BLOOD-FEEDING PATTERN OF MALARIA VECTOR REVEALS MALARIA TRANSMISSION DYNAMICS IN THREE ISLANDS OF LAKE VICTORIA IN KENYA

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EGERTON UNIVERSITY

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DECLARATION AND RECOMMENDATION

DECLARATION

This research thesis is my original work and has not been submitted wholly or in part to any institution for an award of any degree.

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DEDICATION

To Onesmus (M.C.C.J) and Lilian

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LIST OF ABBREVIATIONS AND ACRONYMS

ACTs	Arteminisin combined therapies
Blast	Basic local alignment tool
Bp	Base pairs
Bti	*Bacillus thuringiensis var. israelensis*
CDC	Centre for disease control and prevention
CO1	Cytochrome oxidase subunit 1
CSA	Chondroitin sulphate A
Cyt b	Cytochrome b
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
dNTPS	Deoxyribose nucleoside triphosphates
DOMC	Division of malaria control (Ministry of Public Health and Sanitation), Kenya
EDTA	Ethylenediaminetetraacetic acid
G6PD	Glucose-6-phosphate dehydrogenase
GIS	Geographic information system
GMM	Genetically modified mosquito
GPS	Geographic positioning system
HA	Hyaluronic acid
HRM	High resolution melting
ICAM-1	Intracellular adhesion molecule 1
ICIPE	International Center of Insect Physiology and Ecology
IGS	Intergenic spacer region
IRS	Indoor residual sprays
ITS2	Internal transcribed spacer region 2

KNHMIS	Kenya National Health Management Information System
LLINs	Long lasting insecticides nets
Mb	Megabases
NCBI	National Centre for Biotechnology Information
PCR	Polymerase chain reaction
PFEMP-1	*Plasmodium falciparum* erythrocyte membrane protein 1
PfATP4	*Plasmodium falciparum* ATPase 4
PSC	Pyrethrum spray catches
RBC	Red blood cells
RBM	Roll back malaria
RCF	Relative centrifugal force
Rpm	Revolution per minute
SSA	Sub – Sahara Africa
TAE	Tris-acetate ethylenediaminetetra-acetic acid
TNF	Tumour necrotic factor
WHO	World Health Organisation
WGS	Wideband global sitcom

ABSTRACT

The Lake Victoria region of western Kenya is malaria endemic with a suitable environment throughout the year for the transmission of *Plasmodium* parasites by its primary mosquito vector, *Anopheles gambiae sensu lato* (s.l.), as well as other secondary vectors. Malaria control largely depends on vector control using long-lasting insecticidal nets (LLINs) that protect humans from infectious bites while sleeping. However, the success of this strategy is greatly threatened by resistance to insecticides and changes in mosquito feeding pattern that alter disease transmission dynamics. Therefore, adequate knowledge of mosquito feeding patterns and population structure can inform the efficacy of and allow appropriate deployment of appropriate vector control strategies. In this study, mosquito feeding behaviour, *Plasmodium* infection, and genetic structure of malaria mosquitoes were sought to determine transmission dynamics in three malaria endemic islands of Lake Victoria in Western Kenya namely Mageta, Magare, and Ngodhe. Adult mosquitoes were trapped both indoors and outdoors of 3,081 geo-referenced buildings inhabited by about 7,221 people. Demographics and LLINs usage were also surveyed. The collected mosquitoes were morphologically identified on site and later to species level by PCR product sequencing. Sources of blood-meals in vectors were determined by high resolution melting (HRM) analysis of *cytochrome b* (*cyt b*), *16S ribosomal RNA*, and *cytochrome oxidase 1* (*COI*) genes. Malaria parasites in mosquito's salivary glands were identified by PCR-HRM of *cyt b* and population structure of mosquito vector species characterized using microsatellites. The data show that the islands have an overall LLINs coverage of about 67%, while the abundance of *A. gambiae sensu strictu* (s.s.), *Anopheles arabiensis*, *Anopheles funestus* s.s., and *Anopheles coustani* remain high, enabling high malaria transmission throughout the year. Humans were the most prominent blood-meal hosts of malaria vectors in all study islands, but blood-meal hosts also included diverse non–human sources (*n*=9) such as cow, chicken, goat, and rat among others. Some mosquitoes (3.61%) had fed on humans in addition to cow, goat or chicken. Overall 9.86% (*n*=41) of engorged malaria mosquitoes, including *A. coustani*, harboured malaria parasites. The presence of abundant malaria mosquitoes that harbour *Plasmodium falciparum* and secondary blood-meal sources potentially promote malaria transmission through provision of alternative means for mosquito survival, potentially limiting the effectiveness of LLINs.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Malaria is a febrile illness caused by a single-celled protozoan parasite (Genus: *Plasmodium*), which is transmitted by a female mosquito (Genus: *Anopheles*) and infects red blood cells in mammals, birds, and reptiles (Videvall *et al.,* 2016). Humans with malaria may experience fever, chills, and flu-like illness and if left untreated, may develop severe complications and die (Miller *et al.,* 2002). The geographic distribution of global malaria burden is uneven. The disease is concentrated in more than 97 countries found in poor, tropical and subtropical regions where it continues to be a key contributor to the global health problem and a major obstacle to the socioeconomic prosperity (Sachs & Malaney, 2002; WHO, 2015). In 2015, close to 3.1 billion people were at risk of malaria, with approximately 149 - 303 million cases and about 438 000 deaths, of which an estimated 78% of these deaths occurred in African continent (WHO, 2015). Studies estimate that malaria cost Africa more than US$ 12 billion every year in lost productivity (WHO, 2014). In Kenya, about 28 million people live in areas with high risk of malaria transmission, where young children and pregnant women are the most affected groups (KNHMIS, 2012), with more than 9 million annual cases and 1 in every 20 deaths worldwide occurring in Kenya (KNHMIS, 2012). With such an impact, there is urgent need to improve current and/or develop novel malaria control measures.

Major control strategies for malaria are chemotherapy and blocking of malaria parasite transmission through vector control. In chemotherapy, drugs applied are from compounds such as quinolines, antifolates and artemisinin (Saifi *et al.,* 2013). However, over 50 years reliance on a small group of antimalarial compounds has resulted into development of resistance by the parasite (White, 2008). Though there has been substantial effort in vaccine development, these vaccines have not been rolled out (Moorthy *et al.,* 2004; Neafsey *et al.,* 2015; RTS,S, 2015), mostly because of safety concerns and modest protection (Callaway & Maxmen, 2015).

Main existing front-line vector control measures include indoor residual spray (IRS) and long-lasting insecticides nets (LLINs) (Wallace *et al.,* 2014). IRS involves spraying an active substance to which mosquitoes are fully susceptible to inside households' walls and other mosquitoes resting places, while LLINs involve providing a physical barrier and simultaneously killing and/or repelling of blood-seeking mosquitoes. IRS has contributed massively to malaria reduction, with the greatest sustained success in Africa being in South

Africa (Mabaso *et al.,* 2004). LLINs has been used extensively to reduce mosquito density and biting activity causing a decline in malaria transmission, with 44% reduction of malaria-related mortality in rural Kenya (Phillips-Howard *et al.,* 2003; Fegan *et al.*, 2007; Lim *et al.,* 2011).

However, in addition to increasing cases of resistance to insecticides within mosquito populations, effectiveness of LLINs is threatened by changes in malaria mosquito feeding patterns (Ranson, *et al.,* 2011; Maliti *et al.,* 2014; Christine *et al.,* 2015). Especially, feeding upon humans during active hours, when humans are unprotected by LLINs (Russell *et al*., 2011; Briët & Chitnis, 2013), and opportunistic feeding upon non-human hosts thus minimizing contact with LLINs and IRS (Lefevre *et al.,* 2009). Other strategies that involve killing the vector and blocking transmission have been applied, with the introduction of genetically modified mosquitoes (GMM) into natural populations of mosquitoes as an alternative malaria control strategy being a recent attempt (Okorie *et al*., 2014). However, it remains contentious and very controversial strategy (Mumford, 2012). With these limitations and challenges, it is not only important to improve and/or develop novel vector control strategies, but also there is a need to understand mosquitoes blood-feeding patterns, *Plasmodium falciparum* infection and population structure of malaria mosquitoes in order to provide insight into malaria transmission dynamics and adopt the most effective vector control strategies.

High malaria transmission in Kenya often occurs in rural areas and near large water bodies like lakes (Keiser *et al.,* 2005) with lowland districts of Nyanza, Western provinces and areas along Kenya's coast comprising the most endemic areas (DMOC, 2010). In areas near Lake Victoria, in Western Kenya, water is an important all year round support of the life of malaria mosquitoes. Regardless of the season, the lake provides a suitable environment that maintains high malaria vector abundance and diversity, with various health facilities reporting more than 40% malaria prevalence rates of all hospital visits largely due to *P. falciparum* infections (Noor *et al.,* 2009). The main malaria vectors in this region are *A. gambiae* sensu stricto (s.s.) and *A. arabiensis* (McCann *et al.,* 2014). Additionally, *A. coustani* and *A. funestus* are also present (Minakawa *et al.,* 2012).

Malaria mosquitoes species and their behaviour differ within and between locations, hence transmission is geographically specific (Gallup & Sachs, 2001) and the characteristic of the disease changes from place to place (Okwa *et al.,* 2009). Consequently, different areas

require different tools and strategies for optimal vector control. Areas around large fresh water bodies, which support vector life cycle throughout the year, provide ideal sites for insights on disease dynamics as influenced by vector species and their behaviour. Therefore, this study focused on understanding the role malaria mosquito species and their respective behaviour play in malaria transmission dynamics in Mageta, Magare and Ngodhe islands of Lake Victoria in western Kenya. Malaria mosquito species, their blood-meal sources, *P. falciparum* infection rate, and population structure were determined for geographic information system (GIS) mapped mosquitoes collected over a period of 23 months. In addition human demographics and LLINs usage were also surveyed. The islands have abundant malaria mosquito species, with mosquitoes' densities being highest in Mageta, and comparable in Magare (rate ratio (RR) 0.20; 95 % confidence interval (CI) 0.1-0.38) and Ngodhe (RR 0.15; 95 % CI 0.12-0.2). Overall, humans were the most prominent blood-meal sources of malaria vectors, but other blood-meal sources also included diverse non–human sources including cow, chicken, goat, and rat among others. Some mosquitoes (3.61%) had fed on humans in addition to cow, goat or chicken. About 9.86% of engorged malaria mosquitoes, including *A. coustani*, harboured malaria parasites. Overall, coverage of LLINs was approximately 67%. Secondary blood-meal sources potentially promote malaria transmission through providing alternative means for mosquito survival, potentially limiting the effectiveness of LLINs and facilitating malaria transmission.

1.2 Statement of the problem

The lake environment provides a suitable condition that maintains an abundance of malaria mosquitoes and malaria transmission throughout the year, hence high malaria prevalence. Current malaria control strategies largely depend on chemotherapy and vector control. Chemotherapy is limited by chemoresistance, making vector control important through prevention of infectious bites. Importantly, vector control can be much effective if applied based on more precise knowledge of vector ecology including vector species and their blood-feeding pattern among other factors. This is however lacking, hence knowledge of malaria vector and hosts seeking behaviour in the malaria endemic islands is of significance in understanding vector-host interaction, with insights gathered being important in the implementation of a more focused appropriate and effective vector control strategies. Therefore, there is a need to investigate the blood-feeding pattern of malaria vectors in the three remote islands in order to provide insight into malaria transmission dynamics and recommend appropriate vector control interventions.

1.3 Objectives

1.3.1 General objective

To generate knowledge on malaria vector and host interactions in Mageta, Magare, and Ngodhe islands in Lake Victoria.

1.3.2 Specific objectives

1. To identify malaria vector species and the sources of their blood-meals.
2. To screen the engorged malaria vectors for *Plasmodium* infection.
3. To determine genetic structure of *A. gambiae s.s* and *A. gambiae arabiensis*

1.4 Hypotheses

1. The existing mosquito species do not prefer specific vertebrate host as blood-meal source
2. The identified engorged malaria vectors do not harbour malaria parasite
3. The genetic structure of *A. gambiae s.s* and *A. gambiae arabiensis* are not significantly different

1.5 Justification of the study

Mosquitoes are key determinants of the epidemiology of malaria disease. Hence, investigating the blood-feeding pattern of malaria mosquitoes in Mageta, Magare, and Ngodhe islands of Lake Victoria in Western Kenya is important because of the following reasons. First, chemotherapy is the only available primary malaria control strategy but is limited by chemoresistance, hence there is pressing need to improve and/or develop novel malaria mosquito vector control approaches. Second, the knowledge generated by the present study will provide insight into malaria transmission dynamics, and enable the use of strategies for optimal vector control. Finally, knowledge of malaria mosquitoes' blood-feeding pattern will assist in the implementation of evidence-based malaria vector control interventions, with a potential application on a geographically localized area. Further, accurate description of the malaria mosquito vectors blood-feeding pattern will help in obtaining baseline data important in appraising the success of control strategies in the study area.

CHAPTER TWO
LITERATURE REVIEW

2.1 Malaria and the malaria parasites

Malaria is a febrile illness caused by a single-celled protozoan parasite (Genus: *Plasmodium*), which are transmitted by a female mosquito (Genus: *Anopheles*) and infects red blood cells in mammals, birds, and reptiles (Videvall *et al.,* 2016). Major studies on the disease began after identification of human malaria parasite in 1880s by Laveran (Laveran, 1982), and intensified with the illustration of the parasite development in the mosquito vector and human blood stream in 1898 by Ross and Smyth (Ross & Smyth, 1897), together with elucidation of the human *Plasmodium* entire life cycle in 1948 (Shortt *et al.,* 1948). Recent improvements in bioinformatics (Hume *et al.,* 2003; Hartl, 2004) largely support the hypotheses that human and malaria co-evolved, and that human malaria may have had its origin from African rain forest, and accompanied human migration from the Nile to Mediterranean, Greece and to Italy (Prescott, 1996). Malaria patients may experience fever and flu-like illness and if left untreated, may develop severe complications and die (Miller *et al.,* 2002). There have been efforts towards malaria control with antimalarial drug discovery, vector control and vaccine development being the main efforts. However, malaria continues to be widely spread and having huge health and economic impacts.

2.2 Geographical distribution and economic impact of malaria

The global geographic distribution of malaria burden is irregular (Figure 1). However, the disease is concentrated in more than 97 countries found tropical and subtropical regions where the world poorest lives and is one among the main causes of health problems and a major obstacle to the socioeconomic development (Sachs & Malaney, 2002; WHO, 2015). In 2015, nearly 3.1 billion people were at risk, with about 149 - 303 million cases and approximately 438 000 deaths (WHO, 2015).

