BLOOD-MEAL HOST SELECTION, MALARIA INFECTION, AND GENOTYPING TO DELINEATE HUMAN TO MOSQUITO *PLASMODIUM* TRANSMISSION: IMPLICATION FOR MALARIA PARASITE INFECTIONS IN MALAWI

By

Rex Barnett Marregah Mbewe

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Entomology —Doctor of Philosophy

2021

ABSTRACT

BLOOD-MEAL HOST SELECTION, MALARIA INFECTION, AND GENOTYPING TO DELINEATE HUMAN TO MOSQUITO *PLASMODIUM* TRANSMISSION: IMPLICATION FOR MALARIA PARASITE INFECTIONS IN MALAWI

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Malaria is endemic in Malawi with continuing transmission and high rates of infection in the human population, despite ongoing interventions by the national malaria control program. The two main malaria control interventions in Malawi are long-lasting insecticidal treated nets (LLINs) and indoor residual spraying (IRS). The research described in this dissertation investigated *Anopheles* vector blood host selection among vertebrate hosts and within the human population, and from these investigations inferred pathways of malaria transmission from human to mosquito populations. The primary hypothesis was that biases in mosquito human feeding could drive malaria infection transmission. The study setting was two districts in southeastern Malawi. One district, Balaka, had conventional LLINs, those containing pyrethroid insecticide only; and the other, Machinga, had LLINs containing pyrethroids and the synergist piperonyl butoxide (PBO). Adult female *Anopheles* mosquitoes were sampled indoors in rural villages by CDC light traps, Pyrethrum spray catches and aspiration. Human demographic data and blood spots on filter paper by finger prick were collected from consented participants. *Anopheles* mosquito species were identified by morphological and molecular methods. Mosquito blood meal sources and *Plasmodium falciparum* infection in abdomen or head-thorax of mosquitoes were determined by qPCR with host-specific oligonucleotide probes and parasite specific probes, respectively. Blood meals from humans and human blood spots were analyzed by genotyping 24 microsatellite loci, to generate genetic profiles that were matched one to the other, and between blood meals and blood

spots, using an algorithm executed in RStudio (Version 1.1.456). The results showed similar mosquito species community composition of the three main malaria vectors (*An. arabiensis, An. gambiae, An. funestus*), but different in abundance, with substantial malaria infection rates (ranging from 14-36%) in the mosquitoes at both sites. Potential secondary malaria vector mosquito species were present. Most mosquitoes fed upon humans (81%) and relatively less upon dogs (2%) and goats (5.2%). There were strong biases in human host selection, with a highly nonrandom and aggregated distribution of blood meals, with few individuals contributing most of the blood meals and most individuals contributing few. In human blood meals, males of the age group 6-15 were the most malaria infected group compared to younger and older age groups, and individuals of this group contributed most of the blood meals. These results incriminate males of age 6-15 as drivers of malaria infection from human to mosquitoes. The district with LLINs containing PBO had a lower Human Blood Index (HBI) and Entomological Inoculation Rate (EIR) compared to the district with conventional LLINs. Mixed blood meals of human and non-human hosts suggest interrupted feeding and the adaptability of the mosquitoes to switch amongst host species, possibly circumventing malaria control interventions. Malaria transmission was well sustained despite implementation of LLINs containing synergist PBO or not. Likely, other factors such as net durability and efficacy may be reducing effectiveness of the LLIN program and need further investigation. Continued efforts in malaria control and prevention should include deliberate efforts focused on key demographic population groups, such males of 6-15 years old, which while not the most vulnerable population to malaria, are reservoirs of infection.

This dissertation is dedicated to my precious wife Angela and our two gorgeous daughters Rexilina and Rexangel. They all have been a great pillar of support to me in this arduous academic journey, enduring the unrelenting COVID-19 pandemic, Michigan cold winters, and my absence on some of our family activities. However, we had fun all the way, making new friends, visiting places, enjoying the four seasons and of course taking lots of pictures.

ACKNOWLEDGEMENTS

I would like to acknowledge the following individuals and organizations below for their contribution to the success of the research described in this dissertation and my PhD program. First and foremost, my deepest gratitude goes to Dr. Edward Walker, Professor of Microbiology, Molecular Genetics and Entomology at Michigan State University (MSU), for accepting me into his laboratory, being my academic advisor and for supporting me in various ways throughout my graduate program. His scientific guidance, wisdom, sense of humor and mentorship were instrumental to my academic and research achievements. Ned, you will always be remembered with fondness for having a great impact in shaping my life. Secondly, I would also like to acknowledge the MSU faculty who served on my guidance committee Dr. Eric Benbow, Dr. Jean Tsao, Dr. Jennifer Owen, Dr. Ke dong (past), Dr. Barry Pittendrigh (past) and Dr. Lauren Cohee from University of Maryland. I thank you all for your guidance and oversight on my research project and academic training.

Special thanks also should go to Dr. John Keven who trained me in molecular laboratory and forensic techniques that were relevant to my research. John was an advance graduate student in the lab and finished a year before me. John, we worked together as brothers and I learnt a lot from you. Please continue helping others who may cross your path as I did and help them to shine. May you continue to rise in your profession, and I wish you all the best. My research project was supported through collaborative efforts between Dr. Walker's laboratory at MSU and International Center for Malaria Research (ICEMR) Malawi under Malaria Alert Center (MAC-CDAC). I would like to thank all member of staff in these two organizations who helped in my project starting from field workers doing data collection and management dealing with my financial support. Special

appreciation also goes to Dr. Don Mathanga (MAC), Dr. Terrie Taylor (ICEMR, MSU), and Dr. Mirriam Laufer (University of Maryland) and all staff for all their administrative support during my training.

Last but not least, special recognition to management and staff of my employer Polytechnic, University of Malawi, soon to be Malawi University of Business and Applied Sciences (MUBAS), for allowing me to go on study leave and giving me support in a variety of ways when I needed it.

Finally, I would like to give a very special thanks to my parents for making me believe in my intellectual capabilities, for their words of wisdom and encouragement, and for getting me on the path to arrive at this highly unlikely stage of my life, and to my family, Angela my wife, our daughters Rexilina and Rexangel for their company, emotional support in a foreign country and making this experience enjoyable all the way to my graduation, I could not trade that for anything. Your great sacrifices will only be repaid by our loving father Jehovah.

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KEY TO ABBREVIATIONS

ACT – Artemisinin-based combination therapy

BLAST– Basic Local Alignment Search Tool

CDC – Centre for Disease Control

CI– Confidence interval

COMREC – College of Medicine Research Ethics Committee

DNA – Deoxyribose nucleic acid

EIR – Entomological Inoculation rate

HBI- Human blood index

ICEMR- International Centers of Excellence for Malaria Research

ID – index of dispersion

IRB – Institution Review Board

IRS – Indoor Residual Spray

LLIN – Long-lasting insecticidal net

MSU – Michigan State University

MSU IRB – Michigan State University Institutional Review Board

NIH – National Institutes of Health

NSO – National Statistics Office

PBO – Piperonyl butoxide

PCR – Polymerase Chain Reaction

PSCs – Pyrethroid spray catches

R_o – Basic reproductive number

VC – Vectorial Capacity

WHO – World Health Organization

CHAPTER 1 : INTRODUCTION

1.1. Background

Malaria continues to be a major contributor to worldwide disease burden, linking to poverty and impeding social development. Malaria control efforts have increased tremendously in the past two decades, culminating in appreciable and measurable declines in global burden of the disease (Chanda et al., 2015; Cibulskis et al., 2016; WHO, 2014, 2021b). In 2010, the World Health Organization ranked malaria as the eighth-highest contributor to the global disease burden and was the leading cause of premature death and disability in sub-Saharan Africa (*Http://Www.Healthdata.Org*, 2013). By the year 2020, Malaria cases and deaths had decreased from 216 million and 655,000 in 2010 to 215 million and 386 000 in 2019 respectively (WHO, 2021b).

