EFFECTS OF *PLASMODIUM FALCIPARUM* INFECTED BLOOD ON THE MIDGUT PROTEASES OF *ANOPHELES GAMBIAE S.S*

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A thesis submitted in partial fulfillment of the requirements for the award of the degree of Master of Science in Applied Parasitology in the School of Pure and Applied Sciences of Kenyatta University

KENYATTA UNIVERSITY

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DECLARATION

I, Jackline Milkah Muthoni Mwangi, hereby declare that this research is my original work and has not been presented for a degree in any other University or any other award

JACKLINE MILKAH MUTHONI MWANGI

Signed……Date……

Supervisor's Approval

We confirm that the work reported in this thesis was carried out by the student under our supervision and has been submitted for examination with our approval

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DEDICATION

I dedicate this thesis to my parents, Mr. Paul Mwangi and Mrs. Mary Mwangi, my husband, Mr. Martin Ocholla, son, Michael Imani and to all members of my family who have been extremely supportive and encouraging.

Many thanks and may God bless you all.

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LIST OF ABBREVIATIONS/ACRONYMS

ACUC	Animal Care and Use Committee
ANOVA	Analysis of Variance
CBRD	Centre for Biotechnology Research and Development
CDC	Centre for Disease Control
CO_2	Carbon Dioxide
CS	Circumsporozoite
CTL	Cytotoxic T Lymphocyte
CVR	Centres for Virus Research
DDT	dichlorodiphenyltrichloroethane
DMF	2%*N*-dimethlyformamide
DMSO	Dimethyl Sulfoxide
DOMC	Division of Malaria Control
ESACIPAC	Eastern and Southern Africa Centre of International Parasite Control
HA	Hippuril-arginine
HCL	Hydrochloric Acid
HIV	Human Immunodeficiency Virus
HPA	Hippuril-phenylanine
IFN- γ	Interferon-gamma
IL	Interleukin
iRBCs	Infected Red Blood Cells
IRS	Indoor Residual Spraying
ITNs	Insecticide Treated Nets
KEMRI	Kenya Medical Research Institute
KNMS	Kenya National Malaria Strategy
LLITNs	Long Lasting Insecticide Treated Nets
MHC	Major Histocompatibility Complex
ml	Mililitre
MoH	Ministry of Health
mRNA	Messenger Ribonucleic Acid

MS Excel	Microsoft Office Excel
nm	Nanometer
PM	Peritrophic Membrane
POPs	Persistent Organic Pollutants
RBM	Roll Back Malaria
SOPs	Standard Operating Procedures
SP	Sulphadoxine-pyrimethamine
SPSS	Statistical Package for Social Sciences
SSP2	Sporozoite Surface Protein 2
TDR	Research and Training in Tropical Diseases
WHO	World Health Organization
°C	Degree Celsius

ABSTRACT

Malaria is the leading cause of death in Africa among pregnant women and children under five years. Each year 350-500 million cases of malaria occur worldwide, and over one million people die, most of them young children in sub-Saharan Africa. In Africa, *Anopheles gambiae* mosquito is the main vector of *Plasmodium falciparum*, which causes malaria. All female *Anopheles* mosquitoes are usually haematophagous; they require a blood meal from a vertebrate host, either before or after mating, for egg development. Despite numerous control measures to reduce prevalence of *Plasmodium* transmission, morbidity and mortality rates continue to increase in the tropics and sub-tropics. Control measures have been hindered by the occurrence of insecticide-resistant vectors and drug-resistant malaria parasites; therefore resulting to an urgent need of integrating current strategies with new ones. One effective measure is to block the development and transmission of *Plasmodium* by mosquitoes. Midgut proteases of dipterans are involved in many aspects of the vector-parasite relationship. These proteases are crucial for the parasites' development in the vector. However, they are not fully understood, thus making it difficult to identify a useful transmission blocking mechanism. This study sought to determine the effects of *Plasmodium falciparum* and blood meals on the levels of midgut proteases (trypsin, chymotrypsin and aminopeptidase) as a basis to identify the potential of transmission blocking mechanisms in *Anopheles gambiae.* Replicates of four different groups of female *Anopheles gambiae* were fed on blood containing *Plasmodium falciparum,* human blood alone, hamster blood or 10% glucose in KEMRI, CBRD insectaries. These mosquitoes were then dissected after 6, 12, 18, 24 and 48 hours respectively and their midgut protease levels determined and recorded. The collected data were analyzed using SPSS. Comparisons for replicate test results and the different treatment groups were carried out using analysis of variance (ANOVA). Shapiro Wilk's test was used to check on the normality of the data and Scheffe's test was used for post-hoc testing. The mean differences in overall enzyme trends in the glucose treatment group when compared with those of the human blood and human blood infected with *P. falciparum* treatment groups were significant, thus indicating that human blood and human blood infected with *P. falciparum* alter the level of midgut proteases. Trypsin is essential in protein digestion because in all the blood-fed mosquitoes, trypsin activities peaked at 18-24 hours which coincided with the time taken to digest 80% of the blood meal, unlike in the sugar-fed mosquitoes. When multiple comparisons of chymotrypsin and aminopeptidase activities were made between the human blood treatment group and the human blood infected with *P. falciparum* treatment group with the glucose treatment group, the results showed that the differences were significant thus indicating that human blood and human blood infected with *P. falciparum* alter the levels of chymotrypsin and aminopeptidase in *An. gambiae* mosquitoes. Inhibition of these enzymes in the mosquitoes could lead to transmission blocking mechanisms thus further work needs to be carried out so as to identify the potential of transmission blocking mechanisms in the control of malaria.

CHAPTER ONE: INTRODUCTION

1.1 Background information

Malaria, a potentially fatal vector-borne disease is caused by protozoans of the genus *Plasmodium*. As cited by Budiansky (2002), malaria may result from infection by one of the four species of the genus *Plasmodium* Marchiafava and Celli, namely, *P. falciparum* Welch (1897), *P. malariae* Laveran (1881), *P. vivax* Grassi and Feletti (1890), and *P. ovale* Stephens (1922). *Plasmodium* is transmitted to the human host through the bite of an infected female *Anopheles* mosquito. Other modes of *Plasmodium* transmission are via blood transfusion and through transovarial transmission (Murphy and Breman, 2001).

Individuals with malaria often experience fever, chills, and flu-like illness. Most of the morbidity and mortality cases associated with malaria are caused by the rupture of infected red blood cells (iRBCs) during the asexual reproductive stages of the parasite. Intense fever, occurring in 24-72 hour intervals is accompanied by nausea, headache and muscular pain, retinal damage among other symptoms (Beare *et al.*, 2006). If individuals are left untreated, they may develop severe complications and die. A variety of the potentially fatal symptoms, including liver failure, renal failure and cerebral disease are associated with untreated *P. falciparum* (Murphy and Breman, 2001).

1.2 Impact of malaria

Malaria is a devastating disease, producing nearly 600 million new infections and over one million deaths each year, mostly in young children and pregnant women in sub-Saharan Africa (WHO, 2002; Snow *et al*., 2005). The burden of this disease is heaviest among children below the age of five years in sub-Saharan Africa (Gardner, 2002;

Greenwood *et al.*, 2008). Approximately 40% of the world's population lives in regions where malaria transmission is endemic, mainly tropical and sub-tropical regions (Aultman *et al.*, 2002). Malaria affects a large population in Asia, Africa, south and central America (Figure 1.1), with *Plasmodium falciparum* accounting for the preponderance of morbidity and mortality globally (WHO/RBM, 2001; Bozdech *et al.*, 2003; Snow *et al.*, 2005). The epidemiology of malaria intensity and patterns of transmission are primarily a function of the seasonality, abundance and feeding habits of the mosquito vector (Greenwood *et al.* 2008). *Anopheles gambiae* is the most effective vector of *Plasmodium falciparum* in Africa (Coluzzi, 1992; Collins and Besansky, 1994; Mbogo *et al.*, 2003).

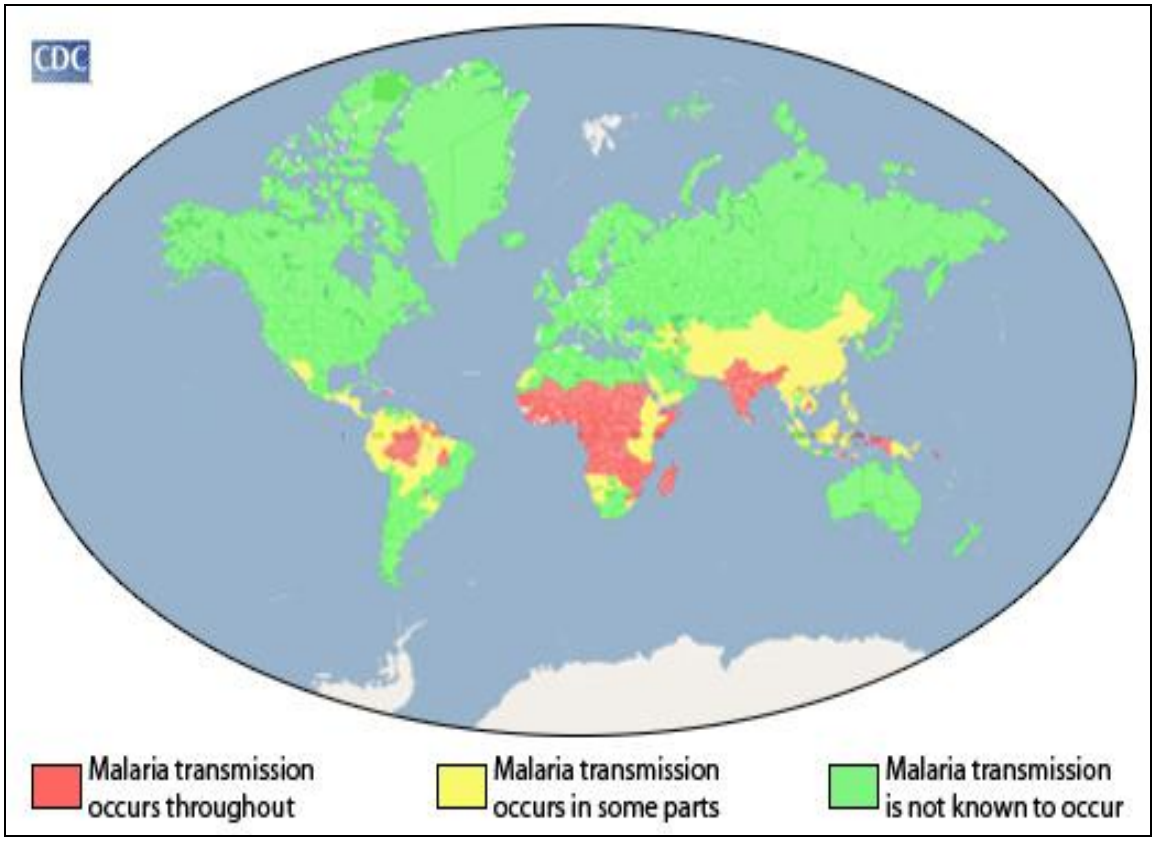

Figure 1.1: Geographic distribution of malaria (Adapted from CDC's Division of Parasitic Diseases, 2010)

The first record of malaria treatment dates 1600 A.D. in Peru; it utilized the quinine-rich bark of the Cinchona tree (Bwire, 2006). Ross and Grassi cited by Good (2001) recognized the mosquito as the malaria vector in 1897. However, despite the enormous and diverse efforts to control malaria, this disease is among the top three most deadly communicable diseases and the most deadly tropical parasitic disease (Sachs and Malaney, 2002). Management strategies today include the development of vaccines and chemotherapeutic agents, vector control, insecticides, awareness, bed nets and the insecticide treated bed nets. Combination therapy has been shown to increase the efficacy of combining drugs (Toure and Oduola, 2004). The number of cases of malaria and their severity has increased in Africa, leading to an increase in the social and economic burden of the disease (Greenwood and Mutabingwa, 2002; Sachs and Malaney, 2002). Due to the occurrence of resistance to the commonly used insecticides in mosquitoes and to chemotherapeutic agents in *Plasmodium*, other strategies are urgently required (Dijk, 1997).