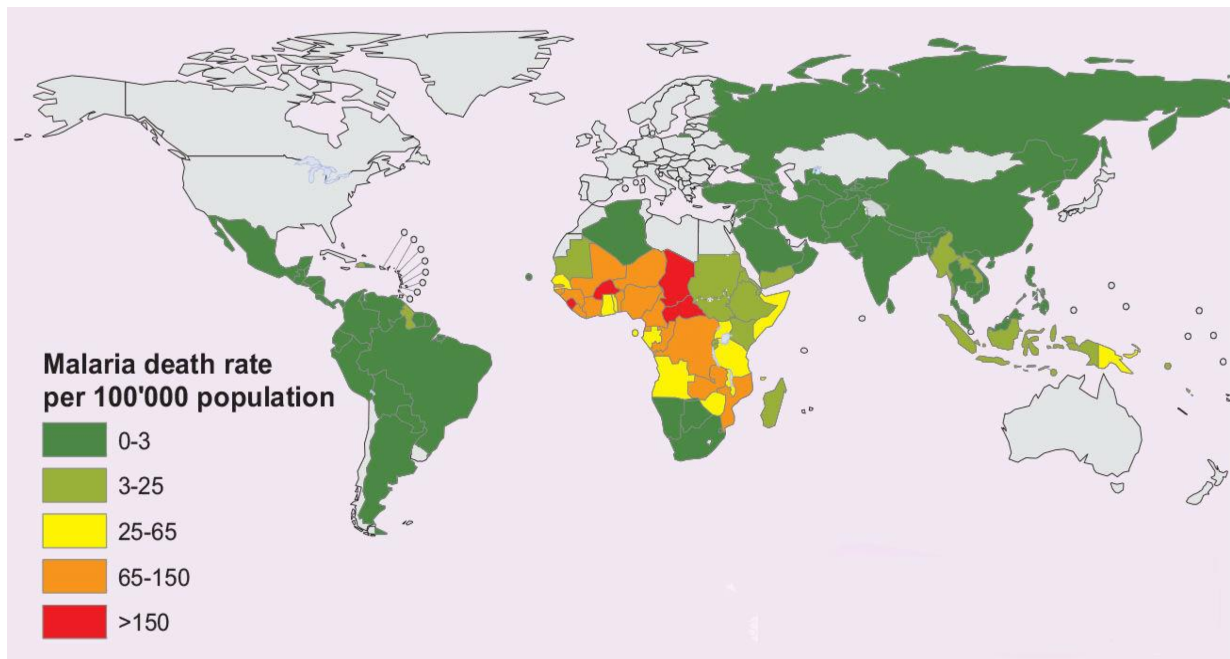

Figure 1: Global distribution of malaria burden. A global map showing malaria death rate per 100 000 cases. The key shows the number of deaths in each zone. There are more malaria deaths in the tropics, where climate favours the most potent vector that carries the deadliest parasite (*Plasmodium falciparum*). The region around tropical Africa highlighted in red has huge disease burden with more than 150 deaths per 100000 cases. Adopted from WHO 2012.

In Africa, malaria is the most prominent parasitic disease and one of the top three killers among infectious diseases (Greenwood & Mutabingwa, 2002). Despite the recent reduction in malaria incidences, studies estimate that in 2015, 80% of malaria cases and 78% of deaths were from 15 malarious countries in sub-Saharan Africa (SSA) (WHO, 2015). It is estimate that malaria lowers Africa's productivity by more than US$ 12 billion every year (WHO, 2014).

In Kenya, nearly 28 million people live in malarious areas with young children and expectant women being the most affected groups (DMOC, 2010). It is estimated that there are about 9 million malaria cases annually in Kenya, with 1 in every 20 deaths worldwide from malaria occurring in Kenya (KNHMIS, 2012). Malaria control approaches targeting malaria parasite are limited by chemoresistance and developed vaccine have not been rolled out (White, 2008; WHO, 2016). Vector control is the most effective measure to prevent malaria parasite transmission, however it is threatened by changes in vector behaviour, together with increasing cases of resistance to insecticides within mosquito populations (Lefevre *et al.,* 2009; Ranson *et al.,* 2011; Briët & Chitnis, 2013; Wanjala *et al.,* 2015). Therefore, there is an

urgent need for improved and/or development of novel control strategies as well as insights into malaria transmission dynamics to enable knowledge-based deployment, and optimal efficiency of current vector control strategies.

2.3 Malaria control

2.3.1 Chemotherapy

Chemotherapy is one of the main control strategies for malaria, with the drugs applied being from compounds such as quinolines, antifolates and artemisinin (Saifi *et al.,* 2013). Chloroquine accumulates in malaria parasite food vacuole interfering with the process of DNA and RNA biosynthesis and subsequent protein synthesis (Saifi *et al.,* 2015). Chloroquine can be used as chemoprophylactic as well as a chemotherapeutic. Quinine (a derivative cinchona tree) inhibits heme polymerization into a less toxic malaria pigment known as hemozoin (Slater & Cerami, 1992), and reduce catalase activity on hemin hence leading to increased reactive oxygen species (Saifi *et al.,* 2013). It is used for the treatment of uncomplicated and severe malaria.

The introduction of artemisinin-based combinatory therapies (ACTs) and their deployment in affected regions have substantially contributed to malaria control. ACTs inhibits mitochondria respiratory chain of the parasite and cysteine proteases activity (Krungkrai *et al.,* 1999), resulting in decreased rate of haemoglobin degradation, reduced levels of amino acids for protein synthesis, and poor cell structure integrity, therefore, useful in malaria parasite control (Wu, 2002). There are fears of malaria parasite resistance to ACTs and a more effective potential drug compound, (+) -SJ733, a dihydroisoquinolones, is still in clinical trial (Jiménez-díaz *et al.,* 2014). (+) -SJ733 acts on *P. falciparum* ATPase 4 (PfATP4) (a cation transporter responsible for regulating parasite' intracellular Na^{+}) inducing death of parasite-infected erythrocytes (Spillman & Kirk, 2015).

2.3.2 Malaria mosquito vector control

The primary vector control strategies include IRS and LLINs (Wallace *et al.,* 2014). IRS control strategy is characterized with spraying an active compound to which mosquitoes are fully vulnerable inside households' walls and other mosquitoes resting places. There are many IRS designed insecticides, but the most common are dichlorodiphenyltrichloroethane (DDT), bendiocarb (a carbamate) and lambda-cyhalothrin (a pyrethroid) (Sibanda *et al*., 2011). IRS has a strong durable protective effect on compounds without IRS located 300 meters. This phenomenon is described as community effect (Sharp *et al.,* 2007) and has

contributed massively to malaria reductions, with the greatest sustained success in Africa being in South Africa (Mabaso *et al.,* 2004). However, the method is increasingly limited by increased insect resistance to available insecticides like pyrethroids (Ranson *et al.,* 2011; Maliti *et al.,* 2014; Wanjala *et al.,* 2015).

Even though untreated mosquito bed net provides a complete physical barrier against mosquitoes, LLINs involve providing a physical barrier and simultaneously killing in case of contact and/or repelling mosquitoes hence preventing blood-feeding and blocking transmission (Fegan *et al.,* 2007). This vector control method has proved practical, highly effective and cost-effective (Phillips-Howard *et al.,* 2003). LLINs has been extensively used to reduce mosquito density and biting activity causing a decline in malaria transmission, with its use being linked to reducing malaria-related mortality by 44% in rural Kenya (Phillips-Howard *et al.,* 2003; Fegan *et al.,* 2007; Lim *et al.,* 2011). However, changes in malaria mosquito feeding patterns, especially upon humans during active hours, when humans are unprotected by LLINs (Russell *et al.,* 2011; Briët & Chitnis, 2013), opportunistic feeding upon non-human hosts (Lefevre *et al.,* 2009), together with increasing cases of resistance to insecticides within mosquito populations (Wanjala *et al.,* 2015; Maliti *et al.,* 2014; Ranson *et al.,* 2011) threaten efficacy of LLINs. Therefore, in addition to improving and/or developing novel vector control strategies, it is necessary to understand vector behaviour changes due to applied strategies, vector, and host interactions, and vector *Plasmodium* infections for a more focused and knowledge-based malaria control intervention.

2.3.3 Alternative malaria control strategies

Various alternative malaria control strategies are in consideration and include the use of genetically modified mosquitoes (GMM), biological methods and vaccines. GMM control strategy involves introduction of GMM into natural populations of mosquitoes (Alphey *et al.,* 2002). Such genetic modification include the development of sterile male mosquitoes by silencing of a germ cell differentiation gene through RNA interference. Sperm-less males induce transcriptional changes in female reproductive genes comparable to those elicited by fertile males thereby reducing the size of field population (Thailayil *et al.,* 2011). The genetic modification can either be self-limiting or self-propagating. In the later, GMM exhibit a gene drive system that enables transgenes to spread rapidly through wild populations and to be continued regularly for generations (Mumford, 2012). However, the use of GMM has been very contentious and remains controversial (Mumford, 2012).

There is also renewed interest in targeting larval stages of malaria mosquitoes by biological methods such as bacteria *Bacillus sphaericus (Bs), Bacillus thuringiensis var. israelensis (Bti)*, entopathogenic fungus, and vector predators like larvivorous fish (Becker *et al.,* 2010; Mwangangi *et al.,* 2011). *Bti* (dead bacterial spores) and *Bs* (live bacterial spores) methods involve the application of *Bti* or *Bs* larvicide on larval breeding sites, where toxins on bacteria spore coat act as stomach poison in the larval and pupal midgut. Overall, *Bti* is the most studied, most successful, and best environmentally friendly (Shililu *et al.,* 2003; Kahindi *et al.,* 2008). Unfortunately, it is costly especially to poor communities where malaria is endemic and more useful in areas where malaria transmission is low, and where mosquito breeding sites are contained and well defined (Worrall & Fillinger, 2011).

There has been a substantial effort in the development of an efficacious, safe and cost-effective malaria vaccine despite several challenges. First, it is difficult to safely grow and manufacture whole parasites in sufficient numbers to induce immunity (Hoffman *et al.,* 2010). Second, parasite antigens exhibit temporal switching of variant genes, for instance, a key blood-stage antigen, *Plasmodium falciparum* erythrocyte membrane protein1 (*PfEMP1)* (Takala & Plowe, 2009). Finally, *P. falciparum* does not infect small animals or old macaques, making direct vaccine evaluation difficult (Hill, 2011). However, a formulation of *P. falciparum* circumsporozoite protein, called RTS,S a subunit vaccine, which targets circumsporozoite protein of *P. falciparum* and is boosted with the potent ASO1 adjuvant has been successfully developed (Ballou, 2009). The vaccine has not been deployed (Moorthy *et al.,* 2004; Neafsey *et al.,* 2015; RTSS, 2015), mostly because of safety concerns and modest protection (Callaway & Maxmen, 2015).

The efforts in development of new alternative malaria control approaches are laudable, but their deployment is still not possible making improvement and proper application of control approaches in current use central to management of the disease. For the methods to be more effective, knowledge of disease transmission dynamics, vector behaviour, and vector – host interactions should guide their deployment.

2.4 Human *Plasmodium*

Five species of *Plasmodium* commonly infect humans and they include *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi* (Coluzzi, 1999). Each is distinct and produces different patterns of symptoms, but the life cycles are very similar. However, two or more species can infect the same person at the same time. *P. falciparum* is the most widely spread, and responsible for huge health and economic loss globally (Coluzzi, 1999; White, 2008). *P. vivax* produces the most widely spread form of malaria. *P. ovale* is a relatively uncommon form of malaria that causes relapses (hypnozoites) and generally occurs in West Africa (Cogswell, 1992). Other human plasmodium parasites are *P. malariae* and *P. knowlesi.* Unusually, *P. knowlesi* is zoonotic and can infect monkeys (Fong *et al.,* 1971; Singh *et al.,* 2004) and also accounts for about 70% of malaria incidences in certain areas in south-east Asia where it is common (McCutchan *et al.,* 2008).

2.4.1 *Plasmodium falciparum* life cycle

Plasmodium falciparum has a complex life cycle that requires both an asexual reproduction in human and a sexual reproduction in insect hosts (Figure 2). An infected female *Anopheles* mosquito transmits malaria parasite during blood-meal. The life cycle has three stages, which include pre-erythrocytic, erythrocytic and sporogonic stages. The feeding mosquitoes deposit saliva containing the infective stage of *P. falciparum* called sporozoites onto the host's bloodstream (Frischknecht *et al.,* 2004). The sporozoite invades liver cells of the vertebrate host, where each sporozoite develops into a schizont, a structure containing about 30,000 merozoites (Kyes *et al.,* 2001). This is the pre-erythrocytic stage. The schizont matures, ruptures and spills merozoites into the bloodstream, which invades erythrocytes marking the beginning of the erythrocytic stage.

The merozoites continue in a repeated cycle of invading erythrocytes, multiplying by consuming haemoglobin and bursting the erythrocytes until it is brought under control, either by antimalarial drugs and/or by the body's immune system defences. During this repeated cycle, some merozoites do not develop asexually into schizonts but instead change into male and female sexual forms known as gametocytes (Kyes *et al.,* 2001). The gametocytes circulate in the vertebrate host bloodstream and during the blood-meal on infected vertebral host, female *Anopheles* mosquito ingests gametocytes along with blood. Once in the mosquito's stomach, gametocytes fertilize (first stage of the sporogonic stage), producing zygotes. The zygotes develop into motile, elongated ookinetes, which penetrate the mosquito's mid-gut wall and mature into oocysts filled with infectious sporozoites (Kyes *et*

al., 2001). When the oocyst matures, it ruptures and the sporozoites migrate to the mosquito's salivary glands, where they will be injected in to mammalian host during blood meal and the cycle starts over again.

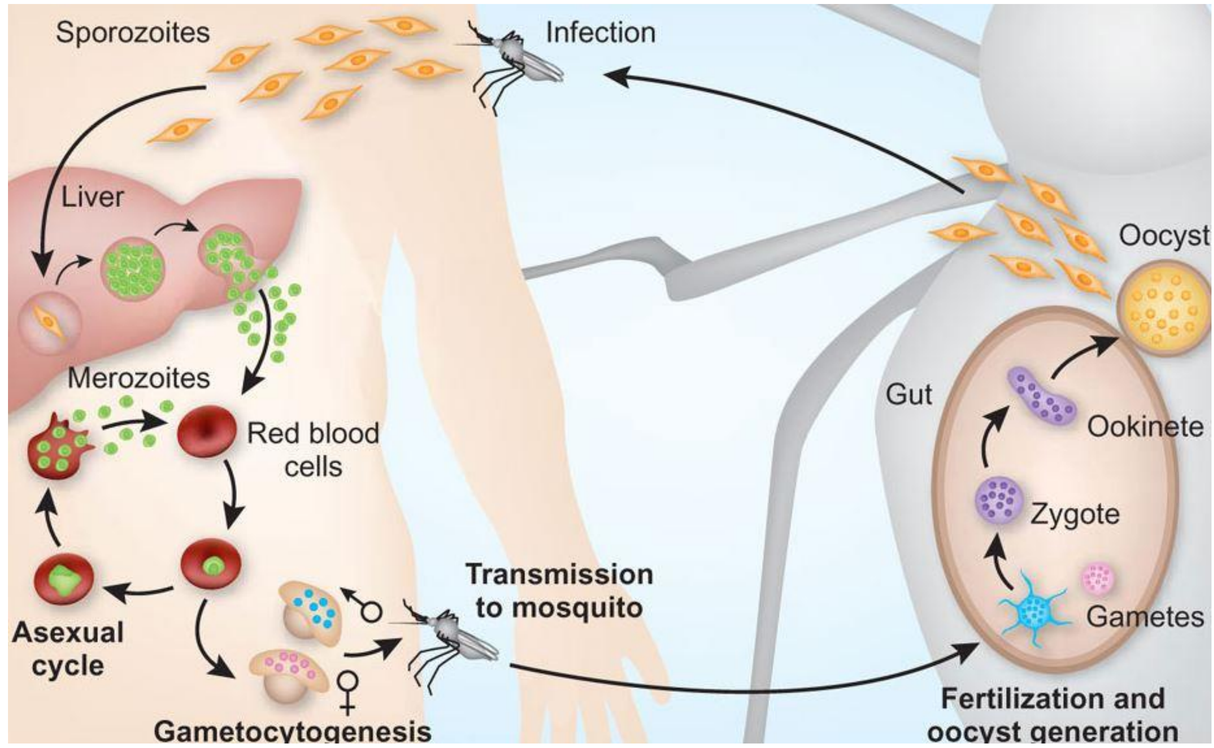

Figure 2: Life cycle of *Plasmodium falciparum*. The sporozoites released with saliva from the salivary glands of infected female *Anopheles* mosquito develop in the hepatocytes and discharge merozoites into the bloodstream. Merozoites continue in a repeated cycle of invading erythrocytes, multiplying and bursting the erythrocytes. During this repeated cycle, some merozoites change into male and female gametocytes. During blood meal on infected vertebral host, mosquito ingests gametocytes along with blood. In the mosquito's mid gut, *P. falciparum* gametocytes emerge and fertilize, producing zygotes. The zygotes develop into ookinetes, which penetrate the mosquito's mid-gut wall and mature into oocysts filled with infectious sporozoites. Sporozoites migrate to the mosquito's salivary glands, where they will be injected into mammalian host during blood meal and the cycle starts over again. Adopted from Pasvol 2010.