Human malaria is caused by sporozoan protists of the genus *Plasmodium*, family Plasmodiidae (Order Haemosporidida, Class Haemosporidea, Phylum Sporozoa) that infect blood tissues and other organs of the body, primarily the liver. These organisms are transmitted from person to person by female *Anopheles* mosquito bites. They are called human malarias because they are naturally known to cause disease in infected humans and not in other mammalian, avian or reptilian hosts (Antinori et al., 2012; H. Gillies, 1993; Schlagenhauf, 2004). These human malaria protozoans are obligate intracellular parasites. There are four species of solely human malaria: *P. falciparum*, causing malignant tertian malaria; *P. vivax*, causing benign tertian malaria; *P. malariae*, causing quartan malaria; and *P. ovale* tertian malaria. The first two species are widespread in the tropics, but *P. vivax* also occurs in some temperate areas. Interestingly, *P. vivax* is noticeably absent in much of sub-Saharan Africa due to the absence of a gene encoding a

receptor protein (the so-called Duffy negative phenotype) that facilitates infection of red blood cells. *P. malariae* also is distributed widely in endemic regions of the world, but in low prevalence. *P. ovale* is rare, occurring mainly in Africa. Recently, a fifth species of *Plasmodium* that causes quotidian malaria, *P. knowlesi*, has been discovered to be relatively common in the human population of some countries of Southeast Asia (28-84% of malaria cases in Malaysian Borneo). This species had been known to occur almost exclusively in monkeys, with only occasional human infections (Foster and Walker, 2009 in Mullen & Durden, 2009). Malaria currently occurs in sub-Saharan Africa, parts of northern Africa, the Middle East, Southeast Asia, Latin America, Central America and South America (Lysenko & Semashko, 1968;Chima et al., 2003; Hay et al., 2004; Guerra et al., 2006; Feachem et al., 2010; Sinka et al., 2012; Griffin et al., 2014; WHO, 2021a). A variety of factors determine the degree of endemicity, typically measured as prevalence of infection of humans by the various species of *Plasmodium* present. These factors include environmental and social as well as species of vectors. Literature abounds on human malaria and its vectors. Among comprehensive sources are Boyd (1949), Macdonald (1957), Molineaux & Gramiccia (1980), Wernsdorfer & McGregor (1988) and Warrell & Gilles (2017).

1.2. *Plasmodium* life cycle

The life cycle of *Plasmodium* species is complex involving both sexual and asexual reproduction and depends on both humans and mosquitoes (Figure 1.1). In the mosquito, the malarial parasite (*Plasmodium spp*.) develops an exogenous sexual phase (sporogony cycle). When the sporozoites have developed, they make their way to the mosquito salivary glands, from which they are transmitted to humans via the mosquito bite. The parasites develop in an asexual cycle within the human host through a series of nuclear division which increases parasite density and eventual

production of gametocytes (gametogony). An infective mosquito is a prerequisite for malaria transmission. It takes between 8 and 35 days to complete the parasite's life cycle in the mosquito host, depending on the environmental conditions and the species of malaria parasites (Ndoen et al., 2012).

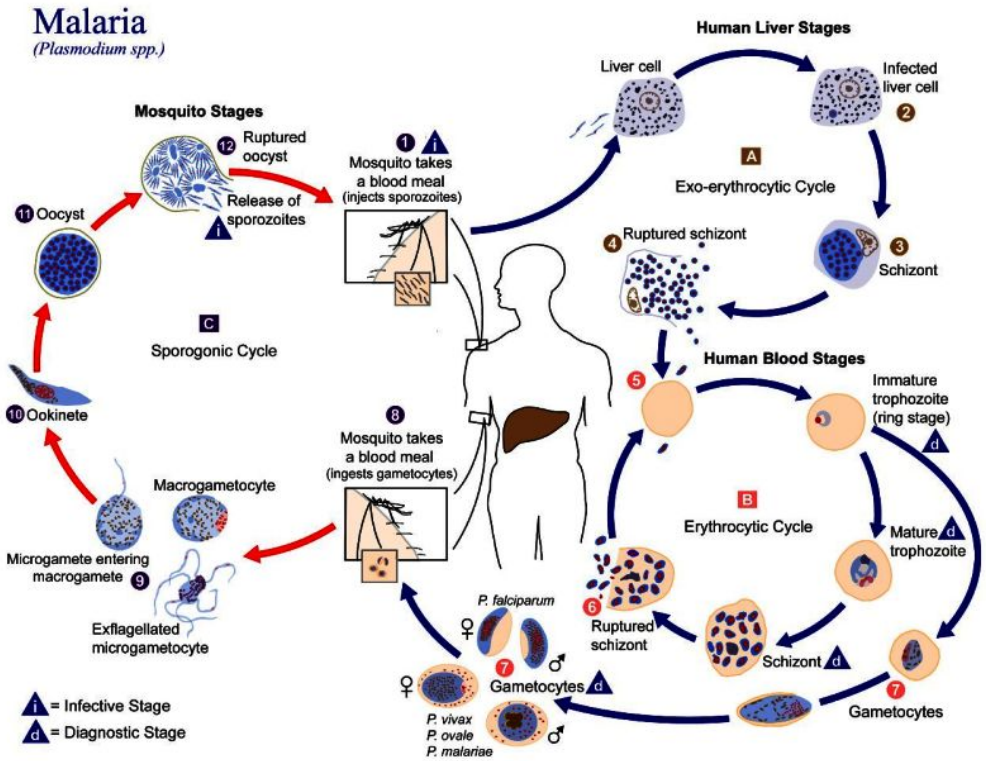

Figure 1.1. Malaria parasite life cycle.
Source: https://www.cdc.gov/malaria/about/biology

1.3. Clinical disease

Malaria is characterized by a syndrome of symptoms signaled by sudden episodes of fever and rigor-like chills (known collectively as a paroxysm) that recur at highly predictable intervals (Warrell & Gilles, 2017). Other acute symptoms include headache, lethargy, fatigue, and profuse sweating after each bout of fever. Episodes of chills and fever are triggered by parasite-mediated events in the circulatory system. After erythrocytes are infected by merozoites, erythrocytic

merogony leads to the synchronous rupture of the erythrocytes and release of new merozoites, toxins, and heme digestion products causing chills and fevers. The next episode occurs in 24, 48, or 72 hours, depending upon the species of *Plasmodium*. From these cyclic episodes come the terms quotidian (daily), tertian (every two days, or days 1 and 3), and quartan (or every four days, or days 1 and 4) (Mullen & Durden, 2009). Malarial infections in humans can range in outcome from asymptomatic infection to chronic infection with anemia, to acutely severe illness and sometimes death. These various outcomes are related to age of the human host, sex (as malaria in pregnancy can lead to maternal anemia, preterm delivery, and low birthweight), immune status, and genetic factors related to race. However, the particular symptoms, including timing and severity, vary with the species of *Plasmodium*. The most severe form of malaria (malignant tertian malaria) is caused by *P. falciparum*, in which merozoites invade both young and old red blood cells. Repeated reinvasions and mass destruction of red blood cells over time may lead to high parasitemia, severe anemia, and anoxia of tissues. In *falciparum* malaria, infected red blood cells stick to the vascular epithelium of capillaries in organs including the brain, impeding blood flow and causing a serious and sometimes lethal end-organ damage including cerebral malaria (Desai et al., 2007; Mullen & Durden, 2009; Idro et al., 2010; Postels & Birbeck, 2013).

