RESTING BEHAVIOUR OF AFRICAN MALARIA VECTORS IN AN ERA OF HIGH INDOOR INSECTICIDE USE

BY

KEVIN OMONDI OWUOR, B.Sc. (Second class Upper)

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DECLARATION

I declare that this thesis is my original work and has not been submitted elsewhere for examination, award of a degree or publication. Where other people's work, or my own work has been used, this has properly been acknowledged and referenced in accordance with the University of Nairobi's requirements.

Student:

Signature: …… Date: ….04th July, 2022……

Kevin Omondi Owuor

I56/7589/2017

This thesis has been submitted with our approval as supervisors

Supervisors:

Signature: …… Date: ….4th July, 2022……

Prof. Wolfgang Richard Mukabana

Department of Biology, University of Nairobi, Nairobi, Kenya

Science for Health Society, Nairobi, Kenya

Signature: …… Date: …4TH JULY, 2022……

Dr. Eric Odhiambo Ochomo

Entomology section- (KEMRI/CGHR), (CDC), Kisumu

DEDICATION

I dedicate this work to my whole support team of kin and kith.

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LIST OF ABBREVIATIONS

1.	CI	:	Confidence Interval
2.	CDC	:	Centers for Disease Control and Prevention
3.	CGHR	:	(KEMRI's) Centre for Global Health Research
4.	CS	:	Circumsporozoite
5.	DC	:	Diagnostic Concentration
6.	DNA	:	Deoxyribonucleic acid
7.	F1	:	1st Filial generation
8.	GABA	:	Gamma-aminobutyric acid
9.	GST	:	Glutathione-S-transferase
10.	IRS	:	Indoor Residual Spraying
11.	KDR	:	Knock Down Resistance
12.	KEMRI	:	Kenya Medical Research Institute
13.	LLIN	:	Long-Lasting Insecticidal Net
14.	MRR	:	Mark-release-recapture
15.	PBO	:	Piperonyl butoxide
16.	PBS	:	Phosphate Buffered Saline
17	PCR	:	Polymerase Chain Reaction
18.	Pf SR	:	*Plasmodium falciparum* Sporozoite rate
19.	SIT	:	Sterile Insect Technique
20.	TE	:	Tris-EDTA buffer (Hydroxymethyl-Ethylenediamine tetra-acetic acid)
21.	TMBZ	:	3, 3',5 ,5'-tetramethylbenzidine
22.	UV	:	Ultra-Violet
23.	VGSC	:	Voltage-Gated Sodium Channel
24.	WHO	:	World Health Organization

DEFINITIONS

1.	Behavioral plasticity	:	The trait of changing a behavioral preference, usually when conditions become unfavorable.
2.	Biparous Mosquito	:	A female mosquito estimated to have undergone two egg-laying cycles
3..	Knockdown Resistance	:	A trait that alters the voltage-gated sodium channel properties which reduce pyrethroid effects. either by reducing pyrethroid binding and/or by altering the gating properties
4.	Mortality	:	This the number of dead mosquitoes 24 hours post durational insecticide exposure, penetration, transversion through tissues into the target site causing death.
5.	Nulliparous Mosquito	:	A female mosquito that has not undergone any egg-laying cycle
6.	Physiological age	:	This is the number of gonotrophic (egg laying) cycles a female mosquito has passed through.
7.	Resting behaviour	:	This is the inactive trait of late-stage blood fed, half gravid or fully gravid states of mosquitoes when they are at the period between end of blood feeding and seeking for oviposition sites.
8.	Sporozoite rate	:	Sporozoite rate (Pf SR) is the number of mosquitoes infected with sporozoites divided by the total number of mosquitoes examined
9.	Stochastic Phenomenon	:	This is an occurrence having a random probability distribution or pattern that may be analysed statistically but may not be predicted precisely.
10.	Synanthropy	:	This is the dwelling and benefiting by an organism in close proximity to human beings.
11.	Uniparous Mosquito	:	A female mosquito estimated to have undergone only a single egg-laying cycle.

ABSTRACT

Long Lasting Insecticidal Nets (LLINs) and indoor residual spraying (IRS) represent powerful tools for controlling malaria vectors in sub-Saharan Africa. The success of these interventions relies on their ability to inhibit indoor feeding and resting of malaria mosquitoes. This study sought to understand the interaction of insecticide resistance with indoor and outdoor resting behavioural responses of malaria vectors from Western Kenya. Mark-release-recapture experiments were used to investigate the plasticity of indoor and outdoor resting behaviour while parity rates were used to estimate the physiological ages of *Anopheles* mosquitoes collected from Kisumu (Kisian) and Bungoma (Kimaeti) counties in Western Kenya. The status of insecticide resistance among indoor and outdoor resting anopheline mosquitoes was investigated in *Anopheles* mosquitoes collected from study sites. The level and intensity of resistance were measured using WHO-tube and CDC-bottle bioassays, respectively. The mutations at the voltage gated sodium channel (Vgsc) knock down resistance (*kdr*) gene and *Ace 1* gene were characterized using PCR. Microplate assays were used to measure levels of detoxification enzymes, if present. Sporozoite rates were assessed by ELISAs for *Plasmodium falciparum* circumsporozoite protein. A total of 1094 samples were discriminated within *Anopheles gambiae s.l.* and 289 within *An. funestus s.l.* In Kisian (Kisumu County), the dominant species was *Anopheles arabiensis* 75.2% (391/520) while in Kimaeti (Bungoma county) collections the dominant sibling species was *Anopheles gambiae s.s* 96.5% (554/574). The *An. funestus s.l* samples analyzed were all *An. funestus s.s* from both sites. Pyrethroid resistance of *An. gambiae s.l* F1 progeny was observed in all sites. Lower mortality was observed against deltamethrin for the progeny of indoor resting mosquitoes compared to outdoor resting mosquitoes (Mortality rate: 37% vs 51%, P=0.044). The intensity assays showed moderate-intensity resistance to deltamethrin in the progeny of mosquitoes collected from indoors and outdoors in both study sites. In Kisian, the frequency of vgsc-L1014S and vgsc-L1014F mutation were 0.14 and 0.19 respectively in indoor resting *An. gambiae s.l* mosquitoes while those of the outdoor resting *An. gambiae s.l* mosquitoes were 0.12 and 0.12 respectively. The *ace 1* mutation was present in higher frequency in the *An. gambiae s.l* F1 of mosquitoes resting indoors (0.23) compared to those of mosquitoes resting outdoors (0.12). In Kimaeti, the frequencies of vgsc-L1014S and vgsc-L1014F were 0.75 and 0.05 respectively for the F1 of *An. gambiae s.l* collected indoors whereas those of outdoor resting ones were 0.67 and 0.03 respectively. The *ace 1* G119S mutation was present in progeny of *An. gambiae s.l* mosquitoes from Kimaeti resting indoors (0.05) whereas it was absent in those resting outdoors. Monooxygenase activity was elevated by 1.83 folds in Kisian and by 1.33 folds in Kimaeti for *An. gambiae s.l* mosquitoes resting indoors than those resting outdoors respectively.

The study recorded high resting behavioural plasticity, physiological (phenotypic, metabolic and genotypic) insecticide resistance and sporozoite rate in indoor resting populations of malaria vectors compared to their outdoor resting counterparts. The indication of moderate resistance intensity and for the indoor resting mosquitoes is alarming as it could have an operational impact on the efficacy of the existing indoor pyrethroid based vector control tools.

CHAPTER ONE: INTRODUCTION

Major declines in the incidence as well as the prevalence of malaria within the Sub-Saharan Africa region have been realized owing to the anti-malarial drug administration campaigns and the augmentation of vector management strategies; principally targeting endophagic and endophilic malaria transmitting mosquitoes (WHO, 2019). However, malaria transmission is still persistent in quite a lot of countries of the Sub-Saharan Africa despite the achieved feats in the mitigative wars against malaria (Mwesigwa *et al.*, 2015; Zhou *et al.*, 2011).

The persistence in malaria transmissions has partly been accredited to mosquito deviations with regards to biting and resting patterns; in response to the increase in the usage of insecticides as vector control tools (Reddy *et al.*, 2011; Russell *et al.*, 2011; Sougoufara *et al.*, 2014; Takken & Verhulst, 2013) and the increased insecticide resistance in the malaria mosquitoes (Hughes *et al.*, 2020; Knox *et al.*, 2014; Omondi *et al.*, 2017). Malaria transmission is dependent upon propensity of malaria mosquitoes successfully obtaining blood meals from humans and their particular predilection to living close to human dwellings (Mandal *et al.*, 2011; Takken & Verhulst, 2013).

Insecticide resistance in malaria mosquitoes may develop from one or more mechanisms which include; increase in metabolic detoxification enzyme systems, target site alterations hence insensitivity and behavioural modifications (Liu, 2015). Metabolic enzyme detoxification (Hemingway *et al.*, 2004) and target site insensitivity (Hemingway & Ranson, 2000) are the most responsible mechanisms when it comes to high levels of resistance to insecticides (Brogdon, 1989). Insensitivity to the toxicity of insecticides rely on one or several variations in the hereditary genes within the mosquito genome (Liu, 2015). Detoxification enzymes that are known to confer insecticide resistance are mainly found in three groups of enzymes namely; monooxygenases

(cytochrome P450s), beta (β) esterases and glutathione-S-transferases. Approximately 80% of the insecticide resistance genotypes reported in Western Kenya are the knock-down (*kdr*) mutations the Voltage-Gated Sodium Channel at locus 1014 of *Anopheles gambiae s.s.*, a primary vector of malaria (Bonizzoni *et al.*, 2012; Mathias *et al.*, 2011; Ochomo *et al.*, 2012; Wanjala *et al.*, 2015). The malaria mosquito, *An. arabiensis,* recently has been reported to developing increments in the levels of knock-down (*kdr*) mutant genotypes (Hemming-Schroeder *et al.*, 2018). A similarly important vector of malaria in most parts of Africa including Western Kenya, *An. funestus* mosquitoes, have currently no records of *kdr* mutants at the locus 1014 in their genome. However, there have been documentation of metabolic resistance in *Anopheles funestus* mosquitoes (Kawada *et al.*, 2011). The increasing levels of resistance to insecticides by malaria vectors have been linked, by countless accounts, to the incessant exposure to Long Lasting Insecticide Nets (LLINs) (Lindblade *et al.*, 2015; Moshi *et al.*, 2017) and in agro-chemicals, mainly due to the formation of selection pressures (Diabate *et al.*, 2002; Nkya *et al.*, 2014; Reid & McKenzie, 2016).

Climatic (environmental) fluctuations have as well been drawn in the mosquito behavioural alterations being witnessed. Mosquitoes adapt to prevailing conditions by expressing phenotypes that are better suited for lowering or averting adverse consequences that may be brought about by environmental conditions (Takken & Verhulst, 2013). For example, many East African region studies have documented amplified instances of zoophagy (Stone & Gross, 2018), early feeding in the evening indoors or outdoors-feeding altogether (Monroe *et al.*, 2015; Monroe *et al.*, 2020; Ototo *et al.*, 2015) and changes in resting behavioural preferences, either indoors or outdoors (Bayoh *et al.*, 2014; Killeen *et al.*, 2006; Pates & Curtis, 2005). These shifts in behaviour could have arisen due to selection pressures created from the increased LLIN coverage (Braimah *et al.*, 2005; Killeen *et al.*, 2017; Mayagaya *et al.*, 2015; Perugini *et al.*, 2020). Wide coverage by LLINs

in Africa has been shown to alter the vector dominance composition; the highly endophilic *An. gambiae s.s.* (hereafter *An. gambiae*) is slowly being replaced by the more exophilic *An. arabiensis* in Western Kenya (Githeko *et al.*, 2012; Mutuku *et al.*, 2011; Zhou *et al.*, 2011). The arising intervention pressures could selectively eradicate the utmost susceptible mosquitoes from within a population thereby the least susceptible (resistant) mosquitoes that can adapt to the new conditions can survive (Lindblade *et al.*, 2006). Even though these field studies have demonstrated the impact of environmental deviations on the behaviour of mosquitoes, little is known on what relationship there is between resistance to insecticides and the resulting malaria vector behaviours.

Mosquito feeding and resting behaviours are very crucial considerations to the success of malaria transmission reduction and vector control. it is therefore paramount that we understand the relationship between physiological resistance and the resting behaviours seen in mosquitoes and the "how?" of these behavioural observations might affect the already existent frontline measures. The underlying mechanisms of the behavioural modifications observed in mosquitoes are currently a grey zone despite the fact that they might bear epidemiological consequences. In any attempt to sustaining effective insecticide-based control of malaria vectors, the resistance to insecticides should continuously be observed and appropriate mitigative stratagems established (Chanda *et al.*, 2011; Hughes *et al.*, 2020; Ochomo *et al.*, 2014; Ranson & Lissenden, 2016; Russell *et al.*, 2013; Sougoufara *et al.*, 2017; WHO, 2012). The study attempted to answer the how on insecticide use and resistance influencing either the indoor or the outdoor resting behaviours and the implications this may have to malaria transmission. The results of this study are important to provide information on the resting behaviour with regards to levels of insecticide resistance in malaria mosquitoes resting either indoors or outdoors and possibly the infectivity rates of the populations.

1.1 Problem statement

Significant morbidity and mortality resulting from malaria, especially among infants and expectant women, is still recorded in Africa. The transmission of malaria is heavily reliant on the tendency, by malaria vectors, to successfully blood-feed on humans and their preference for living within proximity to human dwellings. Vector control is majorly dependent on the disruption of the cycle of malaria transmission. The use of insecticide-based interventions has been able to avert the transmission cycle by hindering human feeding and deterring resting proximal to human dwellings by either lethal action or repellency. However, recent reports of increasing behavioural shifts and levels of insecticide resistance among malaria vectors threatens the attained successes from these insecticide-centered control interventions. The intensive use of these tools has conceivably led to increased insecticide resistance and shift from indoor-feeding to outdoor-biting by malaria mosquitoes. As we are focusing on insecticide resistance management, we need to as well put effort in determining the effects this has on other behaviours of malaria mosquitoes such as resting, which are key in the interaction circles that facilitate malaria transmission and are determinants of appropriateness of interventions. Unlike other mechanisms of insecticide resistance which have proper workable monitoring tools, behavioural mechanisms lack concrete techniques besides mere vector density surveillance.