1.3 Statement of the problem and justification

Malaria remains the leading cause of morbidity and mortality in sub-Saharan Africa despite the availability of control measures (WHO/RBM, 2001). More than 2.6 billion people in the world are at risk of malaria infection and an estimated 5 billion episodes of clinical cases occur each year, 90% in Africa south of the Sahara (WHO, 1996; Snow *et al*., 2005).

Current malaria control strategies primarily targeting the mosquito vector and infections in humans are unsuccessful because mosquitoes have developed resistance to insecticides

while the malaria parasites have developed resistance to the most affordable and effective antimalarial drugs (Dijk, 1997; White, 2008). For successful control of malaria, integrated strategies such as use of Indoor Residual Spraying (IRS) with insecticides, personal protection measures such as ITNs, larval control and environmental management are required.

Mosquitoes are a key element in the transmission of malaria parasites and thus an important target in the control of malaria. The malaria parasite must develop in the midgut of the mosquito before it can be transmitted to the next host (Beier, 1998; Aly *et al.*, 2008). Therefore, interfering with or blocking the development of the parasite in the mosquito midgut is a potential strategy that can be used in the fight against malaria.

Transmission Blocking Vaccines (TBV) against malaria are intended to induce immunity against gametocytes so that TBV-immunized individuals cannot transmit malaria. The effect of TBV-induced immunity is to prevent the fertilization or the subsequent development of malaria parasites in the mosquito midgut. As a consequence, the formation of oocysts and ultimately of infective sporozoites in the mosquito salivary glands is prevented or reduced. As malarial infections are transmitted mainly within a few hundreds of meters from an infectious human source, TBVs used within a community would protect the immediate neighbourhood of the vaccinated individuals (Carter, 2001). A transmission blocking vaccine could move malaria transmission rates towards the range in which eradication could be possible with the deployment of other interventions. However, a useful transmission-blocking strategy is not yet available

because molecules such as proteases that are crucial for malaria parasite development in the vector are not yet fully understood.

The mosquito digestive system contains several enzymes, including a complex set of endoproteases and exoproteases (Clements, 1992). Several studies have documented the detrimental effect of midgut proteases on parasite development (Gass and Yeates, 1979; Shahabuddin *et al.*, 1996, 1998; Kaplan *et al.*, 2001). It is possible that the mechanisms by which the enzymes destroy the parasites might be useful in the attempts to design new transmission blocking strategies. This study evaluated the action of *Plasmodium falciparum* and blood meals on the midgut proteases of *Anopheles gambiae s.s* in order to understand the mechanisms that can be used for transmission blocking. In trying to understand the mechanism by which the enzymes are detrimental to the developing parasites, there is need to understand the changes that take place within the mosquito midgut before and after blood meal ingestion and parasite infection.

Since the blood meal ingested by the mosquito contains different components, it is possible that they might alter the midgut environment and influence the enzyme dynamics (Ribeiro, 1987; Beier *et al.*, 1991; Luo *et al.*, 2000).

1.4 Research questions

a) What are the effects of a human blood meal on midgut proteases in *Anopheles gambiae*?
b) What are the effects of *Plasmodium falciparum* infected blood on midgut proteases in *Anopheles gambiae*?

1.5 Null hypotheses

a) Human blood meal in *Anopheles gambiae s.s* does not alter the levels of midgut proteases.

b) Blood infected with *Plasmodium falciparum* in *Anopheles gambiae* s.s does not alter the levels of midgut proteases.

1.6 Objectives

1.6.1 General objective

To determine the effects of human blood infected with *Plasmodium falciparum* on the levels of midgut proteases in *Anopheles gambiae.*

1.6.2 Specific objectives

a) To determine the effects of a human blood meal on the levels of midgut proteases (trypsin, chymotrypsin and aminopeptidase) of the malaria transmitting mosquito, *Anopheles gambiae*.

b) To determine the effects of *Plasmodium falciparum* infected blood meal on the levels of midgut proteases (trypsin, chymotrypsin and aminopeptidase) of *Anopheles gambiae.*

CHAPTER TWO: LITERATURE REVIEW

2.1 Malaria parasite

Malaria is caused by a protozoon of the phylum *Apicomplexa* (often referred to as sporozoans) and the genus *Plasmodium.* Species of *Plasmodium* that cause malaria are *P. falciparum, P. malariae, P. vivax* and *P. ovale,* where *Plasmodium* exists both in vertebrate hosts and mosquito vectors (Mueller *et al.*, 2007). A fifth species, *Plasmodium knowlesi*, is a zoonosis that causes malaria in monkeys but can also infect humans (Fong *et al.*, 1971; Singh *et al.*, 2004). *P. knowlesi* is an emerging infection that was reported for the first time in humans in 1965 (Chin *et al.*, 1965). It accounts for up to 70% of malaria cases in certain areas in South East Asia where it is mostly found (McCutchan *et al.*, 2008).

Characteristics of *Plasmodium* can influence the occurrence of malaria and its impact on human populations. Areas where *P. falciparum* predominates (such as in sub-saharan Africa) will suffer more disease and death than areas where other species predominate (Rowe *et al.*, 2006). *P. vivax* and *P. ovale* have stages ("hypnozoites") that can remain dormant in the liver cells for extended periods of time (months to years) before reactivating and invading the blood (Cogswell, 1992). This results in the resumption of transmission after apparently successful control efforts, or introduction of malaria in an area that was malaria-free. *Plasmodium falciparum* (and to a lesser extent *P. vivax*) have developed strains that are resistant to antimalarial drugs such as chloroquine and Sulphadoxine-pyrimethamine (SP) (Trigg, 1998; White, 2008). Constant monitoring of the susceptibility of these parasite species to drugs which are used is critical to ensure effective treatment and successful control efforts.

2.2 The Malaria vector

The female *Anopheles* mosquito ingests the gametocytes during a blood meal, thus enabling further development of *Plasmodium*. Various species have been found to be the vectors in different parts of the world. *A. gambiae* complex; *Anopheles gambiae sensu stricto* (hereafter *An. gambiae*), *Anopheles arabiensis* and *Anopheles funestus* transmit most of the human malaria and are all found in Africa (Besansky *et al.,* 2004). *Anopheles gambiae* (Figure 2.1), the most famous and significant of these three species, is one of the sixty Anopheline mosquitoes which are able to transmit malaria to humans (Budiansky, 2002).

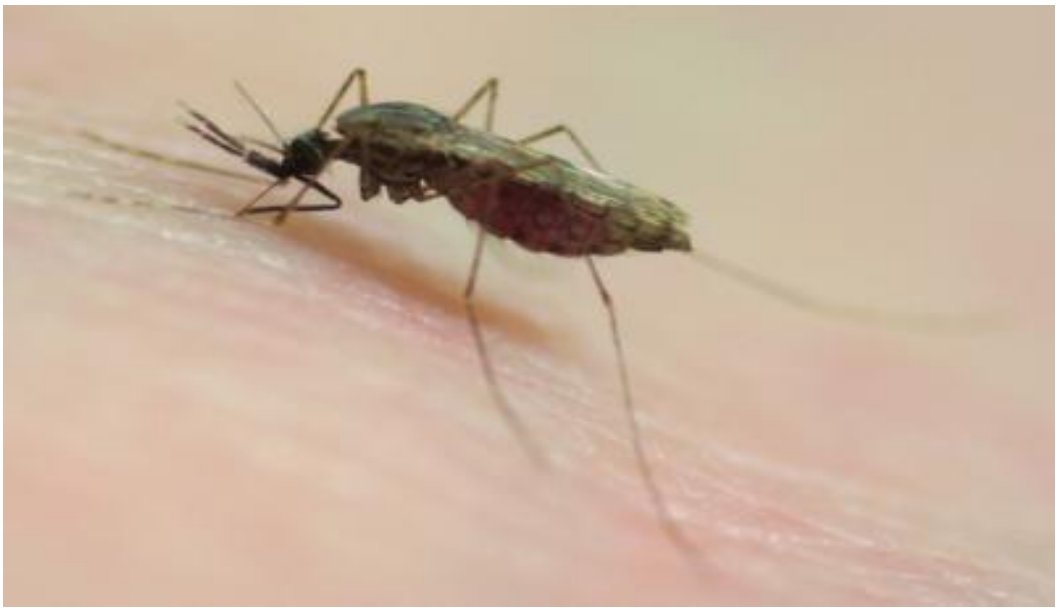

Figure 2.1 *Anopheles gambiae* (Photograph by Inserm/E.Begouen)

Anopheles freeborni was found to transmit malaria in N. America (Vargas, 1950). Nearly 45 species of the mosquito have been found in India where *A. culicifacies, A. fluviatilis, A. minimus, A. philippinensis, A. stephensi, A. sundaicus,* and *A. leucosphyrus* have been implicated in the transmission of malaria (Subbarao *et al.,* 1988). The areas of

distribution are different for these mosquitoes: *A. fluviatilis and A. minimus* are found in the foot-hill regions; *A. stephensi and A. sundaicus* are found in the coastal regions while *A. culicifacies* and *A. philippinensis* are found in the plains (Shukla *et al.*, 2007). Species like *A. stephensi* are highly adaptable and are found to be very potent vectors of human malaria.

2.3 Current vector control strategies

Mosquitoes are a key element in the transmission of *Plasmodium*. The fact that *Plasmodium* must develop in the midgut of the mosquito before it can be transmitted to the next host makes the mosquito an important target in the control of malaria. Efforts to eradicate malaria by eliminating mosquitoes have been successful in some areas such as United States and southern Europe (Brown *et al.*, 1976; Roberts *et al.*, 1997). One method which is used in killing adult mosquitoes in houses is spraying the inside surfaces of the walls and roofs or ceilings with a residual insecticide (IRS). The first insecticide which was used for IRS was DDT (**d**ichloro**d**iphenyl**t**richloroethane) (Kouznetsov, 1977). Although it was initially used exclusively to combat malaria, its use quickly spread to agriculture. In time, pest-control, rather than disease-control came to dominate DDT use; this large-scale agricultural use led to the evolution of resistant mosquitoes in many regions (Roberts and Andre, 1994). In the southern part of the US, by 1951, mosquitoes had been eliminated by use of the pesticide DDT. However, its use was banned due to its effects on the ecosystem. During the 1960s, the awareness of the negative consequences of its indiscriminate use increased, ultimately leading to bans on agricultural applications of DDT in many countries in the 1970s (Roberts *et al.*, 1997).

The consequent insecticidal residues in crops at levels unacceptable for the export trade have been an important factor in recent bans of DDT for malaria control in several tropical countries (Curtis, 1994). It has already been replaced by organophosphate or carbamate insecticides such as malathion or bendiocarb where its resistance has been detected, for instance, in Sri Lanka, parts of India, Pakistan, Turkey and Central America. However, these compounds are considerably more expensive to use than DDT, and malathion does not persist well on mud walls.

The World Health Organization (WHO) advises the use of 12 different insecticides in IRS operations. These include DDT and a series of alternative insecticides such as the pyrethroids permethrin and deltamethrin, to combat malaria in areas where mosquitoes are DDT-resistant and to slow the evolution of resistance (WHO, 2006). Public health use of small amounts of DDT is permitted under the Stockholm Convention on Persistent Organic Pollutants (POPs), which prohibits the agricultural use of DDT. However, many developed countries discourage DDT use even in small quantities. Indoor residual spraying affects endophilic mosquito species only (species that tend to rest and live indoors), leading their descendants to shift to exophilic behaviors (species that tend to rest and live out of doors). This means that they are less affected by the IRS, rendering it somewhat useless as a defense mechanism (Pates and Curtis, 2005).

Pyrethroids such as deltamethrin and lambdacyhalothrin are effective at lower doses than DDT (25 mg/sq. metre compared with 2 gm/sq. metre). Although more expensive per unit weight, these pyrethroids are not much more expensive per house protected per year (Curtis, 1994). Pyrethroids have also been used in the impregnation of insecticide treated

nets (ITNs) and long lasting ITNs (LLITNs), to reduce mosquito bites against humans (Curtis, 1991). Nets have long been appreciated for protection against night biting mosquitoes including malaria vectors. The insecticide-treated nets (ITN) are estimated to be twice as effective as the untreated nets and provide greater than 70% protection compared with the cases where nets were not used (Bachou *et al*, 2006).