2.4.2 *Plasmodium falciparum* pathophysiology

The erythrocytic stage of *P. falciparum* development in the human host is associated with illness. In this stage, the schizont matures, ruptures and spills merozoites into the bloodstream, which invade erythrocytes. The rupture of infected erythrocytes leads to release of endotoxins which trigger the release of tumour necrosis factor (TNF) and Interleukine-1 (Il-1) into blood stream (Deshmukh & Trivedi, 2014). Il-1 and TNF have been associated with fever, severe anaemia and acidosis (Clark *et al.,* 2004; Orengo *et al.,* 2008). Additionally, infected erythrocytes becomes more spherical, rigid and less flexible, resulting to partial obstruction of capillaries and venules (Dondorp *et al.*, 2002). Further, the host responds to malaria by enhancing splenic immune function and filtrative clearance, thus accelerating removal of both infected and uninfected RBCs, causing malarial anaemia (Buffet *et al.,* 2013).

Malaria parasites express a protein called *Plasmodium falciparum* erythrocyte membrane protein 1 (*PfEMP1*) on the RBCs cell surface (Chen *et al*., 2000). *PfEMP1* bind on the surface of vascular endothelial cells, thereby sequestering the infected cells within various tissues (Chen *et al*., 2000). This property result in microcirculatory obstruction, metabolic disturbances, and organ-specific damage. Paradoxical situation exists where *P. falciparum* adhere to chondroitin-sulphate A (CSA), thrombomodulin, and hyaluronic acid (HA) in the placenta during pregnancy in women causing placental malaria (Andrews & Lanzer, 2002; Miller *et al.,* 2002). Similarly, *P. falciparum* can adhere to intracellular adhesion molecule - 1 (*ICAM-1*) in the brain causing cerebral malaria (Idro *et al.,* 2005).

2.5 Malaria mosquito vectors

There are about 3,300 species of mosquitoes belonging to 41 genera (Diptera: *Culicidae*) (Service, 2004). The most important vector species belongs to genera *Aedes, Culex,* and *Anopheles.* However, *Anopheles* mosquitoes spread the deadly *P. falciparum* parasite and pose great challenges to human health. The insect vector ensures the parasites complete an essential lifecycle through the fly and mammalian host. Vector control and subsequent blocking of parasite life cycle is a suitable strategy for malaria control.

2.5.1 Life cycle of female *Anopheles* mosquito

Female *Anopheles* mosquitoes undergo a holometabolous life cycle that includes four distinct aquatic stages, egg, larva and pupa, and a final terrestrial stage, the adult (Becker *et al.,* 2010) (Figure 3 below). The slender, oval eggs, which are usually dark and visible to the naked eye, are laid singly in almost any type of water, but preferably vegetated, slow-moving, or stagnant water. The eggs incubate for 2 – 6 days in tropics and up to 3 weeks in cold climates before hatching into larvae or "wrigglers" (Foster & Walker, 2009). The larvae have a large head and thorax and molts four times in 4 – 10 days, with the last molting resulting into a pupa or the "tumbler." The pupa has a large combined head and thorax, and slender abdomen, giving it a comma shape. It also swims actively, does not feed and lays still for about 2 days in a cocoon as it develops into an adult. The adults are two winged, with long slender legs and have a flight range of about 2km (Service, 1997).

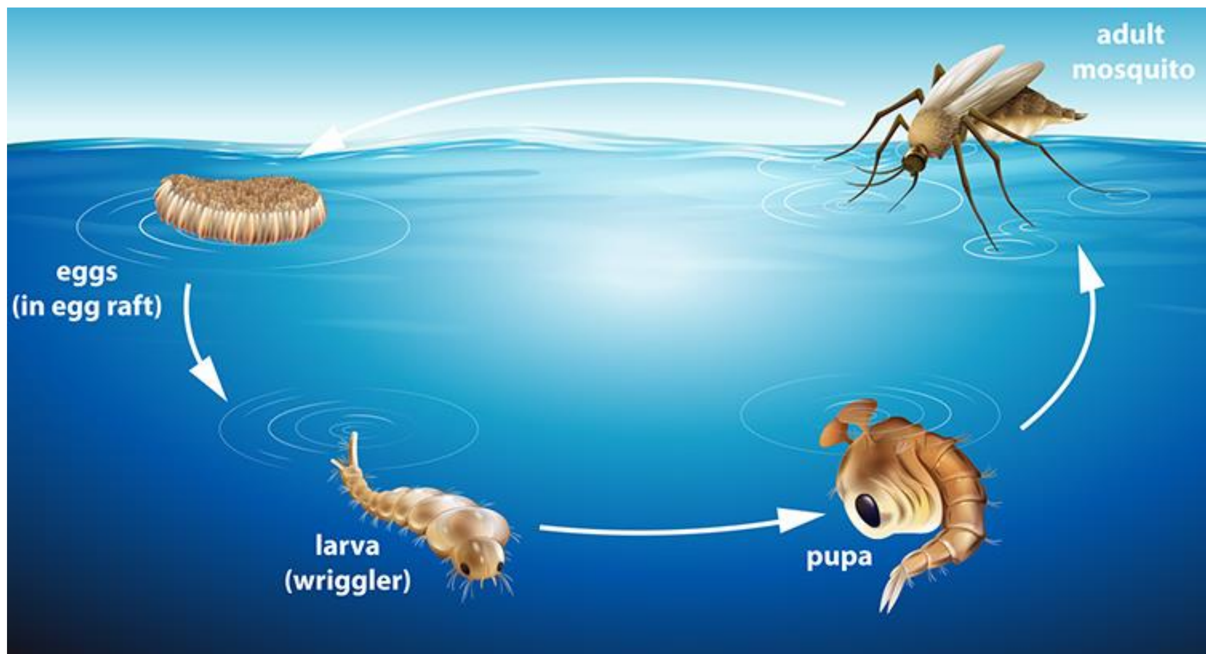

Figure 3: Life cycle of female *Anopheles* mosquito. The life cycle includes four distinct stages egg, larva, pupa, and adult. The eggs, laid singly in slow-moving or stagnant water, hatch into larvae. The legless, "hairy" larvae, with slender abdomen molts four times and the last molting results into a pupa. The coma shaped pupa develops into an adult. Adults are two winged, with long slender legs. This image was adopted from "The science behind the news – why files" website – http://www.whyfiles.org/2014/mosquitoes (accessed December 19, 2015).

Soon after the adult emerges from the pupa, males compete aggressively for large females and female usually mates only once in their lifetime (1 - 2 weeks) (Okanda *et al.,* 2002). Blood- meals are generally taken every 2–3 days. The vertebrate host blood-meal provides nutrients necessary to support biosynthetic burst associated with vitellogenesis (Rono *et al.,* 2010), but sometimes young virgin females also take blood-meal. Breeding sites such as small rock pools, fresh water, running water with shade, are key determinant of adult mosquito distribution, abundance and fitness (Gimnig *et al.,* 2001; Eckhoff, 2011), and mosquito reproduction is only successful if breeding sites remain safe for the growth of immature stages (Barros & Hono, 2011).

2.6 Malaria dynamics

2.6.1 Malaria mosquito vectors

Africa has over 140 *Anopheles* species. Most of the vector species occur in sympatry and their significance in malaria transmission varies depending on their feeding activity, resting preferences, prevalence and vectorial capacity (Fontenille & Simard, 2004). Consequently, malaria epidemiological patterns differ within locations and different areas require different tools and strategies for optimal vector control. Studies show that primary vectors such as *A. gambiae, A. arabiensis* and *A. funestus,* and complementally vectors including *A. pharoensis, A. coustani* and *A. rivurolum* have been reported in Africa (Bekele *et al.,* 2012). Nevertheless, the most efficient vectors for malaria parasite transmission is *A. gambiae* sensu lato (Levine *et al.,* 2004). The high efficiency of malaria transmission by *A. gambiae* can be attributed to its ability to support growth of *P. falciparum*, its tendency to exploit man made environments (endophilly and endophagy) and its preference for humans as source of blood-meal (anthropophilia) (Alavi *et al.,* 2003).

In Kenya *A. gambiae* complex and *A. funestus* are regarded as highly anthropophagic (Githeko *et al.,* 1994). Other complementary malaria vectors including *A. pharoensis* and *A. nili* have also been described in coastal and central Kenya (Okara *et al.,* 2010). Malaria control measures such as LLINs have aimed at selectively reducing *A. gambiae* and *A. funestus*. Consequently, complementary vectors such as *A. coustani* which had been previously considered unimportant and there is little knowledge about their ecology, behaviour, and control are increasingly being implicated in malaria transmission (Meyrowitsch *et al*., 2011; Mwangangi *et al*., 2013).

2.6.2 Environmental factors

Malaria mosquitoes' survival depends largely on favourable environmental factors such as precipitation and temperature, and other aspects including terrain and vegetation cover. High rainfall produce numerous water pools, and swamps that form suitable mosquitoes' habitat (Nkuo-Akenji *et al.,* 2008). High rainfall is also associated with shallow pools of water in cultivated farm that provide good mosquito breeding spots, and existence of fresh green vegetation that serve as mosquitoes' resting sites hence high malaria transmission (Imbahale *et al*., 2011).

Higher temperatures favour the survival and biting rate of malaria vectors, and accelerate the growth of malaria parasite in the mosquito host (Tanser *et al.,* 2005; Reiter, 2008). Nevertheless, temperatures of over 30 °C shorten the lifespan of malaria vector. Therefore, it is speculated that in future global warming will cause to 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 speed and direction determines the spread and direction of carbon dioxide and human odour, thereby influencing mosquito host-seeking behaviour and malaria transmission (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.,* 1998).

2.6.3 Choice of blood-meal sources

The female *Anopheles* mosquito exhibits a diverse choice of blood-meal sources including humans, livestock, birds, and reptiles (Nanda *et al.,* 1996; Muriu *et al.,* 2008), with the prevalence of malaria influenced by mosquito host selection. Host preference is determined by external and inherent factors. While inherent factors are genetic (Dekker *et al.,* 2001), they also appear to be controlled by adaptive advantages that result from feeding on certain host species. However, malaria mosquito feeding habit also depends on density of host species (Lardeux *et al.,* 2007), which by their abundance form a readily accessible blood meal source. Interestingly, while some studies show that females *A. gambiae* infected with sporozoites of *P. falciparum* are more attracted to human odours than uninfected mosquitoes (Smallegange *et al.,* 2013), others showed that female mosquitoes infected with oocyst-stage malaria

parasites were less persistent at blood-feeding and less likely to resume feeding if interrupted (Koella, 2002; Cator *et al.,* 2012). Nevertheless, knowledge of vector blood-meal preference alone is insufficient to achieve an effective vector control approach.

2.6.4 Vector population genetics

Genetic structure of mosquito vectors show spread of genes such as insecticide resistance or refractory genes, hence indicate heterogeneities in malaria transmission (Lehmann *et al*., 2003). Further, studies show that genetic structure of mosquito species determine traits such as vector competence, which has an important impact on malaria transmission (Harris *et al*., 2010) and can be used to infer the likely success of vector control strategies. Arguably, the population genetics of primary malaria mosquito vectors have lower indices of differentiation than secondary malaria mosquito vectors because of their large numbers and higher mobility (Donnelly *et al*., 2002). Various studies have shown high degree of genetic differentiation of *A. arabiensis* populations from Réunion and Mauritius islands (Simard *et al*., 1999) and population substructure of *A. gambiae* on the island of São Tomé, West Africa (Pinto *et al*., 2003). Differences in population structure within species influence biting and resting activities, and preference for humans as source of blood-meal (Lounibos & Conn, 2000) 2000), behaviours that are key determinant of human vector contact and disease transmission. However, little information is available on population genetics structure of malaria mosquito vector in areas with high prevalence of malaria necessitating the investigation in Mageta, Magare and Ngodhe islands of Lake Victoria.

2.7 Malaria in Kenya

Studies show that malaria transmission in Kenya is high in rural areas and in areas near large water bodies such as like lakes and rivers (Keiser *et al.,* 2005) with western Kenya and areas along the coast comprising endemic regions (DMOC, 2010) (Figure 4 below). However, currently, malaria transmission is being reported in Kenyan urban areas and highlands (Hay *et al.,* 2005).

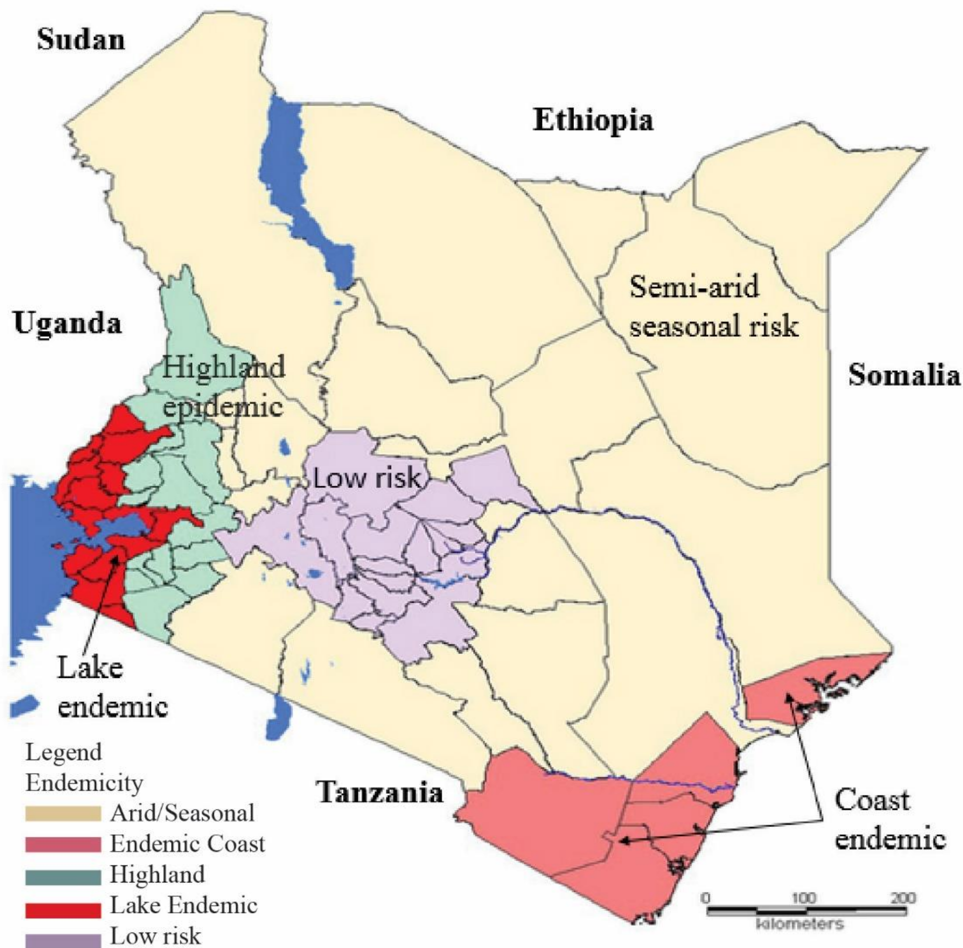

Figure 4: A map showing malaria endemic zones in Kenya. The red area around Lake Victoria (blue region extending into Uganda and Tanzania) and the pink area in the coastal region show areas of stable malaria transmission throughout the year. The light green area in the Western region highlands shows areas with epidemic transmissions with considerable yearly variation. The light brown arid and semi-arid areas of northern and southeastern parts of the country represent areas with short periods of intense malaria transmission during rainy seasons. Lastly, the light purple area that covers central highlands of Kenya including Nairobi has low malaria incidences. Adopted from DMOC 2010.