1.2 Conceptual Framework

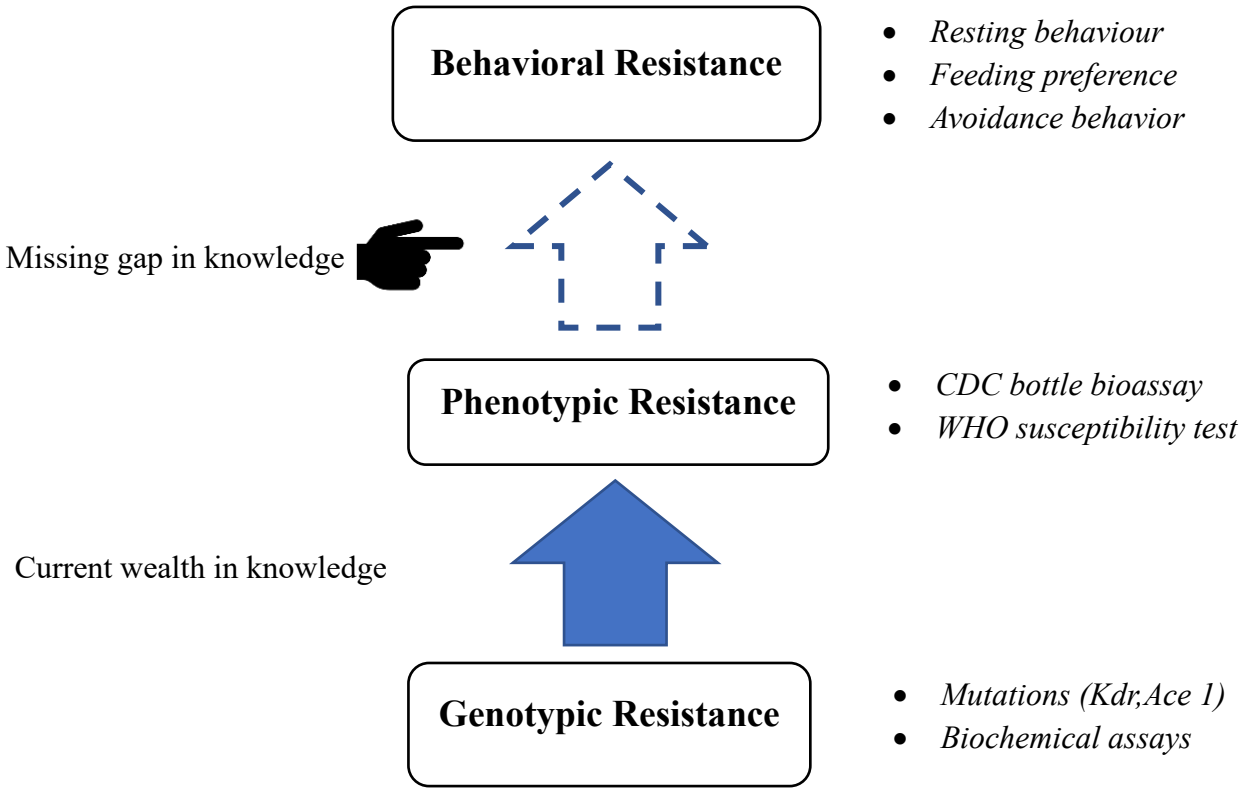

Figure 1: A conceptual illustration of the interactions between genotypic, phenotypic and behavioral aspects of insecticide resistance highlighting monitoring techniques. (**Key focus:** Resting behaviour)

1.3 Justification and significance of the research

Vector control relies heavily on insecticide-based interventions. These depend on the knowledge of biting patterns and resting behaviour of malaria mosquitoes by dictating how much the exposure of the vectors is to these tools (Trung *et al.*, 2005; WHO, 2012). Insecticide resistance is a hindrance to malaria control efforts (Churcher *et al.*, 2016; Hemingway *et al.*, 2016). Globally, new strategies are required to overcome insecticide resistance in the fight against malaria mosquitoes. The reduced susceptibility and behavioural change responses to common insecticides used in indoor residual spraying (IRS) and in long lasting insecticidal nets (LLINs) have been reported previously (Machani *et al.*, 2020; Ochomo *et al.*, 2012; Ochomo *et al.*, 2014; Ochomo *et al.*, 2015; Omondi *et al.*, 2017). This underscores the importance of inventing new tools for management of malaria mosquitoes. The indoor application of IRS and LLINs in vector control have proved to be effective. However, significantly raised levels of indoor malaria transmissions (Mwesigwa *et al.*, 2015; Zhou *et al.*, 2016) and outdoors are still being observed (Monroe *et al.*, 2019; Moshi *et al.*, 2017; WHO, 2019). Behavioural resistance is a compounding factor that affects fitness of malaria vectors in the presence of indoor insecticide-based interventions. The resting behaviour of these vectors could impact on malaria transmission. Despite having insecticide-based interventions in place, malaria vectors have been seen resting in or within proximity of human dwellings (Russell *et al.*, 2013). In order to tackle the issue of resistance, we must have a clear understanding of the link between insecticide use, insecticide resistance and the resulting behaviour change which in this case is the change in resting behaviour of malaria vectors (Ranson & Lissenden, 2016; Russell *et al.*, 2013). The constant transmission of residual malaria has been attributed to the deviations from known biting phenology and the resting behavioural shifts in mosquitoes owing to increased insecticide vector control (Killeen *et al.*, 2017; Reddy *et al.*, 2011; Russell *et al.*, 2011; Sougoufara *et al.*, 2014; Takken & Verhulst, 2013) and the amplified

insecticide resistance (Hughes *et al.*, 2020; Knox *et al.*, 2014; Omondi *et al.*, 2017). Since the resting behaviour is an important consideration when determining appropriate vector control interventions, these findings will be useful in bridging the scientific gap between insecticide-based vector control and the shifts in the resting patterns of mosquitoes. The goal for the study was to find out whether insecticide coverage, through Long Lasting Insecticidal Nets (LLINs), affects the resting behaviour of insecticide resistant mosquitoes and the implications to *Plasmodium* sporozoite transmission. Understanding behavioural mechanisms of resistance would provide better insights into monitoring and management of insecticide resistance.

1.4 Objectives

1.4.1 General objective

- To determine the effect of indoor insecticide coverage on the resting behaviour of malaria mosquitoes in Western Kenya

Specific objectives

1. To find out whether indoor or outdoor resting behavior of malaria vectors is a plastic phenomenon
2. To compare the status of insecticide resistance in indoor and outdoor-resting malaria vector populations in Western Kenya
3. To investigate the *Plasmodium* sporozoite infection rates in indoor versus outdoor resting malaria mosquito populations in Western Kenya

1.5 Hypotheses

H_o: There is no difference in the level of insecticide resistance and the sporozoite infection rates between African malaria mosquitoes resting indoors and outdoors.

H_A: There is a significant difference in the level of insecticide resistance and sporozoite infection rates between African malaria mosquitoes resting indoor and outdoor.

1.6 Research question

- What is the effect of insecticide coverage through LLINs on the resting behaviour and fitness of female African malaria mosquitoes?

1.7 Research assumptions

- Adult female malaria mosquitoes rest indoors or outdoors regardless of insecticide interventions through LLINs in Western Kenya.
- The mosquito samples collected were homogenous and representative of the mosquito population in study regions of Western Kenya.
- The environments from which mosquitoes were collected had different climatic conditions and geographical indices that are a representation of lowlands and highlands of Western Kenya.

CHAPTER TWO: LITERATURE REVIEW

Malaria transmission is dependent upon the success of malaria mosquitoes in biting human hosts. Vector control interventions have been put in place to break the transmission. Insecticide-based control is widely used across the globe (WHO, 2019). The dynamics involved in the interaction of insecticide application with insecticide resistance and how they impact the behaviour of malaria mosquitoes is a growing researched topic. Altogether, the interactions between resistance, behaviour and residual malaria transmission are a growing concern (Machani *et al.*, 2020; Ranson & Lissenden, 2016). There are four recommended lethal classes of insecticides used in LLINs or IRSs. These include pyrethroids, organochlorides, organophosphates and carbamates. Pyrethroids and organophosphates are used in LLINs at operationally effective doses (Ranson & Lissenden, 2016; WHO, 2012). Organochlorides and carbamates on the other hand are used in IRS. However, due to the extensive use in public health and in agriculture, the emergence of resistance has been reported (Ranson & Lissenden, 2016). Resistance to pyrethroid and organophosphate insecticides have been documented for malaria mosquitoes (WHO, 2018).

2.1 Resting behaviour of malaria mosquitoes and vector control

Malaria mosquitoes vary in their resting behaviours. The current vector-control practices rely on human-vector contact. In order to determine what tool to apply, the resting behaviour is a key determinant (Trung *et al.*, 2005). The IRS and LLINs are only effective towards indoor resting mosquitoes (WHO, 2012). *Anopheles arabiensis* are known to exhibit exophilly, having been reported to feed indoors but thereafter escape to rest outdoors (Mahande *et al.*, 2007). In Western Kenya region, the malaria vector *An. arabiensis* had been shown to mostly rest indoors especially around rice irrigation schemes (Githeko *et al.*, 1996) however, the resurgence of *An. gambiae s.s.* around these regions has been reported (Zhou *et al.*, 2011). *Anopheles funestus* mosquitoes are

highly anthropophilic with earlier high reports of indoor resting (Charlwood *et al.,* 1995), however they also rest outdoors (Russell *et al.,* 2011). *Anopheles gambiae s.s.* were previously known to rest and feed indoors exclusively. Recent studies have shown that outdoor feeding by these malaria vectors has increased to substantial levels compared to indoor feeding so does the resting preference. High populations of these vectors are found resting both indoors and outdoors (Reddy *et al.,* 2011; Russell *et al.,* 2011; Zhou *et al.,* 2011). In most regions worldwide, major vector control tools are indoors-based. These interventions aim at reducing human-vector contact indoors using IRS and LLINs (WHO, 2012). The reported changing dynamics in the resting behaviour of malaria vectors away from the indoor interventions affects the widely applied indoor insecticide-based vector control (Reddy *et al.,* 2011). The use of LLINs for vector control is widespread and is effective at reducing malaria incidence, however, it is not known whether their efficacy will stand (Gatton *et al.,* 2013), especially with the emerging insecticide resistance and the shift in mosquito behaviours away from indoor-based interventions (Kiware *et al.,* 2012; Lindblade *et al.,* 2015; Reddy *et al.,* 2011).

2.2 Insecticide resistance and vector control

Insecticide resistance can be defined as the ability of an insect population to become tolerant to dosages of toxicants, which normally could otherwise be lethal for most of the those in a susceptible population comprising of the same species of the insects. Over 60 nations worldwide have reported cases of insecticide resistant mosquito populations (WHO, 2019). The widespread use of some classes of insecticides for public health control has seen cross-resistance to more than one class of insecticide. Insecticide resistance arises from selection pressures due to exposure to these compounds in agro-chemicals (Diabate *et al.,* 2002; Nkya *et al.,* 2014; Reid & McKenzie, 2016). and LLINs that allows malaria mosquitoes to thrive and reproduce even in high insecticide

coverage environments (Alout *et al.*, 2017; Labbé *et al.*, 2011). Both IRS and LLINs for mosquito control rely on a few public health approved classes of insecticides consisting of pyrethroids. The growing resistance to the approved insecticide classes is expected to threaten the globally achieved success in malaria vector control (Omondi *et al.*, 2017) over a period of selection pressures for heritable resistance traits and alleles. Studies have hypothesized a fear of the unknown bearing of growing resistance on adult mosquito controls that are insecticide-based (Lindblade *et al.*, 2015). Insecticide resistance must constantly be observed if we are to up hold the efficacy of insecticide-based vector management and relevant control strategies put in place (Ranson & Lissenden, 2016).

2.3 Mechanisms of insecticide resistance

Insecticide resistance in malaria mosquitoes may develop from mechanisms such as increased metabolic detoxification enzyme activities, genome-based target site insensitivities and behavioural modifications (Liu, 2015). Increased metabolic detoxification (Hemingway *et al.*, 2004) and target site insensitivity (Hemingway & Ranson, 2000), are responsible for high levels of resistance to insecticides (Brogdon & McAllister, 1998). These have been associated with mutations in the genome of malaria mosquitoes. Such allelic mutations have been documented to be responsible for resistance to commonly used insecticides (Labbé *et al.*, 2011). Resistance to organophosphates and carbamates has been derived as coming from a mutation in the acetylcholinesterase-coding *ace-1* gene termed as G119S mutation (Weill *et al.*, 2003). Dieldrin and cyclodiene resistance in mosquitoes is described to arise from a mutation known as the A302G mutation in the *Rdl* gene that codes for GABA (Gamma-aminobutyric acid) receptor subunit (Du *et al.*, 2005). Pyrethroid resistance is associated with single-base pair mutations found in voltage-gated sodium channels (vgsc) that is termed knock-down resistance (*kdr*) mutation. Two such mutations have been reported in African malaria mosquitoes; the West African form and the East

African form differing in the substitution of leucine in both cases to phenylalanine or serine respectively (Martinez-Torres *et al.*, 1998; Ranson *et al.*, 2000).

Insensitivity mechanisms that lower susceptibility to the insecticide toxicity depend on mutations in one or numerous variations in hereditary genes of within the mosquito genome (Liu, 2015). In the presence of insecticides, the frequency of insecticide resistance alleles upsurges in the population due to the conferred advantage, which is expected to hinder the insecticides effect on vector survival and abundance (Alout *et al.*, 2017). Western Kenya has been reported to show the target site vgsc-1014S and the vgsc-1014F alleles responsible for knock down resistance (*kdr*) mutation (Bonizzoni *et al.*, 2012; Machani *et al.*, 2020; Ochomo *et al.*, 2012; Ochomo *et al.*, 2015) Metabolic detoxifying enzymes, monooxygenases and esterases, have also been documented with no reports of glutathione-s-transferase (GST) elevations (Ochomo *et al.*, 2015; Wanjala & Kweka, 2018). The use of the effective insecticide-based control, like chemotherapeutic approaches against the *Plasmodium* parasites, is inevitably bound to lead to the emergence of resistance.

2.4 Monitoring and surveillance of vector behaviour

In epidemiology, vector population surveillance studies have been very important when it comes to understanding population dynamics and parameters for the benefit of controlling arthropod-borne pathogens (Benedict *et al.*, 2018; Guerra *et al.*, 2014; Lindberg, 2012; Mandal *et al.*, 2011; Service, 1993). The difficulty in dealing with populations has been eased by techniques that have been used have relied on measurements of dispersal of vector populations, survival and estimation of vector population size to come up with an understanding vector biology (Cianci *et al.*, 2013; Pollock *et al.*, 1990; Reisen *et al.*, 2003). Mark-release-recapture method has proven to be a reliable tool in ecological studies involving populations (Benedict *et al.*, 2018; Cianci *et al.*, 2013; Lindberg, 2012). Technological advancements such as computing and software have empowered

the MRR studies enabling inclusion of several other variables within populations thereby improving the accuracy of this technique (Conner *et al.*, 2020; Laake, 2013; White & Burnham, 1999; White *et al.*, 2001).

Successful transmission of pathogens by mosquitoes relies heavily on their vectorial capacity. Vector capacity is defined by biological parameters such as survival, dispersion, feeding, inoculation and vector abundance (Mandal *et al.*, 2011; Perkins *et al.*, 2013). This kind of information can be obtained in mark-release-recapture studies of mosquito populations. Estimation of the wild population parameters is limited by environmental and logistical constraints in the laboratory, hence the need for field studies and surveillance of mosquitoes. Field studies enable validation of laboratory findings in order to plan for effective mosquito control programs (Benedict *et al.*, 2018). The abundance of mosquito vector populations has been successfully conducted using the mark-release-recapture method (Cianci *et al.*, 2013; Guerra *et al.*, 2014). The understanding of the biology of male mosquitoes was of critical advantage in implementation and conducting sterile insect technique (SIT) in attempts to suppress the population of mosquito vectors (Cianci *et al.*, 2013; Epopa *et al.*, 2017; Harris *et al.*, 2011; Rafikov *et al.*, 2009; Service, 1993).

