CHARACTERIZATION OF OUTDOOR MALARIA TRANSMISSION AND PLANT FEEDING PATTERN AMONG *ANOPHELES FUNESTUS* AND *ANOPHELES GAMBIAE* MOSQUITOES FROM SELECTED ECOLOGIES OF KENYA

BY

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DECLARATION

This is to testify that this thesis is my original work and has not been presented for award of degree in any other University. Where other people's work has been used, it has been properly acknowledged and referenced in accordance with the University of Nairobi requirements.

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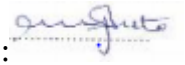
Signature:  Date……11/09/2022….

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DEDICATION

I dedicate this work to Lillian Sangira, Stephen Sangira and Julita Odudo.

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List of Abbreviations

ACTs-Artemisinin-based combination therapies

Bp-Base pairs

BS-Bootstrap

CDC-Centre for Disease Control and Prevention

DDT-Dichlorodiphenyltrichloroethane

DNA-Deoxyribonucleic acid

EID-Emerging Infectious Diseases

GMM-Genetically modified mosquito

HRM-High resolution melting

ICIPE-International Centre of Insect Physiology and Ecology

ID-Identification

IRS-Indoor residual sprays

IR-Infection Rate

ITN-Indoor treated nets

ITS2-Internal transcribed spacer region 2

LLINs-Long lasting insecticidal nets

MOH-Ministry of health

NMCP-National malaria control program

PCR-Polymerase chain reaction

PMI-President’s malaria initiative

RBC-Red blood cells

RbcL-Ribulose- 1,5 bisphosphate carboxylase

Rpm-Revolution per minute

US-United States

USAID-United States Agency for International Development

UK-United Kingdom

WHO-World Health Organization

ABSTRACT

Outdoor malaria transmission remains a major hindrance to effective control and elimination of the disease. However, the anopheline vector composition among outdoor biting fractions is poorly understood including associated bionomic traits especially in dryland ecosystems of Kenya. In this study, adult mosquitoes belonging to *Anopheles gambiae* complex and *Anopheles funestus* group were characterized for their sibling species composition, *Plasmodium falciparum* infection rates and plant feeding profiles. The mosquitoes were trapped outdoors around homesteads using CO_2-baited CDC light traps through a cross sectional survey in selected dryland areas including Kerio Valley, Nguruman and Rabai. Mosquitoes from *An. funestus* group (n=639, 90.6%) were abundant than those of the *An. gambiae* complex (n=66, 9.4%) across the three study areas. Molecular identification of the sibling species in the Funestus group via PCR of the internal transcribed spacer 2 (ITS2) region and infection with non-coding mitochondrial sequence (ncMS) identified *An. longipalpis* C as the dominant vector with a *Plasmodium falciparum* sporozoite rate (*Pf*sp) of 5.2% (19/362) respectively. Other species were *Anopheles funestus* s.s. and *Anopheles rivulorum* with *Pf*sp rate of 8.7% (2/23) and 14.1% (9/64), respectively, varying among the areas. PCR of *An. gambiae* complex samples targeting the rDNA identified *An. arabiensis* and *An. gambiae* s.s with the former (52/66) occurring in higher proportions than the latter (14/66). *An. gambiae* s.s had a *Pf*sp rate of 14.3% (2/14) and *An. arabiensis* 1.9% (1/52). Additionally, six cryptic species associated with *An. funestus* group were uncovered and found to harbor *Pf* sporozoites (cumulative *Pf*sp rate=7.2%, 13/181). The species were identified after PCR of the ITS2 and then sequencing and phylogenetic analysis. Cold anthrone test, used to analyze the mosquitoes for evidence of recent plant feeding, found an overall low fructose positivity rate (2.8%, 19/680). Analysis of a subset of samples including the fructose-positive specimens by DNA barcoding of ribulose-1,5 bisphosphate carboxylase large chain (rbcL) gene, identified plant DNA with a 55.3% (n=262) success rate. Plant DNA sequences were successfully generated from 62.3% (71/114) samples, implicating acacia plants as the predominant plant fed upon by mosquito vectors from Kerio Valley and Rabai. The findings from this study indicate that outdoor malaria vector species composition is dominated by lesser known species which potentially play a role in malaria transmission. The results have implications for persistent malaria transmission and effective malaria control in the study areas. Additionally, findings from the study indicate that cold anthrone test for fructose, grossly underestimates the extent of plant feeding in disease vectors. While plant feeding appears to differ among the species, the basis for seeming preference on acacia requires additional research.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information

Malaria remains one of the most important vector-borne infectious diseases affecting humans globally. Estimates as of 2020 show that 241 million malaria cases were recorded globally spanning 85 countries (WHO, 2021). Sub-Saharan Africa (SSA) accounted for ~95% of this global burden with an estimated 228 million cases and 602, 000 deaths. Between 2000 and 2015, a steady decline in malaria cases and deaths were observed in SSA. However, the current burden represented a slight increase compared to 2019 as a result of service disruption arising from the COVID-19 pandemic.

In Kenya, malaria case incidence and mortality have fallen since the early 2000s. Malaria prevalence decreased from 8% in 2015 to 6% in 2020 although this rate varies greatly by endemicity zone, ranging from <1% in Low risk zone to 19% in Lake endemic zone (KMIS, 2020). The reduction was achieved owing to combined interventions of long-lasting insecticidal nets (LLINs), indoor residual spraying (IRS), prompt diagnosis and treatment with artemisinin-based combination therapies (ACTs) and intermittent preventive treatment of pregnant women (PMI, 2020; PMI, 2021).

Despite this progress, the disease still exerts a huge toll on people accounting for an estimated 19% outpatient consultations (MOH, 2020). This trend potentially indicative of the disease persistence, could have been affected by several factors including house characteristics, use of bednets or other control measures, human activities and behavior and vector-related factors (Bamou *et al*., 2021). However, entomologic determinants remain critical. As observed elsewhere, *Anopheles* vector composition has changed following sustained vector control, as

well as resting and biting habits especially outdoors (Carnevale & Manguin, 2021; Mwangangi *et al*., 2013). Collectively, outdoor biting has been found to limit the effectiveness of LLINs and IRS mainly deployed indoors, resulting in residual malaria transmission (RMT) (Carnevale & Manguin, 2021; Sherrard-Smith *et al*., 2019). This has contributed to malaria transmission as these mosquito populations cannot be monitored using conventional tools(Sougoufara *et al*., 2020). Unfortunately, the effectiveness of these tools is further threatened by rapid spread of insecticide resistance as reported in main malaria vectors across SSA(Tchigossou *et al*., 2020).

Outdoor biting, in particular, has received increasing attention with regards to controlling malaria towards elimination. A recent modeling study predicted that across Africa, outdoor transmission resulted in an estimated 11 million additional malaria cases annually, assuming universal LLIN and IRS coverage (Sherrard-Smith *et al*., 2019). Thus, the contribution of outdoor biting to residual malaria transmission could be much higher. A multicountry study in Cameroon, Kenya and Ethiopia found high malaria transmission outdoors compared to indoors (Bamou *et al*., 2021). While this phenomenon is not new (Kreppel *et al*., 2020; Degefa *et al*., 2017; Okello *et al*., 2006) the profile of vectors implicated in outdoor transmission could differ in different eco-epidemiologic settings (Bamou *et al*., 2021).

Vectoring ability and aspects of mosquito vectorial capacity can be modulated by environmental resources among them plant feeding (Traore *et al*., 2020). Plant feeding is a crucial dietary requirement in arthropod disease vectors including sandflies and mosquitoes (Stone & Foster, 2013a). Studies have shown that different species of mosquitoes feed on plants (Nyasembe *et al*., 2018; Hassanali, 2007). Mosquitoes need sugars to provide them with energy for flight, fecundity and longevity(Foster, 1995; Nyasembe *et al*., 2015). Both males and females frequently feed on plant sugar as floral or extrafloral nectar and honeydew (Foster, 1995).

In addition to sugars, plant feeding provides mosquitoes with water during the dry seasons to cushion them against desiccation (Holmes & Benoit, 2019). The availability of preferred host plants has been reported to have a negative effect on malaria control by extending survival of *An. gambiae* thus, increasing transmission by allowing completion of sporogonic cycle of the parasite both in laboratory and natural settings (Muller *et al*., 2017; Nyasembe *et al*., 2015). In contrast, presence of flowering plants reduces human biting thus positively impacting disease transmission (Beier, 1996). Further studies have shown that mosquitoes as well as sandflies feed on plants for other secondary metabolites of which some reduce the vectors' parasite load (Balaich *et al*., 2016; Nyasembe *et al*., 2015; Schlein & Jacobson, 1994).

Previous studies in *An. gambiae* s.l and *Aedes aegypti*, the dengue virus vector, show that plant feeding is common in nature and appears to be highly selective (Nyasembe *et al*., 2018; Hassanali, 2007; Wanjiku *et al*., 2021). However, its importance remains poorly elucidated for wild populations of *An. funestus* mosquitoes. Knowledge of plant feeding in disease vectors can be exploited in the development plant-derived attractants for vector population surveillance, explored in malaria parasite transmission blocking (Balaich *et al*., 2016) by virtue of secondary plant metabolites, in attractive toxic sugar baits (Gu *et al*., 2020; Traore *et al*., 2020; Fiorenzano *et al*., 2017) and odor baits technology (Nyasembe *et al*., 2014).

1.2 Problem statement

In Kenya, monitoring efforts have allowed for assessment of the malaria epidemiologic landscape and identification of key vector species. Primary malaria vectors in Kenya belong to *Anopheles gambiae* sensu lato (*s.l.*) and *Anopheles funestus* group, like most of East Africa (Kakilla *et al*., 2020; Ogola *et al*., 2019; Okello *et al*., 2006). The sibling species in these complexes are morphologically similar as adults although different ecological preferences, vectorial capacity and behavior, impacting differentially on malaria epidemiology and control

efforts (Coetzee, 2020; Dia & Guelbeogo, 2013). Most comprehensive data in Kenya, on entomologic risk comes from the high Malaria Lake and Coast endemic risk areas. This has limited the understanding of malaria transmission in dry land ecologies which are classified as seasonal malaria risk areas and highly prone to malaria outbreaks. A study (Ogola *et al*., 2019) highlighted the role of previously unreported species in malaria transmission in West Pokot, a dryland ecology. Outdoor species composition in these areas remains poorly understood hence the need to investigate the specific species and their role in residual malaria transmission.

Further, studies have been done to understand the plant feeding behavior in one of the major malaria vectors *An. gambiae* (Nyasembe *et al*., 2018) but knowledge of plant feeding ecology for adult *An. gambiae* and *An. funestus* have been poorly described in dry ecologies. This study purposed to examine evidence of recent sugar feeding based on fructose positivity rates and identify the candidate host plants fed on by wild populations of the *An. funestus* and *An. gambiae* vectors.

