)	Baggerley's test: FDR p-value (norm)	Control RPKM (norm)	Treatment RPKM (norm)
AGAP011984-RA	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 20	1.5363302	1.87166E-11	535.729432	823.0572831
AGAP007534-RC	Protein coding	1.5340349	0.001266299	818.167494	1255.09753
AGAP001109-RA	protein-tyrosine phosphatase	1.5311521	3.26172E-07	1187.6074	1818.40757
AGAP000943-RB	Protein coding	1.5265541	0.038179431	41.9832174	64.08965103
AGAP010390-RA	COE12O: carboxylesterase	1.52376	0.002209565	76.8925639	117.1658163
AGAP004758-RA	Protein coding	1.5231999	0	4014.34675	6114.652538
AGAP012248-RA	ubiquitin carboxyl-terminal hydrolase 5/13	1.517588	0.000526309	2210.5365	3354.683619
AGAP001986-RA	nuclear protein localization protein 4 homolog	1.5154941	6.38714E-14	2166.12852	3282.754948
AGAP005535-RA	26S proteasome regulatory subunit N5	1.5141826	0.001692759	4691.07982	7103.151436
AGAP012693-RA	Protein coding	1.5103854	0.008683695	238.572597	360.3365614
AGAP007007-RA	Protein coding	1.5038581	0.011715811	678.459054	1020.306115
AGAP012641-RA	Aldose reductase	1.5025544	0.000284279	639.322148	960.6162859
AGAP008880-RA	Protein coding	1.5015067	0.01072719	198.809473	298.5137487
AGAP012088-RA	stromal membrane-associated protein	1.5001494	0.001805505	543.636734	815.5363072
AGAP000165-RA	GSTMS1: glutathione transferase microsomal 1	-1.5000728	1.12796E-05	5340.06666	3559.871657
AGAP011912-RA	Protein coding	-1.5016342	0.002860086	10516.9326	7003.658393
AGAP012835-RA	Protein coding	-1.5021013	8.11412E-05	172.978867	115.157922
AGAP007115-RA	Protein coding	-1.5029177	0.000168511	940.296267	625.6472197
AGAP003571-RA	threonine dehydratase	-1.5096026	3.74353E-06	3636.66394	2409.020653
AGAP005076-RC	Protein coding	-1.5098599	0.027138466	6270.89134	4153.293517
AGAP011727-RA	Protein coding	-1.5200349	0.022502822	1342.1693	882.9858556
AGAP007894-RA	Translation elongation factor G	-1.5212121	2.45861E-07	828.698477	544.7619691
AGAP004137-RA	Protein coding	-1.5254345	2.70622E-05	194.797918	127.6999524
AGAP001053-RG	Protein coding	-1.5333122	0.045287556	8338.03648	5437.924819
AGAP011830-RA	myo-inositol-1-phosphate synthase	-1.5352102	2.43685E-07	3464.58345	2256.748524

Feature ID	Description	Fold change (norm)	Baggerley's test: FDR p-value (norm)	Control RPKM (norm)	Treatment RPKM (norm)
AGAP006126-RB	GPROP8: ultraviolet wavelength sensitive opsin	-1.5386362	0.000149886	1844.85771	1199.02138
AGAP007572-RA	All-trans/9-cis	-1.5409379	6.26396E-06	850.269325	551.7868949
AGAP012012-RB	Protein coding	-1.5442563	2.88684E-13	477.051741	308.9200449
AGAP003705-RB	Set1/Ash2 histone methyltransferase complex subunit ASH2	-1.5471929	0.000466437	397.451587	256.8856031
AGAP001737-RA	MOCS3 Molybdopterin-synthase adenylyltransferase Molybdopterin-synthase sulfurtransferase	-1.5489141	0.03045854	1129.93788	729.5032429
AGAP006443-RA	Venom allergen	-1.5489973	2.81703E-24	20506.8059	13238.76184
AGAP000855-RA	Protein coding	-1.549837	4.88373E-07	1891.12296	1220.207612
AGAP003214-RA	Protein coding	-1.5525793	3.93429E-15	465.0198	299.5143645
AGAP007285-RA	alpha-L-fucosidase	-1.5536712	1.50767E-14	1113.72494	716.8343749
AGAP009612-RA	glyoxylate/hydroxypyruvate reductase	-1.554965	7.10576E-05	2324.40299	1494.826597
AGAP003508-RA	ATP-dependent RNA helicase SUPV3L1, mitochondrial	-1.5645066	1.16019E-27	814.411115	520.5545911
AGAP009100-RA	Protein-glutamine gamma-glutamyltransferase E	-1.5661922	0.000130208	134.975774	86.18084962
AGAP009196-RA	GSTE7: glutathione S-transferase epsilon class 7	-1.5781515	3.081E-06	440.825639	279.3303696
AGAP005524-RB	Protein coding	-1.5797857	0.023794065	448.214853	283.7187637
AGAP002409-RA	tRNA pseudouridine38-40 synthase	-1.5835013	0.004912252	2786.61093	1759.778102
AGAP001248-RA	Eupolytin	-1.5842626	0.000139809	3919.30231	2473.896838
AGAP028544-RC	Protein coding	-1.5858563	0.010353869	143.633608	90.57164012
AGAP028448-RA	Protein coding	-1.5861755	0.009305568	558.428129	352.0594798
AGAP008884-RA	protoheme IX farnesyltransferase	-1.5882087	1.1444E-10	8194.32112	5159.473798
AGAP007460-RA	Acyl-CoA-binding protein 2	-1.5938363	0.029606652	120.551349	75.63596799
AGAP001706-RA	Protein coding	-1.5948691	0.033005933	198.063854	124.1881576
AGAP000843-RA	cardiolipin synthase	-1.5950046	0.003795442	1821.24916	1141.845688
AGAP009221-RA	SRPN5: serine protease inhibitor (serpin) 5	-1.5965321	0.024472116	537.244866	336.5074076
AGAP000416-RB	hydroxyacylglutathione hydrolase	-1.6000739	4.54561E-20	2177.28049	1360.73742
AGAP008484-RA	tRNA-dihydrouridine synthase 1	-1.6017423	4.77879E-19	943.682104	589.1597468