2.5 Monitoring insecticide resistance

Standard survey methods have been developed to be used in monitoring of phenotypic insecticide resistance among malaria vectors. These are the WHO susceptibility test and the CDC bottle assay. The two techniques are based on measuring the level of susceptibility to commonly used insecticides by exposure to either of two frequently used insecticides; deltamethrin and permethrin. Knockdown levels for WHO susceptibility bioassays and mortalities are recorded for both bioassays and the proportions are compared against standard indices developed by CDC and WHO for the baseline in susceptibility of mosquito populations (Brogdon & Chan, 2010; WHO, 2012).

In addition to the bioassays, an alternative efficient method for detection of insecticide resistance is through biochemical microplate assays developed by Brogdon (1989). A specific example is the Microplate enzyme activity assays. Newer methods of monitoring insecticide resistance are based on molecular detection of resistance alleles in the genome of the malaria vectors using polymerase chain reactions (Bass *et al.*, 2007; Benedict, 2014; Jones *et al.*, 2012).

2.6 Behavioural insecticide resistance and residual malaria transmission by vectors

Malaria vectors have developed physiological and behavioural adaptations in response to the selection pressures created by insecticides we use such as those in IRS and LLINs (Russell *et al.*, 2011). The role behavioural resistance plays on malaria transmission is difficult to assay compared to the measurable physiological resistance by field populations of malaria mosquitoes (Corbel & N'Guessan, 2013). These behaviours include general avoidance of insecticide covered regions, a shift in the feeding location or times and the changes in the resting behaviour patterns of malaria vectors (Reddy *et al.*, 2011; Russell *et al.*, 2011; Sougoufara *et al.*, 2014; Takken & Verhulst, 2013). A good example of insecticide use attributed behavioural changes of malaria mosquitoes is the shift in biting phenology, increasing the human-vector contact that is limited by IRS or LLINs (Gatton *et al.*, 2013). Residual malaria transmission relies on this human-vector interaction outside of indoor insecticide-based interventions. Insecticide coverage has been acknowledged by several researchers as the leading cause of the rise in insecticide resistant mosquito populations (Lindblade *et al.*, 2015; Moshi *et al.*, 2017). The impacts of indoor insecticide use include changes vector composition, abundance, dominance and behavioural shifts. Insecticide resistance poses direct consequences to these dynamics in mosquito ecology. The ecology through longevity and fecundity of resistant mosquito populations has direct effect on residual malaria transmission and current indoor vector control (Gatton *et al.*, 2013; Omondi *et al.*, 2017).

2.7 Insecticide resistance and Sporozoite infection rates of malaria mosquitoes

The ecological interactions between insecticide-resistant mosquito populations and *Plasmodium* parasites involve very complex and diverse dynamics just as in their susceptible counterparts. It is therefore not easy to measure the connection between resistance to insecticides and the infection of mosquitoes by *Plasmodium* parasites (Alout *et al.*, 2016; Viana *et al.*, 2016). Despite high insecticide coverage through widespread LLINs high sporozoite infection rates have been reported (Churcher *et al.*, 2016; Pombi *et al.*, 2018). Comparisons between the fitness cost of resistance to insecticides and the impact on the that of the *Plasmodium* parasites on malaria mosquitoes is the closest description to this relationship. Survival of insecticide-resistant mosquito populations is greater in comparison with susceptible mosquito populations whereas the endurance of *Plasmodium*-infected resistant populations is sufficiently reduced compared to *Plasmodium*-infected susceptible mosquito populations (Alout *et al.*, 2016; Viana *et al.*, 2016). Insecticide resistance mutations increase the vector competence of *An. gambiae* and possibly malaria transmission (Alout *et al.*, 2014; Alout *et al.*, 2017; Reddy *et al.*, 2011; Russell *et al.*, 2011; Sougoufara *et al.*, 2014; Takken & Verhulst, 2013). The mechanisms of insecticide resistance either phenotypic, biochemical or genotypic have tools that can be used for monitoring the levels (WHO, 2012, 2016). Behavioural resistance, particularly the resting behavioural changes, still lacks proper surveillance tools backed by concrete evidence. We have minimal information on the underlying mechanisms of behaviour change of malaria mosquitoes when exposed to insecticide interventions. Many surveys have shown that the use of insecticides in LLINs causes change in feeding, host selection, avoidance or quick exit from intervention zones.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study sites

The study was done at Kisian (lowland site at longitude 0.0749° S, latitude 34.6663° E, and elevation of 1,137m in Kisumu county) and at Kimaeti (a highland at longitude 0.6029° N, latitude 34.4073° E, and elevation of 1,430m in Bungoma county), Western Kenya (Fig. 1). The two regions are highly abundant in, *Anopheles gambiae s.l.* and *Anopheles funestus s.l.*, malaria mosquitoes. These sites have been reported to have high level of insecticide resistance (Ochomo *et al.*, 2012; Wanjala *et al.*, 2015). Kimaeti, as evident from the visible large tobacco farms with several drying kilns in the area, partakes in extensive cultivation of tobacco. In Kisian, fishing and farming (rice and maize cultivation) are commonplace. Sand harvesting from river beds is extensive as well, which end up mostly enhancing mosquito breeding habitats. Both study sites have commendable coverage of LLINs spread across homes, about by 80% (Zhou *et al.*, 2014). The Western Kenya regions experiences periods of long-rains (March-June) and short-rains (October-November) (Mugalavai *et al.*, 2008).

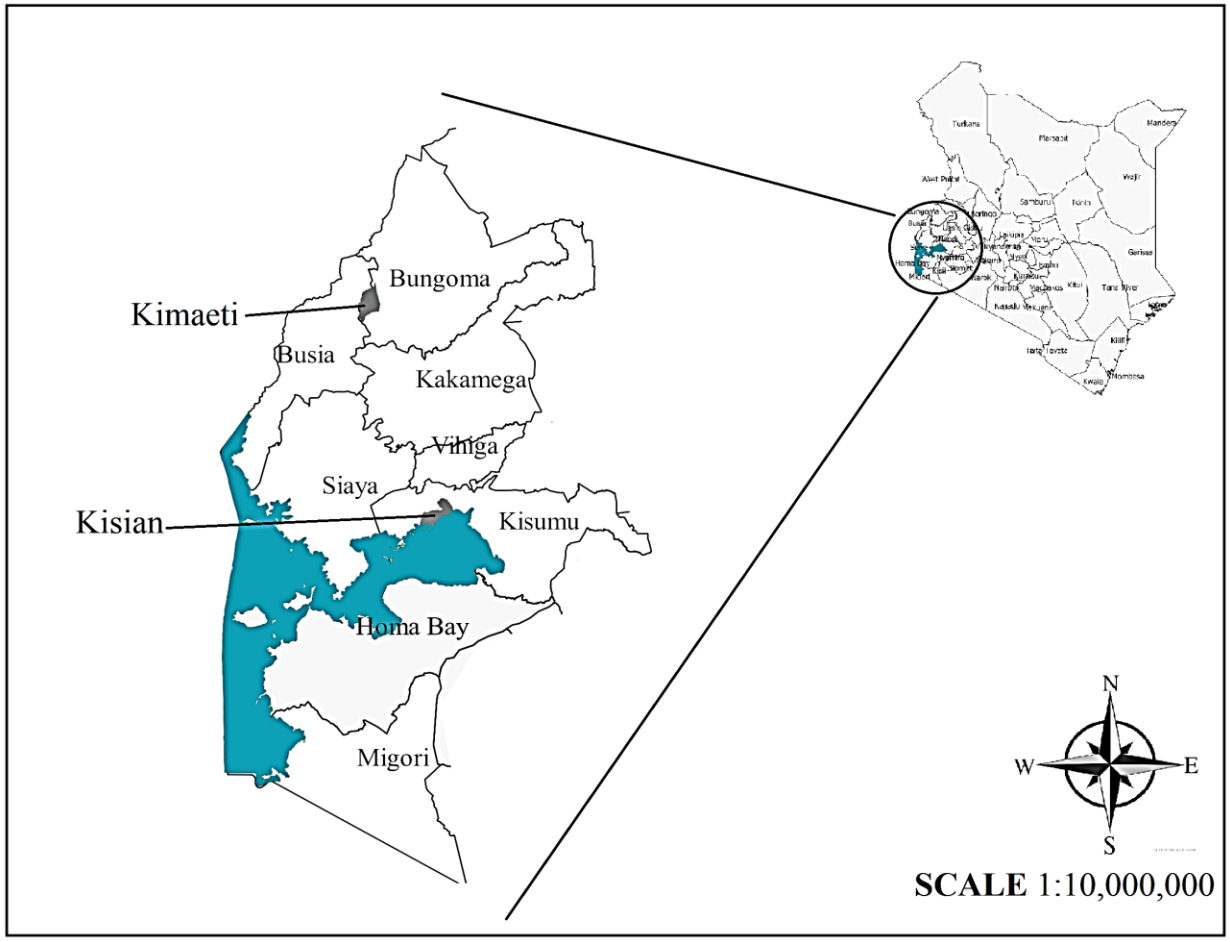

Figure 2: Map highlighting the study sites, (i) Kisian in Kisumu County and (ii) Kimaeti in Bungoma County, both in Western Kenya

3.2 Study design

The study employed a cross-sectional design where sampling was conducted at the start and at the end of the short and long rains. Mosquitoes were sampled from two study sites (a lowland and a highland area) from indoor and outdoor locations. The samples were analyzed in three phases. Phase one comprised of mark-release-recapture experiments and parity rate analyses to understand the plasticity of resting behaviour of malaria vectors. The second phase involved phenotypic, genotypic and metabolic analyses for insecticide resistance mechanisms. The third phase involved sandwich ELISA to detect *Plasmodium* sporozoites in indoor and outdoor resting-mosquitoes.

3.2.1 Mosquito Sampling

Female *Anopheles* mosquitoes were identified by their needle-like proboscis and antennae. The resting mosquitoes were collected from either indoors or outdoors from within or around households that had functional LLINs in use within the study sites in Western Kenya. The late stage blood fed, half gravid and fully gravid states were defined as the resting stages of malaria vectors (WHO, 1995). Mosquito sampling was conducted at the beginning and the end of the wet and dry seasons. Mosquito aspirations were carried out from 0600hrs to 1000 hrs. Mechanical Prokopack and manual mouth aspirators were used to collect mosquitoes resting indoors (human dwellings). Outdoor sampling was done in 30 pit shelter traps dug in the ground measuring $1.5M^3$, at the sites (Muirhead-Thomson, 1958), from empty or half-filled containers such as clay pots placed at least a 5 meters minimum away from the houses or any other proximal outdoor mosquito resting places such as cow sheds and shaded points (Fig 2). Mosquitoes that were sampled were first differentiated morphologically (Gillies & Coetzee, 1987) before further discriminatory speciation between the *Anopheles gambiae s.l.* and the *Anopheles funestus s.l.* by molecular assays. The mosquitoes sampled were transported to the Kenya Medical Research Institute (KEMRI), Center for Global Health Research (CGHR) at the entomology section for rearing, downstream phenotypic, biochemical and molecular evaluation.

Figure 3: Examples of outdoor resting mosquito habitats sampled in Kimaeti, Bungoma County in western Kenya. The habitats in this figure include the pot on the ground and the animal shed that has a roof. These are shown by the red arrows.

3.2.2 Rearing of mosquitoes

Female *Anopheles* mosquitoes collected (fed on blood, half gravid and fully gravid) from both the indoor and the outdoor sampling were introduced into separately labelled holding cages of dimensions 30cm × 30cm × 30cm. The mosquitoes were sustained at temperatures of 25 ± 2°C with a relative humidity (RH) of 80 ± 4%. They were allowed 12-day-hours of light followed by 12-hours of dark in the insectary to mimic the equinox day/night lengths. They were fed on 10% sucrose solution until they became fully gravid. The sugar solution was imbibed from cotton wool balls. Laying trays were provided in the cages for egg laying and easy collection. Eggs laid were

introduced into rearing trays with rain water, they hatched into larvae which were fed on Tetramin™ fish food mixed with brewer's yeast. On a daily basis, the rearing water was changed and the larvae transferred to several rearing trays to prevent overcrowding. Following growth through four larval stages, L1 to L4, pupae were put into small cups and into the holding cages from where they emerged into adults in 10-13 days (Nepomichene *et al.*, 2017).

3.3 Investigating the behavioural plasticity of indoor or outdoor resting of malaria vectors

To investigate whether the choice of resting habitat of malaria mosquitoes is consistent or stochastic, mark-release- recapture experiments were conducted in a semi-field screen house set up with a typical traditional hut, with a bed and LLIN set up, built in one end termed as malariasphere. This work was done at KEMRI-CGHR. The age structure of indoor-resting mosquitoes and outdoor-resting malaria mosquitoes was determined through parity rate dissections.

3.3.1 Mark-release-recapture of the F1 progeny of malaria mosquitoes resting indoors and outdoors

From the F1 progeny of both indoor caught and outdoor caught resting malaria mosquitoes, two hundred 3-5-day old mosquitoes from respective F1 colonies were aspirated into separate paper cups and marked with fluorescent dyes as described by Verhulst *et al* (2013). A pink dye was used for the indoor-resting F1 mosquitoes whereas the outdoor F1 mosquitoes were marked with a green dye. Indoor resting F1 progeny marked pink were released into the screenhouse outside the hut whilst the outdoor-resting F1 mosquitoes marked green were released inside the hut and door closed. After 48 hours of acclimation, the re-capture exercise was done indoors and outdoors, respectively. This was conducted 10 times for the progeny of mosquitoes collected from the two study sites. Following completion of the re-capture exercise, the proportion of indoor-resting

mosquitoes collected outdoors and the proportion of outdoor resting mosquitoes collected indoors was determined using the Lincoln index equation:

$$N = \frac{n_1 \times n_2}{m_2}$$

Figure 4: Interior view of a typical malariasphere at the KEMRI-CGHR centre in Kisumu County, Kenya

3.3.2 Determining the age structure of indoor and outdoor resting wild caught malaria mosquitoes

Wild caught mosquitoes resting indoors and outdoors were age graded by parity rate dissections to investigate the physiological age structure of malaria mosquitoes resting indoors and those found resting outdoors. Mosquito abdomens were dissected individually to determine the parity status based on the ovarian tracheal filament coiling (skein) or uncoiling under a dissecting

microscope (Detinova *et al.*, 1962). The parous ovaries, with uncoiled tracheal filaments, were placed in a drop of PBS buffer solution on a slide and the ovarian sheath removed using dissecting needles to free the ovarioles. They were then individually examined under compound microscope at a 40×/10× magnification for the presence or absence of ovariole dilatations. The number of ovariole dilatations present was used to estimate the number of egg-laying cycles and was recorded.