In the world's largest treated bednet program in Sichuan Province, China, up to 2.25 million bed nets have been treated annually by spraying deltamethrin (Chen *et al.,* 1995). This has been associated with a remarkable decline in the already relatively low level of malaria due to *P. vivax*, which is the less serious of the two main species causing human malaria. It is a cause for concern that large scale use of pyrethroid impregnated nets may select for pyrethroid resistance of a physiological kind or may change mosquito behavior so that they bite outdoors before people go indoors.

Other methods tried in controlling mosquitoes include draining or filling breeding sites permanently as applied in industrialized areas in India or the breeding sites may be stocked with larvivorous fish (Dua *et al.,* 1988). The bacterial toxin from *Bacillus thuringiensis* var. *israelensis* (*Bti*) and *B. sphaericus (Bs)* can be sprayed into breeding sites as a highly specific agent against mosquito larvae (Shukla *et al.*, 1997; Mwangangi, 2011). Unfortunately, the toxin is not self-propagating or long lasting in natural breeding sites and frequent re-treatment is unaffordable in most low income countries where the malaria problem exists.

There is considerable interest among biologists, including molecular biologists, in rendering mosquito populations genetically harmless by introduction of genes which make them non-susceptible to *Plasmodium* or divert them from being strongly attracted to biting humans to biting animals (Collins and Paskewitz, 1995). The real problem with these concepts is not so much producing harmless strains, but propagating their genes extensively. Thus if desirable genes are to be spread in *Anopheles* populations, genetic systems will have to be developed which will reliably cause genes to spread from a small "seeding" of a wild population.

Sterile insect technique is emerging as a potential mosquito control method (Klassen and Curtis, 2005). Progress towards transgenic, or genetically modified insects suggest that wild mosquito populations could be made malaria-resistant (Ito *et al.,* 2002). Researchers at Imperial College London created the world's first transgenic malaria mosquito, with the first plasmodium-resistant species announced by a team at Case Western Reserve University in Ohio in 2002 (Ito *et al.*, 2002). However, this approach contains many difficulties and success is a distant prospect.

Vaccines for malaria are under development, with no completely effective vaccine yet available (Hill, 2011). It has been determined that an individual can be protected from a *P. falciparum* infection if they receive over 1,000 bites from infected, irradiated mosquitoes (Hoffman *et al.,* 2002). The front runner in the race to bring an effective malaria vaccine into widespread use is GlaxoSmithKline’s RTS,S where 12,000 children have already been enrolled in the Phase III trials in Burkina Faso, Gabon, Ghana, Kenya,

Malawi, Mozambique and Tanzania. However, this vaccine has been found to be 53% effective and if successful, it may only be implemented in 2015-2017 (Bejon *et al*, 2008).

It is hoped that knowledge of the *P. falciparum* genome, the sequencing of which was completed in 2002 will provide targets for new drugs or vaccines (Gardner *et al*., 2002). However, these efforts have so far failed to eradicate malaria in many parts of the developing world and the disease is most prevalent in Africa. Other potential strategies such as blocking development of the *Plasmodium falciparum* in *Anopheles* mosquito by regulating midgut proteases could be integrated with the existing strategies so as to decrease malaria infections.

2.4 Malaria in Kenya

In Kenya, malaria is the leading cause of morbidity and mortality, accounting for 30-50% of the outpatient attendance and 20% of all admissions to health facilities. Thus, an estimated 170 million working days are lost to the disease each year (MOH, 2001; 2006). At least 14,000 children are hospitalized annually for malaria, and there are an estimated 34,000 deaths among children under-five each year, that is estimated to be about 20% of all deaths in children under five (MOH, 2006). Annually, an estimated six thousand pregnant women suffer from malaria-associated anemia, and four thousand babies are born with low birth weights as a result of maternal anemia.

Malaria is widespread in the Coast, most of Nyanza, Western and parts of Eastern, North Eastern, Central and Rift Valley Provinces in Kenya. Currently, malaria outbreaks are being reported in some locations that had been previously thought to be at elevations too

high for malaria transmission, such as the highlands of Kenya (Malakooti *et al.*, 1998). All four species of malaria parasites infecting man are found in Kenya. The most prevalent species is *Plasmodium falciparum*, which usually accounts for more than 80% of malaria infections in endemic areas (WHO/RBM, 2003; Janovy and Roberts, 2004). The principal vectors of the malaria parasites in Kenya are members of the *Anopheles gambiae* complex and *An. funestus* (White, 1989). The species of *An. gambiae* complex which are found in Kenya are *An. gambiae sensu stricto*, *An. arabiensis*, which are usually predominant during and after the rains and *An. merus*, which is mainly restricted to the coastal strip. *An. funestus* mosquitoes exist in low densities throughout the year (Coluzzi, 1984).

2.4.1 Malaria epidemiological zones

Kenya has four malaria epidemiological zones:

Endemic epidemiological zone comprises of areas of stable malaria with altitudes ranging from 0 to 1300 meters around Lake Victoria in western Kenya and in the coastal regions. These are endemic areas along the shores of Lake Victoria and the south coast where malaria transmission is perennial but peaks from June to August and again in late November (KMIS, 2010). Rainfall, temperature and humidity are the determinants of the perennial transmission of malaria. The vector's life cycle is usually short with high survival rate due to the suitable climatic conditions. Transmission is intense throughout the year with annual entomological inoculation rates between 30 and 100.

Seasonal malaria transmission epidemiological zones occur in the arid and semi-arid areas of northern and south-eastern parts of the country which are sparsely populated

(Figure 2.2). They experience short periods of intense malaria transmission during the rainfall seasons (KMIS, 2010). Temperatures are usually high and water pools created during the rainy season provide the malaria vectors breeding sites. Extreme climatic conditions lead to flooding in these areas, resulting to epidemic outbreaks with high morbidity rates due to low immune status of the population.

Malaria epidemic prone areas of western highlands of Kenya. Malaria transmission in the western highlands of Kenya above 2,000 meters above sea level is seasonal, with considerable year-to-year variations. The epidemic phenomenon is experienced when climatic conditions favour the sustainability of minimum temperatures around 18^{o}C. Increase in minimum temperatures during the long rains period favours and sustains vector breeding, resulting in increased intensity of malaria transmission. The whole population is vulnerable and fatality rates during an epidemic can be up to ten times greater than what is experienced in regions where malaria occurs regularly (KMIS, 2010).

Low risk malaria zone covers the central highlands of Kenya, including Nairobi. The temperatures are usually too low to allow completion of the sporogonic cycle of the malaria parasite in the vector. However, increasing temperatures and changes in the hydrological cycle associated with climate change are likely to increase the areas suitable for malaria vector breeding, with introduction of malaria transmission in areas it never existed before (KMIS, 2010).

Transmission in the epidemic-prone/seasonal areas is highest from April through June.

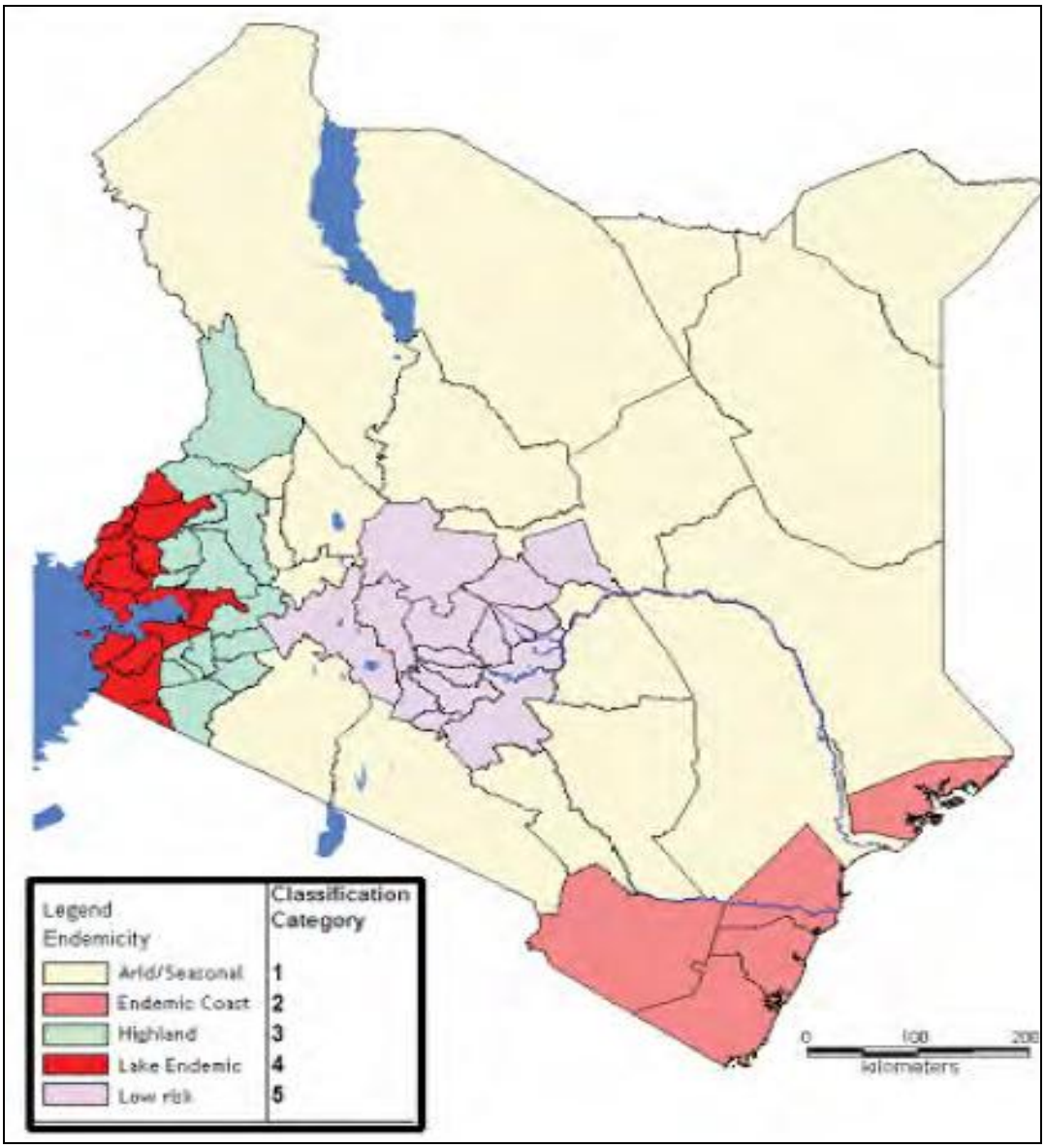

Figure 2.2: Malaria zones in Kenya (Adapted from Kenya Malaria Indicator Survey, 2010)

2.4.2 Malaria interventions

In collaboration with partners, the government has developed the 10-year Kenya National Malaria Strategy which was launched on 4th November 2009 (KNMS, 2009). The goal of the National Malaria Strategy (NMS) is to reduce morbidity and mortality associated with

malaria by 30% by 2012 and to maintain it to 2017. Implementation of the National Malaria Strategy is spearheaded by the Ministry of Health and coordinated by the Division of Malaria Control.

The Division of Malaria Control (DOMC), with support from various partners has been monitoring the burden of malaria through several channels. This include health information data from hospitals and clinics, sentinel site surveys of communities and health facilities in four districts, and national surveys including the Kenya Demographic Health Survey. Evidence from these sources now points towards increased coverage of interventions with a downward trend in disease burden demonstrated by community reported cases of malaria, hospital admissions and deaths due to malaria and childhood deaths from the disease. There is a 44% reduction rate in childhood mortality (MOH, 2006).

The scaling up of malaria control interventions, notably distribution of ITNs has greatly contributed towards reversing the declining health trends in the country specifically between 2002-2007 (KDHS, 2009). Over the period of the operationalization of the NMS 2001-2010, the division has made key achievements in case management such as successful roll-out of the new treatment policy that was launched by the Kenyan Head of State in September 2006 where artemether-lumefantrine combination was recommended as a first line treatment.

Indoor residual spraying with insecticides has been used to prevent the occurrence of malaria epidemics in the western highlands. The proportion of the targeted structures sprayed rose from 27.1% in 2005 to 63% in 2008 (KDHS, 2009).