P. vivax malaria is called benign tertian malaria because symptoms are less severe than *P. falciparum* malaria, and death rarely occurs. Thus, unlike *falciparum* malaria, *vivax* malaria has fewer severe symptoms of anemia and toxemia, making death unlikely. The infected red blood cells do not stick to the epithelial lining of capillaries as they do in *falciparum* malaria. *Vivax* malaria can evolve into chronic infection with development of an enlarged spleen, or splenomegaly. This can happen in other forms of malaria as well when these organs work to

replace red blood cells lost to infection. *P. ovale* is a less common tertian malaria with milder symptoms. It's course of infection is similar to that of *P. vivax*.

P. knowlesi species was previously known only as one of the monkey malarias and is morphologically similar to *P. malariae*. The high frequency of asexual reproduction in red blood cells results in hyperparasitemia, making it especially virulent if left untreated.

The difference between *P. malariae*, which causes quartan malaria, from *P. vivax* and *P. falciparum* is that the parasites invade only mature erythrocytes. Therefore, symptoms can be more severe than in *vivax* malaria in the acute phase. However, infections tend to develop more slowly and become chronic.

In human malaria, there is a clear distinction between relapse and recrudescence of infection. The course of infection of malaria in humans varies with many factors, including history of past exposure and presence of antibodies; age, health, and nutritional status; and genetic resistance (Dechavanne et al., 2016).

1.4. Significance of *Anopheles* mosquitoes

There are nearly 3,500 known species of mosquitoes and about 430 described species of *Anopheles* (Kitzmiller, 1982). Of the *Anopheles* species, only about 40 species are important in malaria transmission as primary or secondary vectors in endemic settings (Besansky, 2008). Among the important malaria vectors are; *Anopheles albimanus* in Central America, *An. darlingi* in South America, *An. gambiae* and *An. funestus* in Africa, *An. culicifacies* in Asia, and *An. dirus* in Southeast Asia. *Anopheles gambiae* is considered the most important of all, because it is responsible for causing large numbers of malaria cases and deaths, mainly in Africa. This species lives in close association with humans, feeds primarily on them, and can complete a gonotrophic

cycle in only two days. A wide variety of sunlit surface pools during the rainy seasons provides places for larvae development. Many of such places are associated with human activity, such as borrow pits, roadside ditches, wheel ruts, and the hoof prints of domestic animals. Larval development normally only lasts one week (Mullen & Durden, 2009).

Several studies have described mosquito contribution to malaria around the world, especially sub-Saharan Africa, where *Anopheles funestus* species assemblage and *Anopheles gambiae* species complex are the primary vectors (Fontenile & Lochouarn, 1999; Sachs et al., 2004; Sinka et al., 2012).

1.5. Vectorial capacity

Anopheles mosquitoes have specialized traits that permit them to function as biological vectors of human malaria (Collins & Paskewitz, 1995). The traits that are important to their role in malaria transmission include susceptibility of the mosquito to the parasite throughout the entire sporogonic stage, mosquito population density, longevity, and blood feeding behavior (Besansky, 2008). Together these traits define the concept of vectorial capacity (VC). VC can be summarized as the total number of potentially infectious bites that would eventually arise from all the mosquitoes biting a single perfectly infectious (i.e., all mosquito bites result in infection) human on a single day (Brady et al., 2016; Kramer & Ciota, 2015). It is understood therefore, that VC describes the potential of a vector to transmit a pathogen (Catano-Lopez et al., 2019). However, in general not all mosquito vectors have the same potential of parasite transmission including malaria pathogens. Other genera like culicine mosquitoes for example, cannot transmit human (indeed, mammalian) *Plasmodium* species due to the fact that they are not susceptible to infection by these parasites. Interestingly though, some members of the *Anopheles* species do not transmit malaria parasites.

This phenomenon acknowledges differences in vector competence even within the same mosquito species. The phenotypic differences in susceptibility to parasites are well-described, both among anopheline species and among individuals of other species (Collins et al., 1986; Vernick et al., 2005). If a mosquito is susceptible to parasite infection, its blood-feeding behavior, population density and longevity become the most important determinants in vector capacity. For a mosquito to transmit malaria efficiently, it must have a high probability of feeding on humans, high enough populations and must live long enough to allow the malaria parasite to complete extrinsic development (Edalat et al., 2015). The average longevity among anopheline species in the tropics ranges widely, from 10 days to over one month (Ndoen et al., 2012). On average the parasites need between 8-35 days to develop in a mosquito and the longer the mosquito's life span, the more effective the mosquito becomes as a vector (Ndoen et al., 2012). Average lifespan can account for differences in vectorial capacity among species, but it is certainly not the only factor, as illustrated by the *An. gambiae* species complex. *An. gambiae* and its sibling species *An. quadriannulatus* represent an example of paired vector and non-vector species that differ profoundly in their roles in malaria transmission. *An. quadriannulatus* is not naturally found infected with malaria parasites, yet can be infected with cultured *P. falciparum* (albeit at lower infection prevalence compared to *An. gambiae*; Takken et al., 1999; Besansky, 2008). The non-vector status of *An. quadriannulatus* in nature is due to its preference for feeding on animals, and very rarely on humans. *An. gambiae* on the other hand, shows an overwhelming preference for human odor (Besansky, 2008).

1.6. Mosquito vectors and epidemiology

Malaria has been viewed in the context of stable or unstable transmission, reflecting in part attributes of an *Anopheles* population that affect its vectorial capacity. **Stable malaria** is most

often associated with *P. falciparum* infection in highly endemic settings. It is characterized by low fluctuations in parasite incidence in human and vector populations, high prevalence, and high seroprevalence for antibodies. Epidemics are unlikely under these conditions because transmission continues at high rates. In such settings, vectors tend to be highly anthropophagic, exhibit greater longevity, and have relatively low, stable densities but still exhibit considerable seasonal variation (Mullen & Durden, 2009). **Unstable malaria** most often tends to be associated with *P. vivax* infections in endemic settings of high fluctuation in disease incidence. In these settings, vectors tend to be zoophagic, have seasonally profound variation in population densities, low or nondetectable field infection rates, and may have shorter longevity than do those in stable malaria settings. Epidemics can occur in conditions of unstable malaria if environmental changes favor increased vector-human contact; e.g., during civil strife, following water projects such as dams or irrigation schemes, or when a new vector species is introduced into an area (Mullen & Durden, 2009). Human-vector contact driven by human availability and exposures to mosquitoes forms the critical point for pathogen transmission in many vector borne disease systems (Martinez et al., 2021; Schaber et al., 2021; Thongsripong et al., 2019, 2020). The same provides an opportunity for vector borne disease prevention and control interventions.