Feature ID	Description	Fold change (norm)	Baggerley's test: FDR p-value (norm)	Control RPKM (norm)	Treatment RPKM (norm)
AGAP007684-RB	Tubulointerstitial nephritis antigen	-1.6064814	0.005458223	797.60192	496.4899943
AGAP005378-RB	Protein coding	-1.608271	2.64705E-06	584.14684	363.2141783
AGAP028686-RA	Protein coding	-1.6103361	0.026529047	208.471284	129.4582479
AGAP008593-RA	Protein coding	-1.616393	0.004867152	418.264168	258.7639055
AGAP002791-RC	tRNA-dihydrouridine synthase 4	-1.6187129	2.62358E-05	172.60166	106.6289546
AGAP006512-RA	Protein coding	-1.6223144	0.010480281	856.914583	528.2049943
AGAP005113-RA	Thiamine pyrophosphate carrier	-1.6293389	0.005522831	125.694784	77.14465361
AGAP003934-RA	battenin	-1.6325541	4.63253E-18	2879.29384	1763.674391
AGAP005653-RA	Solute carrier family 7 (cationic amino acid transporter, y+ system)	-1.6326508	5.13265E-05	940.794675	576.2375301
AGAP004384-RA	Protein coding	-1.6362653	5.69816E-11	811.145717	495.7299647
AGAP002520-RA	short-chain dehydrogenease/reductase	-1.6402042	4.33601E-61	2448.52474	1492.817021
AGAP002378-RA	adenylosuccinate lyase	-1.6460205	1.01907E-12	1959.99841	1190.749713
AGAP028453-RA	Protein coding	-1.6473076	6.93127E-15	1661.9601	1008.894799
AGAP004236-RA	Protein coding	-1.6490678	4.30291E-10	2180.02921	1321.976731
AGAP011183-RA	Protein coding	-1.6492687	0.009920205	32147.4548	19491.94499
AGAP003074-RB	solute carrier family 25 (mitochondrial dicarboxylate transporter), member 10	-1.6549681	8.76028E-21	1407.78548	850.6420738
AGAP000681-RA	tRNA:m4X modification enzyme	-1.6562741	3.28779E-16	1273.29174	768.7687294
AGAP013180-RA	Spindle B	-1.6585571	0.008166633	127.326249	76.76928706
AGAP006971-RA	Protein coding	-1.6611068	3.73936E-70	2403.27723	1446.792762
AGAP011207-RA	Spermine oxidase	-1.6690174	0.007941692	1415.55675	848.1378053
AGAP003688-RA	threonine aldolase	-1.6724127	6.41398E-12	2376.00608	1420.705585
AGAP000447-RA	Protein coding	-1.6756217	9.14837E-13	790.960327	472.039909
AGAP027997-RA	LRIM5: leucine-rich immune protein (Short)	-1.6774915	0.011677807	294.375887	175.4857659
AGAP011603-RB	long-chain acyl-CoA synthetase	-1.6779524	0.002047743	867.192705	516.8160398
AGAP004642-RA	NPF: neuropeptide F	-1.6781901	6.92577E-13	733.26977	436.9408162
AGAP005379-RA	Protein coding	-1.6805371	0.010346882	58.9415857	35.07306497

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AGAP007312-RC	Protein coding	-1.683051	0.000576298	195.928223	116.412528
AGAP007565-RB	Chaperone DnaJ protein	-1.6842771	9.61577E-20	2270.50996	1348.062026
AGAP001053-RB	Protein coding	-1.6891646	4.73976E-38	7255.63133	4295.396181
AGAP028482-RB	Protein coding	-1.6905624	0.004289689	119.170217	70.49146439
AGAP001422-RA	Protein coding	-1.6915875	0.001397281	2416.56795	1428.579891
AGAP005241-RA	Protein coding	-1.6934198	7.79567E-07	826.317745	487.9579935
AGAP005611-RA	Protein coding	-1.6938549	1.31561E-12	2120.42181	1251.832025
AGAP012732-RA	Protein coding	-1.6950604	0.004886363	101.613198	59.94665459
AGAP009633-RA	3-oxoacyl-(acyl-carrier protein) reductase fabG2	-1.6988723	4.4723E-110	3195.29852	1880.835032
AGAP001942-RA	Protein coding	-1.705727	0.020603171	3961.90481	2322.707488
AGAP000672-RB	Obg-like ATPase 1	-1.7121974	1.83416E-25	1208.44311	705.7849359
AGAP009309-RA	Protein coding	-1.7148341	0.007678499	73.5038429	42.86353088
AGAP028742-RA	Protein coding	-1.7195517	0.000135659	383.278761	222.8945856
AGAP000431-RA	Protein coding	-1.7253759	0.011461762	53.7948942	31.17865232
AGAP008301-RA	Protein coding	-1.7265682	1.34199E-05	323.343583	187.2753029
AGAP009489-RA	cytidine deaminase	-1.73447	2.49181E-15	1412.79126	814.5377527
AGAP002609-RA	Protein coding	-1.7381908	0.006870391	410.110918	235.9412569
AGAP005071-RB	calcium release-activated calcium channel protein 1	-1.7415302	0.047222768	363.712896	208.84673
AGAP007343-RA	LYSC2: C-Type Lysozyme.	-1.7544692	0.000818436	74.7593338	42.61079854
AGAP006725-RA	COEAE3H: carboxylesterase alpha esterase	-1.759017	6.22599E-11	1444.02467	820.9270751
AGAP007241-RA	Protein coding	-1.764785	0.004055731	81.4103476	46.13046193
AGAP010311-RA	Dehydrogenase/reductase SDR family member 11 precursor	-1.768358	3.54111E-35	1170.67623	662.0131363
AGAP011845-RA	histone H2A	-1.77181	0.00040902	1702.6874	960.9875935
AGAP001053-RE	Protein coding	-1.7724831	0.003765422	1003.3704	566.0817794
AGAP028555-RA	Protein coding	-1.7766071	2.62144E-09	490.46699	276.0694769
AGAP009054-RA	Protein coding	-1.7790353	0.002913054	573.227208	322.212386

Feature ID	Description	Fold change (norm)	Baggerley's test: FDR p-value (norm)	Control RPKM (norm)	Treatment RPKM (norm)
AGAP006829-RA	CPR59: cuticular protein RR-1 family 59	-1.7816203	2.29044E-09	8371.4255	4698.770949
AGAP002852-RA	Protein coding	-1.7830756	0.002709522	576.98965	323.5923705
AGAP001823-RA	VATG: V-type H+-transporting ATPase subunit G	-1.7921739	0.007165663	16370.1034	9134.216163
AGAP002831-RB	Ribosomal RNA small subunit methyltransferase H	-1.7981968	5.26811E-07	772.402893	429.5430165
AGAP011887-RA	nuclear pore complex protein Nup37	-1.7987575	1.81757E-08	551.159242	306.4110904
AGAP009192-RA	GSTE5: glutathione S-transferase epsilon class 5	-1.801006	1.94955E-59	1716.06599	952.8374798
AGAP005844-RA	Protein coding	-1.8028541	2.53338E-06	640.57677	355.3126047
AGAP028595-RA	Protein coding	-1.8119913	5.23544E-10	858.291267	473.6729403
AGAP003248-RA	Protein coding	-1.8157853	0.004498128	202.954277	111.7721762
AGAP006344-RA	RAG1-activating protein 1-like protein	-1.8162046	0.012210799	2115.95942	1165.044643
AGAP005887-RA	Protein coding	-1.8200766	1.3968E-14	616.118542	338.5124165
AGAP011027-RA	Protein coding	-1.8213779	7.57462E-10	177.994526	97.72520397
AGAP011281-RA	Protein coding	-1.8368889	3.15649E-14	267.292295	145.513588
AGAP001354-RA	Protein coding	-1.8427033	7.34155E-26	4698.73957	2549.91648
AGAP006603-RA	Protein coding	-1.8443898	0.001088335	2077.33176	1126.297554
AGAP005996-RA	CPR21: cuticular protein RR-1 family 21	-1.8636514	4.39565E-11	14942.5547	8017.891398
AGAP012600-RA	COUP transcription factor 2	-1.8641495	0.026674849	60.6997036	32.56160668
AGAP004895-RA	carbonic anhydrase	-1.8744529	6.20406E-13	3852.37926	2055.201918
AGAP011506-RA	Protein coding	-1.8745146	0.007228087	134.725264	71.87208101
AGAP012808-RA	Protein coding	-1.8811397	0.00121893	64.0911432	34.0703785
AGAP005582-RA	pre-rRNA-processing protein TSR4	-1.8844527	1.28164E-09	755.591093	400.960505
AGAP000573-RA	CLIPC4: CLIP-domain serine protease	-1.8949564	0.040548861	857.662605	452.6028121
AGAP005297-RA	F-box and leucine-rich repeat protein 1 (S-phase kinase-associated protein 2)	-1.9060251	0.000341657	642.58286	337.1324194
AGAP006193-RA	Protein coding	-1.9158264	0.000272178	1872.81168	977.5477009
AGAP013078-RA	Plasma glutamate carboxypeptidase precursor	-1.9270389	3.26223E-06	1333.0147	691.7424942
AGAP000690-RA	Protein coding	-1.9279189	3.35685E-15	275.944138	143.1305717