1.3 Justification

The role played by mosquito vectors in determining malaria disease epidemiology is very important with earlier studies implicating previously unrecognized species in malaria transmission (Zhong *et al*., 2020; St. Laurent *et al*., 2016;). This study focused on outdoor malaria transmission by describing outdoor species composition in the *An. gambiae* complex and *An. funestus* group by applying molecular analyses in dry land ecologies. Species complexes are made up of sibling species that are difficult to distinguish based on morphology as adults, yet have unique biologies and exhibit different behaviors that impact malaria transmission and control methods. The natural infection rates with *P. falciparum* in these vectors was also determined using molecular approaches because of the known *Plasmodium* species, it has been reported to be the most virulent.

Knowledge of vector ecology can be exploited for malaria vector control through plant feeding which is an important behavior in mosquitoes that they rely on for metabolic processes and survival. The target rbcL gene widely used in plant DNA barcoding (Wanjiku *et al*., 2021; Hasaballa *et al*., 2021) was employed to identify the plant hosts of wild caught *An. funestus* and *An. gambiae* mosquitoes. Describing plant feeding behavior will highlight the energy source of these malaria vectors as well as provide baseline information for additional studies that will help in developing optimal vector control strategies in future based on their plant feeding behavior.

1.4 Objectives

1.4.1 Main Objective

To characterize outdoor malaria transmission and plant feeding habit among *Anopheles funestus* and *Anopheles gambiae* mosquitoes from Kerio Valley, Nguruman and Rabai, Kenya.

1.4.2 Specific objectives

1. To determine outdoor *Anopheles* species composition and natural *Plasmodium falciparum* infection rates.
2. To identify the plant species fed on by wild populations of *An. funestus* group and *An. gambiae* complex.

1.5 Hypothesis

1. Diverse species of anopheline mosquito vectors contribute to outdoor malaria transmission in dryland ecologies of Kenya.
2. Wild populations of *An. funestus* group and *An. gambiae* complex mosquito vectors feed on diverse host plant species.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Malaria and the malaria parasites

Malaria is a preventable and curable life-threatening disease caused by protozoan *Plasmodium* parasites and transmitted through bites of infected female anopheline vectors (WHO, 2020). Malaria transmission intensity is governed by four related factors including vector, parasite, human host and environment factors. Transmission tends to be more intense in areas where the mosquitoes are more anthropophilic than zoophilic and further associated with warm humid climate (PMI, 2021; WHO, 2020). In Africa, the disease is a major public health risk contributing to approximately 95% of the global malaria burden (WHO, 2021). This has therefore, made malaria to be categorized as one of the public health diseases prioritized for integrated disease surveillance in Kenya (NMCP, 2019).

According to WHO, young children, pregnant women and travelers from areas that have low malaria transmission are the most vulnerable people to the disease due to low immunity (WHO, 2021). There are six *Plasmodium* species that cause malaria in humans: *Plasmodium vivax, Plasmodium malariae, Plasmodium ovale curtisi, Plasmodium ovale wallikeri, Plasmodium falciparum* and *Plasmodium knowlesi* (WHO, 2017; Calderaro *et al.*, 2013). Of these, *P. falciparum* is the most virulent species as it results in most cases of deaths (WHO, 2017).

2.2 Burden of malaria and its geographical distribution

Malaria burden distribution is disproportionate with the disease being concentrated in the sub-Saharan African region (Fig. 1). In the year 2020, 29 countries accounted for approximately 96% of the global malaria cases (WHO, 2021). Fifty-five percent of these global cases were from Burkina Faso (3.4%), Mozambique (4%), Uganda (5%), Nigeria (27%), Democratic Republic of Congo (12%) and Angola (3.4%) (WHO, 2021). On the other hand, China and El Salvador were lately certified by WHO as malaria free zones since they reported zero cases for 4 consecutive years i.e. 2018, 2019, 2020 and 2021 (WHO, 2020; WHO, 2021).

Generally, there has been a reduction in malaria case incidences over the years, with 78% reduction in cases and 74% reduction in deaths from 2000-2019 globally (WHO, 2020). However, in 2020, 14 million cases and 69,000 deaths were additionally reported compared with 2019 (WHO, 2021). The increasing malaria cases highly impacts productivity as more than US$ 12 billion of Africa's productivity is lowered by the disease every year (WHO, 2020; WHO,2021).

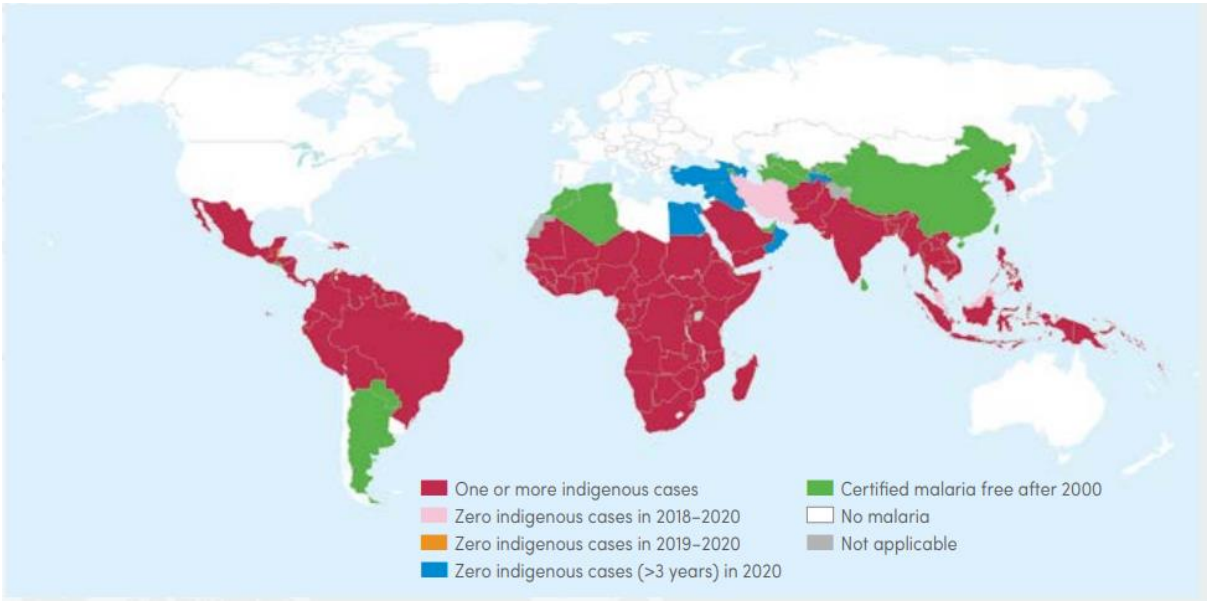

Figure 1: Global distribution of malaria (Source: WHO, 2021)

2.2.1 Malaria burden in Kenya

Kenya is among the countries that is part of President's Malaria Initiative (PMI); a US government project which prioritizes areas of high risk with the aim of greatly reducing malaria morbidity and mortality (PMI, 2021; PMI, 2020). At least, 70% of the Kenyan population is at risk of malaria with infection risks and transmission varying based on temperature, altitude and rainfall patterns (KMIS, 2020; MOH, 2020; PMI, 2020). Malaria burden in Kenya is not homogenous. Kenya has been stratified into four malaria epidemiological zones (KMIS 2020; MOH, 2020; PMI, 2020; PMI, 2021). These include the endemic areas (coastal and lake areas), highland epidemic areas, seasonal malaria transmission areas and low risk areas (KMIS, 2020) (Figure 2).

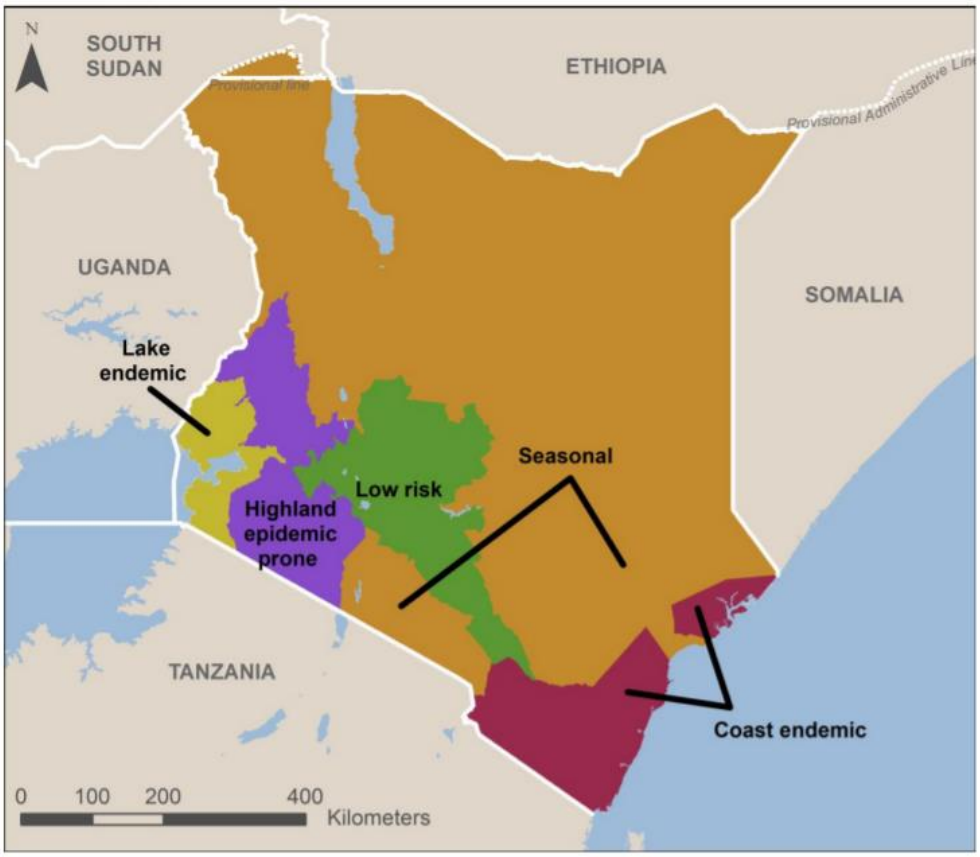

Figure 2: Malaria endemic zones in Kenya (Source: KMIS, 2020)

Coastal and Lake endemic areas**:** These regions have high malaria prevalence throughout the year (Karungu *et al*., 2019; MOH, 2016). Determinants of perennial malaria transmission in these areas include humidity, temperature and rainfall (KMIS, 2020). The annual inoculation rates in these regions range from 30 – 100 (PMI, 2020; KMIS, 2020). An estimate of 29% of the total population lived in the endemic areas in 2020 (PMI, 2020).

Highland epidemic areas: In these areas, seasonal malaria transmission considerably varies from year to year with prevalence ranging between 5-20% (Karungu *et al*., 2019). During the long rains, temperatures tend to rise hence favoring vector breeding, leading to increased malaria transmission (MOH, 2019). An estimate of 19% of the total Kenyan population live in the epidemic areas (PMI, 2020).