1.7. Malaria situation in Malawi

1.7.1. Description and map of Malawi

Malawi is a landlocked country located in southeastern Africa in the Great Rift Valley on the western shore of Lake Malawi, the most southerly lake in the Great African Rift Valley system. Malawi shares international borders with Tanzania, Zambia and Mozambique. It is geographically located on -13° 15' 4.38" S and 34° 18' 5.50" E. The country covers an area of

118,484 km². The nation is divided into three main regions: Northern, Central and Southern. There are also three different geographical regions, the Rift Valley, the Central African Plateau, and the Highlands. The Shire river is the largest in Malawi and the only outlet of Lake Malawi. Mulanje Mountain is the highest at an elevation at 3,002 m (9,849 ft) above sea level. Malawi is one of the world's most densely populated nations with a population of 17.5 million people (NSO, 2019). The study was conducted in Machinga and Balaka districts in in the Southern region of Malawi (Figure 1.2).

Figure 1.2. Map of Malawi showing the study sites by district.

Malaria is highly endemic in Malawi causing an estimated six million annual cases (in a population of approximately 17 million people), and remains a major public health burden, one of the most significant causes of morbidity and mortality (Riveron et al., 2015; Spiers, et al., 2002; Snow et

al., 2013; NSO, 2019; WHO, 2021b). Transmission is mainly determined by climatic factors such as temperature, humidity, and rainfall and the rural landscape which promotes production of *Anopheles* mosquitoes in association with agriculture. The extent and distribution of these factors influence malaria prevalence. In Malawi, transmission is highest in areas of high temperature and frequent rainfall from October through April (Government of Malawi, 2015). Studies have also shown that, although every Malawian resident lives in a region of malaria risk, defined as greater than one case per 1000 residents, the risk is highly uneven and varies by a variety of factors such as landscape (topography, agriculture, urban or rural setting), quality of housing, socioeconomic status (degree of poverty), demographic (age and gender) history of infection and exposure, proximity and access to health care facilities, extent of use of malaria protection measures such as long-lasting insecticidal nets (LLINs), and so on (Buchwald et al., 2016, 2017, 2018; Coalson et al., 2016, 2018). In 2008, over 18% of hospital deaths of children less than five years old, and over one third of all outpatient visits, were attributed to malaria, and the most highly prevalent parasite species was *Plasmodium falciparum*. Illnesses classified as malaria represented one half of all outpatient consultations that occurred in the country (Mathanga et al., 2012; Mzilahowa, et al., 2012). By 2018, it was reported that nearly 4 million people in Malawi were diagnosed with malaria infection every year and the country accounted for 2% of malaria cases worldwide becoming among the top 15 countries with high malaria burden in the world (Chilanga et al., 2020).

1.7.2. Malaria vector control

The push for malaria elimination and eventual eradication is heavily dependent on the ability to reduce parasite transmission. In highly endemic areas the ability to reduce malaria transmission is

dependent on controlling vectors to a low level. It is after achieving low vector populations that control strategies can shift to killing the parasite in infected people (Mendis et al., 2009). Two forms of vector control, indoor residual spraying (IRS) and distribution of long-lasting insecticidal nets (LLINs) have been shown to reduce transmission when properly deployed against insecticide susceptible mosquito populations (Tukei et al., 2017; WHO, 2006).

Endemic countries have deployed efficacious vector control using IRS and LLINs as hallmark interventions, alongside case management with effective treatment using artemisinin-based combination therapy (ACT) guided by definitive diagnosis (Chanda et al., 2015). In Malawi, current malaria control efforts rely heavily on the insecticide-based interventions. The initial malaria interventions are being targeted at protecting those at highest risk of disease (pregnant women and under five children) with the aim to towards universal coverage. However, to move from control to elimination there is need to effectively interrupt transmission in the population, hence the fight against malaria in Malawi is far from over. While there has been substantial research in Malawi on the pathology, immunology and chemotherapy of malaria, knowledge of malaria transmission dynamics and of the vectorial roles (ecology and behavior) of various anopheline species known to occur in Malawi is spars (Spiers, et al., 2002; Mzilahowa, et al., 2012).

Starting in 1911, studies on malaria transmission in Malawi identified two vectors of malaria parasite, *An. funestus* which was the most common, and *An. gambiae* sensu lato (i.e., s.s.) (Mzilahowa et al., 2012). Reports of increasing resistance in these vectors against the main insecticides used in public health are of concern for the continued effectiveness of the control tools in different parts of Africa (Czeher et al., 2008; Djouaka et al., 2008; Hargreaves et al., 2000; Martinez-Torres et al., 1998; Ranson et al., 2009, 2011). In Malawi, the concern is even greater

because of the increasing cases of resistance against pyrethroids (the only insecticide class used in bed nets) in the major malaria vector *Anopheles funestus* s.l. and *An gambiae* s.l. (Mzilahowa et al., 2016; Riveron et al., 2015). Challenges facing malaria vector control strategies in Malawi as in many countries include lack of effective program implementation and management, faulty manufacture and quality control in LLINs, low uptake of LLINs into communities, and elements of the transmission system outside of what coverage provides (Cohen et al., 2012; Lindblade et al., 2015; Vinit et al., 2020; Lindsay et al., 2021).

It is against this background that the current study aimed at furthering the understanding of vector species distribution, host preference and malaria transmission patterns (from humans to mosquitoes) in the local mosquito population. This study also aimed at characterizing human-to-mosquito parasite transmission in detail. This was done by using molecular techniques to link mosquito blood meals to individuals that were fed upon and to determine the mosquito host selection range in few vertebrate hosts (human, dog and goat).

Human availability and vector preferences determine which hosts are most frequently bitten (Keven et al., 2017; Toe et al., 2018). We hypothesize that *An. gambiae* s.l. and *An. funestus* will be the most abundant mosquito species in the study sites. Previous ICEMR-supported research in an urban-rural site in Malawi found that 90% of vectors captured were *An. gambiae* s.l. (primarily. *An. arabiensis*) and *An. funestus* was the only other species detected (Dear et al., 2018). We also hypothesize that humans will be the mostly utilized source of blood meal and that school-age children (6-15 years old) are the predominant source of *Anopheles* blood meals. Previous studies have suggested that a greater proportion of blood meals are taken from adults and older children than young children and infants (Carnevale, Frézil, Bosseno, Le Pont, & Lancien, 1978; Muirhead-

Thomson, 1951) partially because of differences in body surface area. Studies have also shown differential use of LLINs by age, with school-age children being least likely to sleep under nets in Malawi (Buchwald et al., 2016; Walldorf et al., 2015). Thus, school-age children are likely to be bitten more frequently based on size and limited LLIN use. In addition, previous studies reported that *Plasmodium*-infected individuals are more attractive to vectors than uninfected individuals (De Moraes et al., 2014; Lacroix et al., 2005). Given the observations that school-age children have the highest prevalence of both asexual stage infection (Walldorf et al., 2015) and gametocytes (Coalson, 2015), we anticipate that vectors will preferentially take blood meals from them.

We further hypothesize that human transmission reservoirs that are not sufficiently impacted by standard malaria interventions, contribute to malaria being recalcitrant in Malawi. We aim to identify these populations by quantifying the complex interactions that determine the human-to-mosquito *Plasmodium* transmission from different population sub-groups. Exposure to *Anopheles* feeding is one major component of the spread of infection. However, if the person is uninfected or has only asexual parasites, biting frequency is irrelevant to parasite transmission. Thus, the prevalence of gametocyte-containing infections in human sub-groups critically influences their differential contributions. Finally, not all gametocyte infections result in transmission to every feeding mosquito. The population-level dynamics of human-to-mosquito transmission depend on each component: the probability of being bitten, the probability of carrying parasites/gametocytes when bitten, and the probability of successful transmission per blood meal. Estimating all three in the same setting thoroughly quantifies human contributions to malaria transmission and enables the characterization of the predominant transmission reservoirs.