2.7.1 Malaria in Kenyan urban areas

Malaria mosquitoes breeding sites reduce with nearness to highly populated areas like formally developed towns, hence the disease is largely common in rural settings with suitable breeding places (Robert *et al.,* 2003; Omumbo *et al.,* 2005). Though the rate of malaria transmission in Kenyan urban areas is low, it depends on the degree of urbanization (Robert *et al.,* 2003). However, low malaria transmission is more pronounced in urban areas where mean rainfall is low and seasonal, and variation in the level of malaria transmission exists within different areas in the same urban setting (Siri *et al.,* 2008).

Newly emerged high population settlements in the periphery of towns are essentially rural in nature but has a low number of livestock that make zoophilic mosquito species feed on humans. In addition small cultivated farms and excavations resulting from building activities provide vector breeding sites hence stable malaria transmission (Donnelly *et al*., 2005; Warren *et al.,* 1991). The urban poor living mostly in slums areas with poor housing and water disposal practises and lack better vector control approaches such as IRS and LLINs have similar exposure to infective bites compared with those living in rural settings (Keiser *et al.,* 2004). High-income earners living in suburban neighbourhoods and leafy suburbs experience relatively low malaria transmission due to their improved housing and better access to malaria vector control approaches such as IRS and LLINs (Wang *et al.,* 2005).

Generally, malaria mosquitoes breeding sites in urban areas are highly localized and mosquito dispersal is restricted by readily available blood meals. Each person is bitten less leading to irregular and heterogeneous transmission (Hay *et al.,* 2005). Consequently, low exposure to malaria parasite causes low immunity in urban human populations, and high malaria cases and deaths especially in older populations (Doolan *et al.,* 2009).

2.7.2 Malaria in the Kenyan coast

The coastal Kenya provides a favourable environment that maintains a stable malaria transmission throughout the year but differs with endemic lake region because its transmission is low, shows great seasonality and heterogeneity (DMOC, 2010). However, even very low transmission intensity causes high rates of severe malaria (Mbogo *et al.,* 2003). The lower transmission and incidences of severe malaria in coastal Kenya compared to western Kenya can be attributed to reduced number of primary malaria mosquito vectors, and preference of livestock as blood meal source (Mwangangi *et al.,* 2013). Coastal Kenya is characterized with a variety of malaria vector species. The main members of the most efficient malaria vector in this region are *A. gambiae* complex and *A. funestus* (Mbogo *et al.,* 1993). However, in many areas *A. arabiensis,* a known outdoor feeder has substituted *A. gambiae* s.s. as the primary vector (Mwangangi *et al.,* 2013). In Taveta District, *A. coustani* was found to play a major role in outdoor malaria transmission (Mwangangi *et al.,* 2013). The mosquito species are found mostly in older pits with emergent vegetation.

2.7.3 Malaria in Western Kenya Highlands

Western Kenya highlands are transition zones and characterized by low and irregular malaria transmission (Omukunda *et al*., 2013). Additionally, malaria epidemics are localised, with heterogeneous transmission and *P. falciparum* associated with most outbreaks (Hay *et al.,* 2002). *A. gambiae* s.s.*,* a major vector in the region, emerges from modified habitats such as land under agricultural use (Munga *et al.,* 2006). However, *A. funestus* is also present but is regarded as a secondary vector. Emergence of *A. gambiae* s.s. is thought to be due to the higher temperatures in the microclimate of the vector compared to the highland forest (Minakawa *et al.*, 2006). The high temperatures also reduce *Plasmodium* sporogony and gonotrophic cycle of the vector, hence increased malaria transmission and high morbidity and mortality rates (low immunity in the highland human populations) (John *et al.,* 2002). Highlands were earlier regarded as areas with little or no malaria transmission. However, this seems to be changing because of change in climate, ecology, and human activities.

2.7.4 Malaria in Areas near Lake Victoria, Western Kenya

Regardless of the season, the lake environment provides a suitable environment that maintains high malaria vector abundance and diversity, with various health facilities reporting more than 40% of residents harbouring *P. falciparum* infection (Noor *et al.,* 2009). The main members of the most efficient malaria vector are *A. gambiae s.s.,* and *A. arabiensis* (McCann *et al*., 2014). Additionally, *A. coustani* and *A. funestus* are also present (Minakawa *et al.,* 2012). *A. gambiae* s.s. breeds in pools of water along lakeshores especially when there are fluctuations in water level (Minakawa *et al.,* 2008). However, studies show that *A. gambiae* does not breed within the lake water itself (Ndenga *et al.,* 2011). *A. funestus* inhabits diverse ecologic niches including large, stable water pools covered with emergent vegetation, for instance, lake edges (Mbogo *et al.,* 2003). Such habitats exist throughout the year due to constant supply of seepage water from the lake. Studies suggest that *An. funestus* breeding sites in Lake Victoria has increased because of water hyacinths (Ofulla *et al.,* 2010), particularly because the species is closely associated with aquatic vegetation. *A. funestus* are less reliant on rain seasons and multiply even during dry seasons, bridging malaria transmission during dry season when *An. gambiae* densities are low (Mbogo *et al.,* 2003). *A. funestus* is highly anthropophilic, with most biting activity between 0000h, and early in the morning (Githeko *et al.,* 1996).

A. gambiae s.s. is the most efficient vector because of its ability to support growth of *P. falciparum*, its tendency to exploit man made environments and its preference for humans as source of blood meal (Alavi *et al.,* 2003). Extensive use of LLINs has reduced vector density and biting activity and decreased malaria transmission. However, effectiveness of LLINs is threatened by changes in malaria mosquito feeding patterns, especially upon humans during active hours, when humans are unprotected by LLINs (Russell *et al.,* 2011; Briët & Chitnis, 2013), and opportunistic feeding upon non-human hosts (Lefevre *et al.,* 2009). Therefore, it is necessary to generate knowledge on malaria vectors and their behaviour in order to offer insight into malaria transmission dynamics and provide strategies for optimal vector control that can be applied in application of appropriate vector control approaches.

2.8 Summary

Malaria is caused by *Plasmodium* parasite which is transmitted by female *Anopheles* mosquito and cause major public health concerns and hindrances to socioeconomic development. In Kenya, high malaria transmission occurs in the regions near Lake Victoria. The area around the lake provides a favorable environment that maintains high malaria vector abundance and diversity throughout the year, with various health facilities reporting more than 40% of residents harboring *P. falciparum* infections (Noor *et al.,* 2009). Malaria control is primarily by chemotherapy and vector control. Drugs applied are from compounds such as quinolines, antifolates and artemisinin (Saifi *et al*., 2013). However, reliance on a small group of antimalarial compounds has resulted in parasite developing resistance (White, 2008) and there are fears of malaria parasite resistance to the new drug compound ACTs. Further, a more effective drug compound, (+) -SJ733 is still a clinical candidate (Jiménez-díaz *et al.,* 2014). Therefore, main existing front-line malaria vector control includes IRS and LLINs. However, in addition to insecticides resistance, these methods are limited by behaviour change of malaria mosquito vectors within locations. For example, the vector feeding upon humans during active hours, when human are unprotected by LLINs. Additionally, the vectors have become opportunistic by feeding upon abundant alternative hosts over inherently preferred ones. Therefore, there is a need to improve and/or develop new vector control strategies. For current vector control methods, understanding malaria vectors and vector–host interaction, and hence malaria transmission dynamics is important in appropriate deployment and adoption of the most effective vector control strategies. To gather insight into these, studies in geographically isolated areas such as islands with limited interference is important and was undertaken. Here, malaria mosquito species in Mageta, Magare and Ngodhe islands of Lake Victoria were identified and sources of their blood-meal determined. This included determination genetic variations of primary malaria mosquitoes. In addition, demographics and LLINs usage were also surveyed.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study location

The study was conducted in Mageta, Magare and Ngodhe Islands of Lake Victoria in Western Kenya (Figure 5). Mageta and Magare islands are in Siaya County, while Ngodhe Island is in Homa Bay County. The distance between Mageta and Magare is about 0.4 km, and Ngodhe is 28.5 km from Mageta.

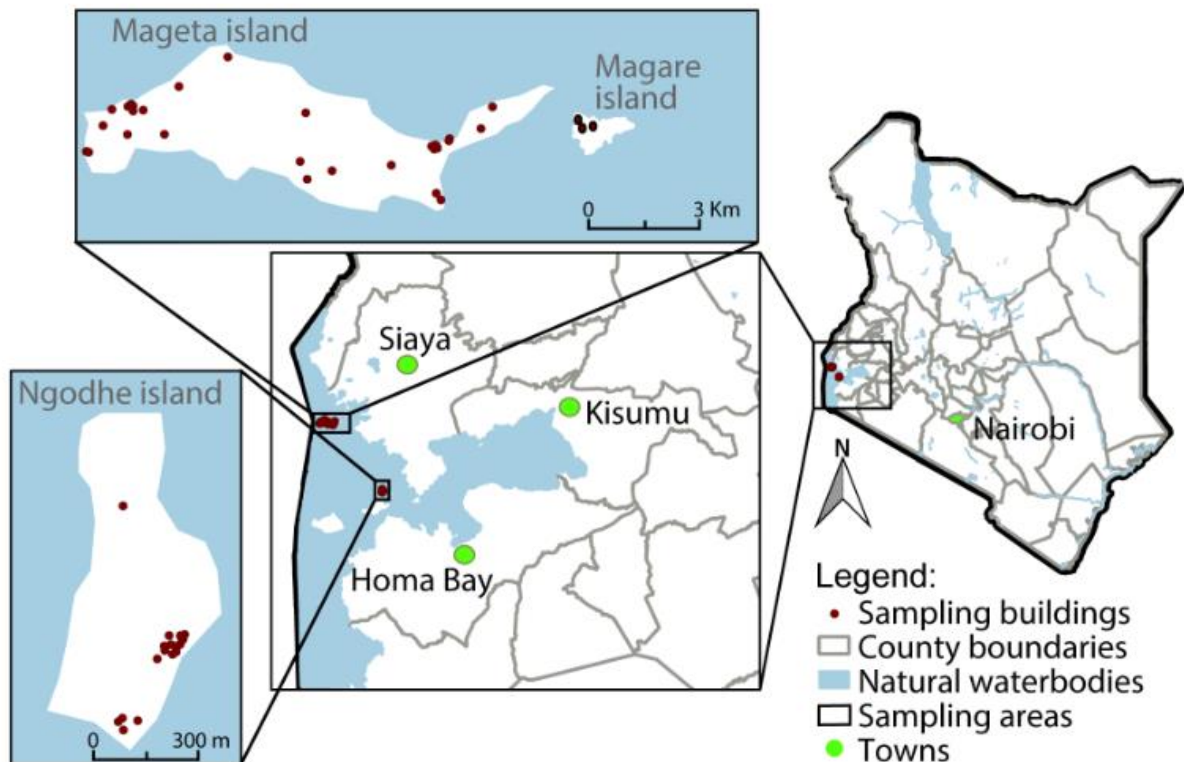

Figure 5: Map showing study location. The three study islands are located in Lake Victoria, western Kenya. Mageta and Magare are located in Siaya County, while Ngodhe is in Homa Bay County. Areas of Mageta, Magare, and Ngodhe are 7.02 km^2, 0.2 $km^{2,}$ and 0.9 km^2 respectively. Mageta and Magare are approximately 0.4 km apart, while the distance between Mageta and Ngodhe is about 28.5 km.

3.2 Ethical approval

Approval for this study was obtained from Kenya Medical Research Institute (KEMRI) ethics review committee (SSC no 388). Informed consent was obtained from village elders on the study activities and from household heads before inclusion of their households in the study.

3.3 Sample Collection

3.3.1 Adult mosquito trapping

Both indoor and outdoor adult mosquitoes were trapped at geo-referenced houses for six consecutive nights each month between November 2012 to September 2014 (23 months) in Ngodhe, and June 2013 to March 2015 (22 months) in Magare and Mageta. The trapping nights coincided with the period before full moon (see Provost, 1959; Bowden & Morris, 1975). CDC light traps, pyrethrum spray catches (PSC) and back-pack aspiration (ASP) trapping methods were used. Through randomization, alternate collection methods were used for every geo-referenced building in a sampling month.

3.3.2 Control samples

Colony-reared sugar-fed *A. gambiae* s.s., and *A. arabiensis* established in 1998 at the International Centre of Insect Physiology and Ecology (*icipe*) Kenya served as standard reference positive controls for *A. gambiae* s.l. sibling species identification and negative controls for blood-meal analysis. Known vertebrate whole blood from cow (*Bos tarsus*), pig (*Sus scrofa*), goat (*Capra hircus*), chicken (*Gallus gallus*), dog (*Canis familiaris*) and human (*Homo sapiens*) clinical samples from Suba district hospital (Kenya) (Omondi *et al*., 2015), as well as Swiss mouse (*Mus musculus*) and rabbit (*Oryctolagus cuniculus*) blood samples sourced from *icipe*'s animal rearing unit served as standard reference positive controls for blood-meal analysis. Non-human vertebrates' whole blood was collected by venal puncture using sterile equipment, with respect for the welfare of the animal. *P. falciparum* DNA acquired from the National Institute for Biological Standards and Control (NIBSC; Hertfordshire, UK) was used as a standard positive reference for malaria parasite detection.

3.4 Identification of malaria mosquito, blood meal source and *Plasmodium*.

3.4.1 Morphological identification

After collection, mosquitoes were anesthetized with chloroform (O'Guinn & Turell, 2002), morphologically identified and sexed (Gillies & Coetzee, 1987). All the female *Anopheline* mosquitoes belonging to *A. gambiae* s.l., *A. funestus,* and *A. coustani* were counted and classified on the basis of their abdominal status as blood-fed (engorged), gravid, half-gravid or unfed (not engorged) (WHO, 1975). Both male and females malaria mosquitoes were preserved individually in bar-code labeled vials containing isopropanol and stored at room temperature in the field and at -20 °C in the laboratory awaiting further analysis. The labelling indicated details of collection method, house identification (ID) number, site ID number, morphological ID number, sex, and collection date.

3.4.2 Dissection

The engorged abdomens of field-collected and laboratory reared adult malaria mosquitoes were separated from the rest of the body (head, thorax, and legs) using sterile forceps and dissection pins, and transferred into individual sterile, 1.5 mL microtube.