Seasonal malaria transmission areas: These areas experience intense malaria transmission especially during and after the rainy seasons (Karungu *et al*., 2019). These regions experience very high rainfall that create water pools, providing the vectors with numerous breeding sites (KMIS, 2020). Malaria prevalence in these areas ranges between 1-5% with approximately 23% of the Kenyan population living in these areas (PMI, 2020).

Low malaria risk areas: In these areas, the temperatures tend to be low, hindering the survival of *Plasmodium* parasite (Karungu *et al*., 2019). However, increase in temperature can introduce/increase transmission in previously unreported areas (KMIS, 2020). These areas experience low malaria prevalence of <1% with approximately 31% of the Kenyan population living in these areas (PMI, 2020).

2.3 Malaria mosquito vectors

There are approximately 3,500 species of mosquitoes and all those known to transmit malaria belong to the genus *Anopheles.* Africa has over 140 *Anopheles* species. Of the vector species

that play a role in malaria transmission, those that pose a great threat are long-lived, abundant, anthropophilic and dwell mostly in proximity to people (Ogola *et al*., 2018). Additionally, their role in malaria transmission greatly depends on favorable environmental factors for larval development, adult survival and parasite development in the vector.

Studies have shown that mosquitoes from two complexes: *Anopheles funestus* and *Anopheles gambiae,* constitute the major vectors of malaria in Africa (Ogola *et al*., 2018; Degefa *et al*., 2017; Ndenga *et al*., 2016; Tchouassi *et al*., 2012). There are currently 9 known species in the *An. gambiae* complex namely: *An. gambiae s.s, An. arabiensis, An. quadriannulatus, An. melas, An. merus, An. bwambae, An. colluzzi*, *An. amharicus,* and *An. fontenillei* (Barrón *et al*., 2019; Coetzee *et al*., 2013). These species are known to differ in their ecological aspects including their breeding sites, host preference, feeding behavior and role in malaria transmission (Besansky, 2011). For example, *An. gambiae* s.s is considered an efficient vector of the malaria parasites due to its highly anthropophilic (White & Kaufman, 2013) and endophilic nature (Pates & Curtis, 2005).

An. funestus group comprises species that are distributed widely or focally in Africa including *An. funestus* s.s, *An. funestus-like, An. parensis, An. longiplalpis C, An. longipalpis A, An. leesoni, An. rivulorum, An rivulorum-like, An vaneedeni, An. aruni, An.confusus, An. fuscivenosus, An. brucei* (Burke *et al*., 2017; Choi *et al*., 2010; Spillings *et al*., 2009; Harbach, 2004). *An. funestus* s.s, the predominant species in this group, is mainly known to bridge malaria transmission during the dry seasons as they prefer permanent swampy areas and their population increases at the end of rainy seasons (Githinji *et al*., 2020). They have also been reported to highly exploit man made environments (Pates & Curtis, 2005).

2.3.1 Malaria vectors in Kenya

In Kenya, *An. funestus* s.s, *An. gambiae* s.s and *An. arabiensis* form the major vectorial system (Zhong *et al*., 2020; Okara *et al*., 2010) but the species abundance varies within and between different areas due to factors associated with human and climatic factors (Shililu *et al*., 2003). For instance, studies done in western Kenya showed that *An. gambiae* s.s is a major malaria vector in Teso North and South counties and Bungoma while in Kisian, *An. funestus* s.s is the predominant species (Githinji *et al*., 2020; Machani *et al*., 2020). The PMI (2020) reported *An. arabiensis* and *An. funestus* s.s to be the major vectors in Siaya, Kisumu, Homa Bay and Migori. Coastal Kenya is characterized with a variety of malaria vector species. The main members of the most efficient malaria vector in this region are *An. gambiae* complex and *An. funestus* (Mbogo *et al*., 1993). However, in many areas *An. arabiensis*, a known outdoor feeder has substituted *An. gambiae* s.s. as the primary vector (Mwangangi *et al*., 2013). *An. pharoensis* and *An. nili* have been described as complementary malaria vectors in coastal and central Kenya (Okara *et al*., 2010). A study by Ogola *et al*. (2018) reported *An. longipalpis C* for the first time, and its' potential role as a secondary vector in the arid areas of Kenya. A study (Okara *et al* 2010) on distribution of vectors in Kenya reported *An. gambiae, An. arabiensis, An. funestus* and *An. pharaoensis* in Eastern Kenya while in Nairobi, *An. arabiensis* was reported as the predominant vector. Consequently, other species that have been given less attention and considered unimportant like *An. coustani*, are being implicated increasingly, with malaria transmission in Taita Taveta and western Kenya (Mustapha *et al*., 2021; PMI, 2020; Mwangangi *et al*., 2013).

Malaria vectors are very important in terms of accurate description and understanding malaria transmission for effective design of vector control interventions (Stevenson & Norris, 2017).

It is therefore, important for mosquito vectors to be accurately identified. With time, molecular techniques have increasingly been applied to characterize vector populations (Mustapha *et al*., 2021; Machani *et al*., 2020; Ogola *et al*., 2018, 2019; St.Laurent *et al*., 2016; Mwangangi *et al*., 2013) Owing to the application of more sensitive molecular techniques, cryptic species and/or lesser known species have been incriminated with malaria transmission (Ogola *et al*., 2019; Zhong et al., 2020).

2.4 Malaria dynamics

2.4.1 Vector behavior

Knowledge of mosquito behavior can be exploited to reduce malaria incidences and even eradicate the disease in temperate areas, based on their resting (Pates & Curtis, 2005) and blood-feeding behaviors(Adugna *et al*., 2021). The anopheline vectors *An. gambiae* and *An. funestus*, are highly endophilic, that is, they prefer resting indoors after feeding and before they can locate their oviposition sites (Gatton *et al*., 2013; Pates & Curtis, 2005). This led to the development of primary control interventions-indoor residual spraying (IRS) and long lasting insecticidal nets (LLINS)-to protect people when they are in bed indoors (Kenea *et al*., 2019; Desalegn *et al*., 2018; Randriamaherijaona *et al*., 2017). With time however, even highly endophilic mosquitoes have displayed divergent behaviors including early biting and resting outdoors, that diminish their exposure to the insecticides used in LLINs and IRS (Pates & Curtis, 2005; Russell *et al*., 2011; Thomsen *et al*., 2017). Published data further show that between 5 - 40% of mosquito bites usually take place when people are not being protected by bed nets or when they are out of bed (Sherrard-Smith *et al*., 2019). Several studies have reported increasing outdoor biting activities by major malaria vectors; for example, *An. gambiae* s.s in Guinea (Overgaard et al., 2012), *An. arabiensis* in Tanzania (Russell *et al*., 2011) and *An. funestus* s.s in Senegal (Sougoufara *et al*., 2014). Such behavioral changes have

contributed to increased levels of residual malaria transmission (RMT), which is defined as ongoing transmission despite 100% implementation of LLINs and IRS fully susceptible to local vectors, as previously documented (Russell *et al.*, 2011; Thomsen *et al*., 2017).

When it comes to feeding on blood, female *Anopheles* mosquitoes have diverse sources including humans, cattle, reptiles and birds (Muriu *et al*., 2008). Blood-feeding behavior is very important in malaria epidemiology as it can influence vectorial capacity and spatial distribution of malaria (Richards *et al*., 2006). Of the known malaria vectors, the most successful are those that primarily feed on humans and secondarily feed on cattle. Host choice by these vectors has also been observed to be influenced by a number of factors including i) size of the host ii) defense mechanisms of the host and iii) proximity to the vectors habitat (Omolade, 2012). Moreover, blood-feeding of malaria vectors is highly dependent on host species density whereby their availability means readily accessible blood meal source. Interestingly a study showed that *Anopheles gambiae* mosquitoes infected with the sporozoite stage of *P. falciparum* tend to be more attracted to humans than those not infected (Smallegange *et al*., 2013). A different study showed that female *Anopheles gambiae* vectors that are infected with oocysts fed less on blood (Cator *et al*., 2013). Knowledge of blood-meal preference and behavior is important and can be used in developing vector control tools.

2.4.2 Insecticide resistance

Recent increase in malaria transmission in different parts of SSA including Kenya has been attributed by increasing insecticide resistance in the mosquitoes. The high levels of insecticide resistance are mostly believed to be caused by increasing use of insecticide treated nets and using agro-chemicals to control pests in agriculture. Four classes of insecticides have been approved for use: carbamates, organophosphates, pyrethroids and organochlorines (Table 1). Unfortunately, continuous use of these chemicals has resulted in insecticide resistance

worldwide, further compromising malaria control and elimination (Rakotoson *et al*., 2017). Out of the four classes of resistance, pyrethroid resistance is of the most concern as this class is used in all bed nets recommended by WHO (WHO, 2016). High levels of resistance in *An. gambiae* and *An. funestus* have been documented in Kenya in the coastal and lake endemic areas further threatening malaria control and elimination (PMI, 2020). Insecticide resistance status among the major malaria vectors in Kenya was reported in all the four classes of insecticides including pyrethroids, carbamates, organochlorines and organophosphates. Resistance to pyrethroids has been detected in *An. funestus s.s, An. gambiae s.s* and *An. arabiensis* while resistance to carbamates was only in *An. gambiae s.s* and *An. arabiensis*. Organochloride resistance was recorded in *An. funestus s.s* and *An. gambiae s.s* while only *An. gambiae s.s* had resistance to organophosphate (Ondeto *et al*., 2017). A pioneer study (Owuor *et al*., 2021) revealed high resistance-associated point mutations in indoor than outdoor populations of *An. gambiae* and *An. funestus*.

Resistance to DDT, the most common organochloride, among these major malaria vectors has been reported in central, western and eastern Africa (Kawada *et al*., 2011; Gambino, 2013; Riveron *et al*., 2016). Insecticide resistance occurs when there are changes in either one or more genes hence reducing the sensitivity of the insecticides or due to enzymatic changes in the targeted insect (Soko *et al*., 2015). Four resistance mechanisms have been described in mosquitoes: i) behavioral resistance by avoiding contact with insecticides, ii) penetration resistance whereby cuticle thickens, reducing insecticide penetration, iii) metabolic resistance due to overexpression of detoxification genes and iv) target site resistance resulting from binding site alterations (WHO, 2016).