1.8. Research significance and objective

1.8.1. Significance

This study aims to increase our understanding of malaria transmission from humans to mosquito, malaria infection reservoirs, and to inform designing of effective malaria control strategies. The linking of human to mosquito infection is not well understood. However, characterizing transmission from humans to mosquitoes is complex. Identifying which humans harbor gametocytes is insufficient because successful transmission involves complex, multidimensional relationships between human and vector behaviors, and human, vector, and parasite biological interactions. Few studies have attempted to fully describe and model the factors that contribute to human-to-mosquito transmission, yet that information is essential to reducing malaria transmission.

Our overall goal is to understand the complex human, parasite, and vector relationships (human and vector behaviors, and human, vector, and parasite biological interactions), identify the most important sources of malaria parasite transmission in the human population. This information could be applied in the designing of new control strategies targeting these malaria reservoirs. It was envisaged that the study will contribute to the current understanding of malaria transmission dynamics and the vectorial roles of mosquitoes (ecology and behavior) and improve the practice of malaria vector control interventions in Malawi.

This research was part of a larger endeavor called the International Centers of Excellence in Malaria Research (ICEMR), a multi-site program funded by the US National Institute of Allergy and Infectious Diseases of the NIH. One of the sites is in Malawi and associated with the College of Medicine and its Malaria Alert Center in Blantyre, with institutional partners in the US being Michigan State University and the University of Maryland School of Medicine in Baltimore. The

project is titled “The Intransigence of Malaria in Malawi: Understanding Hidden Reservoirs, Successful Vectors and Prevention Failures” and the award number is U19AI089683. The overall goal of the ICEMR project in Malawi is to determine why malaria persists in the country despite strong interventions imposed against it (primarily distribution of insecticide treated bed nets, indoor residual insecticide sprays, and distribution of antimalaria drugs to vulnerable groups of people). The aim is to use knowledge gained from the research program to guide Malawi’s malaria policies and intervention strategies to achieve malaria control and, ultimately, elimination. The ICEMR program has three arms or projects: (P1) Epidemiology, (P2) Transmission, and (P3) Infection and Disease. The research reported here falls under P2. The overall aim of P2 was to identify the most important sources of malaria transmission in the human population and to apply this information in the design of new strategies to target these reservoirs.

1.8.2. Objective

The aim of the research described in this dissertation was to delineate human to mosquito *Plasmodium* infection transmission through blood meal and genotyping analysis to understand drivers of malaria infection in different population groups. This aim was broken down into three objectives and their hypotheses as outlined below.

Objective 1 (Chapter 2): To quantify malaria vector species composition, relative abundance and distribution in the study area as they relate to malaria transmission.

Hypothesis 1.a. The composition, distribution and abundance of the different mosquito vector species is the same across the study area.

Hypothesis 1.b. Mosquito infection rates are similar across the study area by species and site.

Objective 2 (Chapter 3): To investigate blood host selection behavior of malaria vector mosquitoes for blood meal sources, addressing the question: which vertebrate hosts are the primary sources of blood meals, amongst the available humans and non-human hosts?

Hypothesis. Mosquito feeding is host specific with regard to human and non-human hosts available in the study area.

Objective 3 (Chapter 4): To determine sources of mosquito blood meals within human population demographic groups.

Hypothesis 3.a. Mosquito feeding on individual humans is nonrandom.

Hypothesis 3.b. Mosquito feeding is preferential on certain human population groups (males and 6-15 years old), classified by age and gender in the study area and these groups drive malaria transmission.

1.9. Research ethical approval

Institutional Research Board (IRB) reviews of this sample design were submitted and approved by the University of Malawi, College of Medicine (COMREC) and Michigan State University (MSUIRB) (IRB00003905 FWA00005976), under the International Center of Excellence in Malaria (ICEMR) research program awarded to these two institutions from the National Institutes of Health (NIH) (U19AI089683). This project was a component of the ICEMR research program under Project 2: Transmission.

APPENDIX

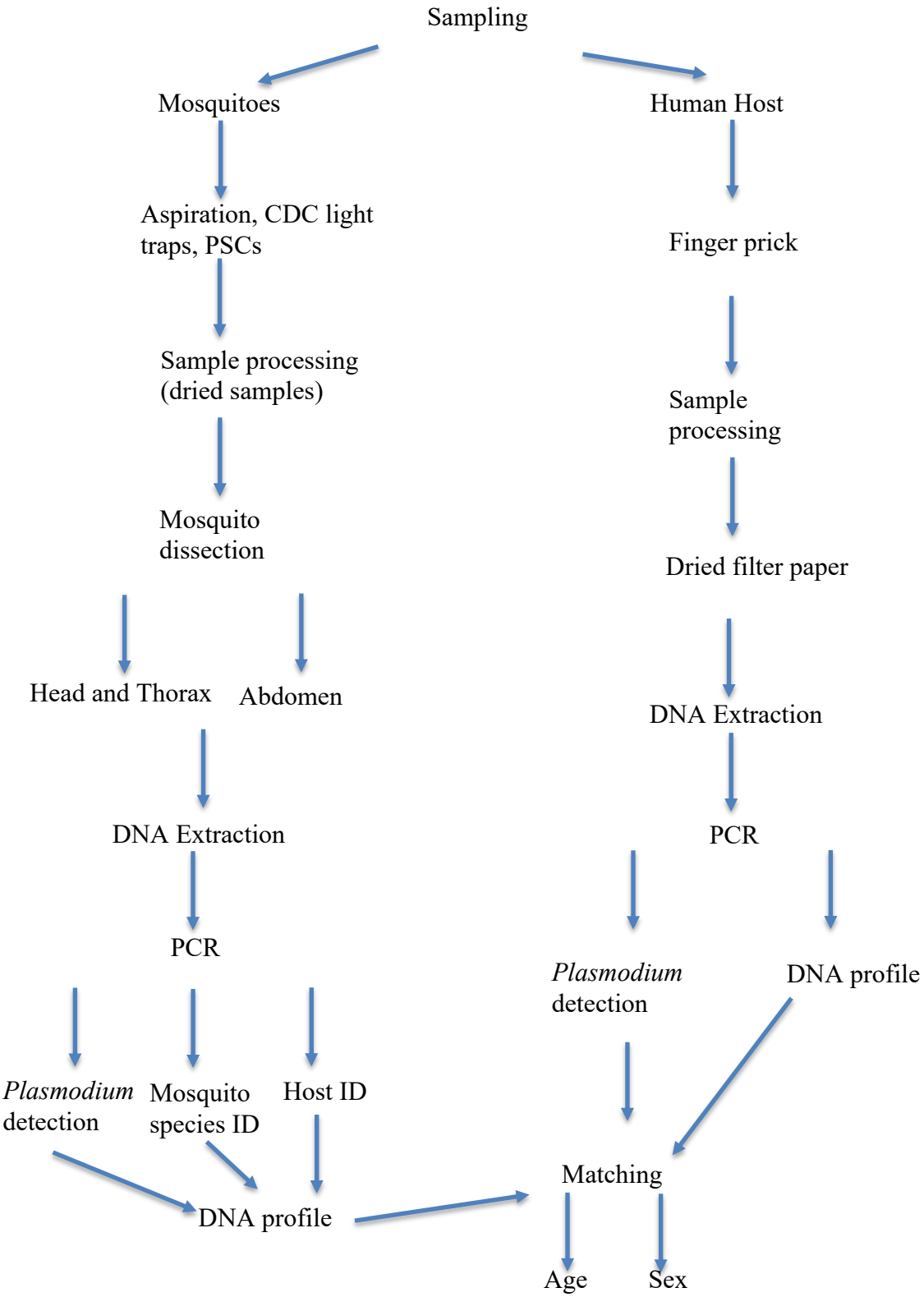

Figure 1.3. Conceptual framework

CHAPTER 2 : MOSQUITO COMPOSITION, ABUNDANCE AND DISTRIBUTION IN RELATION TO *PLASMODIUM* INFECTION TRANSMISSION IN *ANOPHELES* MOSQUITO SPECIES REVEALS PRESENCE OF POTENTIAL SECONDARY MALARIA VECTORS IN SOUTHEASTERN MALAWI