Feature ID	Description	Fold change (norm)	Baggerley's test: FDR p-value (norm)	Control RPKM (norm)	Treatment RPKM (norm)
AGAP028402-RA	Protein coding	-1.928897	3.71453E-10	29239.7935	15158.81538
AGAP013377-RA	Protein coding	-1.9294718	1.26645E-17	1111.34711	575.9851435
AGAP007949-RA	Protein coding	-1.9305489	0.00155068	539.502599	279.4555421
AGAP001459-RA	RpL17: 60S ribosomal protein L17	-1.9353057	1.20236E-06	3137.69848	1621.293488
AGAP002613-RA	CPR8: cuticular protein RR-1 family 8	-1.9486028	8.76886E-08	1662.93411	853.3982124
AGAP011785-RA	CLIPE6: CLIP-domain serine protease	-1.9495883	0.03463278	89.442867	45.8778234
AGAP006602-RA	Protein coding	-1.9592928	0.004666286	4626.79574	2361.462112
AGAP005685-RA	Protein coding	-1.9679928	0.021507986	9106.4104	4627.25803
AGAP007550-RB	Protein coding	-1.9772067	5.92981E-05	564.198454	285.351282
AGAP013105-RA	Protein coding	-1.9777005	4.90935E-05	4512.42189	2281.650733
AGAP004998-RA	Protein coding	-1.9886197	5.92135E-05	1552.17424	780.5284397
AGAP010689-RA	Protein coding	-1.99891	0.034701896	78.3980118	39.22038027
AGAP007233-RA	Protein coding	-2.0092834	0.000130447	4262.45743	2121.381879
AGAP000194-RA	CYP4C25: cytochrome P450	-2.0106332	0.00037646	369.984019	184.0136868
AGAP006869-RA	Eupolytin	-2.0303347	0.031087218	7766.93564	3825.44592
AGAP006806-RA	Mitochondrial thiamine pyrophosphate carrier	-2.0304633	2.74754E-05	520.066231	256.1317994
AGAP005455-RA	CPR14: cuticular protein RR-1 family 14	-2.0304689	0.019041629	298.264242	146.8942656
AGAP001931-RA	Six-cysteine containing astacin protease 3	-2.0371565	0.020917287	47.1346513	23.13747152
AGAP006425-RA	Cyanogenic beta-glucosidase	-2.0411623	0.000309417	5956.99817	2918.434316
AGAP010239-RA	DnaJ homolog subfamily B member 5	-2.0499845	0.027693438	1470.52707	717.3356882
AGAP007226-RA	Protein coding	-2.0709675	0.03380869	11648.5851	5624.706809
AGAP013491-RA	Protein coding	-2.0780447	0.032389943	26.4058894	12.70708424
AGAP011879-RA	Protein coding	-2.0787312	0.025414658	805.86436	387.6712689
AGAP002612-RA	CPR7: cuticular protein RR-1 family 7	-2.0909139	1.38007E-05	1038.1136	496.487977
AGAP007484-RA	Sugar transporter ERD6-like 4	-2.1029886	0.018920577	607.46125	288.8561718
AGAP008372-RA	Protein coding	-2.1030342	5.06612E-10	3466.88061	1648.513631

Feature ID	Description	Fold change (norm)	Baggerley's test: FDR p-value (norm)	Control RPKM (norm)	Treatment RPKM (norm)
AGAP003541-RA	Eukaryotic translation elongation factor 1 alpha 1	-2.1147361	5.89866E-05	18632.562	8810.821226
AGAP011348-RB	Arylsulfatase b	-2.1260981	5.61801E-05	223.774671	105.251338
AGAP004999-RA	Protein coding	-2.1361034	0.000433076	946.197144	442.9547444
AGAP009981-RA	Protein coding	-2.1371412	0.003260751	1180.59827	552.4194111
AGAP012651-RA	Protein coding	-2.1424212	4.47303E-09	929.383712	433.8006481
AGAP011442-RA	serine carboxypeptidase 1	-2.1646528	0.023032056	10231.1332	4726.454723
AGAP002728-RB	Protein coding	-2.172663	0.000778616	889.388275	409.3539882
AGAP001281-RB	Potassium inwardly-rectifying channel subfamily J	-2.173149	9.75035E-07	243.713752	112.1477418
AGAP000859-RA	Protein coding	-2.2031316	0.002295961	40.7266326	18.48579195
AGAP007042-RA	CPR62: cuticular protein RR-1 family 62	-2.2059029	0.024785371	32.1845091	14.59017483
AGAP003977-RA	Protein coding	-2.2074363	0.000136776	222.643761	100.8607856
AGAP001769-RC	Protein coding	-2.2286691	0.001745724	159.05852	71.36928622
AGAP010854-RA	solute carrier family 45, member 1/2/4	-2.2398311	1.4107E-159	5493.99367	2452.860742
AGAP000079-RA	Protein coding	-2.2418578	6.01402E-07	305.41191	136.2316181
AGAP005497-RA	Protein coding	-2.2431701	2.23241E-09	267.043443	119.047343
AGAP007943-RA	Protein coding	-2.24456	9.27152E-09	1467.36182	653.741402
AGAP011098-RA	Protein coding	-2.2625577	1.17639E-07	3354.61047	1482.662947
AGAP007053-RA	Protein coding	-2.2756776	8.90593E-21	266.915585	117.2905955
AGAP004784-RA	fatty acyl-CoA reductase 2	-2.2772488	1.12024E-09	570.977697	250.7313649
AGAP004831-RA	Protein coding	-2.2897879	0.025327023	974.666173	425.6578497
AGAP003993-RA	Protein coding	-2.3147012	1.14231E-56	2840.7452	1227.262186
AGAP001774-RA	Allatostatin receptor	-2.3183549	0.033876569	20.9986403	9.057560784
AGAP002117-RA	Protein coding	-2.324615	1.07219E-05	2030.76859	873.5935081
AGAP013453-RA	Protein coding	-2.3324012	3.06123E-05	35618.127	15271.01193
AGAP005491-RA	Protein coding	-2.3703778	0.005850427	33.6906785	14.21321074
AGAP006424-RA	Cyanogenic beta-glucosidase	-2.4357226	2.30195E-06	16970.4407	6967.312656