Table 1: Major classes of insecticides and their recommended use (IRAC, 2019)

Class	Insecticide	IRS	ITN
Carbamate	Bendiocarb, Propoxur	**Yes**	**No**
Organophosphate	Melathion	**Yes**	**No**
Pyrethroid	Permethrin	**No**	**Yes**
	Deltamethrin	**Yes**	**Yes**
	Alphacypermethrin	**Yes**	**Yes**
	Etofenprox	**Yes**	**Yes**
	Lambdacyhalothrin	**Yes**	**Yes**
	Bifenthrin	**Yes**	**Yes**
	Cyfluthrin	**Yes**	**No**
Organochloride	DDT	**Yes**	**No**

2.4.3 Environmental factors

Survival of malaria mosquitoes largely depends on environmental factors such as temperature, rainfall patterns, humidity and vegetation cover. High rainfall has an influence on mosquito vectors by providing numerous swamps and water pools that are suitable breeding sites and vegetation for their resting, resulting in high malaria transmission (Kipruto *et al*., 2017). Arguably, heavy rainfall can reduce malaria transmission shortly after by washing away their breeding sites (Huang *et al*., 2011). Higher temperatures favor the development of the parasite inside the vector by accelerating its growth (Kipruto *et al.*, 2017). Temperature has an influence

on vector and parasite development leading to increase in malaria with increasing temperature up to an optimum temperature of 30 °C beyond which the lifespan of the vector is shortened (Kipruto *et al*., 2017). Therefore, it is speculated that in future global warming will cause increased malaria transmission in regions where the disease is already present and areas that were not habitable for vector will become habitable due to warmer climatic condition hence the expansion of the disease (Ermert *et al*., 2012).Wind direction influences malaria transmission by providing the vector with the direction of human odor and carbon dioxide, hence promoting the host seeking behavior of mosquitoes (Midega *et al*., 2012). Although environmental modifications such as deforestation, swamp reclamation, brick making, and vegetation clearance may create suitable breeding sites for vectors hence increased rate of malaria transmission (Lindblade *et al*., 2000), others such as pollution and human encroachment destroy mosquito vectors habitat thereby inhibiting *Anopheles* mosquito's multiplication and malaria transmission (Barbazan et al., 1999).

The vectorial capacity and vectoring ability in mosquitoes can also be influenced by the vegetation type in their immediate environment. Plants play an important role in mosquito life and diet by providing them with sugars as their energy source (Van Handel, 1984). Both males and females frequently feed on plant sugar as floral or extrafloral nectar and honeydew (Foster, 1995). Plant feeding behavior in mosquitoes influences certain traits. For example, a pioneer study by Gary & Foster (2001) on *Aedes aegypti* showed that females which have fed on both sugars and blood tend to live longer compared to those that fed on blood only. Additionally, plant feeding influences mosquitoes survival (Nyasembe, *et al*., 2015; Barredo & DeGennaro, 2020) since they can reproduce and survive only on blood but without sugars, they are not able to reach their full life potential (Foster, 1995).

2.5 Impact of plant feeding behavior on malaria transmission

The presence of plants in the localities of mosquitoes has a direct impact on malaria transmission by providing major malaria vectors with habitats as their population drastically dropped when branches of *Prosopis juliflora* were removed from their environment (Muller *et al*., 2017). *Parthenium hysterophorus* is another invasive plant that highly attracts malaria vectors with evidence of improving their survival and fecundity (Nyasembe, *et al*., 2015) by extending their life expectancy, hence increased malaria transmission. A study by Hassanali, (2007) in which lab-reared *An. gambiae* mosquitoes were provided eight plants that commonly grow in western Kenya showed the survival rates of mosquitoes that fed on plants was higher (10 more days) than those that were given only water. The author further observed that infected mosquitoes highly preferred feeding on *Parthenium hysterophorus* (Hassanali, 2007). Parthenin, a major metabolite in *P. hysterophorus*, interferes with *P. falciparum* development through the vector, by inactivating gametocytes (Balaich *et al.*, 2016). Changing the plant communities in an area can increase malaria transmission or removal of preferred nectar-sources in an area can reduce transmission (Ebrahimi *et al*., 2018; Muller *et al.*, 2017). Thus, knowledge of the vegetation type in an area/community can be used to predict high risk areas for malaria (Ebrahimi *et al*., 2018).

2.5.1 Methods for evaluating plant feeding

For a long time, evidence of plant feeding in mosquitoes was based on chemical tests on the gut contents or observing the insects on plants. However, relying on observation was quite challenging because landing and probing did not necessarily mean sugar ingestion (Stone & Foster, 2013b). Additionally, failing to observe the plant feeding and recording as absent was misleading.

Chemical tests are notably less biased than direct observations. Van Handel revolutionized plant feeding studies through the cold anthrone test since fructose reacts with anthrone reagent at room temperature (Van Handel, 1967). The anthrone reagent turns blue-green in the presence of sugars and glycogen (Van Handel, 1985a). Cold anthrone is a colorimetric test which is used as a quantitative determinant of carbohydrates which yields fructose on hydrolysis (Van Handel, 1967). However, the anthrone test only detects the most recent sugars fed upon (fructose). For those sugars that do not react at room temperature, hot anthrone is performed which is a modified cold anthrone by increasing the temperatures to 90°C to react with remaining sugars (Lee, 2019).

Mosquitoes tend to convert sugars to lipid reserves, that act as their energy sources in dry seasons (Beach, 1965) or carry their reserves from larval stages (Ziegler & Ibrahim, 2001). The colorimetric technique used in quantifying lipids in insects is the vanillin phosphoric-acid reagent as described (Van Handel, 1985b). In the presence of lipids, the vanillin reagent turns pink (Lee, 2019). The optical densities of the solutions are then read on an absorbance reader at 625nm (optimum wavelength) to estimate the quantity of sugars and lipids in the mosquito after comparing the calibration curves of the samples with those of the standards (Lee, 2019).

For evidence of plant tissue feeding, the cellulose staining technique has been applied. This technique uses NaOH (pH 8) and 0.1% staining solution of calcofluor in 0.45% saline. The solution is mounted and the dissected gut is placed on a slide for visualization under a microscope (Junnila *et al.*, 2010). However, these assays were limiting as they could not tell the specific plant host.

2.5.2 Plant DNA barcoding

Molecular based techniques were developed which target different markers like trnH-psbA, Maturase K (matK) and ribulose-bisphosphate carboxylase **(**rbcL) as described (Wanjiku *et al*., 2021; Nyasembe *et al*., 2018) to identify the specific plant hosts. Of these markers, the rbcL gene has been widely used in plant DNA barcoding because i) it is located in the chloroplast DNA which is highly conserved and stable, ii) the marker is easily amplified and analyzed and iii) it has low nucleotide substitution rate (Papuangan, 2019).

Specific plants fed upon by *Anopheles gambiae, Aedes aegypti, Aedes ochraceus* and *Aedes mcintoshi* in their natural setting by employing plant DNA barcoding were successfully identified (Nyasembe *et al*., 2018) . The identified plants included *Senna tora* for *An. gambiae, Hibiscus heterophyllus, Senna uniflora* and *Pithecellobium dulce for Ae. aegypt, Opuntia ficus-indica for Ae. mcintoshi* and *Aedes ochraceus.* Recent analysis based on plant DNA barcoding has also highlighted the preference of wild caught *Ae. aegypti* (Wanjiku *et al.*, 2021) and sandflies (Hassaballa *et al*., 2021) to feeding on plants in the Fabaceae family. In laboratory experiments, *An. gambiae* exhibited a feeding choice when exposed to selected locally endemic plants from western Kenya (Hassanali, 2007).

CHAPTER THREE

2.0 MATERIALS AND METHODS

3.1 Study site

Adult female mosquitoes used in this study had previously been collected from three areas: Kerio Valley (Baringo county), Rabai (Kilifi county) and Nguruman (Kajiado county) (Fig 3), as part of disease surveillance and stored at -80 ℃ at the International Centre of Insect Physiology and Ecology (*icipe*). The mosquitoes were surveyed between August 2019 and May 2020.

Nguruman is an agropastoral area located in Kajiado County at the southern end of the Kenyan Rift Valley bordering Tanzania. The area has a semi-arid climate characterized by erratic rains, extreme temperatures, and cyclic and prolonged droughts (Campbell *et al*., 2000). The vegetation is dominated by bushland, grassland and open woodlands along seasonal river valleys. Specific indicator data for malaria is not available for Nguruman except for estimates pertaining to the larger Kajiado county which as of 2019 indicates a malaria incidence rate of 5 per 1000 population (KMIS, 2020).

Collections in Kerio Valley (Baringo County within the Rift Valley) were conducted in Kapluk and Barwesa, both agro-pastoral areas with arid and semi-arid ecology. These two villages are located within the Great Rift Valley and lie 36.277°E, 0.723°N at altitudes ranging between 870 m to 2499 m above sea level. The area receives about 745mm of rainfall annually and annual temperatures range between 18℃ to 33℃. In both Kapluk and Barwesa, semi tropical vegetation is found and mostly thorny bushes such as Acacia and *Cactus* trees cover the area. The major economic activity is farming crops like cotton, maize and animal keeping especially goats and cows. The rainy seasons start from March to November lasting about 8 months.

Malaria is a major vector-borne disease in the areas that occurs perennially in the neighboring riverine areas (Omondi *et al*., 2017).

Rabai is one of the seven administrative sub-counties of Kilifi County in the coastal region of Kenya where malaria is endemic. Rabai lies between 3.9107° S, 39.6093° E and receives an annual rainfall of about 900 to 1,100mm and temperatures between 16℃ to 32℃. A large part of the area is covered by thorny bushland vegetation, savannah and moist deciduous rainforest (Mbuvi *et al*., 2016). The main economic activities in the area include subsistence agriculture, casual labor, crafts and petty trading.

The weather patterns at the sites during the sampling period were as follows: Kerio Valley (mean daily temperature: 21.2℃, mean daily rainfall: 4.1mm, mean relative humidity: 73.4%); Rabai (mean daily temperature: 26.4℃, mean daily rainfall: 2.1mm; mean relative humidity: 78.1%) and Nguruman (mean daily temperature: 22.5℃, mean daily rainfall: 0.9 mm, mean relative humidity: 61.2%).