2.1.Abstract

Introduction. Understanding mosquito species composition, abundance and distribution is vital step in malaria vector surveillance and control. Variations in distribution and abundance of *Anopheles* species are important factors driving spatiotemporal changes in transmission intensity and infection prevalence. This study aimed at understanding differences in species composition and quantifying the malaria vector species abundance and distribution and *P. falciparum* infection rates as they relate to malaria transmission.

Method. Morphologically identified female *Anopheles* mosquitoes were sampled in human dwellings in Namanolo and Ntaja for almost one year using aspiration, CDC light traps and pyrethrum spray catches. Molecular mosquito species identification and *Plasmodium falciparum* infection detection in the mosquito head-thorax and abdominal sections were done by PCR. Statistical data analysis included frequency distribution, chi-square and fisher's exact test with all tests done at 0.05 significance level.

Results. Of 6,763 mosquito samples analyzed and morphologically identified as *Anopheles* only five *Anopheles* species were molecularly identified. The dominant species were *An. arabiensis* (8.8%), *An. funestus* s.s. (25%) and *An. gambiae* s.s. (2.9%). *An. parensis* and *An. vaneedeni* were less abundant (<0.1%); 63.1% were other as yet unidentified *Anopheles* species. There were no significant differences in species composition, but species abundance varied from 0.03 to 65.8%

in the two study sites. The infection rates among the species in the head-thorax section of the mosquitoes were significantly different but no significant difference was observed in the abdominal sections of blood fed samples.

The infection rate was higher in the abdomen (26 and 36%) than the head-thorax section (14 and 28%) across all species in all sites with infection rates higher in Ntaja than Namanolo. The highest *Plasmodium falciparum* infection rate in both abdomen and head-thorax section was observed in *An. gambiae* s.s. In the blood-fed mosquitoes, *Plasmodium falciparum* positive rates in the PCR unidentified *Anopheles* species suggests the presence of potential secondary malaria vectors.

Conclusion. *Anopheles* species composition and distribution except abundance is similar across the study area. The infection rates in *An. arabiensis*, *An. funestus* and *An. gambiae* s.s. in head-thorax and abdomen of mosquitoes point to a potential situation of residual malaria transmission. Presence of potential secondary malaria vectors needs further investigation.

2.2. Introduction

Malaria remains one of the major health burdens in African including Malawi where malaria cases are estimated at four to six million annually (Government of Malawi, 2015; Riveron et al., 2015; WHO, 2021b). The two dominant species of *Anopheles* mosquitoes that transmit malaria in Malawi are *Anopheles gambiae* species complex and *An. funestus* species assemblage (Mzilahowa et al., 2012; Spiers et al., 2002). Since 2000, a decrease in malaria incidence and mortality has been reported in sub-Saharan Africa, mainly due to widespread use of LLINs (Cibulskis et al., 2016; WHO, 2014). However, the gains have been faced with challenges such as the rise of insecticide resistance in the malaria vector mosquitoes which appear to be wide spread across countries and malaria mosquito species (Hunt et al., 2010; Mzilahowa et al., 2016; Ranson et al., 2011; Riveron et al., 2015). Others challenges facing malaria control include lack of effective program implementation and management, faulty manufacture and quality control in LLINs, uptake of LLINs into communities, and elements of the transmission system outside of what coverage provides (Cohen et al., 2012; Lindblade et al., 2015; Vinit et al., 2020; Lindsay et al., 2021).

Therefore, understanding mosquito vector species composition, abundance and distribution is a vital step in malaria vector surveillance and control. Variations in distribution and abundance of *Anopheles* species are important factors driving spatiotemporal variation in transmission intensity and infection prevalence (Eba et al., 2021). Mosquito species composition varies greatly among geographic locations (villages), even among villages that are less than 1 km apart (Keven et al., 2017). Without quantitative estimates, it is difficult to describe the degree to which two localities differ in their mosquito species composition and therefore malaria transmission.

This study aims at understanding the degree of dissimilarities in species composition among localities in the study area (Roberts & Hsi, 1979; Almeida et al., 2008) and quantifying the malaria vector species abundance and distribution as they relate to malaria parasite transmission in Machinga and Balaka districts.

We, therefore, hypothesis that (1) the composition, distribution and abundance of the different mosquito vector species is homogenous and (2) Mosquito infection rates are similar across the study area by species and site.

2.3. Methods

2.3.1. Study sites

Malawi is a landlocked country located in southeastern Africa in the Great Rift Valley on the western shore of Lake Malawi, the most southerly lake in the Great African Rift Valley system. Malawi shares international borders with Tanzania, Zambia and Mozambique. It is geographically located at -13° 15' 4.38" S and 34° 18' 5.50" E (Figure 2.1). The country covers an area of 118,484 km². The nation is divided in three main regions: Northern, Central and Southern. There are also three different geographical regions, the Rift Valley, the Central African Plateau, and the Highlands. The Shire River is the largest river in Malawi and the outlet of Lake Malawi, flowing south to the Zambezi River. Mulanje mountain is the highest at an elevation of 3,002 m (9,849 ft) above sea level. Malawi is one of the world's most densely populated nations with a population of 17.5 million people (NSO, 2019).