Feature ID	Description	Fold change (norm)	Baggerley's test: FDR p-value (norm)	Control RPKM (norm)	Treatment RPKM (norm)
AGAP009879-RA	CPR81: cuticular protein RR-1 family 81	-2.447344	2.72982E-05	56.933128	23.26323066
AGAP005323-RB	D-aspartate oxidase	-2.4671825	3.95516E-21	987.066772	400.0785377
AGAP001825-RA	salivary lipase	-2.4803465	0.003001918	21760.4201	8773.137294
AGAP013303-RA	Protein coding	-2.5464276	0.030830083	23.3888087	9.184949247
AGAP001021-RB	Protein coding	-2.5619198	3.9309E-06	172.226293	67.22548231
AGAP012843-RA	Protein coding	-2.5690426	3.01226E-05	207.218181	80.65969016
AGAP004380-RA	GSTD12: glutathione S-transferase delta class 12	-2.5773501	4.6474E-13	1811.97716	703.0388056
AGAP007188-RA	Protein coding	-2.5779593	0.001597164	28309.0945	10981.20292
AGAP007344-RA	LYSC8: C-Type Lysozyme.	-2.5939807	3.44262E-15	9008.26441	3472.756899
AGAP010614-RA	Female reproductive tract protease GLEANR_896	-2.5988757	0.000200819	1663.18248	639.9622982
AGAP006985-RA	Protein coding	-2.6216055	5.51506E-36	709.940076	270.8035502
AGAP007346-RA	LYSC5: C-Type Lysozyme.	-2.6381168	1.58265E-32	829.203832	314.3165698
AGAP013729-RA	Protein coding	-2.6605395	1.60024E-15	1887.11899	709.2993643
AGAP007345-RA	LYSC3: C-Type Lysozyme.	-2.6615997	9.37652E-28	46426.6157	17443.12502
AGAP012325-RA	OBP28: odorant binding protein 28	-2.6741354	4.5732E-146	3061.13048	1144.717814
AGAP006419-RA	A5R2: antigen 5 related protein 2	-2.7604923	7.66686E-20	2583.5691	935.9088174
AGAP004799-RA	Protein coding	-2.7989584	0.031497103	3405.56204	1216.724798
AGAP007231-RA	Protein coding	-2.8525205	7.8502E-09	252.620051	88.56029385
AGAP003748-RA	Protein coding	-2.9034485	0.01145209	23.0107499	7.925317093
AGAP003358-RA	Sugar transporter SWEET	-2.9080281	0.020649549	1398.62044	480.9514816
AGAP002195-RA	Protein coding	-2.9635227	0.029590119	224.524921	75.76284914
AGAP012983-RA	Protein coding	-2.9947858	1.16769E-07	214.49283	71.62209466
AGAP006002-RA	CPR137: cuticular protein RR-1 family 137	-2.9983545	0.000322422	29.421374	9.81250674
AGAP013146-RA	Protein coding	-3.0788695	5.25197E-07	393.938766	127.9491587
AGAP001717-RA	Protein coding	-3.0932015	0.047927152	1205.33897	389.6736077
AGAP011878-RA	Protein coding	-3.1262731	1.7681E-05	1333.85815	426.6607936

Feature ID	Description	Fold change (norm)	Baggerley's test: FDR p-value (norm)	Control RPKM (norm)	Treatment RPKM (norm)
AGAP013616-RA	18S_rRNA: 18S ribosomal RNA	-3.1748532	0.027995297	63.0840182	19.86990103
AGAP004149-RA	Protein coding	-3.177984	0.03452564	114.656065	36.07823822
AGAP009875-RA	CPR77: cuticular protein RR-1 family 77	-3.3663019	0.007027858	998.133035	296.5072877
AGAP006000-RA	CPR25: cuticular protein RR-1 family 25	-3.5234495	4.32458E-06	14405.9306	4088.587256
AGAP002552-RA	glucose dehydrogenase (acceptor)	-3.5483702	0.035301319	349.422752	98.47415476
AGAP028705-RA	Protein coding	-4.0679381	3.2515E-118	1199.0291	294.7510675
AGAP013511-RC	Protein coding	-4.1787935	0.00023913	1448.54218	346.641242
AGAP008051-RA	SAP1: sensory appendage protein 1	-4.3041337	7.36398E-05	431.417729	100.2333478
AGAP011806-RA	ornithine decarboxylase	-4.3479312	0.036082713	8481.03353	1950.590556
AGAP012818-RA	Protein coding	-4.7666284	0.035301319	1716.35607	360.0775893
AGAP007197-RA	Niemann-Pick Type C-2	-5.2412694	0.02453329	45.5018406	8.681454234
AGAP011807-RA	Antizyme inhibitor 1	-5.8455577	3.14868E-13	1115.2559	190.7869115
AGAP007641-RA	Protein coding	-7.7005874	0.014045811	22.2583501	2.89047431
AGAP008892-RA	Protein coding	-7.7441144	0.01040658	13.585977	1.754361619
AGAP006835-RA	Protein coding	-8.0791475	3.69981E-06	142.248815	17.60690894
AGAP011276-RA	Protein coding	-8.8017208	2.03247E-17	2013.69205	228.7839035
AGAP013106-RA	Niemann-Pick Type C-2	-9.0273528	3.34181E-27	278.075157	30.8036211
AGAP005597-RA	Protein coding	-10.382744	9.42791E-05	3351.94181	322.8377617
AGAP011277-RA	Protein coding	-16.975791	1.5605E-27	1594.60034	93.93378828
AGAP010617-RA	Protein coding	-18.440174	6.67322E-05	1572.74333	85.28896238
AGAP007415-RA	Niemann-Pick Type C-2	-86.4604	0.004979992	2565.19676	29.66903666

Appendix 5: Summary of the top 200 most abundantly expressed (RPKM) genes in the control larvae.