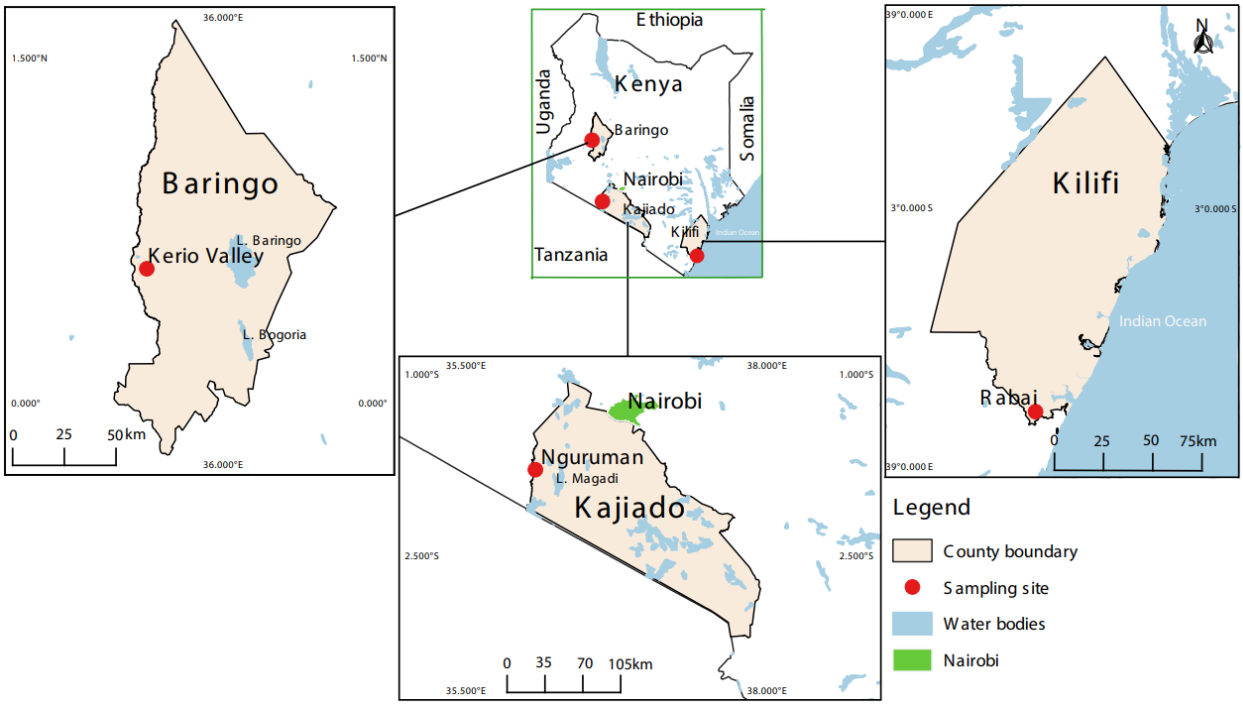

Figure 3: Map of Kenya showing study areas. (ArcMap 10.2.2 was used to design the map with the lakes and ocean base layer derived from a free GIS data source, Natural Earth)

3.2 Sample collection and preparation

A cross- sectional study design was used non-sequentially to generate data for this study. Host seeking mosquitoes were trapped using CDC light traps baited with dry ice (carbon dioxide) attractive to most mosquitoes. Traps were set outdoors in randomly selected homesteads (about 10-15 m away) from 18:00h to 06:00h. Mosquito collection were done immediately after rainy seasons in all the study sites. Repetitive sampling was done across all the study sites. In Kerio valley sampling was done thrice while in Nguruman and Rabai it was done twice. After collection, the mosquitoes were anesthetized with trimethylamine and temporarily stored in liquid nitrogen before transportation to the Emerging Infectious Disease (EID) laboratory at *icipe* and later stored at -80°C. Anopheline mosquitoes were morphologically identified using published taxonomic keys (Coetzee, 2020; Gillies & Coetzee, 1987).

3.3 DNA extraction and *Anopheles* species discrimination

DNA was extracted from the head/thorax of individual mosquitoes using ISOLATE II Genomic DNA Extraction kit (Bioline, UK) following the manufacturer's instructions and used for species discrimination and screening for *P. falciparum* infection (described below). Sibling species of the *Anopheles funestus* group and *Anopheles gambiae* complexes were identified using conventional PCR (Koekemoer *et al.*, 2002; Scott *et al.*, 1993) and sequencing. PCR for *An. funestus* complex in a 15µl reaction volume comprised 0.5µM of each primer targeting: *Anopheles funestus* s.s, *Anopheles vaneedeni*, *Anopheles rivulorum*, *Anopheles parensis*, *Anopheles leesoni*, *Anopheles longipalpis* A and *Anopheles longipalpis* C, 3.0µl of 5X HOT FIREPol Blend Master Mix Ready to Load (Solis BioDyne, Estonia) and 2µl of DNA template. The cycling conditions were initial denaturation at 95°C for 15 min, and then 30 cycles of denaturation at 95°C for 30 s, annealing at 46°C for 30 s and extension at 72°C for 40 s and

final extension at 72℃ for 10 min. Size fragments of each species were scored after separation in 1.5% agarose gel electrophoresis stained with ethidium bromide against a 1 Kb DNA ladder (HyperLadder, Bioline, London, UK).

For *An. gambiae s.l.*, PCR in a 10µl volume consisted of 2µl of 5X Evagreen® HRM Master Mix (Solis BioDyne, Estonia), 1µl of DNA template and 10µM concentration of each primer targeting *An. gambiae* s.s and *An. arabiensis*. The thermal cycling conditions included initial denaturation for 15 min at 95℃ followed by 40 cycles of denaturation at 95℃ for 20 s, annealing at 61℃ for 15 s and extension at 72℃ for 20 s followed by final extension at 72℃ for 7 min.

Few representatives of *An. funestus s.l.* samples that failed to amplify using the established protocol, were further amplified and sequenced targeting the internal transcribed spacer 2 (ITS2) region of the ribosomal DNA (rDNA) (Beebe & Saul, 1995). This target has shown utility in discriminating closely related mosquito species including anophelines (Ogola *et al*., 2018) with sequences from diverse species well represented in reference databases (e.g. GenBank). PCR volumes for rDNA ITS2 were 15µl containing 0.5µM of the forward and reverse primers, 3.0µl of 5X HOT FIREPol Blend Master Mix Ready to Load (Solis BioDyne, Estonia) and 2µl of DNA template. The cycling conditions were initial denaturation at 95℃ for 15 min, followed by 40 cycles of denaturation at 95℃ for 30 s, annealing at 60℃ for 30 s and extension at 72℃ for 45 s and final extension at 72℃ for 7 min. ExoSAP IT rapid cleanup kit (Affymetrix Inc., Santa Clara, CA, USA) was used to clean the PCR product as per the manufacturer's guideline, and then outsourced for bidirectional Sanger sequencing to Macrogen, South Korea.

3.4 Detection of malaria parasites

Plasmodium falciparum sporozoites in individual mosquitoes (head/thorax) were detected by analyzing high resolution melt (HRM) profiles generated from real time PCR products of non-coding mitochondrial sequence (ncMS) (Chaumeau *et al.*, 2016). A *P. falciparum* DNA from National Institute for Biological Standards and Control (NIBSC; London, UK) was used as a reference positive control. PCR was carried out in a 10µl volume consisting of 2µl of 5X Evagreen® HRM Master Mix (Solis BioDyne, Estonia), 1µl of DNA template and 10µM of each primer. PCR cycling conditions were initial denaturation for 15 min at 95°C followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 61°C for 15 s and extension at 72°C for 20 s followed by final extension at 72°C for 7 min. A fraction of RT-PCR-HRM positive samples were further analyzed using conventional PCR in a 10µl volume consisting of 2µl of 5X HOT FIREPol Blend Master Mix Ready to Load (Solis BioDyne, Estonia), 1µl of DNA template and 10µM of each primer. The cycling conditions comprised initial denaturation for 15 min at 95°C followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 61°C for 15 s and extension at 72°C for 20 s followed by final extension at 72°C for 7 min. PCR product of samples positive by RT-PCR were purified using ExoSAP- IT (USB Corporation, Cleveland, OH, USA) and outsourced for sequencing to Macrogen, South Korea. All sporozoite-positive mosquitoes were molecularly identified to species by PCR of the ITS2 region as described above.

3.5 Preparation of mosquitoes for sugar analysis

The abdomen targeting the crop of each specimen was processed for sugars and plant DNA analyses. The abdomen separately in microcentrifuge tubes were crushed using sterile polypropylene pestles. Two hundred microlitres of molecular grade ethanol was added and the tubes incubated for 30 minutes at -20°C. Thereafter, the samples were centrifuged at 12000

RPM at 4℃ for 10 minutes. The supernatant containing sugars and lipids was collected carefully into separate microcentrifuge tubes and analyzed for sugars (section 3.5.1). The remaining pellets were left to dry overnight in a biosafety cabinet and processed for plant DNA extraction and amplification as described in section 3.7.

3.5.1 Separation of sugars from lipids

Two hundred microlitres of chloroform was added directly to the homogenate to extract lipids followed by centrifugation at 1000 RPM at 4℃ for 1 minute. This procedure is a modification of the method described previously (Van Handel, 1985). To enhance phase separation, 100µl of PCR water was added and the homogenate further centrifuged at 1000 RPM at 4℃ for 1 minute. The upper alcohol layer containing sugars was carefully collected for further processing (Section 3.6).

3.6 Analysis of sugars

3.6.1 Cold anthrone

The cold anthrone test was used as described (Wanjiku *et al*., 2021). Briefly, 200µl aliquots of the supernatants were transferred to test tubes, followed by addition of 1mL of anthrone reagent (sulphuric acid and anthrone powder) and incubation of the mixture at room temperature for 75minutes. Glucose dissolved in 70% ethanol was used as positive control. A color change of yellow to blue or green in the sample was scored as positive indicating the presence of fructose and evidence of recent plant feeding.

3.7 Plant DNA extraction

Plant DNA were extracted from the abdominal pellets using ISOLATE II Plant DNA Kit (Bioline, London, UK) following the manufacturer's protocol after slight modifications: 1) The

initial incubation was performed for 1 hour 30 minutes instead of 3 hours and 2) 70µl of DNA was eluted instead of the recommended 100 µl. DNA was extracted from all the anthrone positive samples as well as from the anthrone negative samples.

3.7.1 Plant DNA amplification by Polymerase Chain Reaction (PCR)

PCR was used to amplify plant DNA by targeting the ribulose-1,5 bisphosphate carboxylase gene as described previously (Hassaballa *et al*., 20201). Ten microliter PCR reaction volume using the My Taq DNA Polymerase Kit (Bioline, London, UK) were prepared comprising 5X My Taq reaction buffer, 10 µM final concentrations for each primer, 0.125µl of My taq DNA polymerase and 2µl of DNA template. The thermal cycling conditions were initial denaturation at 94℃ for 5 minutes, followed by 35 cycles of denaturation at 94℃ for 30 seconds, annealing at 55℃ for 30 seconds and extension at 72℃ for 1 minute and final extension at 72℃ for 10 minutes. The amplicons were visualized on a 1.5% agarose gel stained with ethidium bromide (Sigma-Aldrich, GmbH, Germany) against a 1 Kb DNA ladder (HyperLadder, Bioline, London, UK). Based on those that had amplified at the expected band size (400-600pb), a few representatives were cleaned using ExoSAP IT (Affymetrix Inc., Santa Clara, CA, U.S.A.) rapid cleanup kit following the manufacturer's instructions and outsourced for bidirectional Sanger sequencing to Macrogen, South Korea. Sequences were cleaned in Geneious prime (Kearse *et al*., 2012) and compared to GenBank reference sequences using Basic Local Alignment Search Tool (BLAST) and those with >98% percentage identity scored as the host plants.

3.8 Sequence analysis

Sequences (mosquito, *P. falciparum*, plant) were viewed and cleaned in Geneious prime (Kearse *et al*., 2012) and queried in GenBank using Basic Local Alignment Search Tool (BLastn). Plant hosts were identified after BLASTn search with the "Highly similar" option

for those with >98% percentage identity. MAFFT in Geneious Prime (Kearse *et al*., 2012) was used to perform multiple sequence alignment with default parameters. Maximum likelihood (ML) trees were inferred for mosquito ITS2 sequences using the best fit model of sequence evolution with nodal support for different groupings evaluated through 1000 bootstrap replications.