3.4 Testing phenotypic insecticide resistance in the F1 of indoor and outdoor resting mosquitoes

Blood-fed female *Anopheles* mosquitoes aspirated from either indoors or outdoors were reared under ambient conditions in the insectary. Bioassays were used to detect and characterize phenotypic insecticide resistance within the field collected female mosquitoes and the F1 generation raised from the wild captures.

3.4.1 Phenotypic insecticide resistance assays

The emergent adult F1 progeny reared in the insectary were exposed to the WHO bioassays to detect phenotypic insecticide resistance whose intensity was measured by CDC bottle bioassays. These bioassays were used to detect and characterize, respectively, the resistance to the insecticides commonly used in LLINs.

3.4.1.1 WHO bioassay

Adult 3 to 5-day old female mosquitoes drawn from the raised F1 generation were exposed to discriminating concentrations (DC) of deltamethrin (0.05%), permethrin (0.75%), alphacypermethrin (all pyrethroid insecticides), 5% malathion (organophosphate) and 0.1% bendiocarb (carbamate), treated filter papers according to the standard WHO bioassay. One hundred female mosquitoes comprising of four replicates with subsets of 25 mosquitoes per

replicate were assayed for each of the insecticides tested. For each experiment, a control tube was added also with 25 mosquitoes. The control tube had silicone imbibed papers when testing with pyrethroids and olive oil when testing with the bendiocarb and malathion. All the replicates and controls were exposed for a one-hour-period to each of the insecticide impregnated papers and control base solvents respectively, after which the surviving mosquitoes were provided with 10% sucrose solution. The 24-hour post exposure and holding period mortality was recorded. Similar experiments for pyrethroids were conducted with prior 1hour exposure to piperonyl butoxide synergists. The percentage mortalities were tested by the student t-test, after arcsine transformation, to elucidate any significant statistical differences between the indoor and outdoor resting mosquito populations.

3.4.1.2 CDC bottle assay

The CDC bottle bioassay was used to determine intensity of resistance based on the time-mortality data. Five Wheaton bottles were used for each run of the bioassays. Stock solutions of the insecticides to be used were prepared by dissolving the technical grade insecticide in 50ml acetone in conical tubes and stored in a refrigerator at 4 degrees Celsius.

Table 1: Amount of technical grade insecticide required to make 50ml of required concentration of test solutions

Concentration	µg/bottle	mg/ 1000ml	mg/ 50ml
1×	12.5	12.5	0.625
5×	62.5	62.5	3.125
10×	125.0	125.0	6.25

Appropriate concentrations (1×, 5× and 10×) of insecticide solutions were used to coat the inside of four of the Wheaton bottles at varying concentrations from a predetermined diagnostic dosage. Four of these were treated with deltamethrin, while the fifth was the control bottle with just the solvent, acetone. The bottles each received 1ml of treatment, insecticide for the four test bottles and acetone for the control. The bottles were swirled on their side and turned upside down to coat the inside of the lid. The bottles were left to dry overnight with the lids off for the assays the next morning. From the field collections made, 125 female mosquitoes were used for each experiment per insecticide i.e., 25 mosquitoes per bottle. Using a filtered aspirator, 25 female *Anopheles* mosquitoes were introduced into each bottle by gently blowing them in. A timer was started when the final bottle received mosquitoes (Fig 4). The number of dead mosquitoes was recorded into a recording sheet from the diagnostic time after every 15 minutes until it got to 1 hour or until all the mosquitoes in the bottles died after the start of the experiment (Brogdon & Chan, 2010; Benedict, 2014).

Figure 5: CDC bottle bioassay to determine the intensity of insecticide resistance in Wheaton bottles coated with 10× concentration of deltamethrin in progress

3.4.2 Biochemical tests for resistance associated enzymes by microplate assays

Approximately one hundred, 3-day old female malaria mosquitoes were anesthetized by freezing. They were then individually homogenized using a pestle and centrifuged at 4 degrees Celsius at 3000 revolutions per minute. The activities of metabolic enzymes; acetylcholinesterase, glutathione S-transferase (GST), the oxidase 3,3',5,5'-tetramethylbenzidine (TMBZ) peroxidation were detected using microplate enzyme activity assays. The levels of acetylcholinesterase, glutathione S-transferases and cytochrome P450 monooxygenases were measured in microplates using spectrophotometry (Benedict, 2014).

3.4.3 Molecular identification and detection of *kdr* and *ace* 1 resistance alleles

The mosquitoes used in the susceptibility bioassays, both live and dead, were selected randomly and used in the molecular identification of *kdr*-associated resistance gene. The specimens were preserved in individual 1.5µl Eppendorf tubes with silica for PCR analyses of genetic mutations (Martinez-Torres *et al.*, 1998).

3.4.4.1 Genomic DNA extraction

Genomic DNA was extracted by the alcohol precipitation method (Benedict, 2014). Within each of the 1.5µl Eppendorf tubes, mosquitoes were completely macerated using pestles to break the exoskeleton and enhance cell lysis. A prepared extraction lysis buffer (TE buffer, 0.01M pH 7.4) and a protein kinase enzyme were added and incubated in a warm water bath for 30 minutes. To each of the tubes, 14µl of potassium acetate was added and centrifuged for 15 minutes at 4100 revolutions per minute at room temperature. Following centrifugation, the supernant was discarded

and the tubes washed using 70% and then 100% ethanol sequentially. The tubes were air dried for at least 1 hour. Genomic DNA extracted was washed and re-suspended using prepared TE buffer (Benedict, 2014; Collins *et al.*, 1987; Scott *et al.*, 1993).

3.4.4.2 Molecular identification of the wild and genotyping of resistance alleles in the F1 progeny

The extracted *Anopheles* DNA, positive and negative controls were transferred into 96 well microplates in aliquots of 1.5µl. The wells each received the same volume of a prepared master mix (8.5µl) comprising of primers, nucleotides (with probes for real time PCR) and DNA polymerase enzyme and were loaded onto a pre-programmed thermocycler for DNA amplification. The samples were run on agarose gel for visualization in conventional PCR. A computer imaging UV camera was used to capture the bands as images and saved into a computer. TaqMan assays were used in detection of the *kdr* transmutations (*Vgsc*-1014F, *Vgsc*-1014S and *N1575Y*) (Bass *et al.*, 2007; Jones *et al.*, 2012) and the *G119S* mutation in *Ace 1* in the F1 progeny by Real-Time PCR (Bass *et al.*, 2010).

3.5 Investigating *Plasmodium* sporozoite infection rates in indoor-resting versus outdoor-resting malaria mosquito populations in Western Kenya

Enzyme-linked Immunosorbent assays (ELISAs) were used to detect the *Plasmodium* circumsporozoite proteins (CS) for infected mosquitoes captured from the respective indoor or outdoor resting habitats. Sandwich ELISA were used to assay for presence and intensity of the parasite CS antigens. The intensity of the protein through microplate reader absorbance was directly proportional to the sporozoite levels.

3.5.1 Preparation of mosquito antigen

Whole individual mosquito samples were crushed in 1.5µl Eppendorf tubes using pestles. The macerated specimens were suspended in blocking buffer aspirated into the tubes. The mixtures were tested for CS antigens in the sandwich ELISAs.

3.5.2 Preparation of sandwich ELISA plates

The first step was adsorption of capture monoclonal antibodies onto microtiter plates after which, blocking buffer were used to block the binding sites for 30 minutes. After blocking, the antigen aliquots were added together with positive and negative controls to the microtiter plates and incubated for 2 hours at room temperature. A second peroxidase-conjugated monoclonal antibody was added to the microtiter wells and incubated at room temperature for an hour. If the *Plasmodium* circumsporozoite antigen was present, the capture and peroxidase-conjugated monoclonal antibodies formed complexes. Each of the above steps were followed by a washing using TE buffer in a microtiter plate washing machine. A clear substrate solution of peroxidase was introduced into to each well of the ELISA plates followed by a 1-hour incubation at room temperature. A colour change observed in the substrate solution showed positive samples at the screening stage. The intensities of color change, directly proportional to sporozoite levels, were measured at 410nm wavelength using a microplate reader at the quantification stage. Proportions of the sporozoite levels were compared for samples from indoor captures versus outdoor catches (Benedict, 2014).

3.8 Data analysis

The number of mosquitoes recaptured were weighted, as a proportion of the total number of mosquitoes marked and released. using the Lincoln index equation:

$$N = \frac{n_1 \times n_2}{m_2}$$

Where:
N= number of mosquitoes in the initial population

N1= Number of marked mosquitoes released

N2= Number of marked mosquitoes captured

M2= Number of site-specific marked mosquitoes

The recapture rates per site were subjected to t-test, for statistical differences. Parity rate computations were done by weighting the number of parous mosquitoes over the total number of females dissected per location per site multiplied by 100. The phenotypic assays mortality results were stated as proportions around 95% confidence interval as guided by WHO (2016) criteria. The comparisons between indoor and outdoor insecticide resistance were done by the students t-test after transforming the rates to normality using the by arcsine square root transformation (asin(sqrt(x))). Species identification genotypic data was expressed by proportions of all the samples evaluated. Resistance-associated genotype frequencies were calculated using the Hardy-Weinberg equilibrium equation:

$$p^2 + 2pq + q^2 = 1$$

Where;

p^2 = dominant homozygous frequency (FF)

$2pq$ = heterozygous frequency (Ff)

q^2 = recessive homozygous frequency (ff)

ANOVA was used to analyze microplate enzyme readings after which the Turkey-Kramer HSD test to was applied to determine the sources of variation between the biochemical resistance enzyme activities.

The sporozoite rate was calculated as the proportion per class, of mosquitoes positive for *Plasmodium* circumsporozoite proteins weighted against the number of analyzed mosquitoes as illustrated below:

$$\frac{Number\ pf\ Positive\ mosquitoes}{Total\ number\ of\ mosquitoes\ analyzed} \times 100$$

All the statistical data analyses were conducted in R software version 3.6.3.

CHAPTER FOUR: RESULTS

4.1 Plasticity of behaviour in indoor and outdoor resting malaria mosquitoes

Two approaches were used to report on the plasticity of resting behaviour. Mark-release-recapture was conducted and the age-structure was established through ovarian dissections for parity states. Female mosquitoes were marked and released in the malariasphere.

4.1.1 Mark-release-recapture of the F1 progeny of indoor and outdoor resting malaria mosquitoes

Approximately 8,000 sugar-maintained female mosquitoes were powder-marked and then released in the malariasphere. Each of the mark-release-recapture exercises entailed release of 400 female malaria mosquitoes in one go; 200 with pink and the other 200 with green powder dye. The average mosquito recapture rate for the F1 of vectors resting indoors collected from Kisian was significantly higher inside the hut compared to outside the hut following release outside the hut (9.5% vs 4.4%; $t(18) = 3.114$, $P = 0.006$). On the other hand, the outdoor resting mosquito progeny had a higher recapture rate inside the hut (19.5%) compared to outside (4.3%) within the malariasphere following initial release inside the hut ($t(18) = 6.166$, $p < 0.05$) (Table 2). Similarly, the progeny of indoor resting mosquitoes collected from Kimaeti had a higher recapture rate inside the hut compared to that of outside the hut (25.6% vs 7.6%: $t(18) = 3.962$, $p = 0.001$). The outdoor resting malaria mosquito progeny had significantly higher recapture rates inside the hut than outside (42.4% vs 7.1%; $t(18) = 12.086$, $p < 0.05$) (Table 2).

Table 2: Recapture rates on the progeny of indoor and outdoor resting malaria mosquitoes collected from Western Kenya obtained using the Lincoln index equation (Pink colour= F1 of indoor-resting mosquitoes, Green colour= F1 of outdoor-resting mosquitoes)

	Kisian				**Kimaeti**			
	Inside hut		Outside hut		Inside hut		Outside hut	
	Pink	Green	Pink	Green	Pink	Green	Pink	Green
MRR 1	9.0	18.5	6.5	2.5	6.5	41	5.0	2.0
MRR 2	8.0	29.5	10.5	3.5	21.0	49	5.0	7.5
MRR 3	6.5	16.0	6.0	16.0	16.5	34.5	5.5	12
MRR 4	17.5	23.5	4.5	8.0	8.0	37.5	5.0	6.5
MRR 5	16.0	13.5	0.5	1.5	34.5	46.0	10.5	12
MRR 6	6.5	15.0	1.5	3.5	39.0	48.0	7.5	5.5
MRR 7	7.5	11.0	2.0	2.5	20.5	34.5	2.5	8.5
MRR 8	6.0	25.5	1.5	1.5	36.5	49.5	11.5	5.5
MRR 9	9.0	26.5	3.5	2.0	24.5	28.0	15.5	6.5
MRR 10	8.5	15.5	7.5	2.0	49.0	56.0	7.5	5
Average	**9.5**	**19.5**	**4.4**	**4.3**	**25.6**	**42.4**	**7.6**	**7.1**

4.1.2 Age structure of indoor and outdoor resting wild caught malaria vectors

A total of 649 female *Anopheles* mosquitoes were dissected for parity rates for estimation of the physiological age structure of indoor and outdoor resting malaria mosquitoes. In Kisian, the majority of indoor resting mosquitoes dissected were uniparous (42.41%) followed by nulliparous (38.74%) with the biparous (16.23%) and triparous (2.62%) states being the least respectively whereas the outdoor resting malaria mosquitoes were largely uniparous (37.90%) and biparous (33.87%) (Fig 6). In Kimaeti, the bulk were uniparous (40.57% indoor vs 38.99% outdoor) and biparous (22.86% indoor vs 28.93% outdoor).

Physiologically older female *Anophelines* preferred to rest outdoor than indoor. There were no significant differences ($p<0.05$) between the physiological ages of indoor and outdoor resting malaria mosquitoes (Fig 6).