3.4.3 Nucleic acid extraction

Genomic DNA was extracted from the three sample parts, engorged abdomens, remaining body parts (head, thorax and legs), and known vertebrate whole blood samples using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's extraction protocol with some modifications. Briefly, 200 µL of PBS (phosphate-buffered saline, pH 7.4) was added to the sample in a 1.5 mL microcentrifuge tube containing 20 µL proteinase K and the sample mixed by vortexing for 15 seconds. Then, 200 µL lysis buffer was added and the mixture incubated at 56 °C for 2 hours. Subsequently, 200 µL of absolute ethanol was added. The solution mixed by vortexing and homogenate transferred into a mini spin column placed in a 2 mL collection tube before centrifugation for 1 minute at 6 000 x g. The flow-through and collection tubes were discarded and the spin column was transferred to a new 2 ml collection tube, 500 µL of buffer added and centrifuged for 3 minutes at 20,000 x g. The flow-through and the collection tubes were discarded and the spin column was transferred to a new 1.5 mL micro-centrifuge tube. The DNA was finally eluted by adding 30 µL AE buffer to the center of the spin column membrane, incubated for 3 minutes at room temperature (25 °C) and centrifuged for 1 minute at 6,000 x g, and stored at -20 °C.

3.5 Polymerase Chain Reaction

3.5.1 Species identification of engorged malaria mosquitoes

Molecular identification of malaria mosquitoes involved polymerase chain reaction (PCR) amplification and sequencing of the cytochrome oxidase subunit 1 (CO1) region (Folmer *et al.*, 1994), polymorphic internal transcribed spacer 2 (ITS2) region of ribosomal DNA (Koekemoer *et al.*, 2002; Cohuet *et al.*, 2003) and analysing melt curves differences of 165 base pairs (bp) intergenic spacer region (IGS) gene amplicons obtained using *IGS* gene primers (Zianni *et al.*, 2013). Target, primer names and sequences are provided in Table 1.

Ten microliter PCR reactions were prepared with 0.5 µL final concentrations for each primer, 2 µL of 1X Hot Firepol Evagreen HRM Mix (Solis BioDyne, Tartu, Estonia), 1 µL of DNA template, and 6 µL of PCR water. Thermal cycling conditions for *CO1* and *ITS2* were as follows: initial denaturation at 95 °C for 15 minutes, followed by 40 cycles of denaturation at

95 °C for 30 seconds, annealing at 50 °C for 30 seconds and extension at 72 °C for 1 minute 30 seconds, and a final extension at 72 °C for 7 minutes. Whereas thermal cycling conditions used for *IGS* were as follows: initial denaturation at 95 °C for 15 minutes, followed by 40 cycles of denaturation at 95 °C for 30 seconds, annealing at 57 °C for 30 seconds, and extension at 72 °C for 45 seconds and a final extension at 72 °C for 7 minutes. PCR reactions for *CO1* and *ITS2* were conducted on Veriti thermocycler (Applied Biosystems) and for *IGS*, a high resolution melting (HRM) capable Rotor-Gene Q real time PCR thermocycler (Qiagen, Hilden, Germany) was used. Following PCR, HRM analysis of amplicons was conducted by gradually increasing the temperature by 0.1 °C after every 2 seconds from 75 °C to 92 °C, resulting in a plot of the change in fluorescence with time (dF/dT). PCR-HRM protocols were validated for accuracy and sensitivity using standard reference controls.

ExoSAP-IT (USB Corporation, Cleveland, OH) was used to remove unincorporated dNTPs and PCR primers before sequencing. Sequences were edited in Geneious 7.0.5 (Kearse *et al.*, 2012) and used to query GenBank (Altschul, 1990).

3.5.2 Blood-meal source detection

High resolution melting profiles obtained using 383-bp cytochrome b (*cyt b*) (Peña *et al.*, 2012; Lutomiah *et al.*, 2014; Omondi *et al.*, 2015), 200-bp 16S rRNA (Omondi *et al.*, 2015), and 205-bp CO1 gene PCR products were used to distinguish different vertebrate hosts in mosquito blood-meals. Using DNA extracted from known vertebrate whole blood as positive standard reference controls and colony-reared mosquito DNA extracts as negative controls, PCRs was carried out in final volumes of 10 µL, containing 6 µL of PCR water, 0.5 µL final concentrations for each primer, 2 µL of 1X Hot Firepol Evagreen HRM Mix (Solis BioDyne, Tartu, Estonia) and 1 µL of DNA template. Thermal cycling conditions for *cyt b* and 16S rRNA primers (Omondi *et al.*, 2015) were used for all engorged malaria mosquitoes. The thermal cycling conditions used for *CO1* primers were as follows: Initial denaturation for 15 minutes at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 50 °C for 30 seconds and extension at 72 °C for 60 seconds followed by a final extension at 72 °C for 7 minutes. PCR reactions were conducted on HRM capable Rotor-Gene Q real time PCR thermocycler (Qiagen, Hilden, Germany). Following PCR, HRM profile analysis of amplicons was conducted as previously stated with normalization regions between 75.0–78.0 °C and 88.00–95.0 °C. Blood-meal sources were identified by comparison of HRM profiles to the reference control species. Amplicons with unique *cyt b*, *16S* or *CO1* HRM profiles were purified for sequencing as previously stated

3.5.3 Detection of malaria parasites

Malaria parasites in the head and thorax of engorged mosquitoes' were detected by PCR (Fornadel *et al.*, 2011). Target genes and primers sequences are provided in Table 1. Polymerase chain reaction was carried out in final volumes of 10 µL, containing 6 µL of PCR water, 0.5 µL final concentrations for each primer, 2 µL of 1X Hot Firepol Evagreen HRM Mix (Solis BioDyne,Tartu, Estonia) and 1 µL of DNA template. The thermal cycling conditions used for *cyt b* primers were as follows: Initial denaturation for 15 minutes at 95 °C followed by 40 cycles of denaturation at 95 °C for 20 seconds, annealing at 60 °C for 45 seconds and extension at 72 °C for 45 seconds followed by a final extension at 72 °C for 10 minutes. Following PCR, HRM profile analysis of amplicons was conducted as previously stated with normalization regions between 64.0-66.0 °C and 86.00-92.0 °C. *Plasmodium falciparum* infection was detected by comparison of melt curves to those of the standard reference positive control. Representative positive samples were purified for sequencing as previously stated. The sequences were edited in Geneious 7.0.5 software (Kearse *et al.*, 2012) and queried in GenBank using BLAST (Altschul, 1990).

Table 1: List of oligonucleotide primers used.

Target	Primer sequence (5' to 3')	Tm (°C)	Product Size (bp)	Citation
CO1	For: GGT CAA ATC ATA AAG ATA TTG G	50	650	(Folmer *et al.,* 1994)
	Rev: TCC AAT GCA CTA ATC TGC CAT ATT A			(Simons *et al.,* 1994)
ITS2	For: TGT GAA CTG CAG GAC ACA T	50	505	(Koekemoer *et al.,* 2002; Cohuet *et al.,* 2003)
	Rev: GCA TCG ATG GGT TAA TCA TG			(Koekemoer *et al.,* 2002; Cohuet et al., 2003)
	Rev: CAA GCC GTT CGA CCC TGA TT			(Koekemoer *et al.,* 2002; Cohuet *et al.,* 2003)
IGS	For: GTGAAGCTTGGTGCGTGCT	57	165	(Zianni *et al.,* 2013)
	Rev: GCACGCCGACAAGCTCA			(Zianni *et al.,* 2013)
Vert cyt b	For: CCC CTC AGA ATG ATA TTT GTC CTC A	56	383	(Boakye *et al*., 1999; Peña et al., 2012)
	Rev: CAT CCA ACA TCT CAG CAT GAT GAA A			(Boakye *et al.,* 1999; Peña *et al.,* 2012)
Vert 16S	For: GAG AAG ACC CTR TGG ARC TT	55	200	(Omondi *et al.,* 2015)
	Rev: CGC TGT TAT CCC TAG GGT A			(Omondi *et al.,* 2015)
CO1	For: TCC ACT AAT CAC AAR GAT ATT GGT AC	48	205	(Meusnier *et al*., 2008)
	Rev: TAT CAG GGG CTC CGA TTAT			(Lee *et al.,* 2015)
Cyt b	For: ATA CAT GCA CGC AAC AGG TGC TTC TC	60	183	(Fornadel *et al*., 2011)
	Rev: CAA TAA CTC ATT TGA CCC CAT GGT AAG AC			(Fornadel *et al*., 2011)

'For', refers to the forward primer sequence, 'Rev' refers to the reverse primer sequence, 'Tm' refers to annealing temperature, 'bp' refers to base pairs. Abbreviations: *CO1*, cytochrome oxidase subunit 1; *ITS2*, internal transcribed spacer 2; *IGS*, intergenic spacer region; vert cty b, vertebrate cytochrome b; vert 16S, vertebrate *16S rRNA*.

3.5.4 Determination of genetic structure

Determination of genetic structure was done using microsatellite DNA genotyping of 8 populations of *A. gambiae* s.s. and *A. arabiensis.* Each population comprising 48 mosquito species (Hale *et al.,* 2012). Amplification of 16 *A. gambiae* s.s. *and A. arabiensis* microsatellite loci (Zheng *et al.,* 1996) were done using a PCR technique that uses M13 tailed primers. In this technique, a 17 bp (GTAA AACG ACGG CCAG T) and 22 bp (GCGG ATAA CAAT TTCA CACA GG) universal sequences were fused to the 5′-end of forward or reverse primers. The same universal sequences were used to synthesize four oligonucleotides (tails) that were directly-labelled with VIC, NED, FAM, and PET fluorescent dyes (Applied Biosystems).

Fifteen microliter PCR reactions were prepared with 0.5 µL final concentrations for each primer, 3 µL of 1X Hot Firepol blend (Solis BioDyne, Tartu, Estonia), 9 µl of PCR water and 2 µL of DNA template. Thermal cycling conditions were as follows: initial denaturation at 95 °C for 15 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at (52 - 57 °C) for 30 seconds and extension at 72 °C for 45 seconds, and a final extension at 72 °C for 7 minutes. PCR reactions were conducted on Veriti thermocycler (Applied Biosystems). PCR products were resolved in 1% agarose gel in Tris-borate EDTA buffer stained with ethidium bromide. 1 µl of PCR products were centrifuged at pulse and added into a well containing 9 µl of HIDI-LIZ500 mixture. The contents were mixed by vortexing on a plate vortexer, centrifuged for 1 minute and sent for electrophoresis capillary analysis using the ABI 3730 genetic analyser for fragment analysis.

3.6 Statistical analysis

The abundance and density of female mosquitoes, and prevalence of *P. falciparum* infection among engorged malaria mosquitoes were analysed using R version 3.3.0 (R Core Team, 2016). Differences in malaria mosquito density (all three species combined) among the three islands, while adjusting for collection method were compared using the negative binomial model as Poisson model and zero-inflated counterparts were not supported by the data (Vuong test $P<0.001$). Differences in *P. falciparum* infection among engorged malaria mosquito species in each study island were compared in a Bayesian fashion using the Bayesian First Aid package (Bååth, 2014). The Bayesian approach was adopted because these data were sparse, thus rendering the classical Chi-square approach for comparing proportions unreliable. In a Bayesian analysis, prior distributions must be specified for all parameters. In this study, the default prior settings in the package was used. Differences were considered

significant if the 95% credibility interval (Bayesian equivalent of classical 95% confidence interval) did not contain zero.

3.7 Microsatellite data analysis

The microsatellites data analysis has not been analysed yet. The genetic diversity of 16 microsatellite markers will be quantified using heterozygosity, number of alleles and allele frequencies observed in each population using Arlequin (Excoffier *et al*., 2005). Each microsatellite locus will be tested separately for significant departure from Hardy-Weinberg equilibrium, using a Markov-chain algorithm (Guol & Thompson, 1992). The Arlequin software will be used to calculate pair-wise FST values, using 10,000 permutations to determine the significance of FST distance. Gene flow (Nm) will be calculated for FST according to the equation, $Nm \approx (1-FST)/4FST$.

CHAPTER FOUR

RESULTS

4.1 Physical, geographical features, and mosquitoes habitats

All the three islands have a rocky terrain, with Mageta and Magare having a fairly similar terrain off shore, and Mageta's shoreline having some moderately steep sections. The Magare' shoreline is also characterized by large, stable water pools covered with emergent vegetation, while Ngodhe Island is steep althrough.

In the three islands, mosquito-breeding habitats were mainly associated with human economic activities. Such mosquito habitats include cattle hoof-prints, shallow pools in cultivated farms and abandoned fishing boats. In Mageta, mosquito habitant included open rock pools and low-lying plains that end gradually into the lakeshores. In Magare, most mosquito breeding sites were swamps along the low-lying lakeshores that are covered with emergent vegetation and open rock pools. In Ngodhe, mosquito-breeding sites included open rock pools and shallow pools resulting from limited agricultural activity at some sections of the shoreline. Receding lake tidal water created temporary pools at the shoreline, which were also mosquito-breeding habitats in the islands.

4.2 Demographics and LLINs usage

We geo-referenced 3,081 buildings and recorded the number of people in 2,671 households, including their ages and LLINs usage. Mageta had the highest population of 6487, followed by Ngodhe 484 and Magare 250. Magare had the highest LLINs usage of 98.51% (*n*=70), followed by Ngodhe (88.31%; *n*=136) and Mageta (62.34%; *n*=1525) (Table 2). Most residences were made of mud walls with iron-sheet roofing and open eaves between the wall and the roof to facilitate the flow of air in the house. All houses in Magare had open eaves (100%, *n*=71), compared to in Ngodhe 96.73% (*n*=149) and Mageta 95.30% (*n*= 2331). Additionally, majority of doors and windows are usually left open until late afternoon. The open eaves, doors, and windows may serve as the main entry points for host seeking mosquitoes. A compound in a household consists of a residential house, a livestock shed (for cattle, goats, sheep and/or pigs) and a small shed commonly built for dogs. Other domestic animals, such as chicken sleep inside residential houses. The main economic activities are fishing, fish mongering and small-scale agriculture.

Table 2: Demographic information including sampling nights and LLINs usage.

Study area	Area (Km^2)	Population (%)	Households (%)	Buildings (%)	Open Eaves (%)	Sampling Nights	LLINs Usage (%)
Mageta	7	6487 (89.84)	2446 (91.57)	2822 (91.51)	2331 (95.30)	132	1525 (62.34)
Magare	0.2	250 (3.46)	71 (2.66)	86 (2.79)	71 (100)	132	70 (98.51)
Ngodhe	0.9	484 (6.70)	154 (5.77)	173 (5.62)	149 (96.73)	138	136 (88.31)

LLINs, refers to long lasting insecticidal nets.

4.3 Distribution of malaria mosquitoes

The dataset included 7,350 mosquitoes, of which 25.41% (*n*=1,868) were malaria mosquitoes (*A. gambiae* s.l., *A. funestus* and *A. coustani*). In all study areas, *A. gambiae* s.l. *and A. funestus* were collected more frequently indoors by aspirator pyrethrum spray collector, and CDC light traps. However, in all study areas *A. coustani* were collected more frequently outdoors by CDC light traps. Overall, highest numbers of malaria mosquitoes were collected in Mageta (*n*=1515), followed by Ngodhe (*n*=282), and Magare (*n*=70) (Table 3).