3.9 Statistical analysis

Relative abundance was used to estimate the outdoor composition of the anopheline mosquitoes. Daily counts of female mosquito/trap/night for *An. funestus* s.l. and *An. gambiae* s.l. were compared for each site using generalized linear models (GLM) with negative binomial error structure based on best-fit model residuals. The mean catches/trap/night was computed for each of the species complexes. The *P. falciparum* sporozoite infection rates (*Pf*sp) were expressed as the number of positive specimens of the total number of specimens examined. Chi-square test of independence was used to compare proportions among different subspecies. All statistical analyses were performed using R *v.* 4.1.0 software at 95% confidence limit**.**

3.10 Ethical considerations

A research permit (License No: NACOSTI/P/21/10588) was acquired from National Commission for Science, Technology and Innovation (NACOSTI) and ethical approval obtained from Scientific and Ethical Review Unit (SERU) of the Kenya Medical Research Institute (KEMRI) (Protocol No. SSC 2787). Prior to mosquito sampling, the purpose of the study, procedures and associated benefits/risks were provided to the local leadership at county and community levels. Additionally, informed consent to trap mosquitoes around homesteads was obtained from household heads

CHAPTER FOUR

4.0 RESULTS

4.1 Outdoor catches of anopheline mosquitoes

An. funestus s.l was predominant across the three study sites comprising of 90.6% (639/705) of the total captures while *An. gambiae s.l.* registered9.4% (66/705) of the total catches. The breakdown of the catches by sites were as follows: Kerio Valley (*An. funestus s.l.* = 478, *An. gambiae s.l.* = 46); Rabai (*An. funestus s.l.* = 72, *An. gambiae s.l.* = 0) and Nguruman (*An. funestus s.l.* = 89, *An. gambiae s.l.* = 20). Mean trap catches were significantly higher for *An. funestus* s.l. than *An. gambiae* s.l. in Kerio Valley ($\chi^2_{1,86}$=68.6, p<0.0001) and Nguruman: ($\chi^2_{1,110}$=102.7, p<0.0001) (Fig 4a).

4.2 Outdoor species composition

PCR to identify sibling species of *An. funestus s.l.* revealed variation in the species composition by site (Fig. 4b). In Kerio Valley, *An. longipalpis* C was predominant (75.7%, 362/478) followed by *An. funestus s.s.* (4.2%, 20/478) and *An. rivulorum* 0.8% (4/478). Species in this group were represented by *An. rivulorum* (26.4%, 19/72), *An. longipalpis* C (11.1%, 8/72) and *An. funestus* s.s. (2.8%, 2/72) in Rabai. In Nguruman, the identified species were*An. rivulorum* (46.1%, 41/89) followed by 1.1% each of *An. longipalpis* C (1/89) and *An. funestus s.s.* (1/89). A fraction of the *An. funestus s.l.* specimens ranging from 19.2 - 59.7% did not amplify (Unamplified: 28.3%, 181/639) (Fig 4b). *An. arabiensis* and *An. gambiae* were the two species observed in *An. gambiae* s.l. with the former occurring in higher proportion (41/46) than the latter (5/46) in Kerio Valley. Both species were not detected in samples collected from Rabai (Kilifi), but they were fairly represented in the Nguruman samples (9/20 vs 11/20, *An. gambiae* and *An. arabiensis*, respectively).

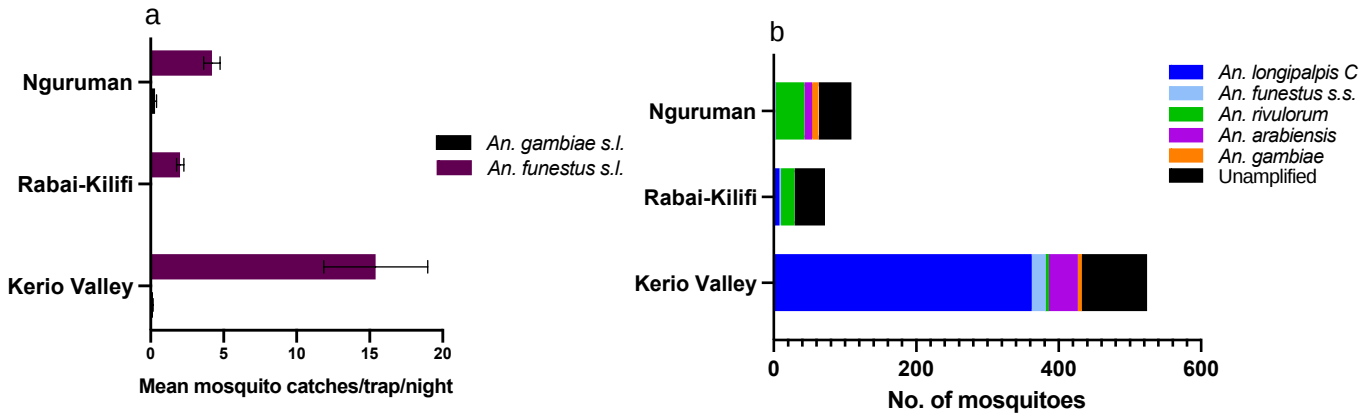

Figure 4: **a**) Outdoor mean catches/trap/day, **b**) Species composition, in three study areas in dryland areas of Kenya. The number of trap nights were 44, 50 and 56 in Kerio Valley, Rabai and Nguruman, respectively.

4.3 *Plasmodium falciparum* infection rates

Forty-six (46) of the 705 female anophelines tested positive for *P. falciparum* sporozoites (*Pf*sp) using ncMS. This translated to an overall *Pf*sp rate of 6.5% but this finding varied with site and mosquito species (Table 2). In the *An. funestus* group, high *Pf*sp rates were recorded in *An. longipalpis* C (5.2% (19/362) in Kerio Valley only; *An. funestus s.s.* in Kerio Valley (5% (1/20) and Nguruman (100% (1/1) and *An. rivulorum* in Nguruman (19.5%, 8/41) and Rabai (5.3%, 1/19). A significant proportion of Unamplified cohorts also tested positive for *Pf*sp in Kerio Valley (12%, 11/92) and Nguruman (4.3%, 2/46) (Table 2). Among *An. gambiae s.l.*, *Pf*sp were detected in *An. gambiae* in Kerio Valley (20% (1/5) and Nguruman (11.1% (1/9) and in *An. arabiensis* from Kerio Valley only (2.4%, 1/41).

Table 2: *Plasmodium falciparum* sporozoite rates (%) by species in selected dryland areas, Kenya. Number in parenthesis indicates fractions of the total that tested positive for each species.

	Species	Kerio Valley	Rabai	Nguruman	Total
***An. funestus* group**	*An. longipalpis* C	5.2% (19/362)	0% (0/8)	0% (0/1)	**5.1% (19/371)**
	An. funestus ss	5% (1/20)	0% (0/2)	100% (1/1)	**8.7% (2/23)**
	An. rivulorum	0% (0/4)	5.3% (1/19)	19.5% (8/41)	**14.1% (9/64)**
	Unamplified	12% (11/92)	0% (0/43)	4.3% (2/46)	**7.2% (13/181)**
An. gambiae s,l,	*An. gambiae ss*	20% (1/5)	0%	11.1% (1/9)	**14.3% (2/14)**
	An. arabiensis	2.4% (1/41)	0%	0% (0/11)	**1.9% (1/52)**
	Total	**6.3% (33/524)**	**1.4% (1/72)**	**11% (12/109)**	**6.5% (46/705)**

Further confirmation of species identity of the infected specimens (for *An. funestus* group only) was conducted by PCR of the ITS2 region and then sequencing. Few samples that tested negative especially for the unamplified cohorts were included. Phylogenetic analysis of the generated sequences showed strong support (BS=98% and 89%) for *An. funestus s.s.* and *An. longipalpis* C, respectively (Fig 5). Two of the infected species showed (BS=100%) for *An. rivulorum*. Additionally, sequenced samples of the Unamplified cohort (positive and negative for *Pf*sp) resolved into well distinct clades of (BS= 100%) *Anopheles sp*.19 DZ 2020 and (BS=100%) *Anopheles sp.* Isolate A MKO43038.1 indicating presence of cryptic species.

Among the Unamplified cohorts, (*An. sp*. 1, *An. sp*. 2, *An. sp*. 4, *An. sp*. 5 and *An. sp*. 7) did not cluster with any significance to any other available anopheline ITS sequence.

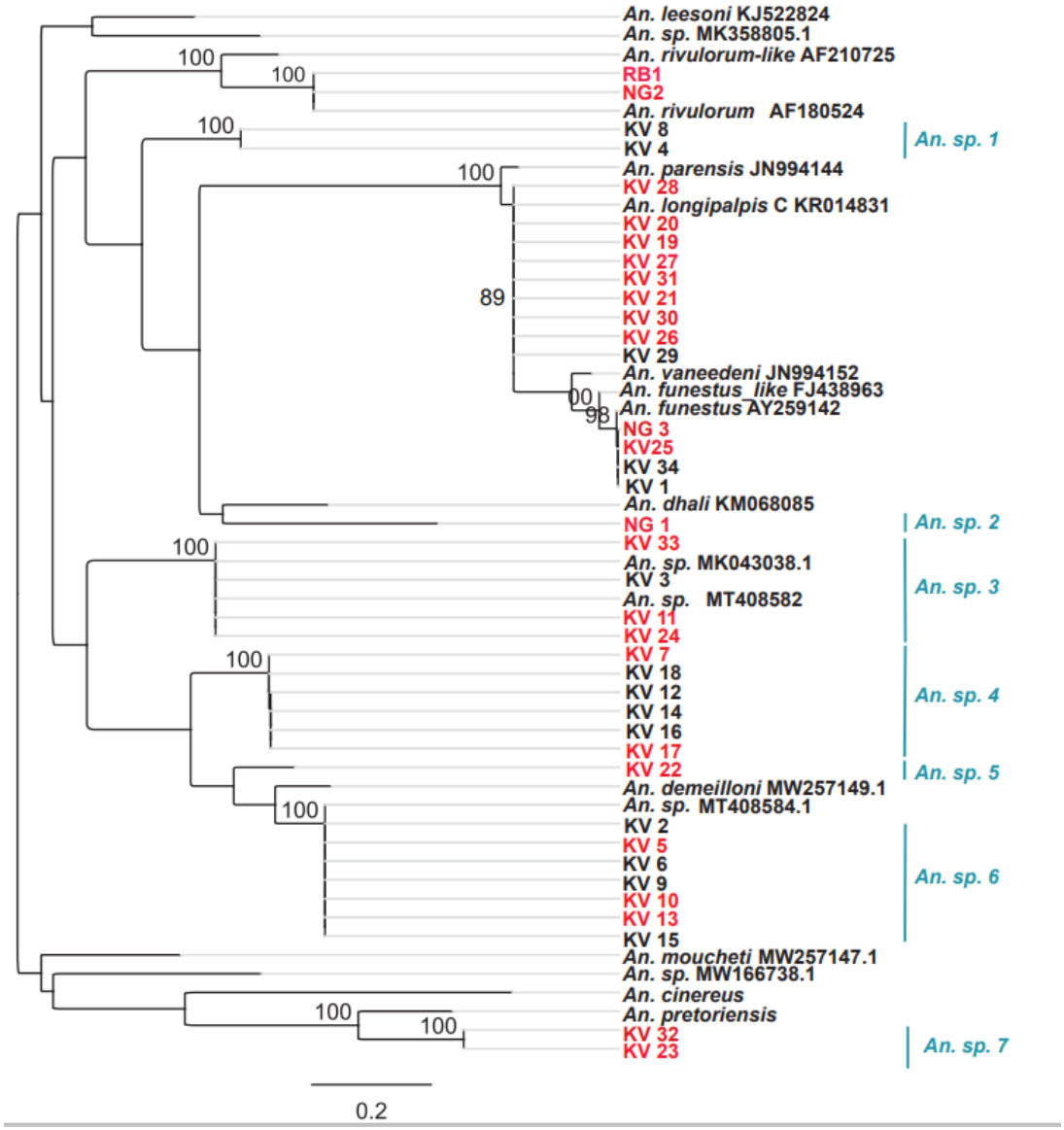

Figure 5: Phylogenetic tree for representative mosquitoes morphologically scored as *An. funestus* group infected with *P. falciparum* sporozoites. Few samples negative for the malaria parasite are also included. Bootstrap support values are indicated above the nodes from 1000 replicates. Highlighted in red are the infected samples.