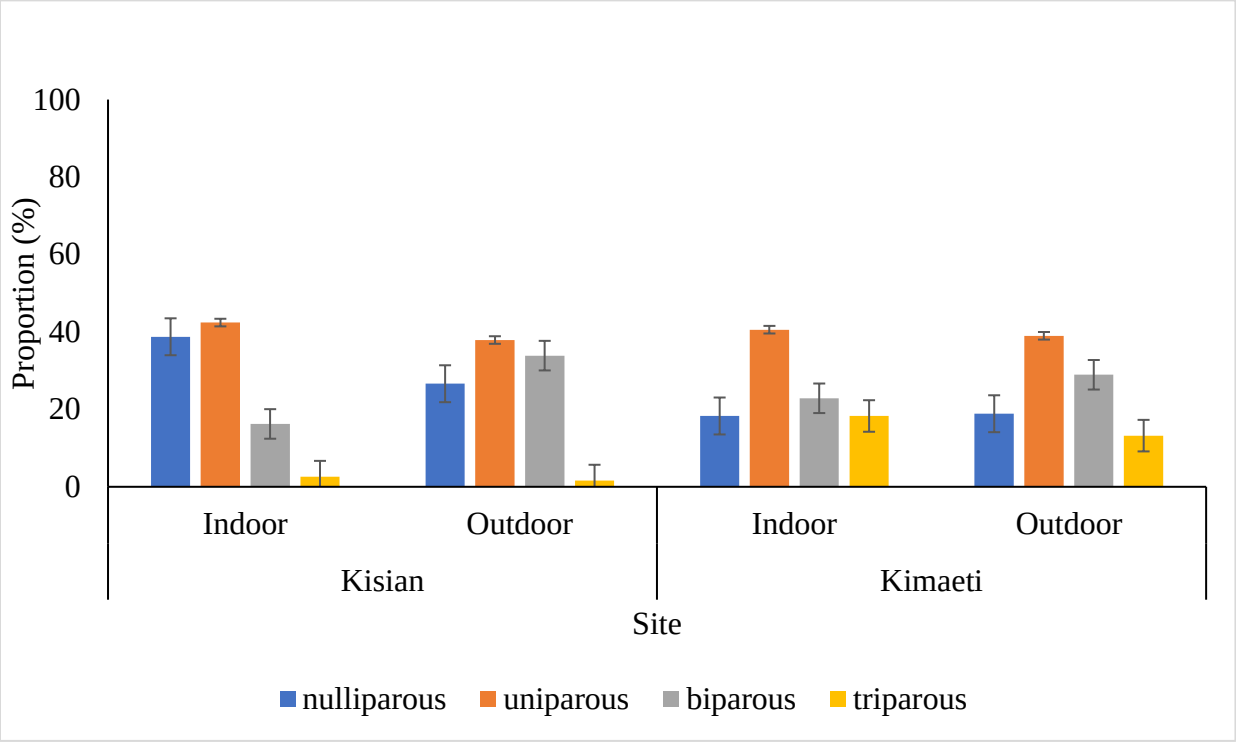

Figure 6: Percentage composition based on the number of egg laying

In Kisian, the number of nulliparous and uniparous females was high especially in *An. gambiae* and *An. funestus* compared to *An. arabiensis* found resting indoors. From the outdoor resting female malaria mosquitoes dissected, the nulliparous mosquitoes were the least in *An. funestus*. The bulk of outdoor resting female malaria mosquitoes were uniparous *An. gambiae*. Biparous mosquitoes were more in *An. gambiae* in comparison to the outdoor resting mosquitoes (Table 3). On the other front, indoor resting malaria vectors from Kimaeti were majorly uniparous *An. gambiae* which were also the bulk of biparous females. The same was observed in outdoor resting malaria mosquitoes dissected. In general, Kimaeti was composed of physiologically older female malaria mosquitoes compared to the Kisian (Table 3).

Table 3: Physiological age composition of indoor and outdoor resting malaria mosquitoes (n=number of mosquitoes sampled, % in brackets)

Site	Location	n	Laying cycles	*An.gambiae* (%)	*An.arabiensis* (%)	*An.funestus* (%)	*Total* (%)
Kisian	Indoor	191	0	28 (14.66)	10 (5.24)	36 (18.85)	74 (38.74)
			1	35 (18.32)	9 (4.71)	37 (19.37)	81 (42.41)
			2	13 (6.81)	7 (3.66)	11 (5.76)	31 (16.23)
			3	3 (1.57)	0	2 (1.05)	5 (2.62)
	Outdoor	124	0	20 (16.23)	10 (8.06)	3 (2.42)	33 (26.61)
			1	12 (9.68)	20 (16.13)	15 (12.10)	47 (37.90)
			2	32 (25.81)	7 (5.65)	3 (2.42)	42 (33.87)
			3	2 (1.62)	0	0	2 (1.61)
Kimaeti	Indoor	175	0	21 (12.0)	4 (2.29)	7 (4.0)	32 (18.29)
			1	59 (33.71)	2 (1.14)	10 (5.71)	71 (40.57)
			2	30 (17.14)	3 (1.71)	7 (4.0)	40 (22.86)
			3	26 (14.86)	0	6 (3.43)	32 (18.29)
	Outdoor	159	0	23 (14.47)	4 (2.52)	3 (1.89)	30 (18.87)
			1	43 (27.04)	3 (1.89)	16 (10.06)	62 (38.99)
			2	30 (18.87)	6 (3.77)	10 (6.29)	46 (28.93)
			3	15 (9.43)	3 (1.89)	3 (1.89)	21 (13.21)

(Number of dissected mosquitoes = n, nulliparous: 0, uni-parous:1, bi-parous: 2, tri-parous: 3)

4.2 Species discrimination of *An. gambiae s.l.* and *An. funestus s.l.*

Species-specific discrimination on a total of 1094 mosquitoes was done within the *An. gambiae s.l.* and on a total of 289 mosquitoes from the *An. funestus s.l.* group from the study sites. Kisian, from the 520 *An. gambiae s.l.* samples analyzed, had *An. gambiae s.s.* turnout of 24.8% (95% CI; 21.1-28.5%) while the composition of *An. arabiensis* was 75.2% (95% CI; 71.5-78.9). All the 122 indoor *An. funestus s.l* samples analyzed turned out to be *An. funestus s.s.* Kimaeti, on the other front, from a total of 574 female mosquitoes, the composition of *An. gambiae s.s.* was 96.5% (95% CI; 95.0-98.0%) while that of *An. arabiensis* was 3.5% (95% CI; 2.0-5.0%). The 167 *An. funestus s.l.* analyzed from Kimaeti were all identified as *An. funestus s.s.* (Table 4).

Table 4: Species composition of *Anopheles gambiae s.l.* and *Anopheles funestus s.l.* from indoor and outdoor resting collections from Western Kenya (% in brackets)

		Anopheles gambiae s.l			***An. funestus s.l.***
Site	**Location**	***An. gambiae s.s (%)***	***An. arabiensis(%)***	**Total**	***An. funestus s.s(%)***
	Indoor	83(33.2)	167(66.8)	250	122(100)
Kisian	Outdoor	46(18.4)	204(81.6)	250	0
	Total	**129(25.8)**	**371(74.2)**	**500**	**122(100)**
	Indoor	304(99.1)	3(0.9)	307	167(100)
Kimaeti	Outdoor	250(93.6)	17(6.4)	267	0
	Total	**554(96.5)**	**20(3.5)**	**574**	**167(100)**

4.2.2 Phenotypic resistance in the F1 progeny of indoor and outdoor mosquitoes

A total of 2,800 *An. gambiae s.l.* and 1,600 *An. funestus s.l* females were evaluated for the detection of insecticide resistance. From both Kisian and Kimaeti, 1,400 *An. gambiae s.l.* and 800 *An. funestus s.l* were assayed in the WHO tube susceptibility test. The mortality rate 24 hours after exposure of mosquitoes from Kisian indoors to deltamethrin was lower (37%, [95% CI; 28-46%]) by a significant amount (t =2.035, df=6, P=0.044) than that of their outdoor-resting counterparts (51% [95% CI; 41-61%]). The mortality rate of indoor-resting *An. gambiae s.l.* progeny was significantly lower than that of the outdoor-resting counterparts (31% [95% CI; 22-40%] vs 51% [95% CI; 41-61%]) 24 hours following their exposure to permethrin (t =2.078, df=6, P=0.042). Alphacypermethrin exposure testing revealed a mortality rate of 30% (95% CI; 21-39%) in the F1 of indoor-resting *Anopheles gambiae s.l.* compared to 60% (95% CI; 50-70%) of their outdoor resting counterparts (t =4.392, df=6, P<0.05). Both indoor-resting and outdoor-resting *Anopheles. gambiae s.l.* were completely susceptible to malathion (Fig 7).

The F1 offspring of indoor resting *Anopheles gambiae s.l.* from Kimaeti showed a 24h-hour post exposure mortality of 49% (95% CI; 39-59%) compared to 53% (95% CI; 43-63%) of those resting outdoors after deltamethrin exposer. Even though the offspring of mosquitoes resting indoors displayed a somewhat lower 24-hour post exposure mortality rate in comparison to that of mosquitoes resting outdoors, the observed difference was not statistically significant (t =0.474, df=6, P>0.05). The F1 that were exposed to permethrin brought about significantly lower 24 hour-post mortality of 7% (95% CI; 1-12%) in the F1 of indoor resting mosquito in comparison with the 51% (95% CI; 41-61%) of the F1 of outdoor-resting mosquitoes (t =6.063, df=6, P<0.001). Exposure testing with alphacypermethrin displayed insignificant difference (t =1.058, df=6, P>0.05) in the 24hour-post mortality rate 70% (95% CI; 61-79%) for the indoor resting mosquito

progeny unlike the 80% (95% CI; 72-88%) mortality rate of the F1 of outdoor-resting malaria mosquitoes. Both of the F1 of either the indoor or the outdoor resting mosquitoes to malathion showed that *An. gambiae s.l.* completely (100% mortality) succumbed to malathion exposure (Fig 7).

Prior exposure of mosquitoes to synergist (PBO) before the insecticide partly reestablished susceptibility of the F1 of indoor-resting mosquitoes in Kisian from a mere 37% to a sweeping mortality rate of 96% with subsequent exposure to deltamethrin ($t = 9.0$, $df = 6$, $P < 0.001$), a low mortality of 31% to a reasonable mortality rate of 79% ($t = 5.908$, $df = 6$ $P = 0.005$) from subsequent permethrin exposure and from a lesser 30% mortality rate to a whooping to 92% ($t = 8.598$, $df = 6$, $P < 0.001$) after sequential exposure testing with alphacypermethrin. This partial restoration of susceptibility by the PBO synergist was correspondingly evident in the F1 raised from the population of mosquitoes resting outdoors with recorded mortality rates of between 98% and 100% for all the tested pyrethroids (Fig 7). In Kimaeti, following the introduction of synergist (PBO) into the testing, the F1 of indoor-resting *An. gambiae s.l.* F1 succumbed significantly ($t = 7.095$, $df = 6$, $P < 0.001$) with increased 24 hour-post mortality rate from 49% to 100% after subsequent deltamethrin exposure, from a mortality rate of 7% to a highly significant ($t = 16.436$, $df = 6$, $P < 0.001$) 95% after permethrin exposure. Lastly, PBO pre-exposure followed by alphacypermethrin recounted a significant ($t = 5.385$, $df = 6$, $P = 0.001$) 24-hour post exposure mortality rate increment from 70% to 99%. The susceptibility reinstating effect of the synergist was similar in the offspring of the outdoor-resting mosquito population whose mortality rate ranged between from 94% to 100% across the board (Fig 7).

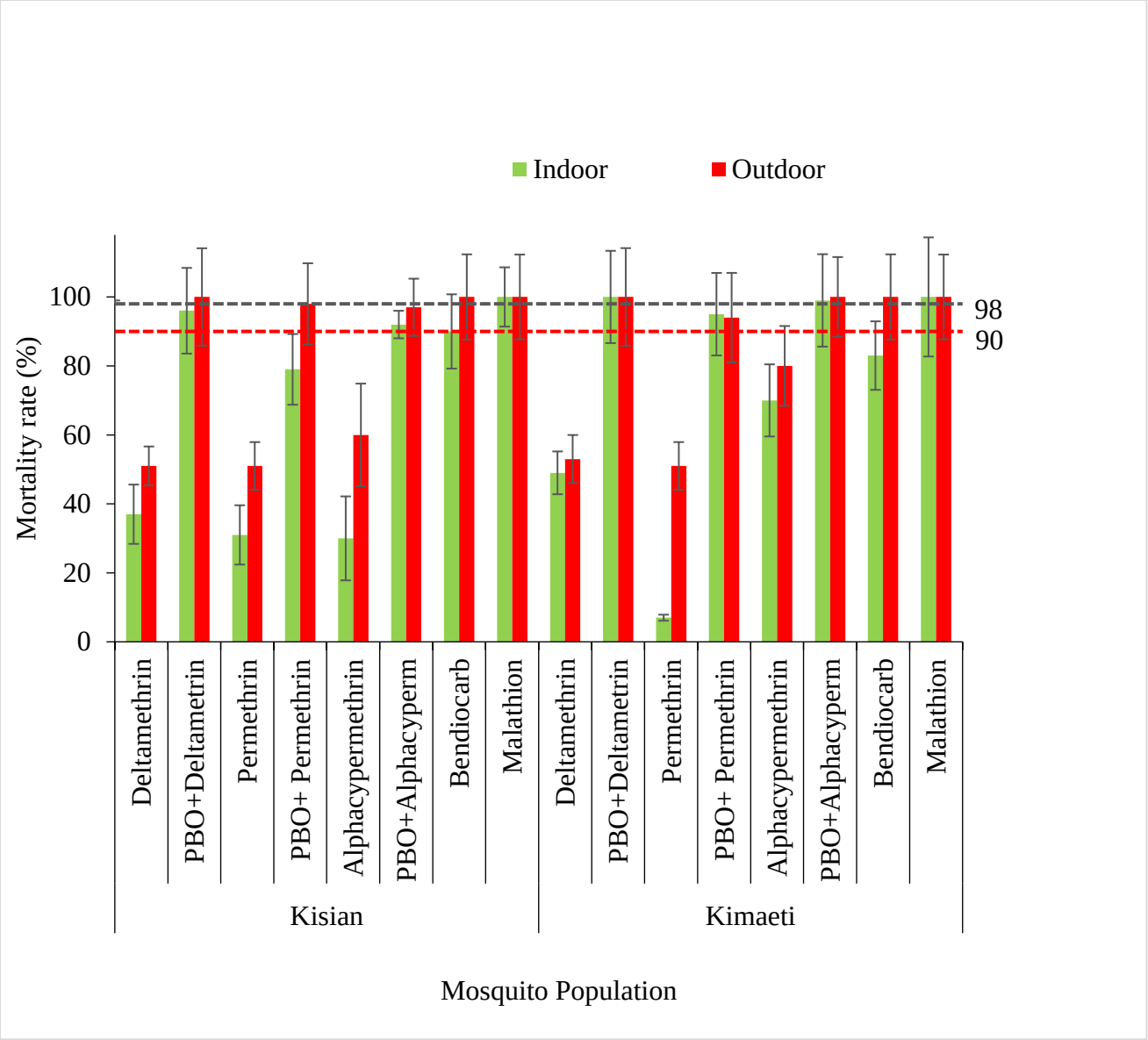

Figure 7: Percentage mortality of *An. gambiae s.l* from WHO tube bioassays with and without PBO. (Green represent indoors and red represent outdoors).

Only the *Anopheles. funestus s.l.* collected indoors were analyzed from both study sites due to their insignificant numbers aspirated outdoors and the technicality of raising the F1. In Kisian, the 24hour-post exposure mortality rate when exposed to deltamethrin, the *An. funestus* succumbed by 68% (95% CI; 59-77%), when exposed to permethrin by 74% (95% CI; 65-83%) and after alphacypermethrin exposure by 77% (95% CI; 69-85%). In Kimaeti, on the other front, displayed 24hour-post mortality of 62% (95% CI; 52-72%) after deltamethrin exposure of the F1 of *An. funestus*, following permethrin exposure testing reported a mortality of 89% (95% CI; 83-95%) and lastly a mortality of 61% (95% CI; 51-71%) was revealed after exposure testing with alphacypermethrin. The PBO pre-exposures revealed that all the mosquitoes from the study sites succumbed to the respective insecticides (Fig. 8).