Table 3: Distribution of malaria mosquitoes

Study area	Mosquito species	Indoor												Outdoor				N (%)
		ASP				PSC				CDC				CDC				
		BF	UF	G	HG	BF	UF	G	HG	BF	UF	G	HG	BF	UF	G	HG	
Mageta	*A. gambiae s.l.*	63	35	14	41	384	53	19	81	29	267	31	7	5	35	2	1	1067 (70.38)
	A. funestus	11	1	5	11	27	0	1	8	8	42	7	2	0	10	1	0	134 (8.84)
	A. coustani	0	1	0	0	0	0	0	0	4	5	0	0	35	266	4	0	315 (20.78)
	Total (%)	182 (12.01)				573 (37.80)				402 (26.52)				359 (23.68)				1515
Magare	*A. gambiae s.l.*	0	0	1	0	4	2	0	1	0	11	0	0	0	1	0	0	20 (28.57)
	A. funestus	0	0	0	0	8	3	1	0	1	8	0	0	0	0	1	0	22 (31.43)
	A. coustani	0	0	0	0	0	0	0	0	0	0	0	0	2	26	0	0	28 (40)
	Total (%)	1 (1.43)				19 (27.14)				20 (28.57)				30 (42.86)				70
Ngodhe	*A. gambiae s.l.*	11	18	6	3	9	11	12	2	14	60	37	4	2	2	2	0	193 (68.44)
	A. funestus	1	1	3	3	7	2	1	2	4	18	15	4	0	0	1	0	62 (21.99)
	A. coustani	0	0	0	0	0	0	0	0	1	6	0	0	2	16	2	0	27 (9.57)
	Total (%)	46 (16.31)				46 (16.31)				163 (57.80)				27 (9.57)				282

N, number of malaria mosquitoes; ASP, aspirator; PSC, pyrethrum spray collector; CDC, CDC light trap; BF, blood-fed; UF, unfed; G, gravid; HG, half-gravid.

The predominant malaria mosquito species in Mageta was *A. gambiae* s.l. (70.38%, *n*=1067), while *A. funestus* (8.84%, *n*=134) was least abundant. In Magare, *A. coustani* (40%, *n*=28) was most abundant, while *A. gambiae* s.l. (28.57%, *n*=20) was least abundant. In Ngodhe, *A. gambiae* s.l. (68.44%, *n*=193) was most abundant and *A. coustani* (9.57%, *n* =27) was least abundant. Overall, malaria mosquitoes' abundance varied in all study areas (Figure 6). Malaria mosquito densities (number of mosquitoes collected per building using four different traps) were highest in Mageta (0.90; standard error of mean (se+/-) 0.10), and comparable in Magare and Ngodhe (0.18; se+/- 0.05 and 0.15; se+/- 0.01 respectively) (Figure 6).

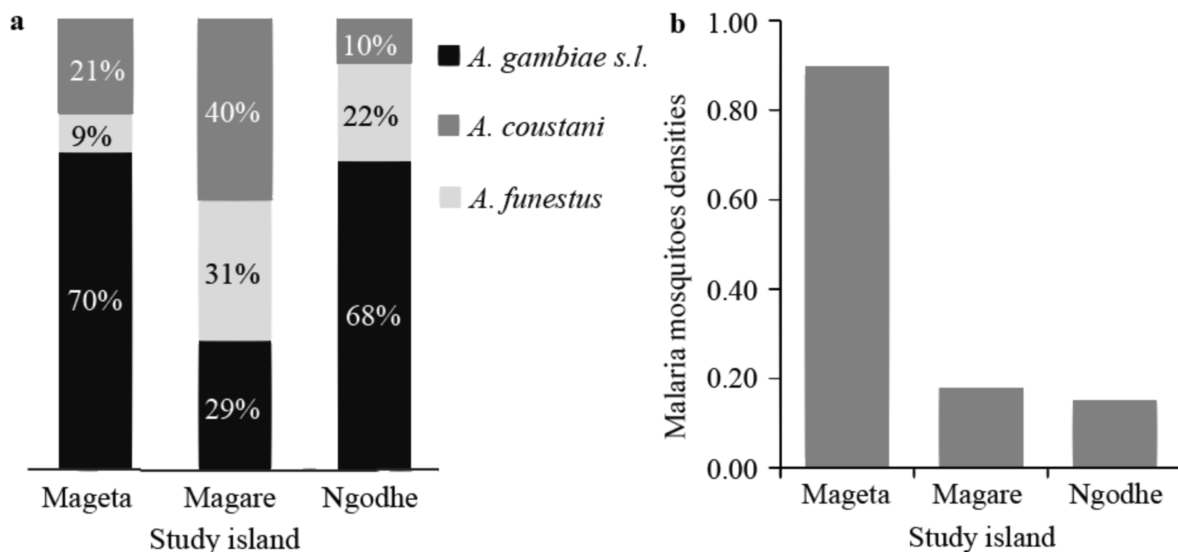

Figure 6: Malaria mosquitoes' abundance and density. (**a**), the stacked bar chart shows the percentage of malaria mosquitoes' abundance by the three islands. Malaria mosquitoes' abundance varied in all study areas. (**b**), shows malaria mosquitoes densities. The y-axis indicate malaria mosquitoes' densities, and the study islands are shown in the x-axis.

There was a significant difference in malaria mosquito densities in Mageta compared to Magare and Ngodhe (P<0.001). However, there was no statistical difference of malaria mosquito densities in Magare and Ngodhe (P<0.4525) (Table 4). In all study areas, *A. gambiae* s.l. and *A. funestus* were collected more frequently indoors by ASP, PSC and CDC light traps, while *A. coustani* were collected predominantly outdoors by CDC light traps. Unfed malaria mosquitoes were caught more frequently by indoor CDC light traps than any other trap. Overall, there were significant differences in malaria mosquito (all three species combined among the three islands) catches by different traps (P<0.0001), with indoor CDC light trap registering the most catches (IRR 3.18; 95% CI 2.35-4.28) (Table 4).

Table 4: Association between malaria mosquito densities and catches by different traps

Variable		IRR (95%CI)	P-value
Island			
	Mageta	ref	ref
	Magare	0.2 (0.1-0.38)	<0.001
	Ngodhe	0.15 (0.12-0.2)	<0.001
	Magare vs Ngodhe	0.56	0.4525
Collection method			
	Aspirator	ref	ref
	PSC	2.04 (1.52-2.73)	<0.001
	indoor CDC	3.18 (2.35-4.28)	<0.001
	outdoor CDC	1.65 (0.94-2.89)	0.081
	PSC vs Indoor CDC	12.09	0.0005
	CDC indoors vs outdoors	6.51	0.0107
alpha		5.87 (4.8-7.15)	

IRR, inter rate ratio; CI, confidence of interval.

4.4 Molecular identification of engorged malaria mosquitoes

Anopheles gambiae s.s. and *A. arabiensis* occur in sympatry and are similar morphologically and hence could not be differentiated by microscopy. The two species were differentiated based on their IGS melt curves (Figure 7). Specifically, IGS melt curves of field-collected *A. gambiae* s.l. were matched to sequenced colony-reared *A. gambiae* s.s. and *A. arabiensis* (Zianni *et al*., 2013). All field-collected *A. gambiae* s.l. melt curves matched with either *A. gambiae* s.s. or *A. arabiensis.* Similarly, *A. funestus* group sibling species could not be identified morphologically because of overlapping distinctive features. In all study areas, only *A. funestus* s.s. were identified among sibling species. In addition, the morphological identification of *A. coustani* by was confirmed PCR and sequencing.

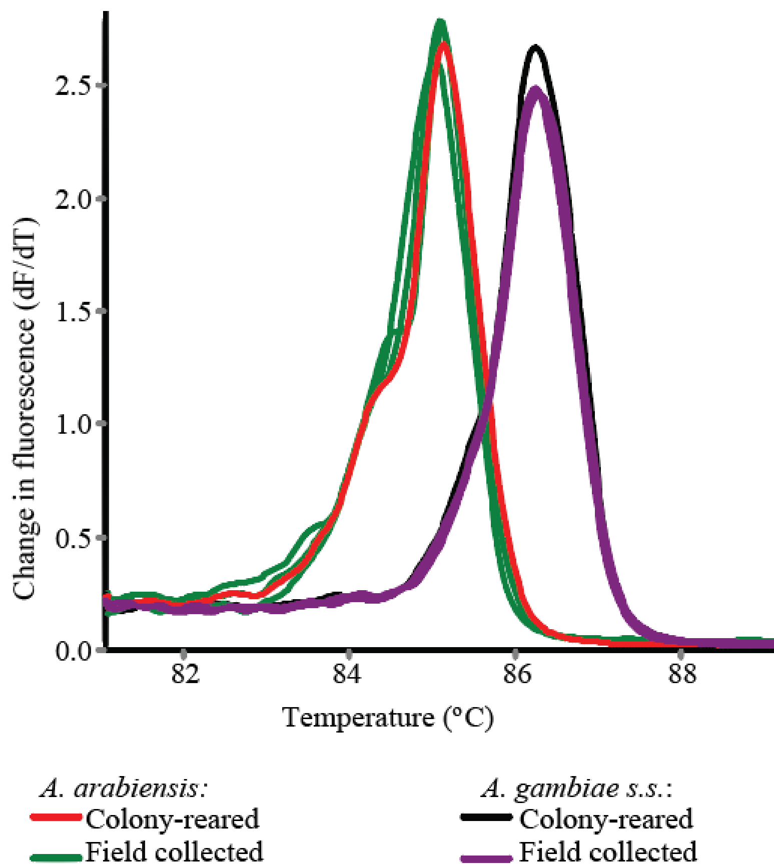

Figure 7: Representative melt curves of *A. arabiensis* and *A. gambiae* s.s. from a subset of field-collected engorged *A. gambiae* s.l. Melt curves with y-axis indicating a change in fluorescence units with increasing temperature (dF/dT). The increasing temperature is shown in x-axis. Each peak of the melt curve shows melting temperature (Tm) values for each mosquito species.

4.5 Distribution of engorged malaria mosquitoes

Anopheles gambiae s.s. (82.89%, *n*=310) was the most frequent blood-fed species collected in Mageta, followed by *A. arabiensis* (10.70%, *n*=40), *A. funestus* s.s. (4.28%, *n*=16) and *A. coustani* (2.14%, *n*=8). In Magare, *A. funestus* s.s. (32%, *n*= 8) and *A. coustani* (32%, *n*= 8) were the most frequent blood-fed species collected, followed by *A. gambiae* s.s. (28%, *n*=7) and *A. arabiensis* (8%, *n*=2). Blood-fed *A. coustani* were not collect in Ngodhe. However, of all blood-fed species collected, *A. arabiensis* (52.94%, *n*=9) was collected in highest numbers, followed by *A. funestus* s.s. (29.41%, *n*=5) and *A. gambiae* s.s. (17.65%, *N*=3). The engorged (blood-fed) species were collected indoors (95.43%, *N*=397) and outdoors (4.57%, *n*=19). In all islands, *A. gambiae* s.s., *A. arabiensis* and *A. funestus* s.s. that were collected more frequently indoors, unlike the majority of *A. coustani*, which were collected more frequently outdoors (Table 5).

Table 5: Distribution of engorged malaria mosquitoes.

Study area	Mosquito species	Indoors			Outdoors	N (%)	ERF (95% CI)
		ASP	PSC	CDC	CDC		
Mageta	***A. gambiae* s.s.**	34 (10.97)	244 (78.71)	28 (9.03)	4 (1.29)	310 (82.89)	0.83 (0.79, 0.86)
	A. arabiensis	5 (12.50)	32 (80.00)	3 (7.50)	0 (0.00)	40 (10.70)	0.11 (0.08, 0.14)
	***A. funestus* s.s.**	6 (37.50)	10 (62.50)	0 (0.00)	0 (0.00)	16 (4.28)	0.04 (0.04, 0.07)
	A. coustani	0 (0.00)	0 (0.00)	3 (37.50)	5 (62.50)	8 (2.14)	0.02 (0.01, 0.04)
	Total (%)	45 (12.03)	286 (76.47)	34 (9.09)	9 (2.41)	374 (100.00)	
Magare	***A. gambiae* s.s.**	0 (0.00)	4 (57.14)	2 (28.57)	1 (14.29)	7 (28.00)	0.29 (0.13, 0.46)
	A. arabiensis	0 (0.00)	1 (50.00)	0 (0.00)	1 (50.00)	2 (8.00)	0.10 (0.01, 0.23)
	***A. funestus* s.s.**	0 (0.00)	7 (87.5)	1 (12.5)	0 (0.00)	8 (32.00)	0.33 (0.16, 0.51)
	A. coustani	0 (0.00)	0 (0.00)	0 (0.00)	8 (100.00)	8 (32.00)	0.33 (0.16, 0.51)
	Total (%)	0 (0.00)	12 (48)	3 (12)	10 (40)	25 (100.00)	
Ngodhe	***A. gambiae* s.s.**	0 (0.00)	0 (0.00)	3 (100.00)	0 (0.00)	3 (17.65)	0.20 (0.04, 0.39)
	A. arabiensis	1 (11.11)	4 (44.44)	4 (44.44)	0 (0.00)	9 (52.94)	0.53 (0.30, 0.73)
	***A. funestus* s.s.**	0 (0.00)	3 (60.00)	2 (40.00)	0 (0.00)	5 (29.41)	0.31 (0.12, 0.52)
	Total (%)	1 (5.88)	7 (41.18)	9 (52.94)	0 (0.00)	17 (100.00)	

N, number of engorged malaria mosquitoes; ERF, estimated relative frequency; CI, credibility interval; ASP, aspirator; PSC, pyrethrum spray collector; CDC, CDC light trap.

4.6 Blood-feeding pattern in malaria mosquitoes

Overall, ten blood-meal hosts including humans and non-humans, namely goat (*Capra hircus*), cow (*Bos taurus*), sheep (*Ovis aries*), dog (*Canis lupus*), chicken (*Gallus gallus*), pig (*Sus scrofa*), rat (*Rattus rattus*) grass frog (*Ptychadena nilotica*) and bird (*Dendrocincla turdina)* were identified (Table 6). Blood-meal sources were identified from 389 malaria mosquitoes, representing 93.51% of all captured engorged malaria mosquitoes (*n* = 416). Malaria mosquitoes collected indoors (*A. gambiae* s.s.*, A. arabiensis* and *A. funestus* s.s.) showed greater tendency to blood-feed on humans. Conversely, *A. coustani* collected both indoors and outdoors showed greater tendency of feeding on cows.

Table 6: Number of blood-meal sources of engorged malaria mosquito species

Study area	Species	N	Vertebrate host										
			Human	Chicken	Sheep	Cow	Goat	Pig	Frog	Rat	Dog	Bird	UN
Mageta	*A. gambiae s.s.*	310	236.5*	9*	1	25.5*	4*	2	1	5	6	1	19
	A. arabiensis	40	26*	1	4	7*	0	0	0	0	0	0	2
	A. funestus s.s.	16	15	0	0	0	0	0	0	0	0	0	1
	A. coustani	8	1	0	0	7	0	0	0	0	0	0	0
	Total (%)	**374**	**278.5* (74.46%)**	**10* (2.67%)**	**5 (1.34%)**	**39.5* (10.56%)**	**4* (1.07%)**	**2 (0.53%)**	**1 (0.27%)**	**5 (1.34%)**	**6 (1.60%)**	**1 (0.27%)**	**22 (5.88%)**
Magare	*A. gambiae s.s.*	7	3	0	0	1	0	0	0	1	0	0	2
	A. arabiensis	2	1	0	0	0	0	0	0	0	0	0	1
	A. funestus s.s.	8	6	0	0	0	0	0	0	0	0	0	2
	A. coustani	8	0	0	0	7	0	0	0	0	0	0	1
	Total (%)	**25**	**10 (40%)**	**0**	**0**	**8 (32%)**	**0**	**0**	**0**	**1 (4%)**	**0**	**0**	**6 (24%)**
Ngodhe	*A. gambiae s.s.*	3	3	0	0	0	0	0	0	0	0	0	0
	A. arabiensis	9	7.5*	0	0	1.5*	0	0	0	0	0	0	0
	A. funestus s.s.	5	2*	0	0	3*	0	0	0	0	0	0	0
	Total (%)	**17**	**12.5* (73.53%)**	**0**	**0**	**4.5* (26.47%)**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Total		**416**	**297**	**10**	**5**	**53**	**4**	**2**	**2**	**6**	**6**	**1**	**27**

N, numbers of engorged malaria mosquitoes analysed; UN, numbers of blood-meals whose sources identification was not successful; * represent mixed malaria mosquitoes blood-meals.