2.5 Life cycle of Plasmodium

An infected female *Anopheles* mosquito transmits malaria during blood meal ingestion from a human host. Female mosquitoes have an absolute requirement to feed at least twice on blood of a vertebrate organism in order to obtain the nutrients required for oogenesis (Clements, 1963). The blood meal provides the nutrients necessary to support the biosynthetic burst associated with the process of vitellogenesis (Clements, 1963). These mosquitoes deposit saliva containing the infective stage of *Plasmodium* sporozoites onto the host's bloodstream (Rosenberg *et al*., 1990; Matsuoka *et al*., 2002; Frischknecht *et al*., 2004).

Together with the blood, mosquitoes may ingest several types of organisms such as *Plasmodium* species, *filariae* and some arboviruses which have evolved to exploit the feeding behavior of *Anopheles gambiae* mosquitoes, to propagate themselves from one organism to another (White, 1974).

Once the mosquito deposits the infective stage of *Plasmodium* sporozoites into the host, the sporozoites move to the liver. In the liver of the vertebrate host, in 1 to 2 weeks, a single sporozoite can give rise to 30,000 merozoites (Kyes *et al.,* 2001). During this pre-erythrocytic stage, no illness is induced by malaria. Asexual amplification occurs with about 6-32 merozoites being produced within one erythrocyte (Kyes *et al.,* 2001). When the erythrocytic schizont ruptures, the merozoites spill into blood; it is during this phase that malaria-associated morbidity and mortality occurs. The merozoites continue in a repeated cycle of infecting erythrocytes, multiplying, and bursting the erythrocytes

(Figure 2.3). During this repeated cycle, some merozoites differentiate into male and female gametocytes. The mosquito vector ingests the gametocytes during a blood meal thus enabling further development of *Plasmodium* (Kyes *et al.*, 2001).

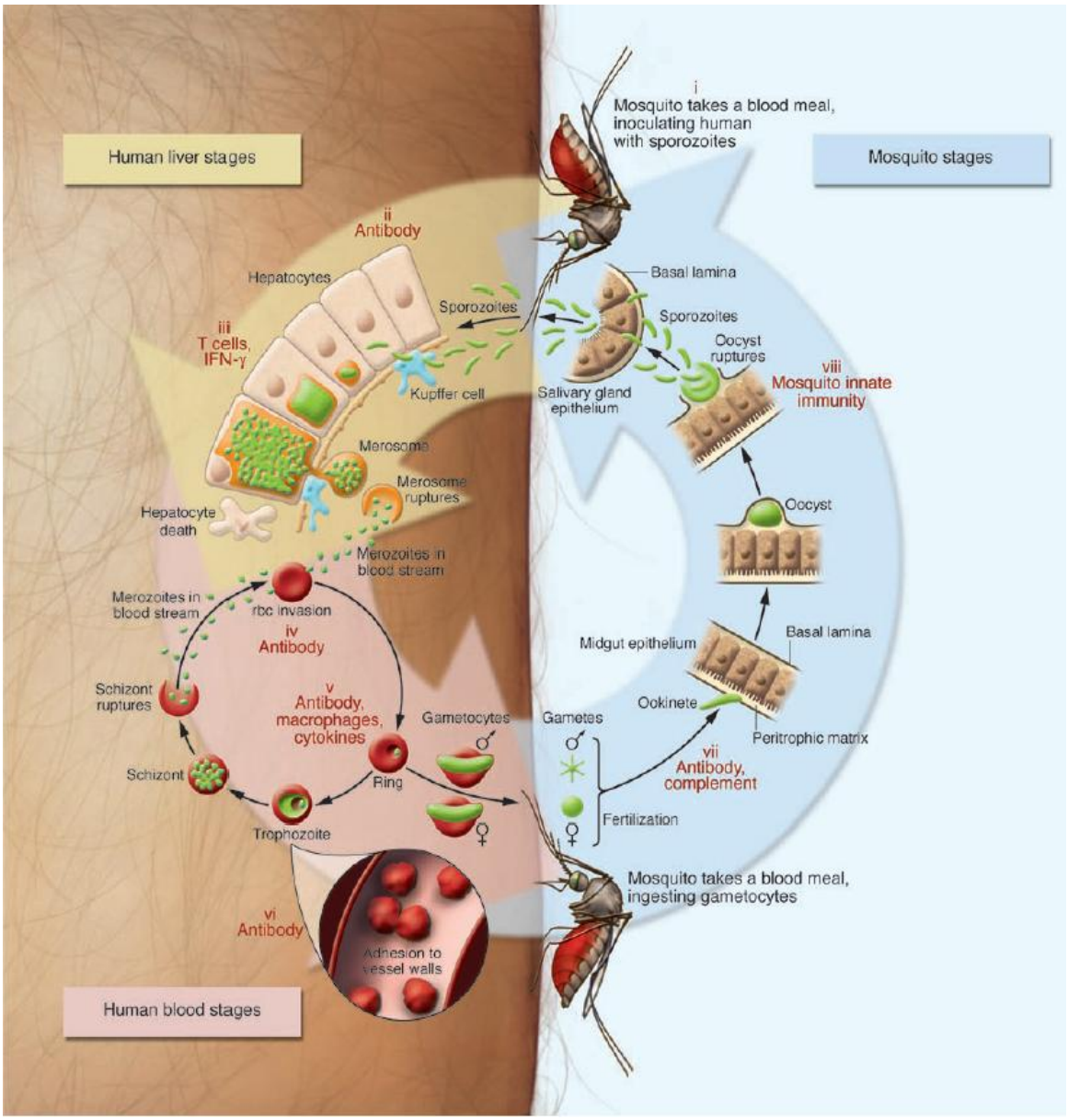

Figure 2.3: Life cycle of *Plasmodium falciparum* (Adapted from Greenwood *et al.*, *2008*)

After blood ingestion by female mosquitoes, *Plasmodium* gametocytes emerge from erythrocytes in the mosquito midgut, where a hostile environment rich in hydrolytic enzymes surrounds them. To survive, the parasites must have defense systems against a variety of digestive enzymes present in the mosquito gut, including proteases. The sporogonic development of *Plasmodium* from gamete to oocyst formation, takes place in the lumen and epithelium of the mosquito midgut (Shahabuddin and Kaslow, 1994a). The outcome of this sporogonic development is most likely determined by the mosquito-specific factors.

In the protease-rich milieu, malaria parasites undergo complex changes that include fertilization, differentiation into a motile ookinete, and about 1 day later, penetration of the midgut epithelium where they divide within oocysts on the external gut wall to form thousands of sporozoites (Shahabuddin and Kaslow, 1994a). At this time, the blood meal is completely surrounded by a thick chitin containing peritrophic matrix (PM). Thus the peritrophic membrane is a potential barrier for *Plasmodium* development. The infective sporozoites are released into the mosquito haemocoele and move to the salivary gland, where they await injection into another human host, thus completing the life cycle.

2.6 Mid-gut proteases

Some mosquito factors are essential for parasite development while others, initiated by blood feeding or the parasite reduce parasite survival (Billker *et al*., 1998; Shahabuddin *et al*., 1998). Mosquito digestive enzymes can affect vector competence; serine proteases are essential mediators of several physiological processes within arthropods. Proteases

are involved in processes such as digestion of food within the midgut, activation of the phenoloxidase cascade leading to melanization of invading microorganisms, detoxification of noxious agents such as insecticides and modulation of embroyonic development (Billingsley and Hecker, 1991; Crews-Oyen *et al.*, 1993).

Once a mosquito ingests a blood meal, proteolytic activity is stimulated and most of the meal is digested within 24 hours (Jahan *et al.*, 1999; Lemos *et al.*, 1996). Previous *in vitro* experiments indicate that *Plasmodium* parasite is not fully able to activate its own chitinase activity (Shahabuddin *et al.,* 1996). To transverse the peritrophic membrane, ookinetes secrete a prochitinase that can be activated by exogenous proteases (Shahabuddin *et al*., 1993). Trypsin produced in the mosquito's digestive tract probably activates parasites' chitinase, facilitating the passage of the parasite through the peritrophic matrix surrounding the parasite-containing blood meal in the mosquito (Shahabuddin and Kaslow, 1994b; Shahabuddin *et al*., 1996). Mosquito midgut trypsin was found to activate *Plasmodium gallinaceum* chitinase thereby ensuring invasion of the peritrophic matrix (Shahabuddin *et al*., 1996). Trypsin also plays a key role in blood meal digestion in mosquitoes (Lehane *et al*., 1996). In sand flies, trypsin in *Phlebotomus papatasi* have been shown to be potential barriers to *Leishmania major*'s growth and development within the midgut (Pimenta *et al*., 1997; Schlein and Jacobson, 1998).

The antibody-mediated inhibition of *Aedes aegypti* midgut trypsins was found to block sporogonic development of *Plasmodium gallinaceum,* therefore indicating that it could work as a transmission blocking mechanism (Shahabuddin *et al.,* 1996). Initial work by

Shahabuddin *et al.* (1993) indicated that inhibition of parasite chitinase blocked further development of oocyst *in vivo* while an addition of trypsin-like protease from the mosquito midgut increased its enzymatic activity.

Chymotrypsins are also known to play a role in digestion in insects (Terra and Ferreira, 1994). In sand flies, chymotrypsin in *Phlebotomus papatasi* has been shown to be a potential barrier to *Leishmania major*'s growth and development within the midgut (Pimenta *et al*., 1997; Schlein and Jacobson, 1998).

Graf and Briegel (1982) reported aminopeptidase activity in the mosquito. Carboxypeptidase A genes have been previously cloned from two haematophagous insects: the black fly, *Simulium vittatum* (Ramos *et al.*, 1993) and *Anopheles gambiae* (Edwards *et al*., 1997). This indicates that other than trypsin, other exopeptidases exist in the mosquito. In both insects, carboxypeptidase A messenger RNA (mRNA) expression is induced by a blood meal. However, the temporal dynamics of this induction differ remarkably between the two insects (Edwards *et al.*, 2000).

Previous research indicated that immature *Plasmodium gallinaceum* ookinetes and zygotes are susceptible to protease digestion in *Aedes aegypti*, while mature forms are relatively resistant (Gass and Yeates, 1979), although some studies have cast doubts over their roles in parasite mortality in the midgut (Chege *et al*., 1996; Kaplan *et al*., 2001). Mosquito saliva also contains glycosidase and other enzymes (Clements, 1992) that may enhance the digestive processes in the midgut.

Proteases also deactivate complement and macrophages in the mid gut, both of which kill parasites (Grotendorst and Carter, 1987) and digest hemoglobin, which is required for ookinete maturation. Midgut proteases can also enhance infection by activating parasite prochitinase to chitinase (Shahabuddin, 1998) or reduce infection when elevated in certain strains (Feldmann *et al*., 1990). Thus, the post-feeding kinetics of enzyme activity affects parasite development and invasion success at a number of levels (Billingsley and Sinden, 1997).

Controlling *Plasmodium* transmission by the mosquito could reduce malaria incidence. A deeper understanding of physiological processes in the mosquito, such as blood meal digestion could contribute to an alternative vector control strategy such as blocking the development of *Plasmodium* in *Anopheles* mosquitoes. No studies have been carried out to quantify midgut proteases in *Anopheles gambiae,* in the presence of *Plasmodium falciparum* though a lot of molecular work has been done on these proteases. Studies to identify differential gene expression between blood fed versus sugar fed mosquitoes have been done (Ribeiro, 2003; Dana *et al.*, 2005) as well as studies on differential gene expression of mosquitoes infected with *Plasmodium* (Dana *et al*., 2005).

This information is very important in developing transmission blocking vaccines in malaria control. Therefore, there is need to identify the effects caused by *Plasmodium falciparum* and blood meal on the midgut proteases in *Anopheles gambiae* s.s mosquitoes as a source of information for the commencement of transmission blocking studies.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Experimental animals

3.1.1 Mosquitoes

Anopheles gambiae s.s mosquitoes (MBITA Strain) were maintained in the insectary at the Centre for Biotechnology Research and Development (CBRD), KEMRI, Nairobi. Mosquito rearing procedures followed standard protocols as described by Benedict (1997). Larvae were reared in 20cm x 15cm rectangular pans and fed on 10% slurry of parts 2:1 of Tetramin E® (Tetra) fish food and yeast. Pupae were collected and kept in 30cm x 30cm x 30cm meshed cages where emerging adults had access to sterile 6% glucose and water.