VectorBase Description	N(%)	Reads Range	RPKM Range
18S rRNA: 18S ribosomal RNA	1(0.5)	99918	63971.23
ADP, ATP carrier protein 1	1(0.5)	198005	126390.20
Alpha amylase: amylase	1(0.5)	87230	59477.93
ATP-dependent RNA helicase DBP2	1(0.5)	67637	41436.43
ATP6: adenosine triphosphatase subunit 6	1(0.5)	98490	164088.01
Ca-P60A: calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type	1(0.5)	292463	188828.17
Chondroitin proteoglycan-2	1(0.5)	25034	29812.60
Chymotrypsin-like protease	1(0.5)	48782	33851.84
citrate synthase	1(0.5)	31373	32064.78
Cofilin	1(0.5)	29308	30429.73
Crc: calreticulin	1(0.5)	49884	35071.56
Creatine kinase	1(0.5)	484371	364891.84
Cysteine-rich venom protein	1(0.5)	33380	45385.09
Enolase	1(0.5)	61602	47881.63
Eukaryotic translation initiation factor 4A, isoform 2	1(0.5)	68654	54331.26
Fatty acyl-CoA reductase	1(0.5)	37093	31569.42
GAPDH: glyceraldehyde 3-phosphate dehydrogenase	1(0.5)	65117	56908.67
GPROP6: long wavelength sensitive opsin 6	1(0.5)	54414	46932.02
Heat shock 70kDa protein 1/8	1(0.5)	226711	133990.43
Lp: lipophorin	1(0.5)	219431	121647.77
lsu rRNA:	1(0.5)	3622409	1555660.76
LYSC3: C-Type Lysozyme.	1(0.5)	43262	46426.62
Metalloendopeptidase	1(0.5)	91643	75559.49
Methylmalonate-semialdehyde dehydrogenase (acylating), mitochondrial	1(0.5)	39391	27966.02
ND4: NADH dehydrogenase subunit 4	1(0.5)	29180	27961.22
Niemann-Pick Type C-2	1(0.5)	47386	38223.33
Nucleoside-diphosphate kinase	1(0.5)	38817	42095.97
OBP9: odorant binding protein 9	1(0.5)	60601	60021.76
Paramyosin	1(0.5)	74960	44719.26
polyubiquitin	1(0.5)	42761	40618.00
Rack1: guanine nucleotide-binding protein subunit beta-like protein	1(0.5)	88765	72719.91
SAP3: sensory appendage protein 3	1(0.5)	125887	92834.29
Sarcoplasmic calcium-binding protein	1(0.5)	225682	147398.66
Sterol carrier protein 2 variant 2	1(0.5)	61154	51193.50
SUI1: protein translation factor SUI1	1(0.5)	39199	30985.34
Tctp: translationally-controlled tumor protein homolog	1(0.5)	198883	171138.33
Voltage-dependent anion-selective channel protein 2	1(0.5)	40442	37535.23
28S rRNA: 28S ribosomal RNA	2(1)	60464 - 89224	38285.66 - 56301.23
Aldo: fructose biphosphate aldolase	2(1)	52332 - 55332	34836.30 - 35816.33
Tubulin	2(1)	42260 - 49138	30758.81 - 40832.85
Eupolytin	3(1.5)	41458 - 89676	33049.34 - 72869.08
F-type H+-transporting ATPase subunit alpha	3(1.5)	23109 - 90309	30009.90 - 60113.50
Tropomyosin	3(1.5)	52778 - 138386	42342.49 - 88422.05
Troponin protein	3(1.5)	36721 - 155381	36212.72 - 105250.18
Cytochrome protein	4(2)	115029 - 541120	233826.20 - 506246.73
Elongation factor	4(2)	31457 - 463346	33721.33 - 331844.62
Hexamerin	4(2)	118304 - 318480	77148.51 - 223497.57
Myofilin	4(2)	45532 - 291052	28668.31 - 310103.48
Actin	5(2.5)	49804 - 164018	30295.72 - 116855.82
Cuticular protein	9(4.5)	12771 - 79072	29609.24 - 217254.75
40S ribosomal protein	33(16.5)	21301 - 129865	27984.80 - 197468.90
Unknown protein	36(18)	16101 - 410493	28107.72 - 363059.30
60S ribosomal protein	46(23)	29759 - 151443	32785.75 - 243071.45

Appendix 6: Percentage area under peak of chemical compounds from *Murraya koenigii* essential oils collected at four study sites within Eastern and Coastal Kenya

			Percentage area under peak‡			
Index	**Compound Name**	**Rt (min)***	**Kibwezi**	**Malindi**	**Mombasa**	**Makindu**
1	α-Pinene	9.78	0.00	0.00	0.00	**0.74**
2	**Myrcene**	11.02	0.00	0.00	0.00	**9.02**
3	α-Phellandrene	11.26	0.00	0.00	0.00	0.32
4	δ-2-Carene	11.51	0.00	0.00	0.00	0.03
5	β-Phellandrene	11.73	0.00	0.00	0.20	0.00
6	Sabinene	11.73	0.32	0.05	0.00	1.75
7	trans-β-Ocimene	12.09	0.20	0.04	0.08	1.08
8	γ-Terpinene	12.29	0.00	0.00	0.00	0.04
9	Linalool oxide (furanoid)	12.54	0.00	0.00	0.00	0.03
10	trans-Linalool oxide (furanoid)	12.81	0.00	0.00	0.00	0.06
11	Linalool	13.01	0.10	0.00	0.00	0.27
12	p-Menth-2-en-1-ol	13.37	0.00	0.00	0.00	0.09
13	Allo-Ocimene	13.48	0.00	0.00	0.00	0.17
14	Lavandulol	14.08	0.22	0.00	0.17	0.30
15	trans-α-Necrodol	14.24	0.07	0.00	0.00	0.00
16	p-Nitrophenyl bromoacetate	14.24	0.00	0.00	0.00	0.21
17	3-Cyclohexene-1-carboxaldehyde, 1,3,4-trimethyl-	14.33	0.12	0.00	0.00	0.00
18	Terpineol<alpha->	14.49	0.40	0.04	0.36	0.35
19	Safranal	14.64	0.00	0.00	0.00	0.05

			Percentage area under peak‡			
Index	**Compound Name**	**Rt (min)***	**Kibwezi**	**Malindi**	**Mombasa**	**Makindu**
20	Piperitol<trans->	14.76	0.00	0.00	0.00	0.07
21	Piperitone	15.47	0.00	0.00	0.00	0.04
22	Terpineol<trans-beta->	15.72	0.00	0.00	0.00	0.06
23	Geranyl propanoate	15.88	0.28	0.24	0.52	0.00
24	Lavandulyl acetate	15.88	0.00	0.00	0.00	0.36
25	1,5-Diacetoxypentane	16.12	0.00	0.00	0.19	0.07
26	Cyclobutane, 1,2-diethenyl-3,4-dimethyl-	16.12	0.05	0.00	0.00	0.00
27	1,5-Cyclooctadiene, 3,4-dimethyl	16.12	0.00	0.08	0.00	0.00
28	Mentha-1(7),8-diene<para->	16.23	0.00	0.00	0.00	0.06
29	Sylvestrene<iso->	16.23	0.05	0.11	0.16	0.00
30	Camphene	16.48	0.07	0.00	0.00	0.00
31	Santolina Triene	16.48	0.00	0.08	0.22	0.00
32	1,5,5-Trimethyl-6-methylene-cyclohexene	16.50	0.00	0.43	0.00	0.16
33	Isobazzanene	16.64	0.84	0.00	0.94	0.00
34	Sabina ketone<dehydro->	16.64	0.00	0.00	0.00	1.35
35	Cyclohexa-1,3-diene, 5,6-diethyl-	16.75	0.49	0.00	0.00	0.63
36	**α-Cubebene**	16.79	0.00	**3.15**	**0.39**	0.00
37	Isoledene	17.13	0.00	0.12	0.00	0.00
38	Guaiadiene<6,9->	17.20	0.37	0.00	0.81	0.00
39	Selina-3,7(11)-diene	17.20	0.00	0.40	0.00	0.00
40	1,5-Cycloundecadiene, 9-(1-methylethylidene)-	17.26	0.13	0.00	0.00	0.00
41	**β-Elemene**	17.38	**2.09**	**0.73**	**1.19**	**2.13**