4.4 Fructose positivity rates

Plant sugar analysis was limited to samples from Kerio Valley and Rabai areas only. Nineteen of 680 mosquitoes tested positive (2.8%) for fructose using the cold anthrone test (Table 3). Low positivity rates were recorded across the species: *An. longipalpis C* (2.75%, 10/364), *An. rivulorum* (3.13%, 2/64), *An. arabiensis* (3.85%, 2/52) and the unidentified mosquitoes (3.07%, 5/163). No *An. funestus* s.s and *An. gambiae* s.s tested positive for fructose.

Table 3: Summary of fructose positivity and successfully sequenced plant DNA among different species from Kerio Valley and Rabai

Species	Fructose positive	No. amplified (No. sequenced)
An. longipalpis C	10	78(47)
An. funestus	0	4(2)
An. rivulorum	2	11(3)
An. arabiensis	2	20(5)
An. gambiae	0	1(0)
Unamplified	5	31(14)
TOTAL	**19**	**145(71)**

4.5 Plant DNA and PCR success rates

Plant DNA was extracted from 262 samples inclusive of fructose positive (19) and negative samples (243) only from Rabai and Kerio Valley. One hundred and forty-five of the 262 samples amplified successfully (Table 3) which translated to an overall PCR success rate of 55% (145/262). The PCR success rate varied among the fructose positive 68.4% (13/19) and fructose negative samples 54.3% (132/243). When compared among the different species, PCR success varied significantly ($\chi 2$ =0.78, df = 5, P=0.038).

Seventy one specimens were successfully sequenced and the plant host identified after BLASTn search with the "Highly similar " option. Sequenced were assigned as plant hosts for those with >97% percentage identity. Few samples (n=4) had more than one band, potentially indicating multiple plant hosts. However, attempts to identify plant hosts by sequencing were not successful. The dominant plant host was acacia plant. Distribution of the identified plants by species are presented in a heatmap (Fig 6). Additionally, of the 71 successful sequences, results showed that the key plant family was Fabaceae in which Acacia plant was the most commonly fed plant (Table 4).

Table 4: Summary of host plant families identified in the different study areas

Plant family	Identity species >97%	Kerio valley	Rabai
Poaceae	*Cynodon dactylon*	2	0
Fabaceae	Acacia plant	57	6
Poaceae	*Newtonia duparquentiana*	1	0
Fabaceae	*Senna tora*	1	0
Musaceae	*Musa ornata*	1	1
Fabaceae	*Parkia filicoidea*	1	0
Fabaceae	*Dichrostachys cinerea*	1	0

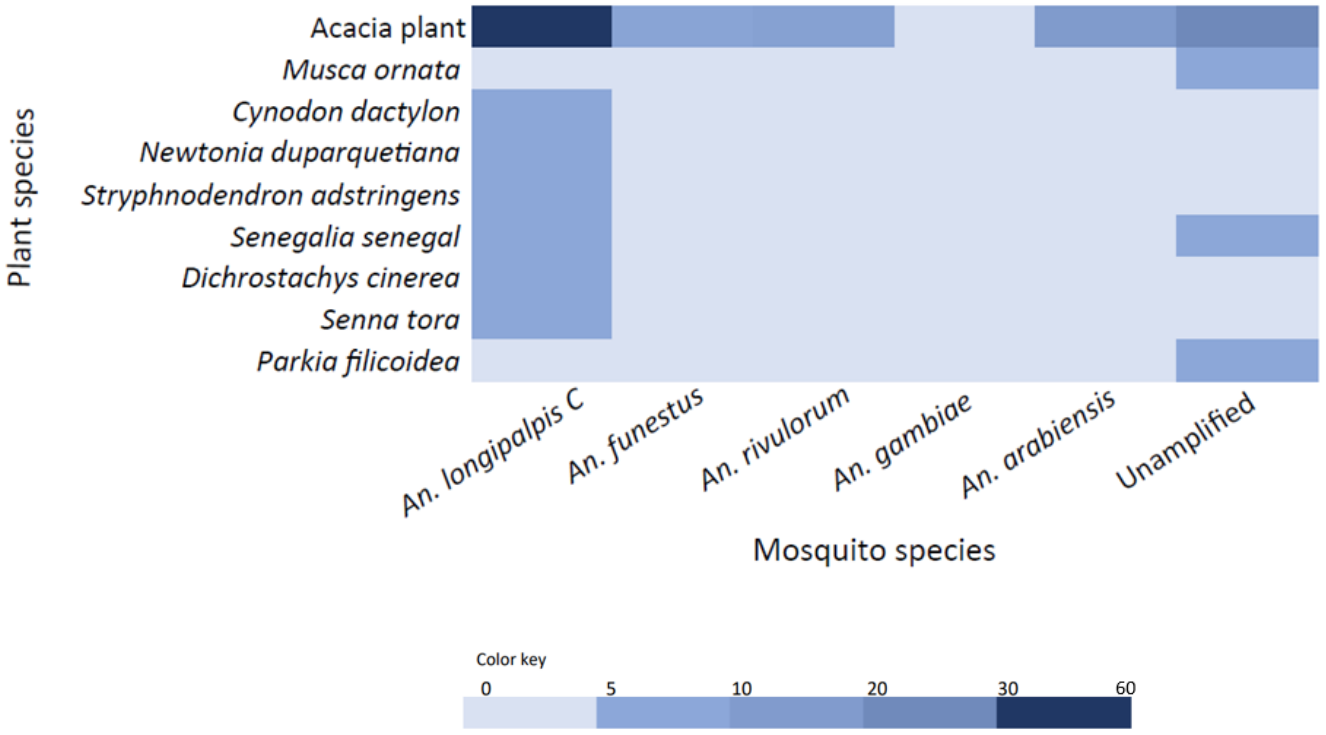

Figure 6: Heatmap showing identified plant hosts for anopheline mosquitoes from Kerio Valley and Rabai.

CHAPTER FIVE

5.0 DISCUSSION

Outdoor transmission of malaria is a dynamic process and the vectors involved may vary from one ecological setting to another. Here, diverse known and cryptic anopheline species were collected outdoors and found to harbor *Plasmodium falciparum* sporozoites. These species could play crucial roles in residual malaria transmission (RMT), given that parasite infection rate represents the most sensitive indicator in assessing importance of a vector species in this phenomenon (Okumu & Finda, 2021).

This study observed that *An. funestus* s.l dominated the mosquito species collected from the study areas. Further identification of the samples by molecular techniques revealed the abundance of other sibling species including *An. longipalpis* C and *An. rivulorum* in the *An. funestus* complex and a multitude of other unidentified cryptic species although there were site-specific differences. Previous studies have largely implicated *An. funestus s.s.* as the most important vector in this group and its adaptation to survive in dry seasons (Charlwood *et al*., 2000). This leads to the question of whether the low numbers signal dwindling importance of this species in certain ecological areas. Being a highly anthropophilic and endophilic mosquito (Dia & Guelbeogo, 2013; Ogola *et al*., 2018; Okumu & Finda, 2021), the low numbers encountered outdoors is not surprising.

It could also be argued that the low numbers reflect ITN-related effectiveness on their populations. The replacement of *An. funestus s.s.* by more outdoor resting/biting *An. rivulorum* after implementation of IRS in Kenya has been reported in literature (Gillies & Smith, 1960). This could be the likely scenario following its absence in Rabai or enhanced contribution of *An. rivulorum* to malaria transmission in Nguruman and to lesser extent in Rabai. Despite the low numbers, this species was still associated with parasite infection supporting its high

susceptibility and efficient role in malaria transmission. Its contribution to malaria transmission in the study areas can be further assessed from additional data of longer-term surveys both indoors and outdoors as well as data on bed net coverage and use patterns in the target communities.

Findings from this study revealed a high representation of lesser known species, *An. longipalpis* C, in the Funestus group. Although the non-malaria vector status accorded to *An. longipalpis* C in southern Africa was attributed to absence of parasite infection and its highly zoophilic yet endophilic habits (Kent *et al*., 2006), A high human blood meal index observed for this mosquito in Ethiopia (Adunga N. and Peteros B., 1996) lend support for its potential role as a malaria vector. This is further supported by previous detection of *P. falciparum* infection in indoor populations in Kerio Valley (Ogola *et al*., 2018). The present findings of high *Pf*sp rate and occurrence in high densities, even surpassing those of known vectors, suggest it could assume major role as a malaria vector in certain foci in Kenya. Therefore, understanding aspects of its biology and ecological adaption including breeding structure, resting and biting habits and vectorial capacity are warranted.

Exophilic habits among mosquitoes do not preclude indoor activities and outdoor biting behavior could be a response to indoor insecticidal interventions (Carnevale & Manguin, 2021). Endophilic behaviour is well documented in *An. funestus s.s.* (Dia & Guelbeogo, 2013; Machani *et al.*, 2020) and *An. longipalpis* C (Ogola *et al*., 2018). These species could play a crucial role in RMT as parasite presence represents the most sensitive indicator in assessing vector importance in this phenomenon (Okumu & Finda, 2021). Kenya is intensifying its national efforts in malaria control to achieve malaria elimination and success requires surveillance of all potential vector species. This could be facilitated by integration of molecular tools in routine entomologic surveillance (Ogola *et al*., 2019) to guide malaria control interventions.