3.1.2 Syrian hamsters

Syrian hamsters (*Mesocricetus auratus*) were used for feeding mosquitoes. The hamsters were bred and maintained at KEMRI's animal house facility under standard hygienic conditions. The experiments were carried out in compliance with the Animal Care and Use Committee (ACUC) guidelines of KEMRI. Standard operating procedures (SOPs) available at the CBRD laboratories included restraining the hamsters in specially designed cages during mosquito feeding and killing them by using CO_2 or by cervical exsanguination. These killed animals were sterilized by dipping into 70% ethanol and then disposed in appropriate biohazard bags before transfer to the incinerator at KEMRI.

3.2 *Plasmodium falciparum* culture

Parasites of *P. falciparum* (NF54 strain) were maintained at CBRD, KEMRI, Nairobi to generate gametocytes. Gametocytes were grown in cultures at 0.3% parasitemia in 6% heamatocrit of blood group O erythrocytes in 25cm^3 flasks. The flasks were aerated with a gas mixture of 92% Nitrogen, 5% Oxygen and 3% Carbon dioxide and incubated at 37.8 ^{0}C for 3-5 days. Culture media was changed daily. The parasitemia of the cultures was monitored by microscopic examination of Giemsa-stained thin films. When the culture parasitemia reached 5-6%, fresh red blood cells and media were added so that the parasitemia changed to 0.5% at 6% heamatocrit. 5ml of the suspension was dispensed into 25cm^3 flasks, incubated at 37.8 ^{0}C and the culture medium changed for 3 days.

3.3 Determining the effects of a human blood meal on the levels of midgut proteases in *Anopheles gambiae*

Three groups of the laboratory-bred 5-7 day old female mosquitoes were artificially fed with either 10% glucose, human blood only or with hamster blood only. Fifty female mosquitoes were fed per group. In each group, 4 female mosquitoes were dissected after 6, 12, 18, 24 and 48 hours respectively and the midguts were isolated from mosquitoes by dissection on ice. The midguts were put in 1ml vials containing physiological saline which were stored at -70 ^{0}C until use. Stored midguts were then homogenized. The protease levels were determined and recorded as described by Bradford (1976) and Ortego *et al.* (1996).

3.4 Determining the effects of *Plasmodium falciparum* infected blood meal on the midgut proteases of *Anopheles gambiae*

Two groups of laboratory-bred 5-7 day old female mosquitoes were artificially fed with either human blood only or human blood containing 15 to 17 day old cultured gametocytes (0.8% gametocytes). Fifty female mosquitoes were fed per group. In each group, 4 female mosquitoes were dissected after 6, 12, 18, 24 and 48 hours respectively and the midguts were isolated from mosquitoes by dissection on ice. The midguts were put in 1ml vials containing physiological saline and stored at -70 ^{0}C until use. Stored midguts were then homogenized. The protease levels were determined and recorded as described by Bradford (1976) and Ortego *et al.* (1996).

3.5 Midgut protease assays

For trypsin assays, *N*-benzoyl-DL-arginine-*p*-nitroanilide (BapNA) was dissolved in dimethylfluoride (DMF) while synthetic substrates of chymotrypsin (*N*-succinyl-ala-ala-*0*-nitroanilide) and aminopeptidase (L-leucine *p*-nitroanilide) were dissolved in dimethyl sulfoxide (DMSO) as described by Ortego *et al.* (1996). Substrate buffers contained 2 mM substrate, 7% DMF or 3% DMSO in 100Mm Tris-HCL, pH 8.0. Ten µl samples of the midgut homogenates were added to the synthetic substrates in microwell plates containing buffer (Tris-HCL at a pH 8.0) and the reaction left to run. The experimental reactions were performed in triplicates. The change in absorbance was monitored at 405nm and 30 ^{0}C for 20 mins in an Anthos III microplate reader and the optical densities were recorded (Moore, 1968).

3.6 Ethical considerations

This study required blood samples from volunteers for gametocyte culture. The donors were informed of the purpose of the project after which they were asked to read the consent form (see appendix V for consent form) and if satisfied, they were requested to fill the form. The donor was then asked for his/her blood group and thereafter blood was collected from the tip of the finger to get two drops of blood; one for the blood group confirmation using the antibody coagulation test and one to check the presence of *Plasmodium* by making smears on a glass slide. If the donor did not have any *Plasmodium*, the upper part of the arm was tied with a tourniquet to expose the blood veins. The part to be pricked was sterilized with cotton wool soaked in 70% alcohol and a size 18-21 gauge needle was used to draw blood from an identified vein into a 20ml syringe. Twenty mls of blood from one donor of blood group O +ve was required to culture the cells. *Plasmodium* parasites were cultured in the blood group O +ve red blood cells. For the blood used in obtaining the serum, the same procedure was followed though the blood was drawn into a 500ml plastic bag. One donor each of blood group A +ve, B +ve or O +ve was required for the serum donation. Serum was added to the culture media to supplement the nutrients in the media. Pressure was applied on the pricked area using a sterile cotton wool to stop the bleeding. The KEMRI Ethical Review Committee approved the proposal.

3.7 Data analysis

Data was entered into laboratory notebooks and MS Excel where it was analysed using SPSS. Comparisons for replicate test results were carried out using analysis of variance

(ANOVA). ANOVA was also used to compare the different treatment groups; glucose, hamster blood, human blood and human blood infected with *Plasmodium falciparum* by time (post-blood feeding). Data was analyzed using ANOVA to compare the glucose fed mosquito group (control) and the blood fed mosquito groups (experimental) data. Shapiro Wilk's test was used to check on the normality of the data and Scheffe's test was used for post-hoc testing.

CHAPTER FOUR: RESULTS

4.1 The effects of a human blood meal on the levels of midgut proteases in *Anopheles gambiae*

Overall enzymes' optical density value distribution demonstrated high enzyme activities in the hamster blood and the glucose treatment groups compared to the human blood treatment group. In the human blood treatment groups the overall enzyme activity increased steadily from 6 hours, declined at 12 hours and reached its peak at 18 hours after the blood meal and then declined steadily (Figure 4.1.1). The overall enzyme activities in the glucose treatment group remained constant and the maximum activity of the treatment was achieved by the mosquitoes 18 hours after the blood meal.

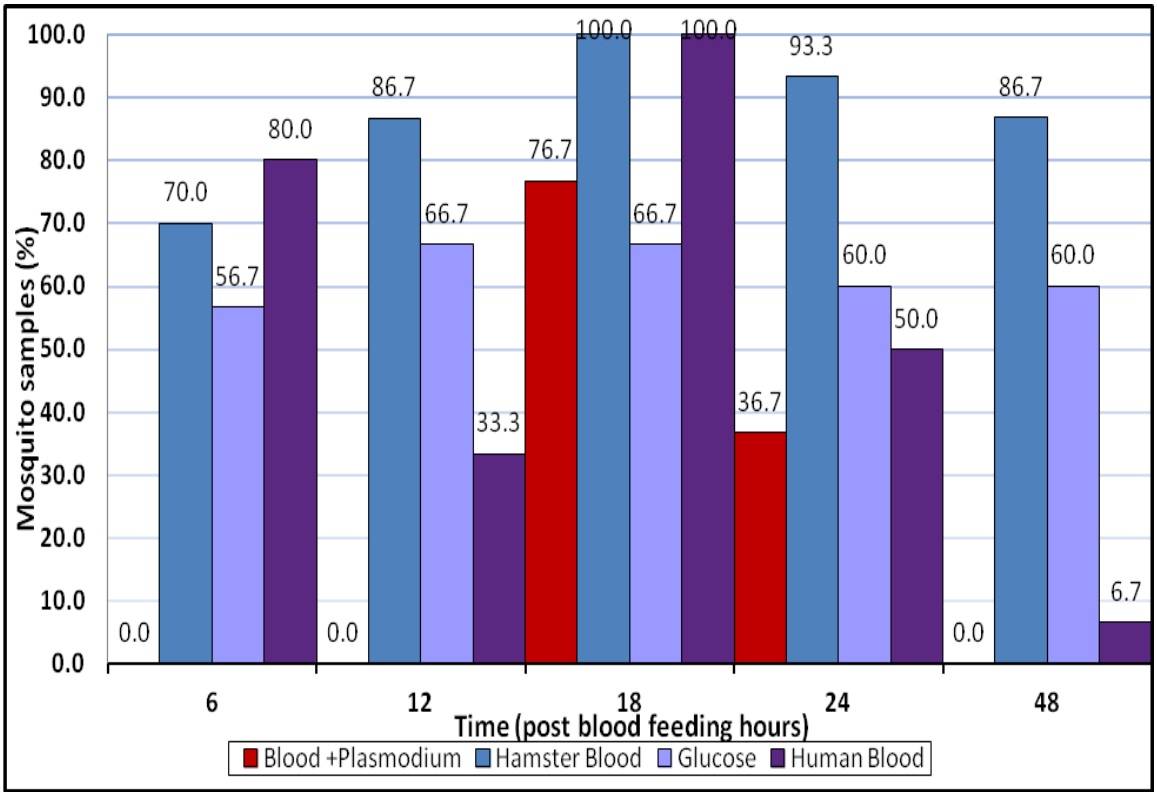

Figure 4.1.1: Percentage of mosquitoes with high enzyme activities (≥ 0.18) by time (post blood feeding hrs) and treatment

Further analysis using Scheffe's method indicated that overall, the enzyme activities were highest in the hamster blood treatment, followed by the glucose treatment and least in the human blood group (Table 4.1.1). The mean differences in the overall enzyme activity trends in the glucose treatment group compared to the hamster treatment group were not significant (-0.0627) but they were significantly different when compared to the human blood treatment group (0.1684).

Table 4.1.1: Multiple comparisons of the overall enzyme activities across treatments

(I) Treatment	(J) Treatment	Mean Difference (I-J)	SD	95% C. I.		P value
Glucose	Hamster Blood	-0.0627	0.0231	-0.134	0.009	0.119
	Human Blood	0.1684	0.0207	0.105	0.232	0.000(*)

*:The mean difference is significant at the .05 level; C.I: Confidence interval;
SD: Standard deviation

4.1.1 Distribution of trypsin activity by time and treatment

Trypsin activities were highest in the hamster blood treatment, followed by the human blood treatment and least in the glucose treatment (Figure 4.1.2). The median optical density value of the hamster treatment group was 0.18, 16% of the mosquitoes were found to have the highest trypsin optical densities (> 0.25) as shown in table 4.1.2. In the human blood treatment group, only 2% of the mosquitoes showed high trypsin activities while 0% of the mosquitoes showed high trypsin activities (> 0.25) in the glucose treatment group.

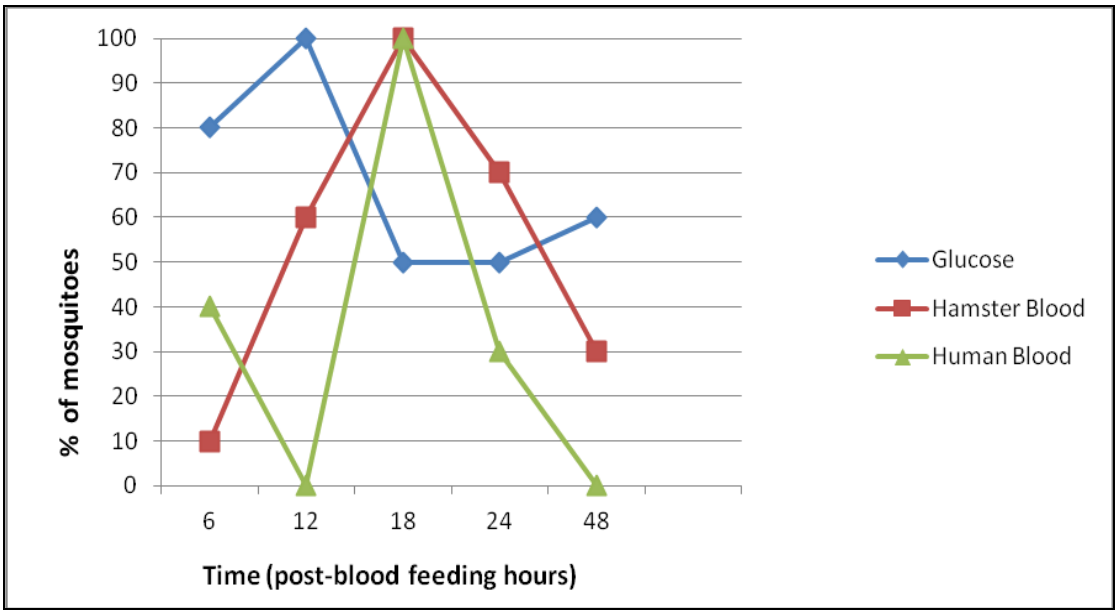

Figure 4.1.2: Percentage of mosquitoes with high trypsin activities (≥ 0.18) by time (post blood feeding hrs) and treatment

Table 4.1.2: Overall optical density values distribution of trypsin

Treatment	Total no. of mosquito dissected	< 0.1		0.11 - 0.15		0.16 - 0.20		0.21 - 0.25		> 0.25	
		n	%	n	%	N	%	N	%	n	%
Trypsin											
Glucose	50	14	28.0	32	64.0	4	8.0	0	0.0	0	0.0
Human Blood	50	1	2.0	24	48.0	17	34.0	7	14.0	1	2.0
Hamster Blood	50	0	0.0	0	0.0	34	68.0	8	16.0	8	16.0

n= No. of mosquitoes within that optical density value
%= percentage of mosquitoes within that optical density value

Further analysis using Scheffe's test indicated that mean differences of trypsin activity trends in the glucose treatment when compared to the hamster blood (-0.0967) and human blood (-0.0477) treatments were significantly different as shown in table 4.1.3.