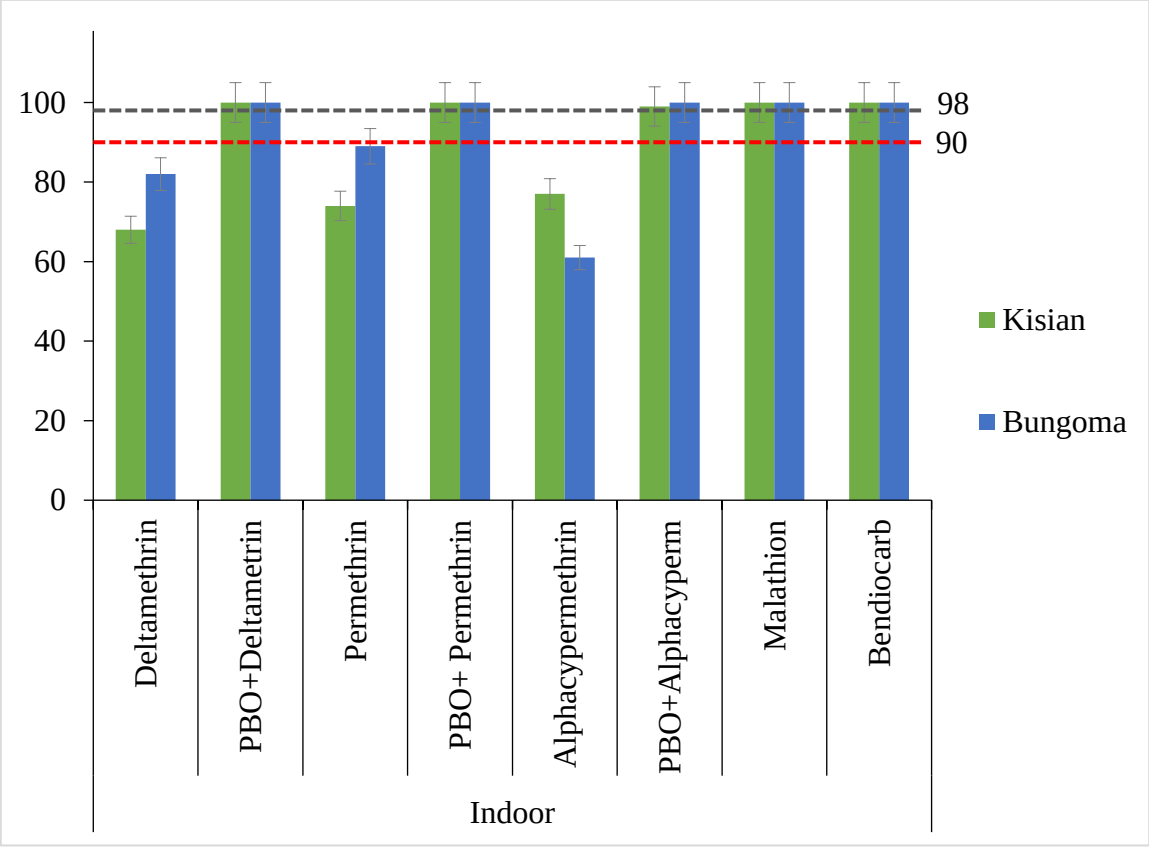

Figure 8: Percentage mortality of *An. funestus s.l* mortality from WHO tube bioassays with and without PBO

4.2.3 Intensity of insecticide resistance in F1 of *An. gambiae s.l.* resting indoors and outdoors

From the CDC intensity bioassays, the mortality rates displayed by the F1 of indoor resting *Anopheles gambiae s.l.* from Kisian subjected to concentrations of 1×, 5× and 10× the diagnostic dose of deltamethrin showed 42% (95% CI; 32-52%) at 1×, 78% (95% CI; 69-84%) and 100%, respectively, while for the progeny of mosquitoes resting outdoors the mortalities rates revealed were 51% (95% CI; 41-61%), 83% (95% CI; 76-90%) and 100% following exposer to 1×, 5× and 10× deltamethrin respectively. This, with guidelines from WHO 2016 criteria (WHO, 2016) directed towards moderate-intensity insecticide resistance across both resting locales (Fig. 9**).** In spite of the fact that the mortality rate turned out lower for the F1 of indoor resting mosquitoes when compared to their outdoor resting counterparts at 1× concentration (t=1.269, df=6, P=0.130) and at 5× concentration (t=0.823, df=6, P=0.221), the differences were statistically insignificant (Fig 9).

The F1 raised from population of *Anopheles gambiae s.l.* resting indoors from Kimaeti reported a mortality of 31% (95% CI; 22-40%) at 1× concentration, 75% (95% CI; 67-83%) at 5× concentration and 100% at 10× concentration of deltamethrin. The F1 of mosquitoes resting outdoors showed mortality rates of 48% (95% CI; 38-58%) at 1× concentration, 80% (95% CI; 72-88%) at 5× concentration and a whopping 100% at 10× the diagnostic concentration of deltamethrin thereby, indicating a moderate-intensity insecticide resistance based on a guided judgement by the WHO 2016 criteria in both locations (WHO, 2016). Likewise, regardless of the mortality rates being lower for the F1 of mosquitoes resting indoors than those resting outdoors, there was no statistically significance in the difference between their mortality rates at 1× concentration (t=1.512, df=6, P>0.05) and at 5× strength (t=0.808, df=6, P>0.05) (Fig. 9**).**

From both study sites, only the F1 raised from indoor resting *Anopheles funestus* were assayed due to challenges in raising offspring from the few numbers sampled from the field. The mortality across both sites was significantly increased as the concentration of deltamethrin was strengthened (Fig 10).

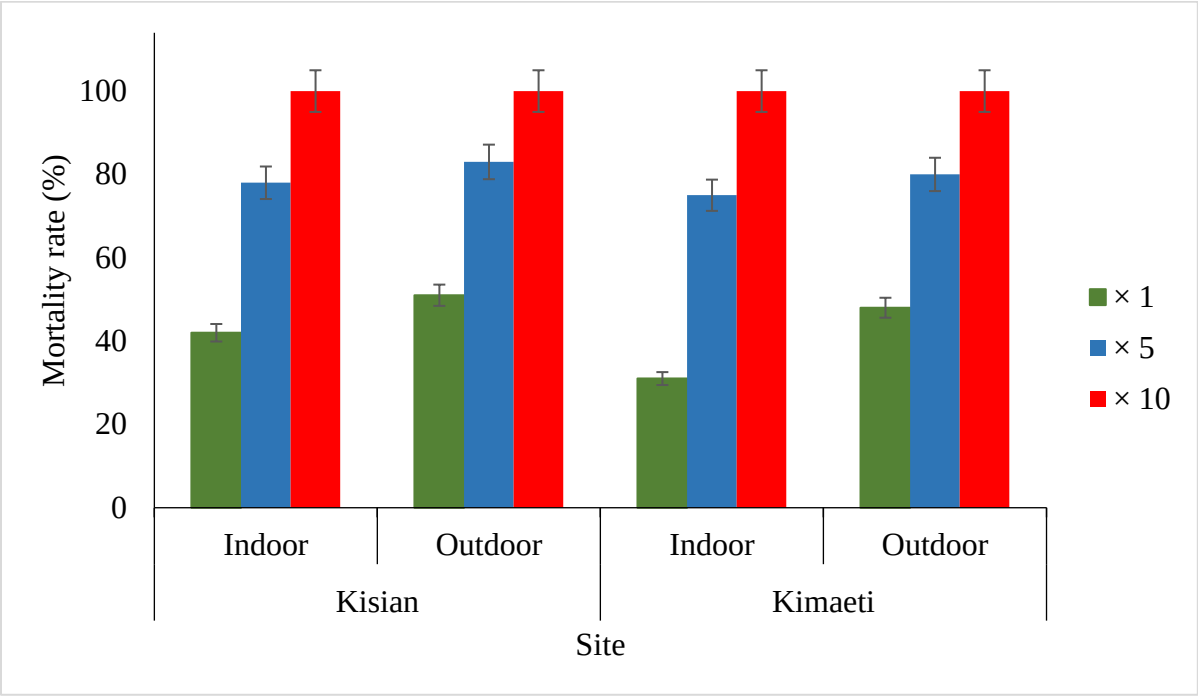

Figure 9: Mortality rate of *An. gambiae s.l.* exposed to ×1, ×5, and ×10 concentration of deltamethrin in CDC intensity bottle bioassays

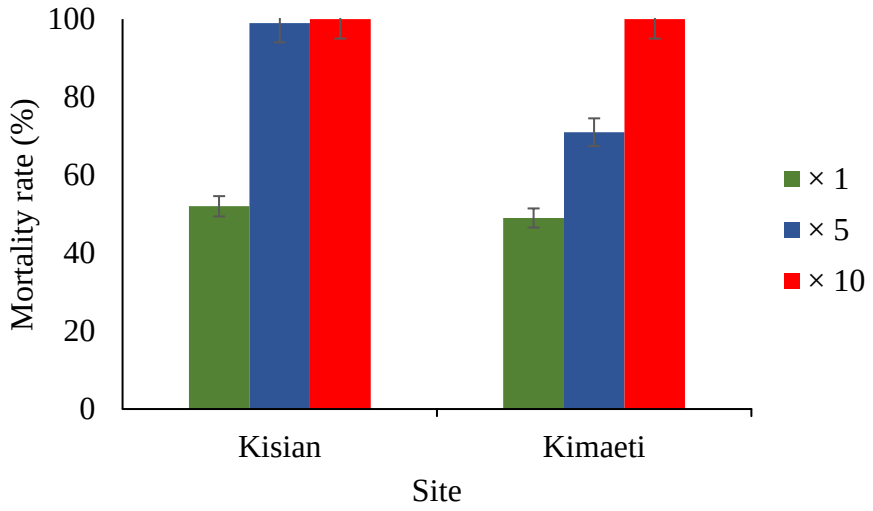

Figure 10Mortality rate of *An. funestus s.l.* exposed to ×1, ×5, and ×10 concentration of deltamethrin in CDC intensity bottle bioassays

4.2.4 Target site genotyping for resistance alleles in the indoor and outdoor resting *An. gambiae s.l.*

In Kisian, the frequencies of the *kdr* East and *kdr* West (vgsc L1014S and vgsc L1014F respectively) allelic mutations in indoor-resting malaria vectors were 0.14 and 0.19 respectively whereas that of outdoor-resting vectors detected were 0.14 and 0.12 respectively. The detected *ace 1* mutation in the indoor resting mosquitoes was higher (0.23) compared to that of the mosquitoes resting outdoors (0.12). Most probable owing to the small number of *An. gambiae* sample size from Kisian, the vgsc-1014S and *ace 1* mutations were not found (Table 5).

The allele frequencies of *kdr* East and *kdr* West detected in mosquitoes sampled resting indoors from Kimaeti were 0.75 and 0.05 respectively. The allelic frequencies detected in outdoor-resting malaria vectors were 0.67 and 0.03 respectively. On another front, the *ace 1* (G119S) mutation presented with a frequency of 0.05 in the mosquitoes resting indoors and was undetected in

outdoor-resting malaria mosquitoes. The *kdr* mutation at locus 1575Y was absent in sampled mosquitoes from both study sites and locales (Table 5**).**

Table 5: Frequency of *Kdr* and *Ace 1* resistant alleles in indoor and outdoor-resting *An. gambiae* s.s and *An. arabiensis* populations from Western Kenya (n= number of analyzed mosquitoes, p^2 + *2pq*, Where; p^2 = homozygous resistant, *2pq* =heterozygous resistant)

				Vgsc (*kdr*)			Ace 1
				Locus 1014		Locus 1575	Locus 119
Site	Location	Species	n	L1014S	L1014F	1575Y	G119S
Kisian	Indoor	*An.gambiae*	8	0	0.25	0	0
		An.arabiensis	36	0.14	0.19	0	0.23
	Outdoor	*An.gambiae*	1	0	0	0	0
		An.arabiensis	43	0.14	0.12	0	0.12
	Total	*An. gambiae*	9	0	0.33	0	0
		An.arabiensis	79	0.08	0.06	0	0.19
Kimaeti	Indoor	*An.gambiae*	43	0.75	0.05	0	0.05
		An.arabiensis	1	0.01	0	0	0
	Outdoor	*An.gambiae*	39	0.67	0.03	0	0
		An.arabiensis	5	0.60	0	0	0
	Total	*An.gambiae*	82	0.72	0.06	0	0.02
		An.arabiensis	6	0.07	0	0	0

4.2.5 Biochemical enzyme levels in the F1 of indoor and outdoor resting *An. gambiae s.l.*

The metabolic enzymes whose activities are involved in detoxifying insecticides (monooxygenases, beta-esterases and glutathione S-transferases) were evaluated to find out their role in insecticide resistance in the F1 of *Anopheles gambiae s.l.* In mosquitoes from Kisian, the monooxygenases levels were increased by 1.83-folds in the F1 of indoor-resting *Anopheles gambiae s.l.* while it was raised by 1.66-folds in the F1 of outdoor-resting mosquitoes with reference made to the susceptible laboratory Kisumu strain ($F_{2,134}$=105.20, P<0.05, Fig. 11). The β-Esterases levels were not significantly different for the F1 progeny raised from indoor resting compared to those of *An. gambiae s.l.* mosquitoes resting outdoors ($F_{2,134}$=188.50, P<0.05, Fig. 12). The level of GSTs was elevated by a 2.3-folds in the F1 of indoor-resting mosquitoes from Kisian. This turned out significantly higher than that of the F1 of outdoor-resting mosquitoes ($F_{2,134}$=95.14, P<0.05, Fig. 13) by reference to the insectary susceptible Kisumu strain.

In mosquitoes from Kimaeti, the enzyme activity of monooxygenases was 1.3-folds higher in the F1 of indoor resting mosquito population compared to the F1 of outdoor resting mosquitoes ($F_{2,134}$=51.43, P<0.05, Fig 11). The levels of β-esterases from Kimaeti were elevated by 1.2 folds in the F1 of indoor-resting mosquitoes. This was significantly higher than that of the offspring of the outdoor resting mosquitoes ($F_{2,134}$=36.66, P<0.001, Fig. 12). The activity levels of Glutathione S-transferase in the F1 progeny of indoor-resting mosquitoes were elevated by a 3.0-folds in the progeny of mosquitoes found resting indoors than those of mosquitoes found resting outdoors ($F_{2,134}$=119.9, P<0.05) in comparison to insectary susceptible Kisumu strain as reference (Fig. 13**)**.