Specifically, HRM profiles obtained using *CO1*, *16S ribosomal RNA*, and *cyt b* genes from engorged abdomens of field-collected mosquitoes were matched to those obtained from standard reference positive controls (Peña *et al*., 2012; Lutomiah *et al*., 2014; Omondi *et al*., 2015). Representative melt curves obtained using *CO1*, 16S ribosomal RNA, and *cyt b* genes are shown in Figure 8.

This approach also allowed for high sensitivity identification of mixed blood-meals from individual mosquitoes. Mixed blood-meal melt curves showed double peaks with melting temperatures similar to those of more than one positive controls (Figure 8, c, f, and i). Overall, 3.61% of all analysed blood-meals (*n*=15) were from mixed blood-meals. The mixed blood-meals were observed in *A. gambiae* s.s., *A. arabiensis* and *A. funestus* that had fed on a human in addition to cow, goat or chicken, respectively, and of an *A. gambiae* s.s. mosquito that had fed on cow and goat.

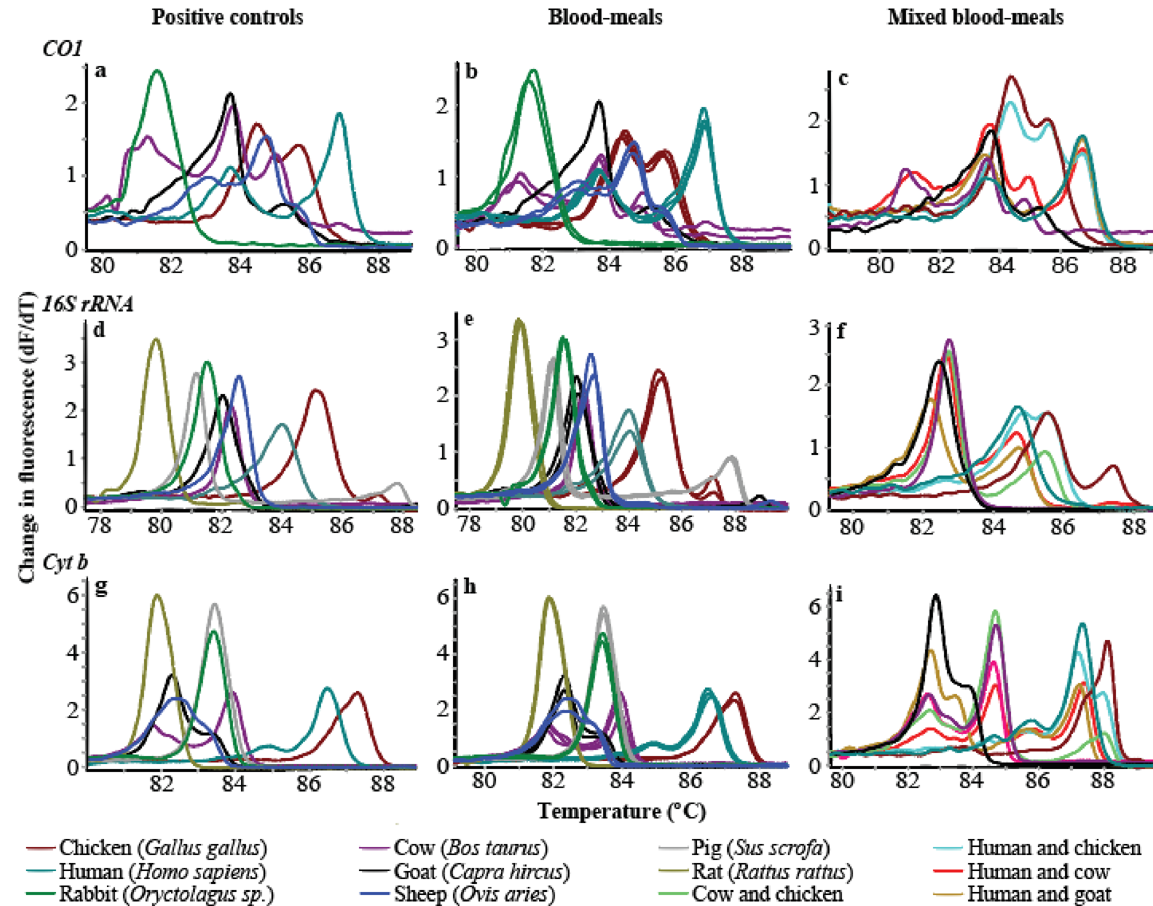

Figure 8: PCR-HRM melt curves of blood-meal sources. (**a-c**), *COI*, a, positive controls, b, blood-meals, c, mixed blood-meals. (**d-f**), *16S rRNA*, d, positive controls, e, blood-meals, f, mixed blood-meals. (**g-i**), *cyt b*, g, positive controls, h, blood-meals, i, mixed blood-meals. y-axis indicate a change in fluorescence units with increasing temperature (dF/dT), while the increasing temperature is shown in the x-axis. Each peak of the melting curve shows melting temperature (Tm) value for each blood-meal source.

In Mageta, blood-meal sources were 74.46% human and 19.65% non-humans. In Magare, 40% of blood-meal sources were from humans and 36% from other vertebrate species. In Ngodhe, 73.53% of blood-meal sources were from humans and 26.47% from non-humans (Figure 9). Further, the results showed that the range of host species varied in all study areas (Figure 9).

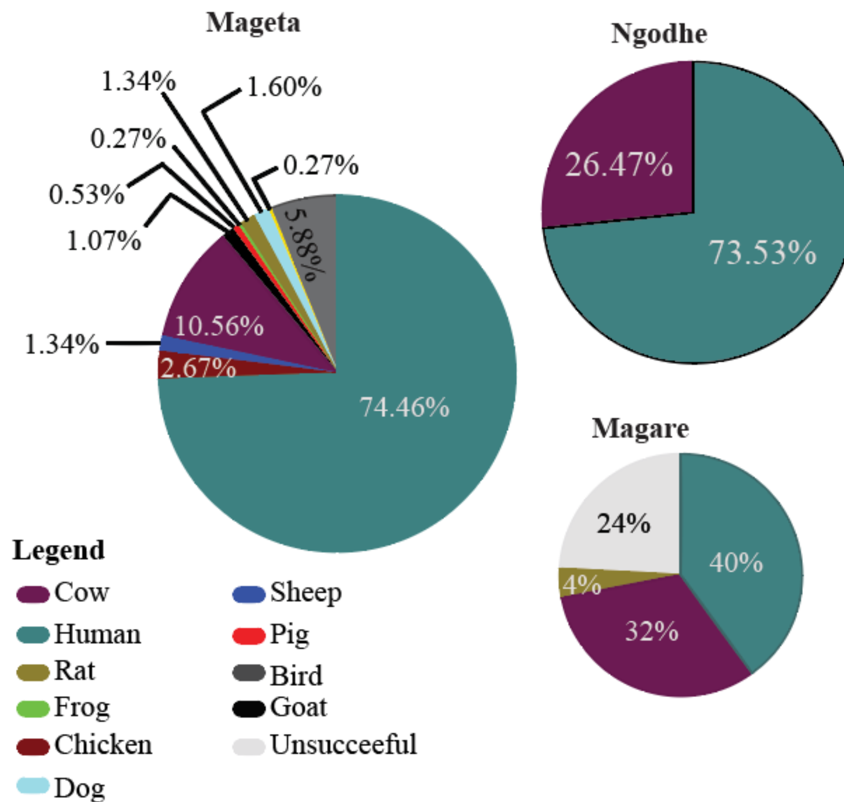

Figure 9: Proportions of blood-meal sources. The range of blood-meal sources varied in all study locations with malaria vectors feeding prominently on humans in all study areas. In addition, the prominent non-human blood-meal source in all study areas was the cow. Mixed blood-meals were divided equally between identified blood-meal sources.

4.7 *Plasmodium falciparum* infection of engorged malaria mosquitoes

A total of 416 engorged malaria mosquitoes (320 *A. gambiae* s.s., 51 *A. arabiensis,* 29 *A. funestus* s.s. and 16 *A. coustani*) were tested for presence of malaria parasites using PCR-HRM (Table 7) (Fornadel *et al*., 2011). Overall, 9.86% of engorged malaria mosquitoes harboured *P. falciparum* infection (*N*=41). However, there were no significant differences in *P. falciparum* infection in engorged mosquitoes of all four species or among the three islands (P-value = 0.4999) (Table 7). Representative melt curves of *P. falciparum* among engorged malaria mosquito species is shown in figure 10.

Table 7: *Plasmodium falciparum* infection of engorged malaria mosquito species.

Study Area	Mosquito species	N	Pfal (%)	ERF (95% CI)
Mageta	*A. gambiae* s.s.	310	30 (9.68)	0.10 (0.067, 0.13)
	A. arabiensis	40	4 (10.00)	0.11 (0.032, 0.21)
	A. funestus s.s.	16	0 (0.00)	0.04 (3.9e-06, 0.16)
	A. coustani	8	2 (25.00)	0.29 (0.055, 0.57)
	Total	374	36 (9.63)	
Magare	*A. gambiae* s.s.	7	2 (28.57)	0.32 (0.059, 0.62)
	A. arabiensis	2	1 (50.00)	0.50 (0.085, 0.89)
	A. funestus s.s.	8	0 (0.00)	0.07 (5.4e-07, 0.28)
	A. coustani	8	1 (12.50)	0.18 (0.011, 0.44)
	Total	25	4 (16.00)	
Ngodhe	*A. gambiae* s.s.	3	0 (0.00)	0.16 (5.4e-07, 0.53)
	A. arabiensis	9	1 (11.11)	0.16 (0.0068, 0.39)
	A. funestus s.s.	5	0 (0.00)	0.11 (3.4e-06, 0.40)
	Total	17	1 (5.88)	

Abbreviations: N, number of engorged malaria mosquitoes species analyzed; Pfal, *P. falciparum*; ERF, estimated relative frequency of success; CI, Credibility interval.

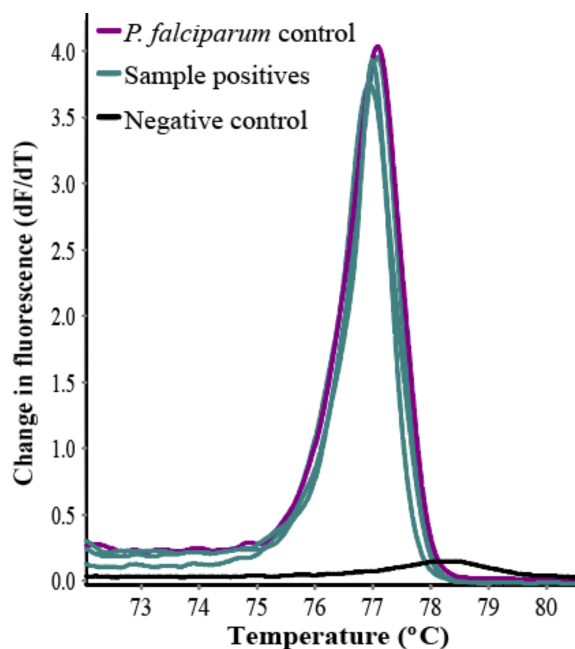

Figure 10: Representative melt curves of *Plasmodium falciparum* among engorged malaria mosquito species. y-axis indicate a change in fluorescence units with increasing temperature (dF/dT), while the increasing temperature is shown in the x-axis.

4.8 Genetic variation of *A. gambiae* s.s. and *A. arabiensis*

Sixteen polymorphic *A. gambiae* s.s. and *A. arabiensis* microsatellite markers were optimized. 1% agarose gel in Tris-borate EDTA buffer stained with ethidium bromide revealed 10 markers were polymorphic (Figure 11). This work is pending fragment analysis.

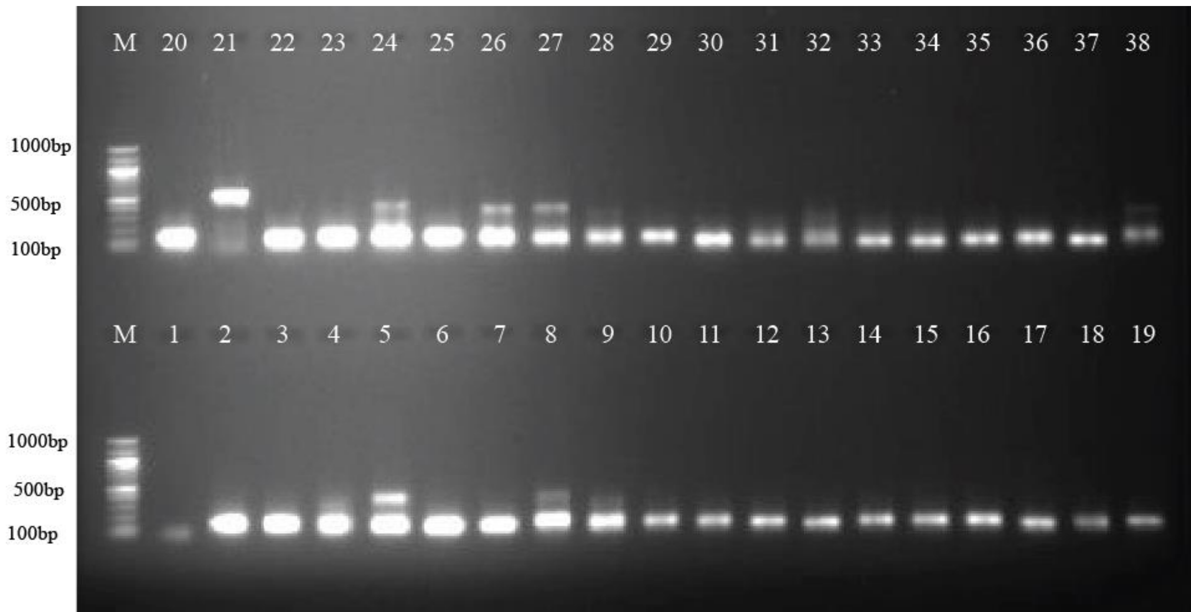

Figure 11: Agarose gel image showing DNA bands of amplified *A. gambiae s.s.* microsatellite marker. The image was obtained after electrophoresis of PCR products of target marker (XH164) on a 1% ethidium bromide-stained agarose gel. Lane M represent DNA ladder in base pairs while the following lanes marked 1-38 represent negative control (lane marked 1) and amplified microsatellite marker. Lane 5, 21, 24, 26 and 27 shows *A. gambiae s.s.* are polymorphic.

CHAPTER FIVE

DISCUSSION

5.1 Suitable lake environment maintains high malaria mosquitoes abundance

Though a suitable lake environment ensures high malaria mosquito abundance throughout the year, the results indicate that the malaria mosquito density also depends on island terrain, vegetation cover, human economic activities, and LLINs usage. Mosquito breeding habitats such as open rock pools, hoof-prints, and shallow pools in cultivated farms relied on rainfall for water supply and dried out in absence of rain. However, seepage of water from the lake to low-lying swamps along the shores of lake Victoria prevents these habitats from drying up thereby supporting mosquito breeding even during the dry season. In Mageta and Ngodhe, *A. gambiae* s.l. was the most abundant, possibly because of open rock pools and low-lying plains that end gradually into the lakeshore, forming favorable mosquito habitats (Minakawa *et al*., 2008). In Magare, *A. coustani* were present in highest numbers compared to other species, possibly due to the presence of large, stable water pools covered with aquatic vegetation that provide a suitable habitat for the species (Minakawa *et al*., 2012). Overall, malaria mosquito densities were highest in Mageta and comparable between Magare and Ngodhe (p-value < 0.001), likely due to Mageta's relatively low LLIN coverage and suitable breeding habitats.