Table 4.1.3: Multiple comparisons of each enzyme activities across treatments

(I) Treatment	(J) Treatment	Mean Difference (I-J)	SD	95% C. I.		P value
Trypsin						
Glucose	Hamster Blood	-0.0967	0.0088	-0.124	-0.069	0.000(*****)
	Human Blood	-0.0477	0.0078	-0.072	-0.023	0.000(*****)
Chymotrypsin						
Glucose	Hamster Blood	-0.0510	0.0156	-0.099	-0.003	0.033
	Human Blood	0.5444	0.0139	0.501	0.588	0.000(*****)
Aminopeptidase						
Glucose	Hamster Blood	-0.0404	0.0079	-0.0649	-0.0159	0.000(*****)
	Human Blood	0.0084	0.0071	-0.0136	0.0303	0.842

*:The mean difference is significant at the .05 level; C.I: confidence interval;
SD: Standard deviation

4.1.2 Distribution of chymotrypsin activity by time and treatment

The activity of chymotrypsin was highest in mosquitoes dissected 12 and 18 hours after feeding in the glucose and hamster blood treatments while in the human blood treatment the activities were constantly low (< 0.74) (Figure 4.1.3).

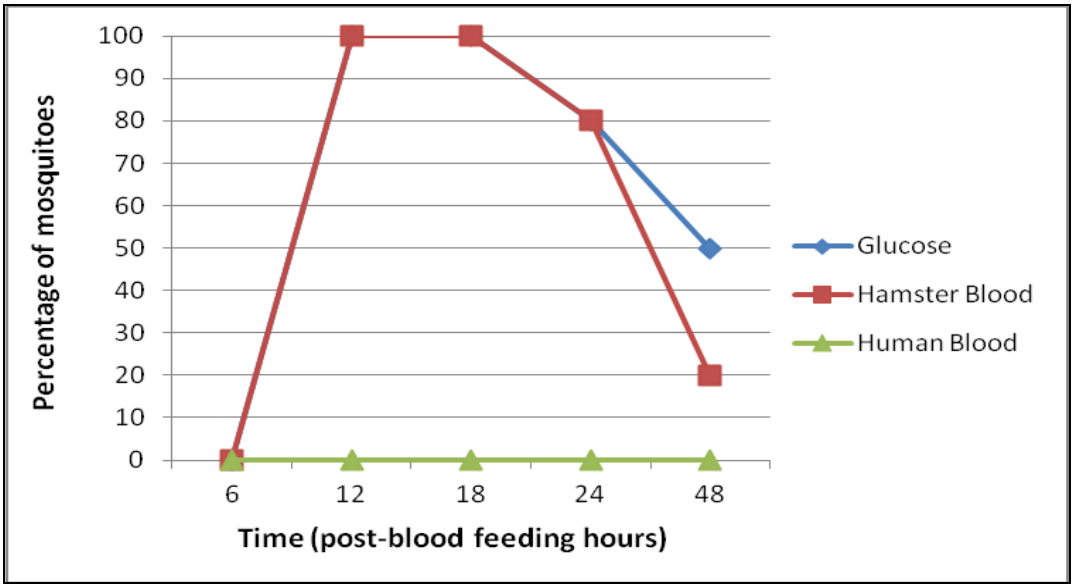

Figure 4.1.3: Percentage of mosquitoes with high chymotrypsin activities (≥0.74) by time (post blood feeding hrs) and treatment

As indicated in table 4.1.4, chymotrypsin activities were highest in the hamster blood-fed group which had an optical median value of 0.78 with 100% of the mosquitoes indicating an optical density value greater than 0.25. Glucose also had a higher median optical density value of 0.75 when compared to the human blood-fed treatment group (0.17) thus indicating that the human blood treatment had the lowest activity.

Table 4.1.4: Overall optical density values distribution of chymotrypsin

Treatment	Total no. of mosquito dissected	< 0.1		0.11 - 0.15		0.16 - 0.20		0.21 - 0.25		> 0.25	
		n	%	n	%	n	%	n	%	n	%
Chymotrypsin											
Glucose	50	0	0.0	0	0.0	0	0.0	0	0.0	50	100.0
Human Blood	50	8	16.0	4	8.0	17	34.0	10	20.0	11	22.0
Hamster Blood	50	0	0.0	0	0.0	0	0.0	0	0.0	50	100.0

n= No. of mosquitoes within that optical density value
%= percentage of mosquitoes within that optical density value

As illustrated in table 4.1.3, further analysis indicated that the mean differences of the chymotrypsin activity trends in the glucose treatment when compared to those of the hamster blood treatment were not significant (-0.0510) but they were significantly different (0.5444) when they were compared with those of the human blood treatment group.

4.1.3 Distribution of aminopeptidase activity by time and treatment

In the human blood treatment group, aminopeptidase was at its peak in the 6 hour and 18 hour post blood-fed mosquitoes. In the hamster treatment group, aminopeptidase peaked at 12 and 18 hours post blood-feeding (≥ 0.19) while in the glucose treatment group, the enzyme was highest in the 12 hour post blood-fed group (Figure 4.1.4). There was a significant association between time (post blood feeding hrs) and sample activity ($P<0.05$) for all the other treatment groups except the glucose treatment group ($P=0.650$).

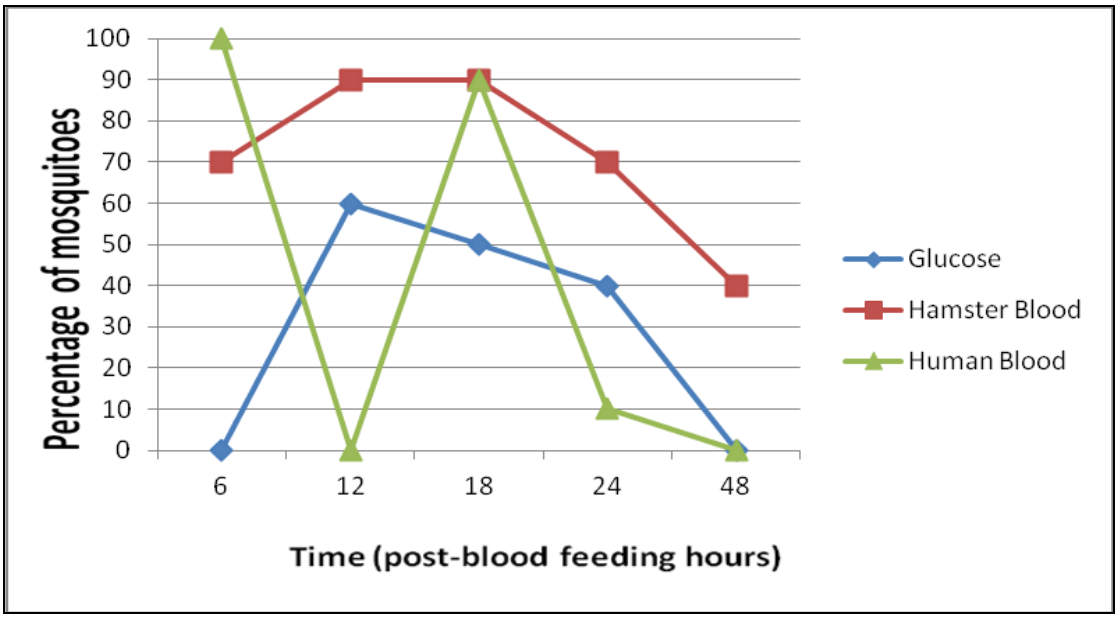

Figure 4.1.4: Percentage of mosquitoes with high aminopeptidase activities (≥0.19) by time (post blood feeding hrs) and treatment

Table 4.1.5: Overall optical density values distribution of aminopeptidase

Treatment	Total no. of mosquito dissected	< 0.1		0.11 - 0.15		0.16 - 0.20		0.21 - 0.25		> 0.25	
		n	%	n	%	N	%	N	%	n	%
Trypsin											
Glucose	50	14	28.0	32	64.0	4	8.0	0	0.0	0	0.0
Human Blood	50	1	2.0	24	48.0	17	34.0	7	14.0	1	2.0
Hamster Blood	50	0	0.0	0	0.0	34	68.0	8	16.0	8	16.0
Aminopeptidase											
Glucose	50	0	0.0	0	0.0	37	74.0	13	26.0	0	0.0
Human Blood	50	0	0.0	9	18.0	30	60.0	11	22.0	0	0.0
Hamster Blood	50	0	0.0	0	0.0	19	38.0	23	46.0	8	16.0

n= No. of mosquitoes within that optical density value
%= percentage of mosquitoes within that optical density value

The activities of aminopeptidase enzyme were similar to those of trypsin where the highest activity was in the hamster treatment followed by the human blood treatment and were least in the glucose treatment as shown in table 4.1.5. An optical density value of greater than 0.25 in the 16% of the mosquitoes fed on the hamster blood meal indicated a high activity of aminopeptidase unlike in the glucose fed mosquitoes.

The mean differences of the aminopeptidase activity trends in the glucose treatment group when compared to the hamster blood treatment group were significant (-0.0404) but were not significantly different (0.0084) when compared to those of the human blood treatment group (Table 4.1.3).

4.2 The effects of *Plasmodium falciparum* infected blood meal on the midgut proteases of *Anopheles gambiae*

In the blood and *Plasmodium*, hamster and human blood treatment groups, the overall enzyme activity increased steadily from 6 hours, declined at 12 hours and reached its

peak at 18 hours after the blood meal and then declined steadily (Figure 4.1.1). The overall enzyme activities in the glucose treatment group remained nearly constant. Maximum activity of the treatments was achieved by the mosquitoes 18 hours after the blood meal.

Further analysis using Scheffe's test indicated that overall, enzymes activities were highest in the glucose treatment group followed by the human blood treatment group and least in the blood + Plasmodium treatment group. In table 4.2.1, enzyme activities were seen to be suppressed in the blood + Plasmodium treatment group when compared to that of the human blood treatment. Comparisons of the mean differences in the overall enzyme activities in the glucose treatment group with the blood + Plasmodium and the human blood treatment groups were significantly different ($P < 0.001$).

Table 4.2.1: Multiple comparisons of overall enzymes activities across treatments

(I) Treatment	(J) Treatment	Mean Difference (I-J)	SD	95% C. I.		P value
Glucose	Blood +Plasmodium	0.2119	0.0207	0.148	0.276	0.000(*)
	Human Blood	0.1684	0.0207	0.105	0.232	0.000(*)

*:The mean difference is significant at the .05 level; C.I: Confidence interval;
SD: Standard deviation

4.2.1 Distribution of trypsin activity by time and treatment

Trypsin activities were lower in the glucose treatment group as shown in table 4.2.2 when compared to the activities of the human blood treatment and the blood + Plasmodium treatment groups.