The uncovering of diverse species in the Funestus group highlights the importance of application of molecular tools in routine entomologic surveillance (Ogola *et al*., 2018, 2019). Not only were multiple species uncovered, they were found associated with *P. falciparum* infection. One of the species in a well-supported clade (BS= 100%) had 100% sequence identity with *Anopheles sp*. 19 DZ-2020 (GenBank accession number MT408584.1) recently reported in western Kenya as a novel cryptic species (Zhong *et al*., 2020). Another species shared (BS=100) 99.9% sequence identity with *Anopheles sp*. Isolate A (GenBank accession number MK043038.1) previously reported in the dryland area of West Pokot Kenya, as a potential secondary malaria vector on the basis of harboring malaria parasite and predominance among outdoor *An. funestus* mosquitoes (Ogola *et al.*, 2019). Its vectoring ability was confirmed in a recent study in western Kenya (Zhong *et al*., 2020).

The results suggest an adaption to dry ecological areas but highlight the potential for wider distribution and greater impact of these cryptic species on malaria transmission with growing effect of climate change and global warming. These could provide new, favorable and suitable environmental conditions for the survival of these species, but this needs further investigations. As control targets primary vector species, these species could fill the gap and emerge to become important vectors. In addition, the observed number of unamplified specimens in the *An. funestus* group calls for improvement in the existing molecular identification protocol to discriminate members of this group other than the commonly known malaria vectors.

Plant utilization can also contribute to malaria transmission as it impacts the survival of mosquito vectors as well as the vectorial ability. This study recorded quite low fructose positivity rates compared to previous studies (Wanjiku *et al*., 2021; Hassaballa *et al*., 2021) done on plant feeding behavior in mosquito vectors. Potential difference related to species analysed and ecological origin of the samples cannot be ruled out given similarity in the methodology employed. Just like the above previous studies, mosquito's abdomen was used

to test for fructose in the mosquitoes. Further, fructose stability is not affected by the method of storage (Swan *et al*., 2021). Results from a previous study showed that there was no significant difference in fructose content based on the different storage methods in *Ae. albopictus* (Scott *et al*., 1993).

Previous studies have attempted to detect plant DNA only from fructose positive samples (Nyasembe *et al*., 2018; Wanjiku *et al*., 2021). This study however, went further to incorporate fructose negative samples and results from plant DNA extraction revealed that the anthrone test underestimates plant sugar feeding evidence. The anthrone negative samples were seven folds higher than the anthrone positives with plant DNA. Utilization of plant seems to differ among different species since the plant DNA detection rate was different among the species. As much as this is a trait implicated in vector species, the variation of plant utilization among different species needs validation. After performing PCR, the high PCR success rates contrasted with previous studies that targeted the same gene (rbcL) (Wanjiku *et al*., 2021; Hassaballa *et al*., 2021).

This study is believed to be the first to investigate the plants that *An. funestus* mosquitoes feed upon in nature in Kenya through DNA barcoding. Plant DNA barcoding analysis is a sensitive technique of identifying plant sugar sources of arthropods in nature (Wanjiku *et al*., 2021; Hassaballa *et al*., 2021; Nyasembe *et al*., 2018; Smith, 1961). Sequence analysis revealed a broad host range for these mosquito vectors. The identified species mostly belonged to the family Fabaceae resembling previous studies of mosquito preference to plants in this family (Weber & Keeler, 2013; Nyasembe *et al*., 2018; Wanjiku *et al*., 2021; Müller *et al*., 2010; Barredo & DeGennaro, 2020).

Furthermore, this study recorded high PCR success rates of rbcL gene and acacia as the most preferred host plant in Kerio Valley and Rabai. The overall finding demonstrated that

mosquitoes from Kerio Valley and Rabai, in the *An. funestus* group had fed on acacia plant. Field ecology from the two study areas confirmed the presence of these plants. Acacia is an indigenous species that extends in the arid and semi-arid parts of Kenya. Acacia plants have been reported to have high levels of tannins especially in their fruits and leaves; tannins affect the fecundity, growth and microbe interaction of some insects (Hasaballa *et al.*, 2021; Elgailani & Ishak, 2014). These plants also contain sugars which vectors potentially seek to supplement their nutritional budget (Stone & Foster, 2013). It is however not clear if the overwhelming preference to acacia plant is due to attraction from specific compounds. Mosquitoes like other insects are attracted to volatile organic compounds emitted by plants (Nyasembe *et al.*, 2018; Nyasembe & Torto, 2014). Further studies to identify the semiochemicals mediating attraction to acacia plants by these mosquito species are warranted. Understanding this can pave way for developing surveillance tools for monitoring vector populations.

5.1 CONCLUSION AND RECOMMENDATION

5.1.1 Conclusions

This study demonstrated that a previously unappreciated vector, *An. longipalpis* C, plays a role in outdoor malaria transmission in a dryland ecology of Kenya. The outdoor malaria vector profile included other known species such as *An. funestus* s.s. *An. rivulorum*, *An. gambiae* s.s, and *An. arabiensis*. In addition, at least six cryptic potential malaria parasite vectors most of which have not been previously described in Kenya were identified by molecular tools. The study also revealed cold anthrone test underestimates vector feeding in comparison with the more sensitive molecular tools. The high detection rate of acacia plant feeding as noted in different species is an indication of potential role of the plant regulating the survival and vectoring capacity of malaria vector. Additionally, the seemingly overwhelming feeding on

acacia suggests the need of future studies to understand the plant-vector interactions and possible exploitation of this interaction for future vector control approaches.

5.1.2. Recommendations

This study was exclusively based on samples collected outdoors and the collection was done over a short duration. Future studies should incorporate longer-term surveys both in and outdoor. Longer term studies will also lead to better comparisons and understanding vector species composition by incorporating the effect of different seasons. Only CDC light traps were used in this study. It would be important to incorporate other trapping methods such as human landing catches (HLC) because mosquito composition can be affected by the trapping method used.

Findings from this study indicated vast diversity of species. However, the analysis was only centered on a few subsets. Analyzing a larger data set could unravel an even higher diversity. There is also the need to study the biology of lesser known vector species and generate data on their bionomic traits especially traits relating to insecticides resistance. Molecular tools, which are more sensitive in identifying species-specific interactions, should be incorporated in malaria disease surveillance.

In addition, future studies should determine the nutritional benefit of acacia, and how the plant-vector interaction can be exploited for developing surveillance tools for monitoring vector populations.

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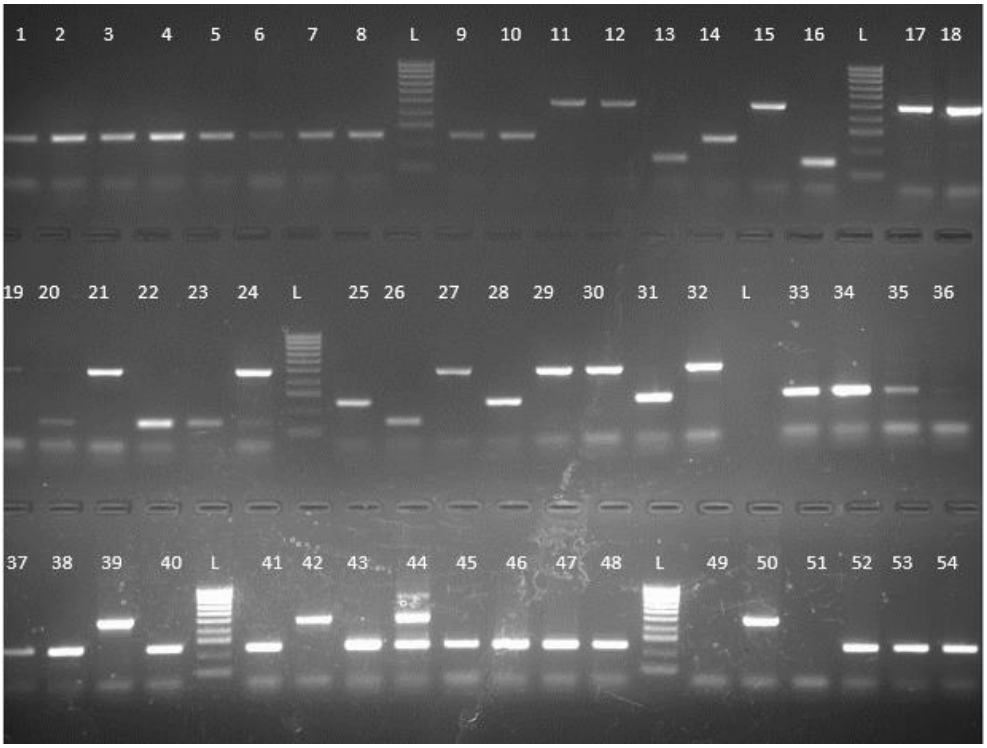
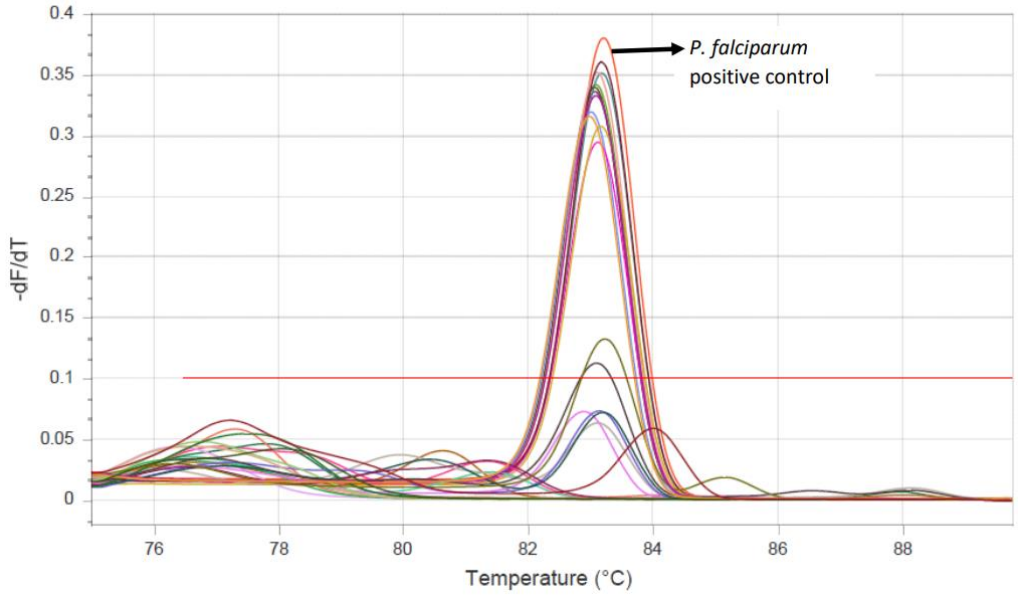
APPENDICES



Appendix 1: Gel electrophoresis image of *An. funestus* samples speciated using cocktail of primers(L=100bp ladder)



Appendix 2: Representative melt curves of *P. falciparum* infected samples. y-axis shows change in fluorescence units with increasing temperature (dF/dT), x-axis shows increasing temperature