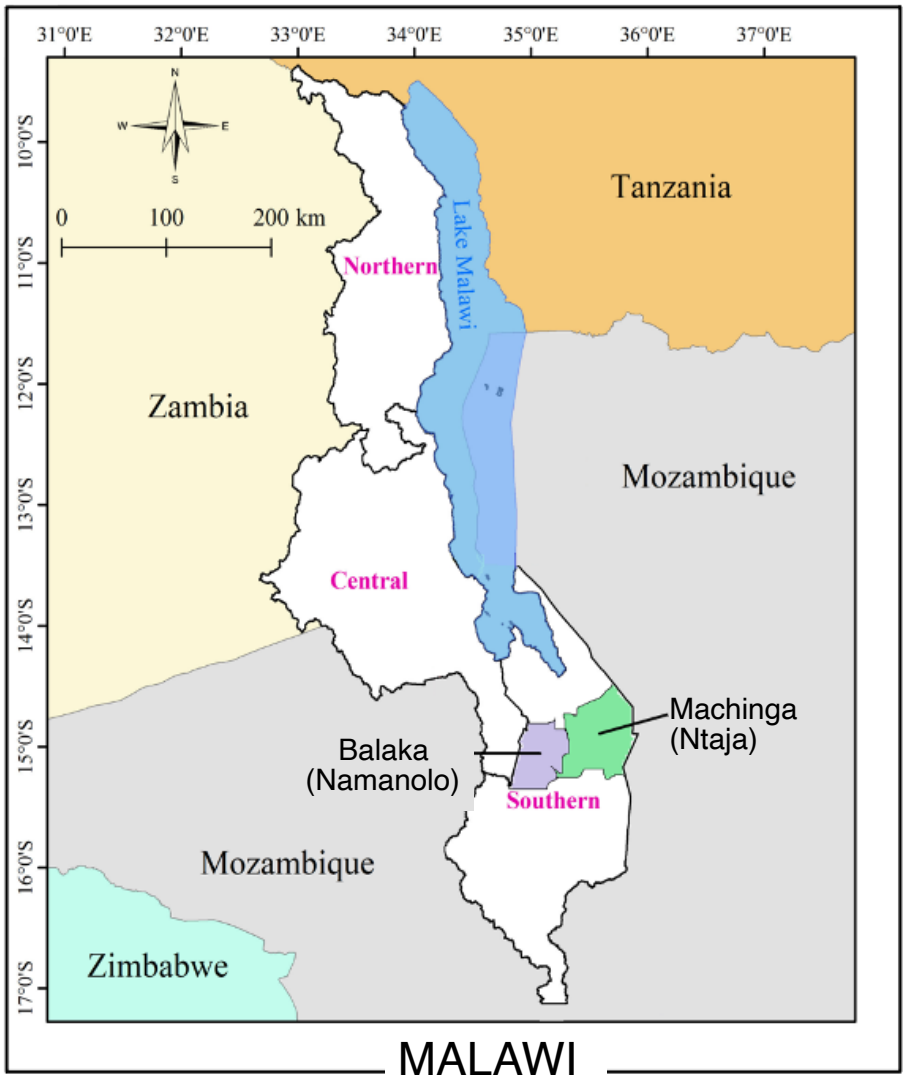

Figure 2.1. Map of Malawi showing the study sites.

2.3.2. Description of Study areas

The study was conducted in Machinga and Balaka districts in the Southern region of Malawi. Machinga district covers an area of 3,771 km² and has a population of 735,438 (NSO, 2019). Balaka is a township in the southern region of Malawi and headquarters for Balaka District. The township was formerly a boma of Machinga District, before Balaka District was created in 1998. The district covers an area of 2,193 km² and has a population 438,379 (NSO, 2019). Both districts are mostly rural areas with the majority of the people sustaining their livelihood through

subsistence farming. Balaka district is located on a lower elevation and close to the Shire River, the largest river in the country, while Machinga is on a higher elevation further away from the river but has small semi-permanent and temporally water bodies more prominent in the rainy season.

2.3.3. Study design

The overall study approach was a longitudinal cohort study in communities located in Balaka and Machinga districts within the catchment area of two health centers, Namanolo health center in Balaka and Ntaja in Machinga district. Mosquito sampling was conducted in these catchment areas from which ~100 households in each site (total 200) were sampled. Index houses were randomly sampled within an ecogeographic quadrant in a 10 kilometers radius from the health center. Then neighboring houses were identified to create clusters. On each selected household, the household head was consented. Households were visited for entomological sampling for one year after the universal Long-Lasting Insecticidal Nets (LLINs) distribution campaign in 2018 (Government of Malawi, 2018).

2.3.4. Sample collection and processing

Mosquito sampling and analysis

Indoor resting female *Anopheles* mosquitoes were sampled until there were none found resting on walls in the sleeping spaces of human dwellings on any sampling morning. The households were selected by the randomized cluster sampling method, monthly for three times each period from May-June 2019, October-November 2019, and December 2019-January 2020. Beginning in February to April of 2020, only a subset of households in each site were sampled once every two

weeks. Mosquito sampling tools including hand and battery powered aspirators, standard miniature CDC Light Trap (Model 512; John W. Hock Company, Gainesville, Florida, USA) and pyrethrum spray catches (PSCs) (WHO, 1995; Odiere et al., 2007) were used to maximize sampling effort. Mosquitoes were morphologically identified (Gillies & Coetze, 1987) into *Anopheles* mosquito species with emphasis on identifying major malaria vectors *Anopheles gambiae* sensu lato (Giles,1902) and *Anopheles funestus* sensu lato (Giles, 1900). The mosquito samples were stored in silica gel and processed. Blood feeding status was scored by visual inspection into fully engorged (fed), gravid, half-gravid and unfed mosquito. The abdomen was separated from the head and thorax of each mosquito using sterile techniques, and these two body parts were processed separately.

Genomic DNA was extracted from mosquito abdomens and mosquito head-thorax using Qiagen extraction kits (DNeasy® Blood & Tissue Kit; Cat. No. 69506) following the manufactures standard protocol. The mosquito abdomens (a subset which included all blood fed and a random sample of unfed mosquitoes) were analyzed for *P. falciparum* blood-stage and oocyst infection while the head-thorax was analyzed for *P. falciparum* (presumably, sporozoite) infection and mosquito species identification.

Molecular mosquito species identification

Mosquitoes of the *Anopheles gambiae* species complex were identified to species using real time quantitative PCR method of Walker et al., (2007). The universal primers were forward 5'-GTG AAGCTTGGTGCGTGCT-3' and reverse 5'-GCACGCCGA CAAGCTCA-3'. The probe for *An. gambiae* s.s. (Giles, 1902) was 5'-VIC-CGGTA GGAGCGGGACACGTA- MGBNFQ-3' and the probe for *An. arabiensis* (Patton, 1905) was 5'-6FAM-TAGGATGGAGAAGGACACTTA-

MGBNFQ-3'. Mosquitoes of the *Anopheles funestus* species assemblage (Giles, 1900) were identified to species using the real time PCR method of Scott et al., (1993) and Koekemoer et al., (2002). The universal primers were forward 5'-AGAACACTATGGCGAGCAGC-3', and the reverse was 5'-TTACGACGGATACGGTCAACG-3'. The probe for *An. funestus* s.s. was 5'-6FAM - CATGGGGAAATTCAATCGAAAACCTCT-QSY-3', the probe for *An. parensis* (Gillies, 1962) was 5'-ABY-TGGCGTGCTCGGAACCTAGC-QSY-3', and the probe for *An. vaneedeni* (Gillies, 1968) was 5'-VIC-CGTTGTGAAAAATGGAGATTCATTTGAAAACC-QSY-3'. PCR amplifications for *An. gambiae* s.l. and *An. funestus* s.l. were carried out in a volume of 10 µL reaction with 5 µL of 2X Taq Universal Master Mix (Cat. No. 4304437), 0.6 µL of each primer (10 µM), 0.4 µL of each probe, 2 µL of DNA, and nuclease-free water. PCR cycling conditions for the amplification of DNA from *An. gambiae* s.l. and *An. funestus* s.l. sources on QuantStudio™ RT-PCR System were as follows: one cycle at 50 ºC for 2 minutes, 95 ºC for 10 minutes, followed by 40 cycles at 95 ºC for 15 seconds, denaturization, and 60 ºC for 1 minute annealing/extension temperatures. Positive DNA controls for *An. arabiensis An. gambiae*, *An. funestus* obtained from beiresources.org (MRA-495, MRA-142 and MRA-1027 respectively) and negative controls as blank were included within each experiment. The amplification curves were visualized on QuantStudio software.