Table 4.2.2: Overall trends in trypsin levels across treatments (mean optical densities)

Enzymes	Treatment	N	Mean	SD	95% C. I.	
Trypsin	Glucose	50	0.11	0.02	0.11	0.12
	Human Blood	50	0.16	0.04	0.15	0.17
	Human Blood + Plasmodium	50	0.14	0.02	0.13	0.15

N: No. of mosquitoes within that optical density value; C.I: Confidence interval; SD: Standard deviation

Further analysis indicated that the mean differences of trypsin activity trends for the glucose treatment group when compared to those of the blood + plasmodium treatment group were not significant (-0.0258) but the mean differences when compared to those of the human blood treatment group were significant (-0.0477) as shown in table 4.2.3.

Table 4.2.3: Multiple comparisons of each enzyme activities across treatments

(I) Treatment	(J) Treatment	Mean Difference (I-J)	SD	95% C. I.		P value
Trypsin						
Glucose	Blood +Plasmodium	-0.0258	0.0078	-0.050	-0.001	0.032
	Human Blood	-0.0477	0.0078	-0.072	-0.023	0.000(*****)
Chymotrypsin						
Glucose	Blood +Plasmodium	0.6242	0.0139	0.581	0.667	0.000(*****)
	Human Blood	0.5444	0.0139	0.501	0.588	0.000(*****)
Aminopeptidase						
Glucose	Blood +Plasmodium	0.0372	0.0071	0.0152	0.0591	0.000(*****)
	Human Blood	0.0084	0.0071	-0.0136	0.0303	0.842

*:The mean difference is significant at the .05 level; C.I: Confidence interval; SD: Standard deviation

4.2.2 Distribution of chymotrypsin activity by time and treatment

In table 4.2.4, chymotrypsin activity was slightly suppressed in the blood + Plasmodium and in the human blood treatment groups when compared to the glucose treatment group. Further analysis indicated that the mean differences of chymotrypsin activity trends for the glucose treatment group when compared to those of the blood + Plasmodium and the human blood treatment group were significantly different ($P < 0.001$) (Table 4.2.3).

Table 4.2.4: Overall trends in chymotrypsin levels across treatments (mean optical densities)

Enzymes	Treatment	N	Mean	SD	95% C. I.	
Chymotrypsin	Glucose	50	0.74	0.04	0.73	0.76
	Human Blood	50	0.20	0.08	0.18	0.22
	Human Blood + Plasmodium	50	0.12	0.05	0.10	0.13

N: No. of mosquitoes within that optical density value; C.I: Confidence interval; SD: Standard deviation

4.2.3 Distribution of aminopeptidase activity by time and treatment

For aminopeptidase, activity was at its peak in 24 hour post blood-fed mosquitoes for the blood + Plasmodium treatment group while in the human blood only the enzyme was at its peak in the 6 hour and 18 hour post blood-fed mosquitoes. In table 4.2.5, aminopeptidase activity was slightly suppressed in the blood + Plasmodium and in the human blood treatment groups when compared to the glucose treatment group. Using Scheffe's method, the mean differences of the aminopeptidase activity trends for the glucose treatment group when compared to those of the blood + Plasmodium treatment group were significant (0.0372) but were not significantly different (0.0084) when compared to those of the human blood treatment group (Table 4.2.3).

Table 4.2.5: Overall trends in aminopeptidase levels across treatments (mean optical densities)

Enzymes	Treatment	N	Mean	SD	95% C. I.	
Aminopeptidase	Glucose	50	0.19	0.02	0.18	0.19
	Hamster Blood	50	0.23	0.05	0.21	0.24
	Human Blood	50	0.18	0.03	0.17	0.19
	Human Blood + Plasmodium	50	0.15	0.04	0.14	0.16

N: No. of mosquitoes within that optical density value; C.I: Confidence interval; SD: Standard deviation

CHAPTER FIVE: DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Effects of the source of blood meal on the midgut proteases in *Anopheles gambiae* s.s

Although mosquitoes feed from both man and animals, *Anopheles gambiae* s.s strictly feeds on humans while *An. arabiensis* are zoonotic. This study determined whether there was a significant effect of the source of blood meal on the levels of midgut proteases. The overall enzyme trends demonstrated high activity in the glucose and hamster blood treatments compared to the human blood treatment. Activity was highest in the hamster-fed treatment group followed by the sugar-fed and the human blood treatment groups and was least in the blood and plasmodium treatment group.

The mean differences in the overall enzyme activity trends in the human blood treatment group when compared to that of the hamster and the glucose treatment groups were significantly different, thus indicating that the presence of human blood meal in *An. gambiae* altered the levels of the midgut proteases. Also, the mean differences in the overall enzyme trends in the glucose treatment group when compared with those of the human blood and blood + Plasmodium treatment groups were significantly different thus indicating that human blood and blood + Plasmodium altered the level of midgut proteases. The midgut proteases in the mosquitoes were affected by the type of the ingested meal. When compared to the blood + Plasmodium treatment group, the human blood treatment had higher enzyme activities thus indicating that plasmodium suppressed the enzymes activities.

5.1.2 Distribution of trypsin across treatments

A lot of studies have been carried out on the midgut proteases of the malaria vector *Anopheles stephensi* based on the meal ingested. Previous studies have described a peak in trypsin-like midgut activity 28-32 hours after a blood meal (Billingsley, 1990; Feldmann *et al.,* 1990; Hörler and Briegel, 1995) which concur with the trypsin activities found in the blood fed *Anopheles gambiae* s.s mosquitoes in this study.

Trypsin activities in the blood fed mosquitoes (hamster blood, human blood and human blood + Plasmodium treatment groups) peaked between 12 and 24 hours post-blood feeding and then reduced at 48 hours, which coincided with the complete digestion of blood. The activities of trypsin in the human blood-fed mosquitoes were higher when compared to those of the blood infected with the Plasmodium, thus indicating that plasmodium may have inhibited the enzyme's activity. The increase of trypsin at 24 hour post blood-fed mosquitoes concurs with Muller *et al.*(1995) findings which indicated that trypsin 1 and 2 are induced by a blood meal and peaks at 24 hours post blood feeding .

In the *An. gambiae* mosquitoes which were fed on the blood infected with *Plasmodium falciparum,* trypsin activities peaked at 18 hours post blood-fed mosquitoes while the mosquitoes which were fed on the human blood had mild activity in the 6 hour post blood-fed which decreased and peaked in the 18 hour post blood-fed mosquitoes. Thus mild activities of trypsin in the human blood treatment group in the 6hr PBF mosquitoes indicated the presence of constitutive trypsin that may have been suppressed by *Plasmodium falciparum* in the infected blood treatment group.

Ookinete migration across the peritrophic membrane and invasion of the midgut occurs at the same period (18-24 hrs PBF) thus indicating that trypsin may activate the parasite's chitinase. Shahabuddin and Kaslow (1994b) and Shahabuddin *et al*. (1996) indicated that trypsin produced in the mosquito's digestive tract, probably activates parasites' chitinase, thus facilitating passage of the parasite through the peritrophic matrix. In glucose, trypsin activities did not alter significantly in the 6, 12, 18, 24 and 48hr PBF mosquitoes. This indicates that trypsin is essential in protein digestion because in all the blood-fed mosquitoes, trypsin activities peaked at 18-24 hours which coincides with the time taken to digest 80% of the blood meal (Jahan *et al.,* 1999) unlike in the sugar-fed mosquitoes.

The presence of human blood meal in the midgut of *An. gambiae* altered the levels of trypsin (Table 4.2.3). However, the presence of the human blood infected with *P. falciparum* did not alter trypsin levels.

Ookinete migration across the peritrophic membrane and invasion of the midgut occurs at the same period (18-24 hrs PBF) thus indicating that trypsin may activate the parasite's chitinase. Previous *in vitro* experiments indicate that *Plasmodium* parasite is not fully able to activate its own chitinase activity (Shahabuddin, *et al.,* 1996). Inhibition of the midgut trypsins would block activation of the parasite's chitinase thus blocking the development of *Plasmodium falciparum,* therefore indicating its potential for a transmission blocking vaccine. Initial work by Shahabuddin *et al.* (1993) indicated that inhibition of parasite chitinase blocked further development of oocyst *in vivo* while an

addition of trypsin-like protease from the mosquito midgut increased its enzymatic activity.

5.1.3 Distribution of chymotrypsin across treatments

The increase in chymotrypsin activities in the 18 hour post blood-fed mosquitoes coincided with the time taken for protein digestion, thus indicating that the chymotrypsin may have been produced for protein digestion. However, in the human blood treatment group, mild activities of chymotrypsin in the 6hour post blood-fed mosquitoes may indicate the presence of constitutive chymotrypsin that may have been suppressed by *Plasmodium falciparum* in the infected blood treatment group. The hamster and glucose fed mosquitoes had constantly high chymotrypsin activities which coincided with Hörler and Briegel (1997), who found that after a human blood meal, inhibitor activity against chymotrypsin was more than double that of the sugar-fed mosquitoes. Human blood infected with *P. falciparum* alters the level of chymotrypsin in *An. gambiae* mosquitoes. The activities of chymotrypsin in the human blood-fed mosquitoes were higher when compared to those of the blood infected with the plasmodium treated mosquitoes, thus indicating that plasmodium may have inhibited the enzyme's activity.

Several studies have reported that midgut chymotrypsin is induced in *An. stephensi* fed on blood meal and that activity peaks within 36 hours post-feeding. However, in the sugar-fed mosquitoes, chymotrypsin activities are not detected (Hörler and Briegel, 1995). In *An. gambiae* mosquitoes fed on the blood infected with *Plasmodium falciparum*, chymotrypsin activities peaked in the 18hours post blood-fed mosquitoes

while those mosquitoes which were fed on the human blood had mild activity in the 6 hour post blood-fed which decreased and peaked in the 18 hour post blood-fed mosquitoes. This peak coincides with protein digestion thus indicating that inhibition of this enzyme might inhibit development of the *Plasmodium falciparum* and thus could be a potential transmission blocking mechanism.

5.1.4 Distribution of aminopeptidase across treatments

In *An. gambiae* mosquitoes which were fed on the blood infected with *Plasmodium falciparum*, the aminopeptidase activities peaked in 24 hours post blood-fed mosquitoes while those of the mosquitoes which were fed on the human blood had mild activity in the 6 hour post blood-fed, which decreased and then peaked in the 18 hour post blood-fed mosquitoes. The mild activity in the 6 hour post blood-fed human blood treatment group could have been due to the constitutive aminopeptidase which was then replaced by the induced aminopeptidase that resulted in the maximum peak at 18 hour post blood-fed. The presence of *P. falciparum* may have resulted in the suppression of the constitutive aminopeptidase in that treatment group. Further work is required to determine whether boosting production of the constitutive aminopeptidase would block the development of *Plasmodium falciparum* in the *Anopheles gambiae* midgut.

In the blood-fed females, the maximal aminopeptidase activity was reached at 24hours post blood-feeding, and this concurred with the previous reports of aminopeptidase activity in *An. gambiae* (Billingsley and Hecker, 1991; Lemos *et al*., 1996). Hörler and Briegel (1995) reported constitutive activities in the midguts of the sugar-fed *An.*

albimanus females. A similar constitutive aminopeptidase activity has been seen in the glucose treatment group of *An. gambiae* in this study.

The presence of a human blood meal and the human blood infected with *P. falciparum* in the midgut of *An. gambiae* altered the levels of aminopeptidase.

The activities of aminopeptidase in the human blood-fed mosquitoes were higher when compared to those of the blood infected with the plasmodium treated mosquitoes, thus indicating that plasmodium may have inhibited the enzyme's activity. Generally, *Plasmodium* suppressed the activities of trypsin, chymotrypsin and aminopeptidase enzymes because their trends in the blood and Plasmodium treatment group were lower when compared to the human blood treatment group.

5.2 Conclusions

a) The presence of human blood meal in *An. gambiae* alters levels of the midgut proteases.
b) The presence of human blood infected with *P. falciparum* in *An. gambiae* alters levels of the midgut proteases.

5.4 Recommendations

Data from this study indicates that;

a) Inhibition of trypsin in the mosquito midgut is a potential transmission blocking strategy.

b) Midgut proteases are potential targets in transmission blocking mechanisms.

c) Further work on inhibition of chymotrypsin and trypsin and promotion of aminopeptidase in the mosquitoes needs to be done so as to identify the potential of transmission blocking mechanisms in the control of malaria.

d) Further work needs to be carried out to identify the constitutive and the induced midgut protease enzymes.