			Percentage area under peak‡			
Index	**Compound Name**	**Rt (min)***	**Kibwezi**	**Malindi**	**Mombasa**	**Makindu**
42	Jasmone<E->	17.49	0.00	0.31	0.49	0.00
43	**(Z)-Caryophyllene**	17.62	**4.24**	**1.68**	0.00	0.00
44	**(E)-Caryophyllene**	17.87	**19.18**	**22.18**	**25.28**	**14.04**
45	**Acetonitrile, bromo-**	17.94	**2.34**	0.00	0.00	0.00
46	**Premnaspirodiene**	18.05	**3.00**	0.00	0.00	0.00
47	**γ-E-Bisabolene**	18.07	0.00	**3.34**	0.00	0.00
48	**Eudesma-6,11-diene<cis->**	18.20	0.00	0.00	0.00	**4.16**
49	**α-Humulene**	18.25	**8.27**	**8.38**	**7.82**	**5.85**
50	Aristolochene<4,5-di-epi->	18.43	0.74	0.00	0.00	0.00
51	**γ-Muurolene**	18.50	**2.69**	0.00	0.00	0.00
52	**β-Selinene**	18.68	**8.16**	**7.36**	**6.34**	**16.09**
53	**α-Selinene**	18.81	**13.73**	**11.38**	**9.27**	0.00
54	Cadina-1(6),4-diene<trans->	19.06	1.38	0.00	0.00	0.00
55	Cadinene<delta->	19.06	0.00	0.00	1.63	0.00
56	Calamenene<trans->	19.06	0.00	1.49	0.00	0.00
57	**Amorpha-4,7(11)-diene**	19.24	0.00	**2.81**	**0.65**	**1.81**
58	Patchoulene<gamma->	19.24	0.50	0.00	0.00	0.00
59	Calacorene<beta->	19.32	0.00	0.34	0.40	0.00
60	1,6(2H,7H)-Naphthalenedione, 3,4,8,8a-tetrahydro-8a-methyl-	19.32	0.22	0.00	0.00	0.00
61	Isocaryophillene	19.41	0.00	0.00	0.00	0.35
62	**(E)-Nerolidol**	19.46	**1.90**	**1.69**	**1.69**	**3.50**

			Percentage area under peak‡			
Index	**Compound Name**	**Rt (min)***	**Kibwezi**	**Malindi**	**Mombasa**	**Makindu**
63	**β-Patchoulene**	19.55	0.00	0.00	**4.32**	**4.37**
64	Viridiflorol	19.55	0.62	0.00	0.00	0.00
65	Intermedeol	19.64	0.00	0.00	1.13	0.00
66	Maaliol	19.64	0.74	0.00	0.00	0.00
67	Pogostol	19.64	0.00	0.31	0.00	0.00
68	7-Amino-4-(methoxymethyl)coumarin	19.80	0.00	0.00	**2.25**	0.00
69	**Globulol**	19.86	**3.23**	**0.81**	**4.38**	**5.69**
70	**Caryophyllene oxide**	19.86	0.00	**7.56**	0.00	0.00
71	Cubeban-11-ol	19.95	1.18	0.00	0.00	0.00
72	2-Propenal, 3-(2,6,6-trimethyl-1-cyclohexen-1-yl)-	19.95	0.00	0.91	0.00	0.00
73	1-Bromo-3,5-dimethyladamantane	19.97	0.00	0.00	1.41	0.00
74	Adamantane, 1-isothiocyanato-3,5-dimethyl-	20.02	0.00	0.00	0.00	1.00
75	Benzene, 1-butyl-4-methoxy-	20.04	1.25	0.00	0.00	0.00
76	o-Isopropylphenetole	20.06	0.00	0.00	1.92	0.00
77	Rosifoliol	20.06	0.00	1.62	0.00	0.00
78	(3E)-3-ethylidene-3a-methyl-2,4,5,6,7,7a-hexahydro-1H-indene	20.13	0.00	0.00	0.00	1.56
79	**Selin-11-en-4-α-ol**	20.29	0.00	0.00	0.00	**10.41**
80	**Valerianol**	20.31	**2.15**	0.00	**2.57**	0.00
81	Sibirene	20.38	0.00	0.00	0.00	**3.29**
82	2(3H)-Benzothiazolone, 3-methyl-, hydrazone	20.40	0.00	0.00	0.70	0.00
83	**Cadina-1(6),4-diene<cis->**	20.47	**1.93**	0.00	**2.00**	**0.81**
84	**Bicyclo[4.4.0]dec-1-ene, 2-isopropyl-5-methyl-9-methylene-**	20.47	0.00	**2.87**	0.00	0.00