The majority of engorged *A. gambiae* s.s., *A. arabiensis* and *A. funestus* s.s. were collected indoors by PSC, CDC light, and ASP traps, revealing their tendency of feeding indoors. Further, like in previous studies, these species' blood-meals were fresh, showing that they had fed and rested indoors (Faye *et al*., 1997). Even though there are few records of exactly where malaria mosquitoes rest within a house, one would expect these vectors to be more affected by LLINs use than *A. coustani*, which exhibits outdoor resting behaviour and plays a major role in outdoor malaria transmission (Mwangangi *et al*., 2013). The high abundance of indoor resting mosquito species collected in this study, despite extensive use of LLINs, may be as a result of growing resistance to LLINs insecticides like pyrethroids (Ranson *et al*., 2011; Maliti *et al*., 2014; Wanjala *et al*., 2015).

In summary, the study islands have a suitable environment, with a variety of mosquito breeding habitats, and alternative blood-meal sources thus ensure high malaria mosquito abundance throughout the year despite high LLINs usage in the study area.

5.2 Malaria vectors' multiple blood-meal sources suggest opportunistic feeding as an important factor in malaria transmission

It is necessary to establish mosquito blood-feeding patterns to understand malaria transmission dynamics and provide strategies for optimal vector control. The study demonstrates that malaria vectors exhibit a diverse host-feeding pattern. In total, eleven blood-meal hosts were identified by matching HRM profiles obtained using *CO1*, 16S ribosomal RNA, and *cyt b* genes from engorged abdomens of field-collected mosquitoes to those obtained from standard reference positive controls. Although HRM profiles of *cyt b* and 16S ribosomal RNA amplicons had been earlier used for blood-meal analysis (Omondi *et al.,* 2015), *CO1* is the universal barcode region in vertebrates species for various reasons. *COI* evolves more slowly than *cyt b* (Meiklejohn *et al.*, 2007) and has higher resolution than 16S ribosomal RNA in rodents (Nicolas *et al.,* 2012). Additionally, there are more documented (GenBank) *CO1* sequences for vertebrate species than 16S rRNA sequences (Lee *et al.,* 2015). Therefore, using *CO1* to differentiate potential blood-meal host species can expand the certainty of vertebrate species identification.

Mosquitoes make short-range, appetitive flights seeking blood-meal and feed on diverse hosts (Nanda *et al.,* 1996). Hence, this HRM of three distinct genetic markers can resolve a greater potential diversity of vertebrate hosts. However, a major impediment is a very effective hematophagous digestion system in mosquitoes (Calvignac-Spencer *et al.,* 2013). Studies show that vertebrate host DNA is detectable in frozen engorged mosquito abdomens a day after blood-feeding (Ngo & Kramer, 2003; Kent & Norris, 2005) and that this period varies depending on the length of the DNA fragment of interest. Longer DNA fragments have a better resolution but lower recovery from processed biological product such as blood-meals. In this study, a shorter *CO1* fragment (205-bp) that is convenient for analysing degraded DNA samples from digested blood-meals (Lee *et al.,* 2015) was used to generate HRM profiles. Some blood-meals showed HRM profiles with low variability for either *CO1*, 16S rRNA or *cyt b* only, but not in all the three markers. Such blood-meal sources were discriminated by comparing HRM profiles using other genes. For instance, *CO1* gene showed low variability in HRM profiles of cow and pig (Figure 8). Similarly, human and cow blood-meals could not clearly discriminate by analysing HRM profiles obtained using *16S rRNA*

gene (Figure 8). Further, *cyt b* gene HRM profiles could not clearly resolve blood-meal from pig and rabbit, and from sheep and goat (Figure 8). By utilizing HRM profiles of *CO1*, *16S rRNA* and *cyt b*, an exhaustive strategy that provided for a cheap, reliable and robust way to delimit and resolve diverse blood-meal sources from abundant malaria mosquito hosts was developed.

Diverse mosquito blood-meal hosts in both study areas were identified. Most engorged malaria mosquitoes collected indoors had fed on humans. This result extends the observation of a previous study, which established that the majority of malaria mosquitoes have indoors resting behaviour and could be associated with indoor feeding on humans (Paaijmans & Thomas, 2011). Diverse non-human hosts in *A. gambiae* s.s. blood-meals were identified and included a rat and a bird, with humans making the highest percentage of this diversity. Blood-meals from both *A. arabiensis* and *A. funestus* collected indoors were substantially from humans, unlike in a study in Mwea which showed bovine feeding for indoor collected *A. arabiensis* to be significantly higher, while indoor collected *A. funestus* also preferentially fed on humans (Muriu *et al.,* 2008). Overall, *A. coustani* preferred feeding on cows, outdoors, therefore having an alternative blood-meal sources that ensure their survival and further promoting malaria transmission.

Overall, 7.21% of host DNA from engorged abdomens were not detected and the reason for this failure was not investigate. Malaria mosquito feeding patterns depend on density and diversity of host species (Lardeux *et al.,* 2007), which by their availability form readily accessible blood-meal sources. By placing traps indoors and outdoors, the study aimed at maximise the recovery of data on feeding patterns to enhance understanding of a localised malaria mosquito feeding dynamics. The study showed that the range of host species varied in all study areas. Mageta had the highest numbers of engorged malaria mosquitoes and broadest range of host species. Contrastingly, Ngodhe had the fewest engorged malaria mosquitoes and narrowest range of host species. The range of host species did not differ significantly in Magare compared to Ngodhe (estimated group difference -0.07 (95% CI, -0.4-0.26). This is an extension of a finding which showed that malaria mosquitoes feed opportunistically upon available hosts over inherently preferred ones, reducing their contact with LLINs (Lefevre *et al.,* 2009).

The study approach also allowed for high sensitivity identification of mixed blood-meals from individual mosquitoes (Figure 8). Malaria prevalence is influenced by mosquito host

selection (Garret-jones, 1964) and detection of mixed blood-meals is important for understanding disease transmission pathways because multiple blood-feeding leads to possible multiple *Plasmodium* species infections (Pollitt *et al.,* 2015). Mixed blood-meal sources were as a result of blood-feeding malaria mosquito resuming blood-feeding on a different host in an effort to complete a successful blood-meal, a characteristic that is common with malaria mosquitoes infected with sporozoites-stage malaria parasites (Koella, 2002; Cator *et al.,* 2012). The finding that mixed feeding on vertebrate hosts included blood-meals from cow, chicken and goat, further confirm malaria vectors feeding on readily available blood-meal sources (Lardeux *et al.,* 2007), and underline economic activities of study population, which provide other blood-meal sources important for vector survival. The analysis was not able to differentiate malaria mosquitoes that had fed on multiple hosts of the same species like in Gambia where one malaria vector was suspected to have taken blood-meals from children sharing rooms on one night (Lindsay, 1990).

Together, using HRM profiles of *CO1*, 16S rRNA and *cyt b* to differentiate potential blood-meal sources the study robustly expanded the certainty of identifying vertebrate host species and identified diverse blood-meal sources. This included identification of mixed blood-meals, underlining malaria vectors opportunistic feeding pattern, that threaten the effectiveness of LLIN, and ensure their survival, and subsequent malaria transmission.

5.3 Secondary vector could play an increasing role in malaria transmission

The study found 9.86% of engorged malaria mosquitoes to be harbouring malaria parasites. Our study implicated *P. falciparum* infection in *A. coustani* (Table 7). The association of secondary vector *A. coustani* with *P. falciparum* transmission has been recently been reported in Taveta District (Kenya) and in Madagascar (Mwangangi *et al*., 2013; Nepomichene *et al*., 2015). Hence, it is likely that the vector is playing an important role in residual malaria transmission, especially outdoors, by harbouring malaria parasites and surviving on alternative blood-meal sources. The present study did not investigate malaria vector parasite density, and the possibility that malaria vector species survived long enough to transmit *P. falciparum*. The study did not find significant differences between engorged malaria mosquito species with *P. falciparum* infection in all four mosquitoes species and among the three islands (p-value = 0.4999). However, the vector species may play an increasingly important role in residual malaria transmission, especially by harbouring malaria parasites, surviving on alternative blood-meal sources and feeding upon humans during active hours when humans are unprotected by LLINs.

Combined, the malaria vectors harbouring *P. falciparum*, included those collected inside houses with LLINs and *A. coustani*, a secondary vector and an outdoor feeder. Hence, it is likely that the vector species are playing an increasingly important role in residual malaria transmission, especially by harbouring malaria parasite, surviving on alternative blood-meal sources and feeding upon humans during active hours, when humans are unprotected by LLINs.

In summary, the study present substantial information on malaria transmission dynamics in three islands of Lake Victoria, Western Kenya. The islands have a suitable environment, a variety of mosquito breeding habitats, and alternative blood-meal sources that ensure year long malaria vector abundance despite high LLINs coverage. Additionally, the blood-meal analysis approach robustly increased the certainty of identifying vertebrate host blood-meal sources, revealing diverse blood-meal sources that ensured survival malaria vector survived long enough to transmit malaria parasite, threatening the effectiveness of LLIN and facilitating malaria transmission. Further, this study identified *P. falciparum* in malaria mosquito species collected inside houses with LLINs, and in *A. coustani*. Together, these dynamics facilitate and efficient malaria parasite transmission. Moving forward, for a successful vector control intervention, it is necessary to reassess the role of main malaria control approach (LLINs), this should include improvement and/or development of novel vector control strategies.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

This study shows that Mageta, Magare, and Ngodhe islands have a high abundance of primary malaria mosquitoes that harbour *P. falciparum* thereby enabling continued malaria transmission all year round. It also reveals that the effectiveness of main existing method of disrupting malaria parasite transmission, LLINs, is potentially threatened by mixed blood-meals from individual mosquitoes and blood-feeding upon humans during active hours, when humans are unprotected by LLINs, demonstrating opportunistic feeding patterns of malaria vectors with residual malaria transmission. The finding is important in appraising the success of LLINs as main vector control approach in the region.

6.2 Recommendation

In this study, the observed presence of abundant malaria mosquitoes that harbour *Plasmodium* and blood-feeding on diverse sources of blood-meals potentially limiting the effectiveness of LLINs. However, this knowledge alone is limited in achieving an effective vector control approach. Therefore, improvement of knowledge on current and/or development of novel vector control measures and proper application of control approaches remains central to complete eradication of malaria. Towards this end, the study recommends that it is necessary to reassess the use of LLINs, by integrating other measures of malaria control. This should include a regular survey of mosquitoes' behaviour, which potentially changes disease transmission dynamics.

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APPENDICES

Appendix 1: Mapping of malaria mosquitoes

Buildings where adult malaria mosquitoes were trapped were marked using a hand-held geographic positioning system (GPS). Coordinates of mosquitos' collection points were later be integrated to a GIS database using software QGIS 2.14 (Hugentobler, 2008) to quantify spatial heterogeneity in the associated area. Features like water bodies and towns were extracted and digitized from the raster image as feature layers. Raster images of the site were overlaid with feature layers and entomological data in a GIS database. All digital data in the GIS was displayed in the wideband global satcom (WGS) 1984 coordinate system.

Appendix 2: Content of DNeasy blood and tissue extraction Kit

Kit Contents	250 preps
DNeasy mini spin columns (colourless) in 2 mL collection tubes	250
Collection tubes (2 mL)	500
Buffer ATL	50 mL
Buffer AL	54 mL
Buffer AW1 (concentrate)	95 mL
Buffer AW2 (concentrate)	66 mL
Buffer AE	2 @ 60mL
Proteinase K	6 mL

Appendix 3: 1X TAE buffer

Prepare 50X TAE by mixing 242 g of Tris base, 57.1 mL of glacial acetic acid, 100mL of 0.5M EDTA (pH 8.0) and diluting to 1X TAE with PCR water.

Appendix 4: ExoSAP-IT amplicons purification

Mix 5 µl of PCR product with 1 µl of fast P and 0.5 µl of exonuclease I. Mix and incubate at 37 °C for 15 minutes, incubate further at 85 °C for 15 minutes to deactivate the enzyme.

SUPPLEMENTARY DATA

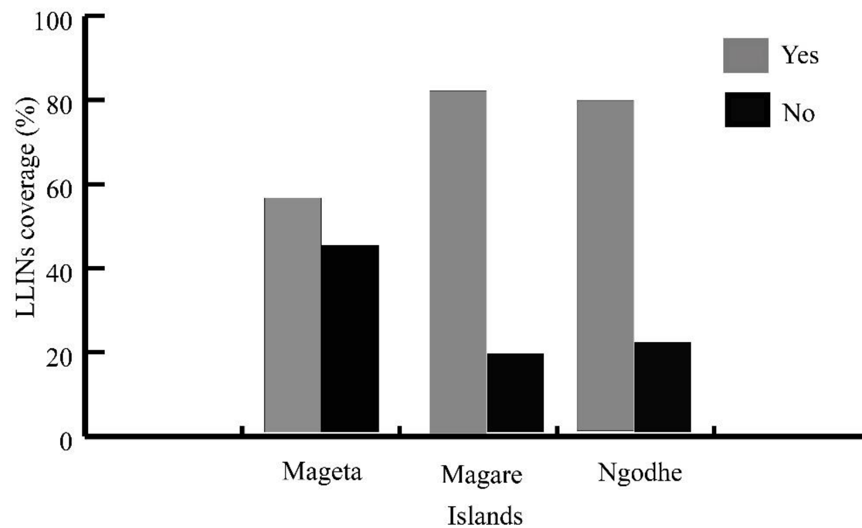

Figure 12| LLINs coverage. The y-axis shows percentage LLINs coverage, while x-axis indicates the study area. Black bars shows LLINs coverage while grey bars shows percentage that is not covered with LLINs.

Table S1: Representative sequence identities and e-values of *A. gambiae* s.s. and *A. Arabiensis* malaria mosquitoes (Accessed December 19, 2015).

Sample	*Anopheles* mosquito	*ITS* (% identity; e-value)
HEG071	*A. gambiae* s.s.	AF470116.1 (98%; 2e-52)
HEG043	*A. gambiae* s.s.	AF470116.1 (100%; 3e-55)
HEG001	*A. arabiensis*	EU091308.1 (100%;7e-59)
HEG003	*A. arabiensis*	AF470110.1 (99%;1e-56)

ITS, means internal transcribed spacer

Table S2: Representative sequence identities and e-values of malaria mosquito blood-meal sources (Accessed December 19, 2015).

Sample	Blood-meal sources	*C01* (% identity; e-value)	*Cyt b* (% identity; *e*-value)
HEG202	Human (*Homo sapiens*)	KT932092 (100%; 0.0)	
HEG058	Human (*Homo sapiens*)		KJ801974 (99%; 2e-152)
HEG380	Human (*Homo sapiens*)	KP635239 (100%: 0.0)	
HEG061	Human (*Homo sapiens*)		KJ801974 (99%; 8e-157)
HEG007	Cattle (*Bos taurus*)	JN817348 (99%; 0.0)	EU365345 (99%; 8e-156)
HEG007	Cattle (*Bos taurus*)	JN817348 (100%; 0.0)	
HEG044	Cattle (*Bos taurus*)		EU365345 (100%; 6e-157)
HEG067	Pig (*Sus scrofa*)	KM101042 (100%; 1e-57)	

CO1, means cytochrome oxidase subunit 1; *cyt b*, means cytochrome b.