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Appendix I: Overall enzyme distribution of the optical density values

Treatment	Total no. of mosquitoes dissected	< 0.1		0.11 - 0.15		0.16 - 0.20		0.21 - 0.25		> 0.25	
		n	%	n	%	N	%	n	%	n	%
Trypsin											
Glucose	50	14	28.0	32	64.0	4	8.0	0	0.0	0	0.0
Human Blood	50	1	2.0	24	48.0	17	34.0	7	14.0	1	2.0
Hamster Blood	50	0	0.0	0	0.0	34	68.0	8	16.0	8	16.0
Chymotrypsin											
Glucose	50	0	0.0	0	0.0	0	0.0	0	0.0	50	100.0
Human Blood	50	8	16.0	4	8.0	17	34.0	10	20.0	11	22.0
Hamster Blood	50	0	0.0	0	0.0	0	0.0	0	0.0	50	100.0
Aminopeptidase											
Glucose	50	0	0.0	0	0.0	37	74.0	13	26.0	0	0.0
Human Blood	50	0	0.0	9	18.0	30	60.0	11	22.0	0	0.0
Hamster Blood	50	0	0.0	0	0.0	19	38.0	23	46.0	8	16.0

Where n= No. of mosquitoes within that optical density value
%= percentage of mosquitoes within that optical density value

Appendix II: Overall trends in enzyme levels across treatments (mean optical densities)

Enzymes	**Treatment**	**N**	**Mean**	**SD**	**95% C. I.**	
Trypsin	Glucose	50	0.11	0.02	0.11	0.12
	Hamster Blood	50	0.21	0.06	0.19	0.23
	Human Blood	50	0.16	0.04	0.15	0.17
	Human Blood + Plasmodium	50	0.14	0.02	0.13	0.15
Chymotrypsin	Glucose	50	0.74	0.04	0.73	0.76
	Hamster Blood	50	0.79	0.08	0.76	0.83
	Human Blood	50	0.20	0.08	0.18	0.22
	Human Blood + Plasmodium	50	0.12	0.05	0.10	0.13
Aminopeptidase	Glucose	50	0.19	0.02	0.18	0.19
	Hamster Blood	50	0.23	0.05	0.21	0.24
	Human Blood	50	0.18	0.03	0.17	0.19
	Human Blood + Plasmodium	50	0.15	0.04	0.14	0.16

Appendix III: Distribution of activity of each enzyme by treatment and time

Trypsin

	Blood +Plasmodium				Glucose				Hamster Blood				Human Blood			
	< 0.18 (Low)		>= 0.18 (High)		< 0.18 (Low)		>= 0.18 (High)		< 0.18 (Low)		>= 0.18 (High)		< 0.18(Low)		>= 0.18 (High)	
Time post blood feeding (Hours)	N	%	N	%	n	%	n	%	N	%	n	%	n	%	n	%
6	10	100.0	0	0.0	2	20.0	8	80.0	9	90.0	1	10.0	6	60.0	4	40.0
12	10	100.0	0	0.0	0	0.0	10	100.0	4	40.0	6	60.0	10	100.0	0	0.0
18	6	60.0	4	40.0	5	50.0	5	50.0	0	0.0	10	100.0	0	0.0	10	100.0
24	9	90.0	1	10.0	5	50.0	5	50.0	3	30.0	7	70.0	7	70.0	3	30.0
48	10	100.0	0	0.0	4	40.0	6	60.0	7	70.0	3	30.0	10	100.0	0	0.0
Total	45	90.0	5	10.0	16	32.0	34	68.0	23	46.0	27	54.0	33	66.0	17	34.0

Chymotrypsin

	Blood +Plasmodium				Glucose				Hamster Blood				Human Blood			
	< 0.74 (Low)		>= 0.74 (High)		< 0.74 (Low)		>= 0.74 (High)		< 0.74 (Low)		>= 0.74 (High)		< 0.74 (Low)		>= 0.74 (High)	
Time post blood feeding (Hours)	N	%	N	%	n	%	n	%	n	%	n	%	n	%	n	%
6	10	100.0	0	0.0	10	100.0	0	0.0	10	100.0	0	0.0	10	100.0	0	0.0
12	10	100.0	0	0.0	0	0.0	10	100.0	0	0.0	10	100.0	10	100.0	0	0.0
18	10	100.0	0	0.0	0	0.0	10	100.0	0	0.0	10	100.0	10	100.0	0	0.0
24	10	100.0	0	0.0	2	20.0	8	80.0	2	20.0	8	80.0	10	100.0	0	0.0
48	10	100.0	0	0.0	5	50.0	5	50.0	8	80.0	2	20.0	10	100.0	0	0.0
Total	50	100.0	0	0.0	17	34.0	33	66.0	20	40.0	30	60.0	50	100.0	0	0.0

Aminopeptidase

	Blood +Plasmodium				Glucose				Hamster Blood				Human Blood			
	< 0.19(Low)		>= 0.19(High)		< 0.19(Low)		>= 0.19(High)		< 0.19(Low)		>= 0.19(High)		< 0.19(Low)		>= 0.19(High)	
Time post blood feeding (Hours)	N	%	N	%	n	%	n	%	n	%	n	%	n	%	n	%
6	10	100.0	0	0.0	10	100.0	0	0.0	3	30.0	7	70.0	0	0.0	10	100.0
12	10	100.0	0	0.0	4	40.0	6	60.0	1	10.0	9	90.0	10	100.0	0	0.0
18	4	40.0	6	60.0	5	50.0	5	50.0	1	10.0	9	90.0	1	10.0	9	90.0
24	1	10.0	9	90.0	6	60.0	4	40.0	3	30.0	7	70.0	9	90.0	1	10.0
48	10	100.0	0	0.0	10	100.0	0	0.0	6	60.0	4	40.0	10	100.0	0	0.0
Total	35	70.0	15	30.0	35	70.0	15	30.0	14	28.0	36	72.0	30	60.0	20	40.0

Appendix IV: Distribution of high activity by end-point (post-blood feeding time) and treatment

Treatment	< 0.18 (		>= 0.18	
	n	%	n	%
Blood +Plasmodium				
6hr	30	100.0	0	0.0
12hr	30	100.0	0	0.0
18hr	7	23.3	23	76.7
24hr	19	63.3	11	36.7
48hr	30	100.0	0	0.0
Total	116	77.3	34	22.7
Blood +Plasmodium +saliva				
6hr	30	100.0	0	0
12hr	30	100.0	0	0
18hr	30	100.0	0	0
24hr	30	100.0	0	0
48hr	30	100.0	0	0
Total	150	100.0	0	0
Glucose				
6hr	13	43.3	17	56.7
12hr	10	33.3	20	66.7
18hr	10	33.3	20	66.7
24hr				
48hr				
Total	33	36.7	57	63.3
Hamster Blood				
6hr	9	30.0	21	70.0
12hr	4	13.3	26	86.7
18hr	0	0.0	30	100.0
24hr				
48hr				
Total	13	14.4	77	85.6
Human Blood				
6hr	6	20.0	24	80.0
12hr	20	66.7	10	33.3
18hr	0	0.0	30	100.0
24hr	15	50.0	15	50.0
48hr	28	93.3	2	6.7
Total	69	46.0	81	54.0

Appendix V: Informed consent form

Project Title: Effects of *Plasmodium Falciparum* Infected Blood On Midgut Proteases Of *Anopheles Gambiae* S.S

PI: J. Milkah Muthoni Mwangi

Introduction and participation information

Kenya Medical Research Institute (KEMRI) in collaboration with Kenyatta University is conducting a study on malaria control. Malaria causes untold sufferings and economic losses to majority of populations through out the world. In Kenya, malaria is the leading cause of morbidity and mortality. It is caused by parasites of the genus *Plasmodium* and is transmitted by the bite of an infected female *Anopheles* mosquito. The investigators are requesting your participation by donating blood for the study. Participation is entirely voluntary and you may be included as a participant in this study if you sign the section at the end of this form. You may withdraw from the study if you so wish, at any time without penalty. When you've read this explanation, please feel free to ask questions on any issues related to this study or your participation in it.

Purpose of study

The current malaria control strategies targeting mosquito vectors and the malarial infection in humans are proving to be unsuccessful. This is due to occurrence of resistance in mosquitoes against insecticides and resistance of parasites to commonly used antimalarial drugs. Therefore, there is an urgent need to come up with other strategies that can be integrated with the current methods to control malaria. The *Plasmodium* parasite develops within the mosquito's gut and it is transmitted when the infected mosquito ingests a blood meal from a host. This indicates that the *Anopheles* mosquito could be an important target in the control of malaria. The midgut proteases digest the blood meal in the *Anopheles* mosquito. However, these molecules that are crucial for the parasites development are not yet fully understood. In this study, we seek to determine changes in levels of the midgut proteases that may be caused by the

presence of a blood meal and the presence of *Plasmodium falciparum* infected blood in *Anopheles gambiae s.s* mosquitoes.

Procedures to be used

The prospective participant will read the consent form and if satisfied, he/she will be requested to fill the consent form. **The donor would then be asked for his/her blood group. This donor will then be pricked at the tip of the finger to get two drops of blood; one for the blood group confirmation using the antibody coagulation test and one to check the presence of gametocytes by making smears on a glass slide. If the donor does not have any gametocytes,** the upper part of the arm will be tied with a tourniquet to expose the blood veins. The part to be pricked will be sterilized with cotton wool soaked in 70% alcohol and a size 18-21 gauge needle will be used to draw blood from an identified vein into a 20ml syringe. 20mls of blood from a donor of blood group O+ve will be required to culture the cells. For the blood used in obtaining the serum, the same procedure will be followed though the blood will be drawn into a 500ml plastic bag. Donors of blood group A +ve, B +ve or O+ve will be required for the serum donation. Pressure will be applied on the pricked area using a sterile cotton wool to stop the bleeding.

Maintenance of confidentiality

All information collected in this study is confidential. Any blood collected will be used for the sole purpose of the study on *Effects of Plasmodium falciparum infected blood on midgut proteases of Anopheles gambiae*. The names of the donors will not be linked with the serum or the whole blood sample. The original information provided by the donor and the signed informed consent forms will be kept under lock and key in the offices in KEMRI. The blood collected will be screened for *Plasmodium* (malaria parasite), HIV and Hepatitis B viral antigens before use. The donors' identity and test results will remain anonymous and confidential.

Risks

The risks of participation in this project are minimal. Possible risks of drawing blood include infection and minimal bruising. There may be a small amount of pain caused by the pricking that will last for only a few minutes. The sample will be assigned an anonymous numerical identifier and kept privately to ensure that chances of tracing back the sample to the donors' information are minimal.

Benefits

The donor will not benefit directly from giving a sample because their identity will be anonymous. Although the experiment is not designed to benefit the donor personally, by participating, the donor will contribute to scientific knowledge about the effects of *Plasmodium falciparum* infected blood on midgut proteases of *Anopheles gambiae*.

If you have any questions about your rights as a participant in this study, you may contact Ms. Milkah Mwangi (0722 805127) or Dr. Willy Tonui (020 2722541) of the Centre for Biotechnology Research and Development in KEMRI, or Dr. Rashid Juma, the secretary National/KEMRI Ethical Review Committee (ERC) on Tel: 2722541/2713349

Consent

I_____ (Name of donor), hereby state that I am over 18 years of age, in good physical health, not pregnant, and wish to participate in a program of research on *Effects of Plasmodium falciparum infected blood on midgut proteases of Anopheles gambiae* s.s being conducted by J. Milkah M. Mwangi, at the Centre for Biotechnology Research and Development, Kenya Medical Research Institute, Nairobi.

The contents of this study have been explained to me and I have had the opportunity to read the consent information for the study. I have understood the procedures that will be used, the benefits and the risks associated with the procedures to be done have been clearly explained to me. All the questions have been answered to my full satisfaction in a

language that I understand. I therefore consent to participate in this study and as a show of my approval, I have signed on the space below.

I also understand that I may revoke this consent at any time without penalty or loss of benefits, if any.

Donor's details

Name: _____ Date: _____

Signature: _____

ID No.: _____

Address: _____

_____

Witness

Name: _____ Date: _____

Signature: _____

Investigator

Name: _____ Date: _____

Signature: _____