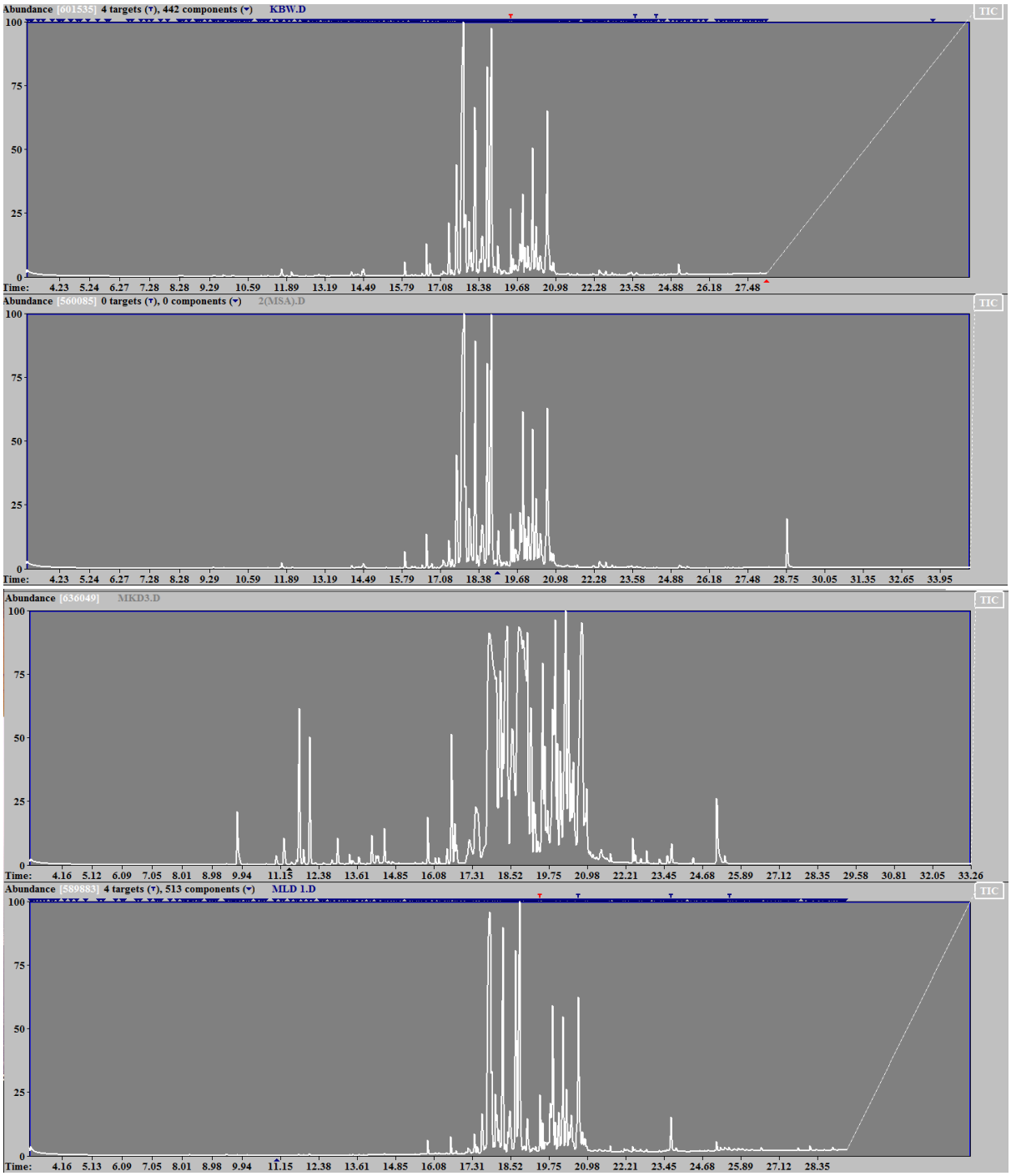
			Percentage area under peak‡			
Index	**Compound Name**	**Rt (min)***	**Kibwezi**	**Malindi**	**Mombasa**	**Makindu**
85	**neo-Intermedeol**	20.69	**12.50**	**11.15**	**11.17**	0.00
86	Caryophyllene<14-hydroxy-9-epi-(E)->	20.83	0.00	0.70	0.00	0.00
87	α-Bisabolol	20.91	0.78	0.96	0.92	1.38
88	α-Eudesmol	21.30	0.00	0.00	0.00	0.18
89	**Spathulenol**	21.39	**1.50**	**0.33**	**3.62**	0.00
90	Amorpha-4,7(11)-diene<2-alpha-hydroxy->	21.41	0.24	0.00	0.00	0.46
91	Tetradecanoic acid	21.61	0.00	0.00	0.00	0.13
92	Cyclocolorenone<epi->	21.72	0.12	0.37	0.20	0.00
93	Cyperotundone	21.74	0.00	0.00	0.00	0.19
94	.alpha.-Farnesene	22.26	0.11	0.00	0.00	0.00
95	alpha-Bergamotene	22.26	0.00	0.00	0.23	0.00
96	1-Isopropyl-2,2-dimethylpropylideneamine	22.28	0.00	0.29	0.00	0.00
97	Trichloroacetic acid, 2,7-dimethyloct-7-en-5-yn-4-yl ester	22.46	0.33	0.00	0.00	0.00
98	9-Octadecyne	22.46	0.00	0.00	0.50	0.00
99	1,1'-Bicyclopentyl	22.46	0.00	0.00	0.00	0.23
100	Citronellyl isobutyrate	22.46	0.00	0.21	0.00	0.00
101	2-Pentadecanone, 6,10,14-trimethyl-	22.53	0.00	0.23	0.00	0.12
102	**Calamenene<cis->**	22.68	**0.16**	0.00	**0.23**	**3.29**
103	1,2-Dioctylcyclopropene	22.68	0.00	0.22	0.00	0.00
104	trans-2-Undecen-1-ol	22.71	0.00	0.00	0.00	0.07
105	7-Heptadecyne, 1-chloro-	22.89	0.00	0.00	0.00	0.13
106	Cyclohexanol, 1-ethynyl-	22.89	0.11	0.00	0.00	0.00

			Percentage area under peak‡			
Index	**Compound Name**	**Rt (min)***	**Kibwezi**	**Malindi**	**Mombasa**	**Makindu**
107	Phytol acetate<E->	22.89	0.00	0.13	0.15	0.00
108	Farnesyl acetone<5E,9E->	23.31	0.00	0.00	0.00	0.07
109	Aromadendrene epoxide-<allo->	23.42	0.00	0.00	0.09	0.00
110	Cyclopentane-3'-spirotricyclo[3.1.0.0(2,4)]hexane-6'-spirocyclopentane	23.42	0.14	0.00	0.00	0.00
111	1,7,7-Trimethylbicyclo[2.2.1]hept-5-en-2-ol	23.49	0.00	0.00	0.06	0.00
112	Cyclopentadecanone, 2-hydroxy-	23.49	0.00	0.13	0.00	0.00
113	Isophytol	23.56	0.13	0.00	0.07	0.10
114	n-Hexadecanoic acid	23.69	0.00	1.27	0.00	0.00
115	Undec-10-ynoic acid	23.71	0.14	0.00	0.00	0.00
116	(3,6-dimethylcyclohex-4-ene-1,2-diyl)dimethanol	23.71	0.00	0.00	0.07	0.00
117	Cyclodecasiloxane, eicosamethyl-	23.85	0.00	0.39	0.00	0.00
118	Geranyl linalool<E,E->	24.39	0.00	0.00	0.00	0.08
119	Phytol	25.13	0.42	0.51	0.17	0.99
120	3-(4-N,N-Dimethylaminophenyl)propenoic acid, 2-(diethoxyphosphinyl) -, ethyl ester	25.26	0.00	0.25	0.00	0.00
121	Dihydroisojasmone	25.39	0.00	0.00	0.00	0.10
122	Cyclopentanone, 2-(5-oxohexyl)-	25.39	0.00	0.30	0.00	0.00
123	Cyclopentanone, 2-(1-methylpropyl)-	25.42	0.07	0.00	0.00	0.00
124	2H-Azepin-2-one, 3-(dimethylamino)hexahydro-	25.42	0.00	0.00	0.05	0.00
125	Octadecanoic acid	25.55	0.00	0.20	0.00	0.00
126	Azetidine, 1-benzoyl-3-ethenyl-	25.71	0.00	0.20	0.00	0.00

			Percentage area under peakł			
Index	**Compound Name**	**Rt (min)***	**Kibwezi**	**Malindi**	**Mombasa**	**Makindu**
127	N-(2-Chloroethyl)benzamide	25.82	0.00	0.20	0.00	0.00
128	Bicyclo[3.1.1]hept-3-ene-spiro-2,4'-(1',3'-dioxane), 7,7-dimethyl-	25.93	0.00	0.17	0.00	0.00
129	1,3,5[10]-Estratriene-2,3-diol-17-one	26.56	0.00	0.22	0.00	0.00
130	Azetidine, 1-benzyl-3,3-dimethyl-2-phenyl-	28.13	0.00	0.17	0.00	0.00
131	1,2-Benzenedicarboxylic acid, diisooctyl ester	28.80	0.00	0.00	1.31	0.00
132	Cyclononasiloxane, octadecamethyl-	28.84	0.00	0.51	0.00	0.00
	Total identified (%)		**99.98**	**99.60**	**98.30**	**99.88**

Major compounds with ≥2% area under peak from at least one site are in bold font. * - Retention times in minutes. ł - Percentage area under curve correlating to the abundance of a chemical compound.