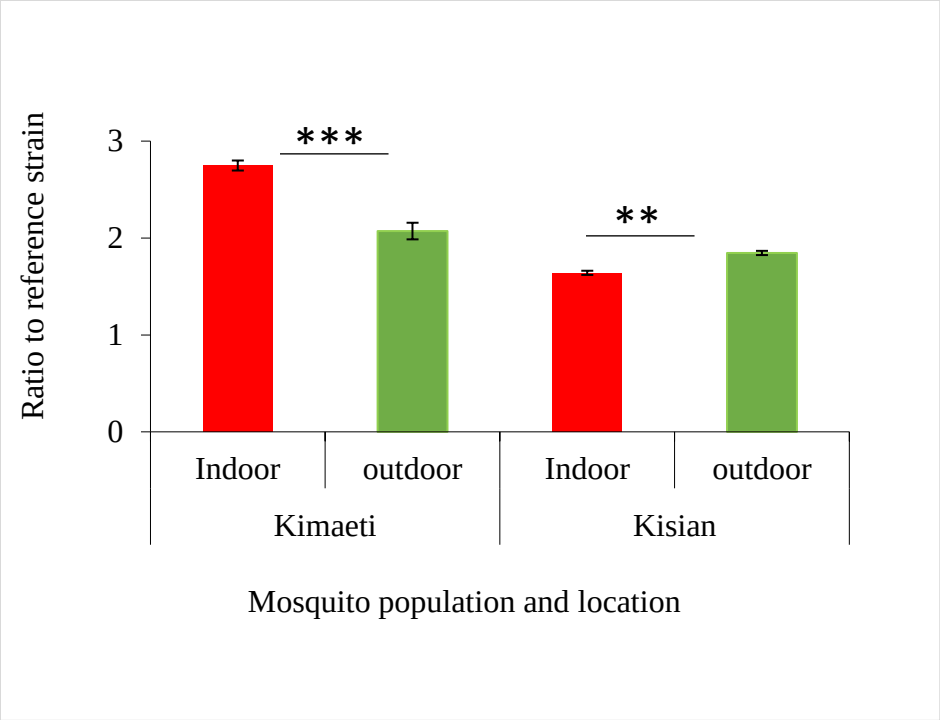

Figure 11:Monooxygenase enzyme activity in *An. gambiae s.l.* (**P<0.05, ***P<0.001).

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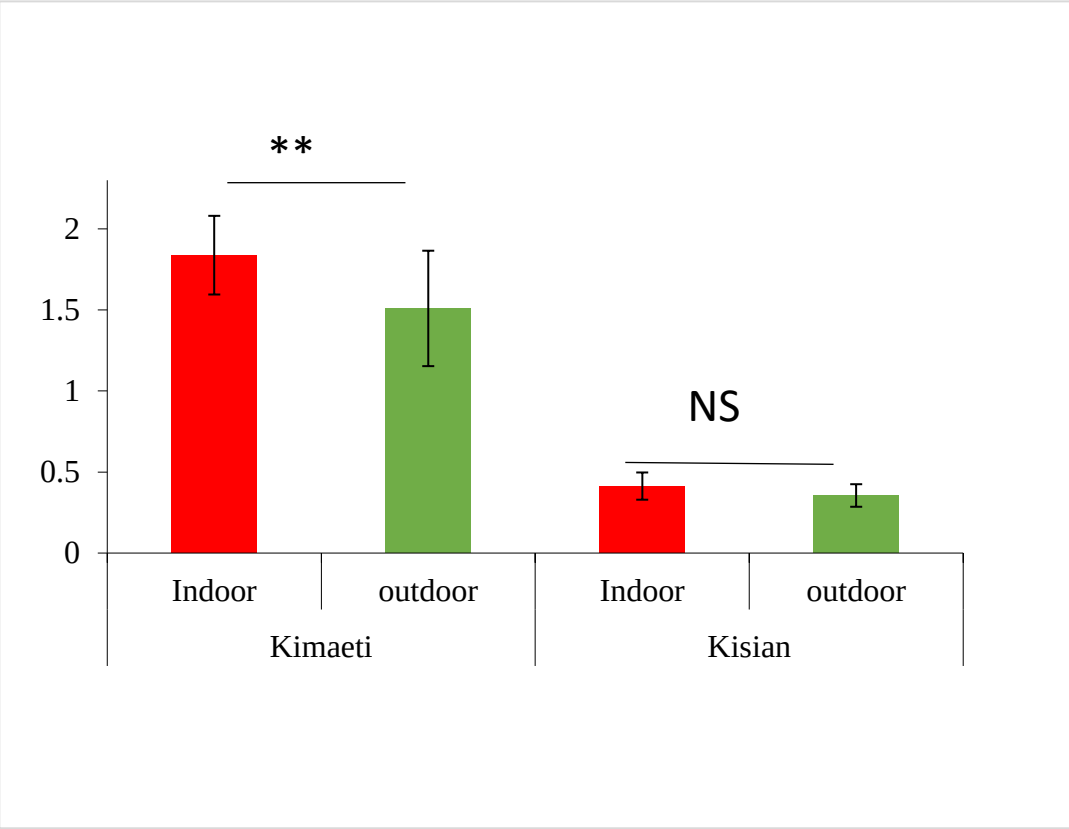

Figure 12: Esterase enzyme activity in *An. gambiae s.l.* (**P<0.05, NS not significant).

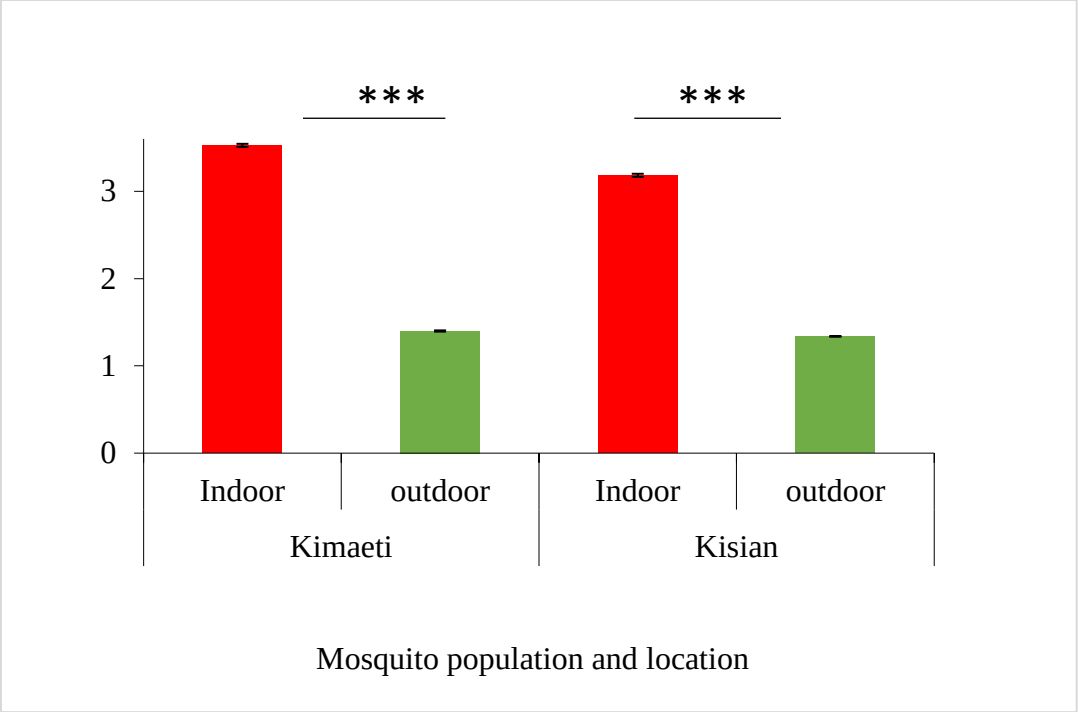

Figure 13: Glutathione S-transferase enzyme activity in *An. gambiae s.l.* (**P<0.05, ***P<0.001).

4.3 Investigating *Plasmodium* sporozoite infection rates in indoor vs outdoor resting malaria mosquito populations in Western Kenya

Mosquito samples were assayed for *Plasmodium* circumsporozoite protein presence by CS-ELISA. A total of 1,000 female mosquitoes comprising of 522 *An. gambiae*, 412 *An. arabiensis* and 66 *An. funestus* were used in the assay between both sites and respective indoor or outdoor locations in batches of 250 female mosquitoes. In Kisian, out of 250 mosquitoes that were caught resting indoors, the sporozoite rate was 8.8% (22/250) while for those that were collected outdoors was 3.6% (9/250) (Table 6). The sporozoite rate for mosquitoes resting indoor from Kimaeti was 20.4% (51/250) whereas that of their outdoor resting counterparts was 10.4% (26/250). From both study sites *An. gambiae* had higher proportions of *Plasmodium* sporozoite infections both in indoor resting and outdoor resting mosquitoes (Table 6).

Table 6: Sporozoite rate between mosquitoes resting indoors and those resting outdoors (n=number of mosquitoes analyzed, % in brackets)

			Mosquito species			
			An. gambiae	*An. arabiensis*	*An. funestus*	Total
Site	location	n	No. pf positive (sporozoite rate %)			
Kisian	Indoor	250	12(4.8%)	7(2.8%)	3(1.2%)	22(8.8%)
	Outdoor	250	6(2.4%)	3(1.2%)	0	9(3.6%)
Kimaeti	Indoor	250	38(15.2%)	5(2.0%)	8(3.2%)	51(20.4%)
	Outdoor	250	24(9.6%)	2(0.8%)	0	26(10.4%)

CHAPTER FIVE: DISCUSSION

This study sought to determine the effect of indoor insecticide use on the indoor-resting and outdoor-resting behaviour of malaria mosquitoes in Western Kenya. Firstly, the plasticity of resting behaviour was investigated. Secondly, the status of resistance to insecticide by female *Anopheles* mosquito species that are either indoor-resting or outdoor-resting was determined. Finally, the sporozoite rates in malaria mosquitoes resting indoors and outdoors was compared to understand the implication on malaria transmission. The resting behavioural plasticity was generally observed in the malaria vectors. There was commendable phenotypic as well as physiological insecticide resistance by malaria vectors from Western Kenya resting indoors compared to those resting outdoors. The sporozoite rate was similarly higher in indoor-resting malaria mosquitoes compared to the ones resting outdoors.

Across both study sites, *An. funestus* was found mostly resting indoors. The *An. arabiensis* malaria vector was more abundant in Kisian compared to its sibling species *An. gambiae s.s.* The *An. gambiae s.s*. mosquito was dominantly abundant in Kimaeti, an observation similar to prior accounts (Bayoh *et al.*, 2014; Degefa *et al.*, 2017; Machani *et al.*, 2020; Ochomo *et al.*, 2013; Ochomo *et al.,* 2012).The high temperatures and low humidity in lowlands tend to favour *An arabiensis* which is more resilient whereas the low temperatures and high relative humidity present in highlands favour *An. gambiae* (Afrane *et al.*, 2007). The different ecological conditions of the two study sites have been reported to be predominated by *An. arabiensis* in the lowlands and *An. gambiae s.s.* in the highlands of Western Kenya. The abundant presence of *An. funestus* indoors across both study sites as well has been reported previously (Bayoh *et al.*, 2010; Degefa *et al.*, 2017; Machani *et al.*, 2020; Ochomo *et al.*, 2012; Ototo *et al.*, 2015; Wanjala *et al.*, 2015).

The resting indoor or outdoor resting behaviour of malaria vectors determines, alongside the feeding patterns, the effectiveness of LLINs by the potential proportional exposure to these insecticide-based interventions (Trung *et al.*, 2005; WHO, 2012). The study has shown that the plasticity in the resting behaviour of malaria mosquitoes. The mosquitoes either maintained the same place or moved to the location of recapture. This kind of behavioral plasticity has been reported for host-selection behaviours (Gillies 1964) (Takken & Verhulst, 2013), indoor or outdoor feeding (Russell, 2011, Reddy 2011) biting times (Magesa 1991, Mbogo 1996). The proportion of indoor resting *Anopheles* mosquito progeny that were recaptured inside the hut and the outdoor resting progeny counterparts suggest a maintenance of behavioural pattern from the mother population. This has been demonstrated in some cases where more than a single recapture was conducted in the field and significant proportions of the marked mosquitoes were recaptured in the same locations the second time (Russell *et al.*, 2016).

The movement of the indoor resting mosquito progeny into the hut could be attributed to the synanthropic nature of malaria mosquitoes having a tendency to be attracted to human dwellings. The movement of the outdoor resting progeny from the initial release inside the hut to outside could be in expedition for sugar sources in the plants in the malariasphere (Gu *et al.*, 2011). Studies have shown that malaria vectors are capable of changing their behavioural patterns especially after the successive deployment of interventions such as LLINs (Killeen *et al.*, 2017; Russell *et al.*, 2011). The LLIN could have repelled some of the outdoor resting mosquito progeny to exit the hut, an effect expected during development of LLINs. The plasticity of the resting behaviour of the malaria vectors that were recaptured at the same location of release demonstrates that indoor or outdoor resting subpopulations occur within the same populations of malaria vectors as has been reported previously in Solomon Island by Russell *et al.,* (2016). Repeated studies within Western

Kenya have reported *Anopheles gambiae s.s*, *Anopheles arabiensis* and *Anopheles funestus s.l* collections in both indoor and outdoor sampling thereby demonstrating plasticity of malaria vectors (Bayoh *et al.*, 2014; Degefa *et al.*, 2017; Machani *et al.*, 2020).

The majority of physiologically older malaria vectors were resting outdoors than indoors across both study sites. Physiological insecticide resistance in malaria mosquitoes declines with increase in age (Saddler & Koella, 2015). This could have led to increased toxicity of LLIN approved insecticides to the physiologically older indoor resting malaria mosquitoes hence their repulsion to resting outside or even killing and reduction. This could be the reason for the drastic decline in the number of female malaria mosquitoes that had laid eggs twice (biparous). The majority of the nulliparous *An. funestus* were found resting indoors in Kisian. This could be due to their endophilic nature as studies have reported previously by several studies (Bayoh *et al.*, 2014; Charlwood *et al.*, 1995; Degefa *et al.*, 2017). The *An. gambiae s.l.* complex at the various gonotrophic cycle stages were homogenous in both the indoor and the outdoor resting malaria mosquitoes dissected. This suggests that there are different behavioural adaptations exhibited by the same pool of these malaria vectors. Several prior studies in have reported different behavioral responses especially to the intervention tools within the same population of mosquitoes (Machani *et al.*, 2020; McCann *et al.*, 2014; Ndiath *et al.*, 2014; Reddy *et al.*, 2011; Russell *et al.*, 2011; Sougoufara *et al.*, 2014; Trape *et al.*, 2011).

The study demonstrates that despite the use of LLINs in indoor vector control, malaria vectors are still able to thrive to physiological ages (triparous) where the incubation period of malaria parasites lies. Physiologically older mosquitoes have been reported to be the ages of high malaria transmitters (Cook *et al.*, 2008). This is especially in Kimaeti (Bungoma county) where high sporozoite positivity rates have previously been reported in the major malaria vectors, *Anopheles*

gambiae and *Anopheles funestus* (Degefa *et al.*, 2017; Machani *et al.*, 2020). The physiologically older a female malaria mosquito gets, the more it will have taken more blood meals hence increased chances of being infected with and transmitting *Plasmodium* parasites. Similar reports have been brought forward by several studies (Cook *et al.*, 2008; Mayagaya *et al.*, 2009; Uttah *et al.*, 2013).