Appendix 7: Total ion chromatogram for essential oil analysed by GC-MS.



Appendix 8: Research authorization from National Commission for Science, Technology, and Innovation (NACOSTI)

NATIONAL COMMISSION FOR SCIENCE, TECHNOLOGY AND INNOVATION

Telephone:+254-20-2213471,
2241349,3310571,2219420
Fax:+254-20-318245,318249
Email:dg@nacosti.go.ke
Website: www.nacosti.go.ke
when replying please quote

9th Floor, Utalii House
Uhuru Highway
P.O. Box 30623-00100
NAIROBI-KENYA

Ref: No.

Date:

NACOSTI/P/16/51278/13367

15th February, 2017

Clarence Maikuri Mangera
Kenyatta University
P.O. Box 43844-00100
NAIROBI.

RE: RESEARCH AUTHORIZATION

Following your application for authority to carry out research on ***"Physiological and molecular responses in adult and larvae anopheles gambiae S. S. to Murraya Koenigii Phytochemicals,"*** I am pleased to inform you that you have been authorized to undertake research in **Kilifi, Makueni and Mombasa Counties** for the period ending **15th December, 2017.**

You are advised to report to **the County Commissioners and the County Directors of Education, Kilifi, Makueni and Mombasa Counties** before embarking on the research project.

On completion of the research, you are expected to submit **two hard copies and one soft copy in pdf** of the research report/thesis to our office.

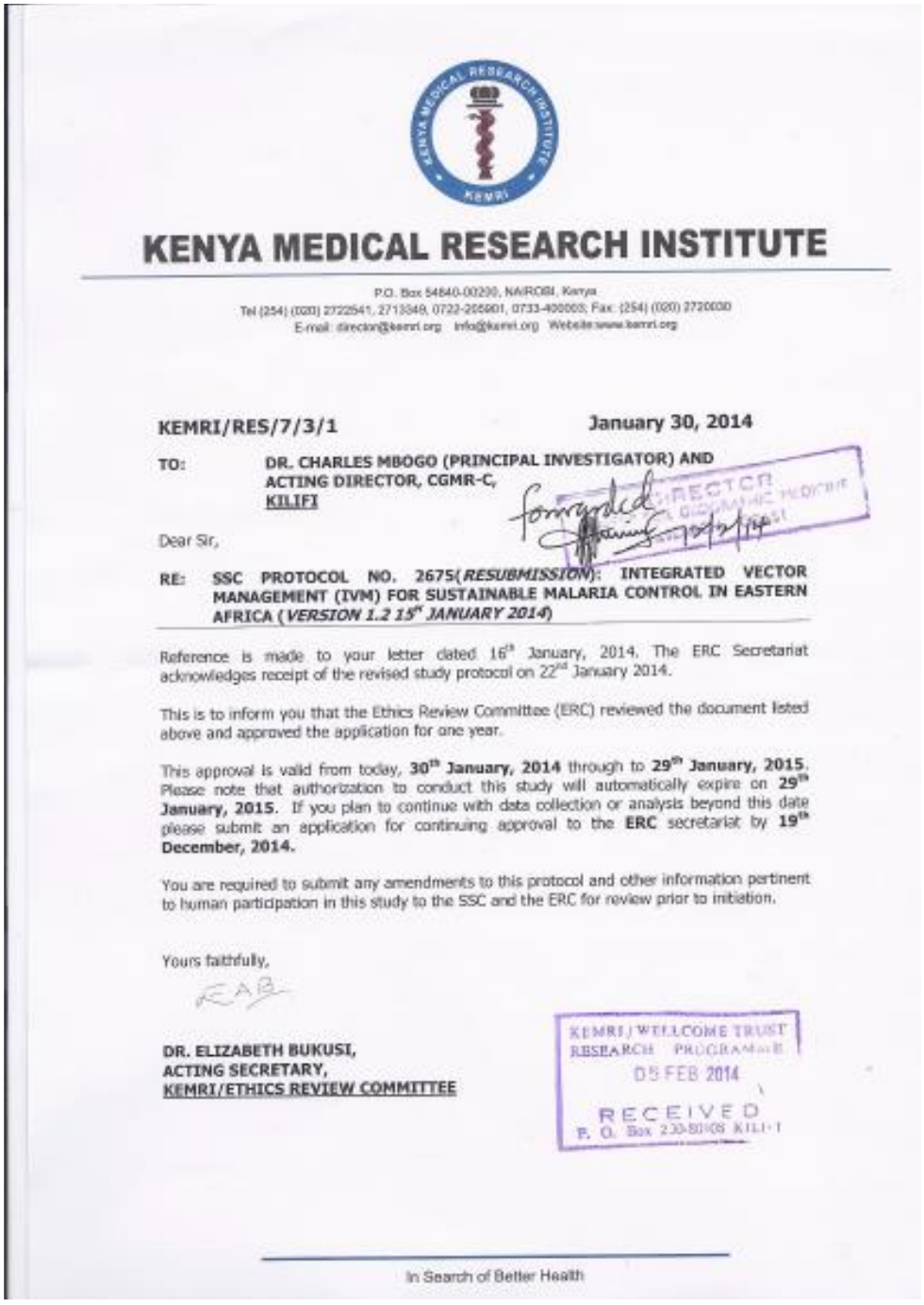
BONIFACE WANYAMA
FOR: DIRECTOR-GENERAL/CEO

Copy to:

The County Commissioner
Kilifi County.

The County Director of Education
Kilifi County.

Appendix 9: Approval for feeding mosquitoes on mice obtained from the Kenya National Ethical Review Board (protocol number KEMRI/RES/7/3/1).



KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1 **January 30, 2014**

TO: **DR. CHARLES MBOGO (PRINCIPAL INVESTIGATOR) AND ACTING DIRECTOR, CGMR-C, KILIFI**

Dear Sir,

RE: SSC PROTOCOL NO. 2675(*RESUBMISSION*): INTEGRATED VECTOR MANAGEMENT (IVM) FOR SUSTAINABLE MALARIA CONTROL IN EASTERN AFRICA (*VERSION 1.2 15th JANUARY 2014*)

Reference is made to your letter dated 16th January, 2014. The ERC Secretariat acknowledges receipt of the revised study protocol on 22nd January 2014.

This is to inform you that the Ethics Review Committee (ERC) reviewed the document listed above and approved the application for one year.

This approval is valid from today, **30th January, 2014** through to **29th January, 2015**. Please note that authorization to conduct this study will automatically expire on **29th January, 2015**. If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the **ERC** secretariat by **19th December, 2014.**

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the SSC and the ERC for review prior to initiation.

Yours faithfully,

DR. ELIZABETH BUKUSI,
ACTING SECRETARY,
KEMRI/ETHICS REVIEW COMMITTEE

KEMRI/WELLCOME TRUST RESEARCH PROGRAMME
05 FEB 2014
RECEIVED
P. O. Box 230-80108 KILIFI

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