The study detected higher pyrethroid phenotypic insecticide resistance in malaria mosquitoes resting indoors compared to the outdoor-resting mosquitoes. Phenotypic resistance to pyrethroid insecticides in Western Kenya *An. gambiae s.l.* mosquitoes is evidently widespread. This observation has been made by reports of previous studies (Hemming-Schroeder *et al.*, 2018; Ochomo *et al.*, 2012; Wanjala *et al.*, 2015). The pyrethroid resistance by *An. funestus* was detected and has as well been reported by previous findings (McCann *et al.*, 2014). Many reports of rising levels of resistance to pyrethroid insecticides in the class of public-health approved use in LLINs have been made in these regions of Western Kenya (Kawada *et al.*, 2011; Ochomo *et al.*, 2012; Ochomo *et al.*, 2014; Wanjala *et al.*, 2015). There was total susceptibility to both malathion and bendiocarb by the malaria mosquitoes, an observation similar to prior studies such as findings from Ghana (Majidah *et al.*, 2020). It was observed that pre-exposure to synergist (PBO) partially restored susceptibility to pyrethroids by the malaria mosquitoes (resting both indoors and outdoors). This revealed the greater role of the metabolic enzymes in detoxifying insecticides in these areas. The finding coincides with earlier reports from Western Kenya of there being more factors contributing to insecticide resistance (Hemingway *et al.*, 2004; Martinez-Torres *et al.*, 1998; Ochomo *et al.*, 2015). Increased deltamethrin concentration in the CDC bottle bioassays restored susceptibility to 100%. This finding suggests that exposure to the current dosage concentration in LLINs is becoming inadequate. This may also be due to interactions with non-lethal dosages in agro-chemicals may be contributing to development of resistance to pyrethroids. This has previously been demonstrated in

malaria mosquitoes

(Lindblade *et al.*, 2006). These results depicted moderate intensity insecticide resistance levels according to set guidelines for monitoring and evaluating insecticide resistance in malaria

mosquitoes (WHO, 2016), i.e. they were fully susceptible to the highest concentration of deltamethrin. The phenotypic insecticide resistance, that is especially higher in mosquito populations resting indoors, could perhaps get to levels threatening the present-day insecticide-based vector control tools. This should be a cause for concern just as suggested by prior studies (Churcher *et al.,* 2016; Protopopoff *et al.,* 2018).

The detection of insecticide-resistance-associated alleles was observed in both the indoor and outdoor-resting malaria mosquito population. Even though it was higher indoor than outdoors, this could be attributed to favourable adaptations that may be triggered by selection pressures from the repeated exposure to insecticide-based tools such as LLINs or even in the extensive agro-chemical usage in tobacco farms for example in Kimaeti study site. Previous studies have reported resistance genotypes in malaria mosquitoes (Lindblade *et al.,* 2015; Ochomo *et al.,* 2012; Ranson & Lissenden, 2016; Trape *et al.,* 2011). The study also recorded substantial proportions of the vgsc-1014F and vgsc-1014S in *An. Arabiensis* in lower frequencies. This has previously been reported in Western Kenya by preceding studies (Hemming-Schroeder *et al.*, 2018; Ochomo *et al.*, 2012; Ochomo *et al.*, 2015). This manifestation of more than one *kdr* mutations at a given locus (1014) within an *An. gambiae s.l.* population of is in line with studies that have already shown and reported previously (Kabula *et al.*, 2014; Kawada *et al.*, 2011; Machani *et al.*, 2020; Ochomo *et al.*, 2012; Ochomo *et al.*, 2015). The significant *kdr* mutations observed are attributable to the buildup of pressures of selection arising from the widespread contact with insecticides particularly through vector control measures based indoors (Machani *et al.,* 2020; Ochomo *et al.*, 2012; Ochomo *et al.*, 2014; Ochomo *et al.*, 2015; Ranson & Lissenden, 2016). The ace 1 (*G119S*) mutation was detectable in Kisian despite the fact that it was at lower frequencies this was higher in indoor resting mosquitoes in comparison with outdoor-resting malaria mosquitoes. The ace 1

mutation was detected more in Kisian compared to Kimaeti. With these findings, the suggestion is pointing towards different pressures of selection that might be present in the lowland but completely missing from the highland or contrariwise. These allelic mutations could be originating from such variations of selection pressures.

The activities of detoxifying metabolic enzymes, (β-esterases, GSTs and monooxygenases) associated with insecticide resistance were quite elevated. This was by higher margins in indoor-resting malaria vectors in comparison with those of mosquitoes resting outdoors from both sites. Malaria mosquitoes pre-exposed to PBO synergist in phenotypic experiments exhibited partial susceptibility restoration to commonly used pyrethroids by public health e.g., in LLINs. Phenotypic experiments with prior PBO exposures revealed that monooxygenases play a superior role in abetting metabolic insecticide resistance. Implication of monooxygenases in the pyrethroid resistance has been reported by previous studies in Western Kenya (Ochomo *et al.*, 2012). Mosquitoes from Kisian did not elucidate the involvement of β-esterases in contributing to insecticide resistance as is seen by the analogous activity levels in both the indoor-resting and the outdoor resting mosquito populations. However, in mosquitoes from Kimaeti, the β-esterases enzyme levels were increased. This was higher in the F1 of mosquitoes resting indoors mosquitoes compared to resting outdoors. The glutathione-S-transferase enzymes feasibly had a role in the observed insecticide resistance similar to a previous account from different geographical zones (Nardini *et al.*, 2012). With these observations, we can conclude therefore, that monooxygenases in Kisian, were the primary machinery of resistance to insecticides more likely despite having less detection of the of resistant allele frequencies. In Kimaeti however, the observations lean towards a blend of both genotypic and metabolic machineries playing part.

The study recorded high *Plasmodium falciparum* sporozoite infectivity rates in indoor resting mosquito captures than the outdoor ones across both study sites. Prior findings have reported high sporozoite infectivity in Western Kenya and many parts of Sub-Saharan Africa (Degefa *et al.*, 2017; Machani *et al.*, 2020). It was observed that the malaria vectors sampled were all important in malaria transmission, with *An. gambiae* having the highest positivity for circumsporozoite protein compared to *An. arabiensis* and *An. funestus* across both study sites similar to what has been reported by previous studies (Bayoh *et al.*, 2014; Machani *et al.*, 2020; Mayagaya *et al.*, 2015). The major malaria vector *An. gambiae* and *An. funestus* have maintained the anthropophagic behaviour. In comparison, *An. arabiensis* whose feeding preference is diverse has been reported to feed on non-human hosts (Degefa *et al.*, 2017; Machani *et al.*, 2020; Muriu *et al.*, 2008) hence reduced chances of acquiring and transmitting malaria parasites. This could be the reason the study together with previous reports recorded lower sporozoite rates in *An. arabiensis* compared to *An. gambiae*. The ability to rest close proximity to humans despite having interventions such as LLINs in place due to insecticide tolerance they have developed creates a risk of continued malaria transmission as similar studies have reported (Hughes *et al.*, 2020; Knox *et al.*, 2014). The different mechanisms of insecticide resistance may make it possible for mosquitoes to rest within the households waiting for potential host once they exit the bed nets. This shift in resting behaviour supports other studies reporting shifts expose persons during unprotected times to the insecticide tolerant malaria vector bites in proximity thereby creating risk of malaria transmission. Behavioural changes in biting patterns (Ototo *et al.*, 2015; Wamae *et al.*, 2015), biting phenology and host preference on the implications on disease transmission have been reported (Takken & Verhulst, 2013). Similarly, outdoor resting malaria mosquitoes sampled were sporozoite positive meaning that outdoor malaria transmission could be maintained by the

population of vectors that prefer resting away from indoor-based interventions. Previous studies have reported malaria vectors avoiding intervention areas (Killeen, 2014; Kleinschmidt *et al.*, 2007; Ndiath *et al.*, 2014; Reddy *et al.*, 2011; Sougoufara *et al.*, 2014).

The malaria mosquitoes displayed plasticity in resting behaviour study showing that the behaviours can be elicited by random factors. The presence of phenotypic, metabolic and genotypic resistance in Western Kenya was observed higher in the vectors resting indoors compared with the malaria mosquitoes that prefer to be resting outdoors. Predominant LLINs use of in controlling malaria mosquitoes could be a double-edged sword. This together with the extensive agro-chemical usage could be firming up the increase of insecticide resistance around these regions (Machani *et al.*, 2020; Ochomo *et al.*, 2013). The higher levels of resistance to insecticides in indoor-resting malaria vectors suggests that they might be indoor-resting because they have gained enough tolerance (or resistant) to LLINs-embedded insecticides. This levels of insecticide resistance have been brought forward as potential threats to the broad coverage of these insecticide-based mitigations (Ochomo *et al.*, 2013). The resistance mechanisms were present as well in the mosquitoes resting outdoors, suggestive of the exposures in just enough pressures to these insecticide-based interventions capable of eliciting the expression of the insecticide resistance trait. Reduction in mortality or insecticide susceptibility by resistant malaria vectors due to the insecticide resistance levels being sufficient to provoke upsurge of malaria incidences. Insecticide resistance has been implicated in sustaining malaria incidence and hindering achieved successes of current vector control interventions (Churcher *et al.*, 2016). The levels of sporozoite infectivity rate among the major malaria vectors in the indoor and outdoor resting mosquito populations is a clear indicator that in presence of indoor intervention transmission still persists just as it does where there is no intervention outdoors. This sporozoite infectivity of malaria vectors

in the insecticide era has well been documented (Machani *et al.*, 2020; Reddy *et al.*, 2011). The major concern remains to be the rapidly growing behavioural resistance. This needs be given more attention in order to successfully improve on the fight against vectors of malaria as concluded by earlier studies (Russell *et al.*, 2013).

5.1 Conclusions

The study aimed at determining the effect of indoor insecticide use on the resting behaviour of African malaria mosquitoes. The results presented herewith attempt to give a clear picture of what the resting behaviour of these malaria mosquitoes is in the era of high indoor insecticide use. The results collected on the plasticity of resting behaviour opens up a potential for exploring and monitoring the biology of the mosquitoes in efforts to strengthen interventions or find alternative tools for vector control. The status and profiles of insecticide resistance observed in the different resting locations (indoors and outdoors) further alleviates the need to improve current insecticide-based vector control tools to target both the insecticide resistant indoor-resting and the outdoor resting mosquito populations. Despite having indoor-based insecticide vector control, the risk for malaria infection has higher probability indoors than outdoors. The study therefore implies that the use of insecticides indoors, besides impacting biting phenology and host seeking behaviour, also alters the resting behaviour and therefore, a sustenance in malaria transmissions. This information bridges the gap between resting behaviour being a part of the dynamics of insecticide resistance which now opens up an important front for further exploitation through monitoring and research. The results also dictates a need to monitor outdoor malaria transmissions.

5.2 Recommendations

1. Behavioural insecticide resistance surveillance and monitoring should be initiated to better be in a position to mitigate their impacts.
2. The widespread usage of LLINs programme should incorporate use of synergist (PBO) in such regions reporting high physiological insecticide resistance.
3. Urgent enhancement of indoor insecticide-based interventions and development of alternative tools is called for in order to strengthen indoor vector control.
4. Monitoring and surveillance systems should be strengthened for outdoor malaria transmission.
5. Integrating outdoor vector control through eliminating potential breeding and resting habitats such as proper disposal or storage of empty containers.

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APPENDICES

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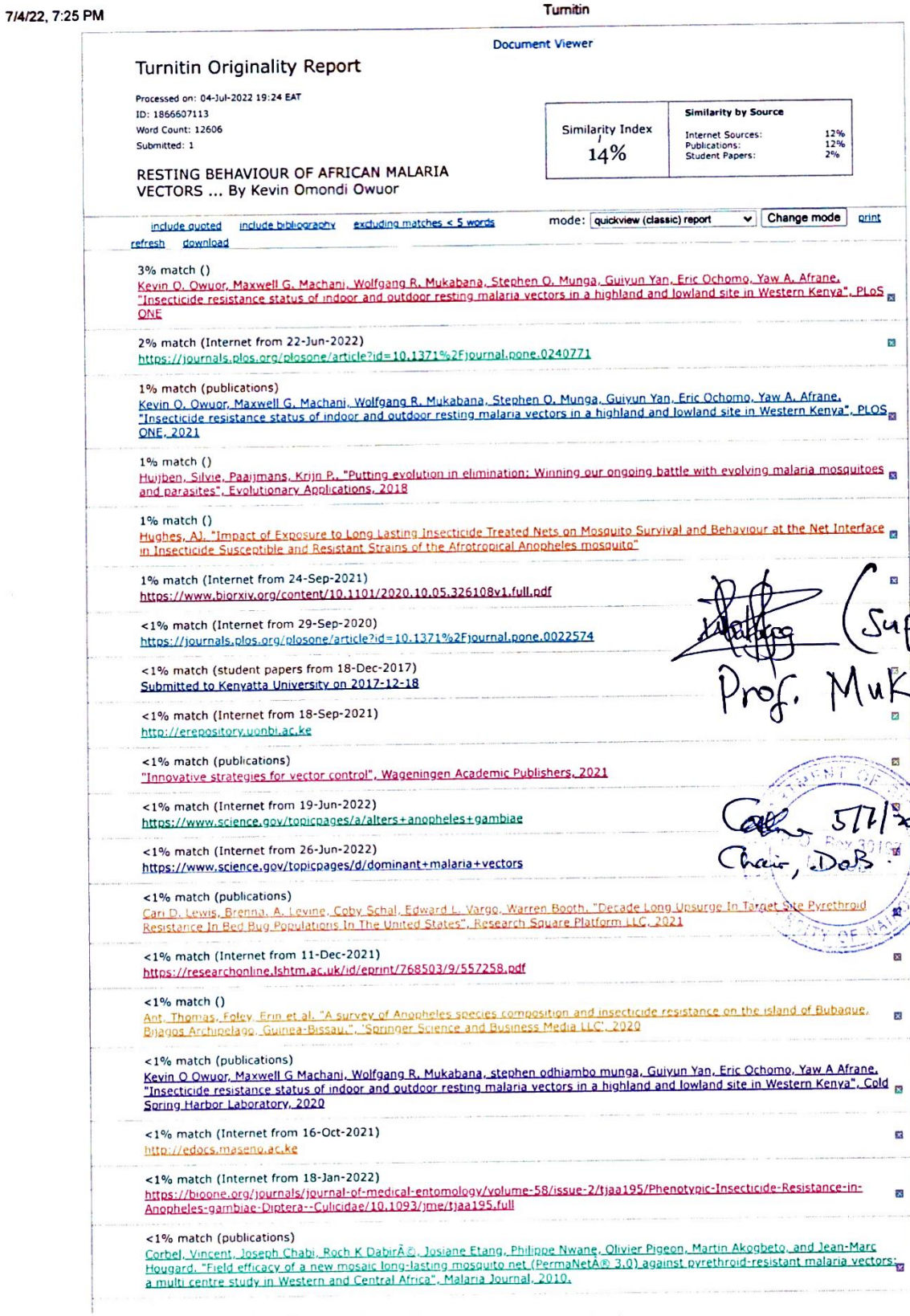

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