Molecular identification followed morphological identification for the following crucial reasons. 1) non-*Anopheles* species need to be separated from the sample, 2) morphological identification can be challenging in the field especially where a species complex is involved, 3) morphological identification is prone to human error and 4) the two methods complement each other (Friedheim, 2016).

Cytochrome oxidase subunit 1 (COI) gene barcoding (Beebe, 2018; Maekawa et al., 2021) was applied to a subsample of the mosquitoes that were not identifiable using the above PCR method.

Molecular identification of Plasmodium falciparum

Plasmodium falciparum was detected in nucleic acid extractions of mosquito abdomens, or heads-thoraces targeting 18S rRNA gene (Bass et al., 2008; Mangold et al., 2005). The primers used were forward 5'-ATTGCTTTTGAGAGGTTTTGTTACTTT-3' and reverse 5'-GCTGTAGTATTCAAACACAATGAACTCAA-3'. The probe was 5'-FAM-CATAACAGACGGGTAGTCAT-MGBNFQ-3' adopted from Kamau et al., (2013). PCR reactions for *Plasmodium falciparum* detection were performed in a volume of 10 µL with 5 µL of 2X Multiplex Master Mix (Cat. No. 4461882), 0.6 µL of each primer (10 µM), 0.4 µL of each probe, 2 µL of DNA, and nuclease-free water. PCR amplifications were with the QuantStudio™ RT-PCR System as follows: one cycle at 95 ºC for 20 seconds, 40 cycles at 95 ºC for 1 second, and 60 ºC for 20 seconds. Positive genomic DNA controls for *P. falciparum* obtained from beiresources.org (MRA-506) and negative controls as blank were included within each experiment. The amplification curves were visualized on QuantStudio software.

The number of *Plasmodium falciparum* positive mosquito samples was used to estimate human infection (using mosquito abdomen for asexual stages and gametocytes) and mosquito infection prevalence (using mosquito head and thorax for sporozoites).

2.3.5. Data analysis

An estimate of community mosquito composition was assessed. Relative abundance and species distribution were calculated based on absolute numbers and proportions of specimens collected per location (household) and mosquito species in each site (Mbokazi et al., 2018). The infection rate for sporozoites in salivary glands (i.e., the sporozoite rate) and abdomen (mosquito infection; asexual stages and gametocytes) was compared for each species between districts. Statistical analysis included frequency distribution analysis to access abundance and distribution of mosquito species, chi-square, and contingency table fisher's exact test where applicable was used to compare the mosquito proportions, all tests were carried out at 0.05 significant level.

2.4. Results

2.4.1. Mosquito species composition, distribution and abundance

A total of 6,763 *Anopheles* mosquitoes were collected, including 3,766 (56%) from Namanolo and 2,997 (44%) from Ntaja (see supplementary data Appendix 2.1). Head-thorax sections from all 6,763 were analyzed, however, only a subset of samples, 1386 comprising of all blood fed mosquitoes and a few randomly selected nonblood feds, were analyzed in the abdominal sections. A total of 2,566 (37.9%) were identified as *An. arabiensis*, *An. gambiae* s.s., *An. funestus* s.s, *An. parensis* and *An. vaneedeni* (Table 2.1). *An. funestus* s.s. was the most abundant while *Anopheles vaneedeni* was only detected in Namanolo. Molecular identification did not detect 4197 (62.1%) of the entire samples to species using the five set of primers and probes in the scope of the study and were referred to as *Anopheles spp*. Those species identified molecularly were in agreement to morphological identifications done prior to extraction of nucleic acids. In addition, the morphological identifications also revealed the presence of *An. pretoriensis* (Theobald, 1903), *An. maculipalpi*s (Giles, 1902), and *An. coustani* (Laveran, 1900). The results by Cytochrome oxidase subunit 1 (COI) gene barcoding on a subsample of the non-identified samples revealed the presence of other *Anopheles* species and that most of the non-identified mosquitoes were of the *Anopheles gambiae* complex (Figure 2.3).

Mosquito relative abundance across species expressed as mean number of mosquitoes per household showed significant difference between the two sites ($X^2 = 396.3$, $P<0.001$) (Figure 2.2). The non-identified species (*Anopheles spp.)* did not show significant differences between the two sites.

Table 2.1. *Anopheles* mosquito species abundance and distribution per site based on molecular identification.

Number of mosquitoes collected per site (n (%))			
Species	**Overall (n=6763)**	**Namanolo (n=3766)**	**Ntaja(n=2997)**
An. arabiensis	593 (8.8)	502 (13.3)	91 (3.0)
An. funestus s.s.	1,694 (25.0)	730 (19.4)	964 (32.2)
An. gambiae s.s.	198 (2.9)	50 (1.3)	148 (4.9)
An. parensis	8 (0.1)	6 (0.2)	2 (0.1)
An. vaneedeni	1 (0.01)	1 (0.03)	0 (0.0)
Anopheles spp.	4,269(62)	2,477 (65.8)	1,792 (59.8)

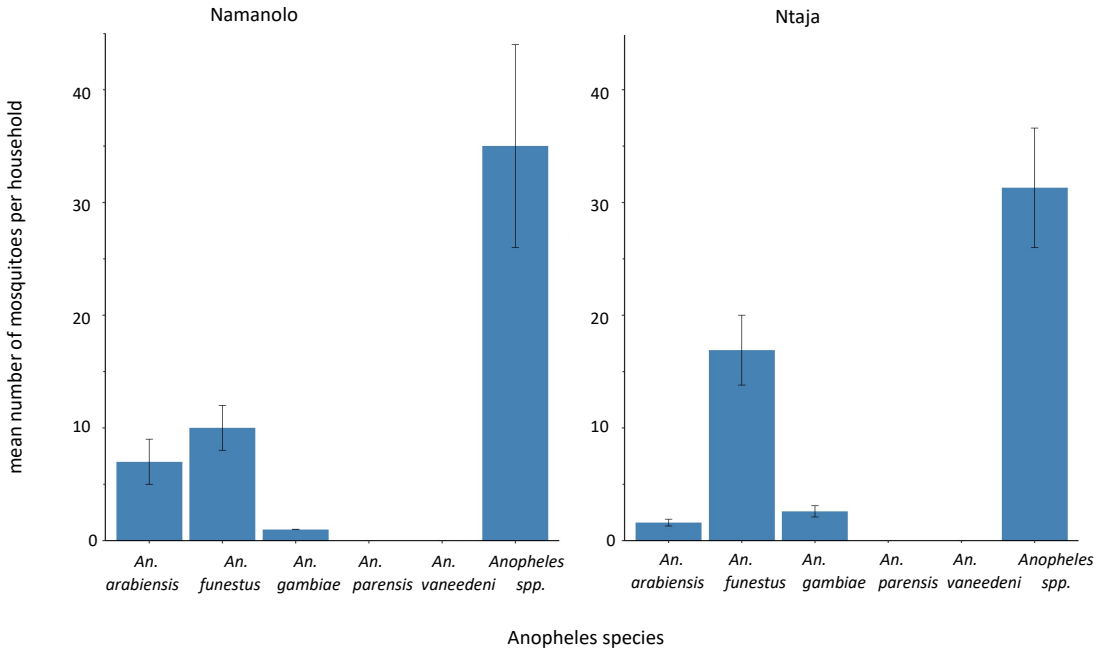

Figure 2.2. *Anopheles* mosquito species abundance and composition per site. Abundance calculated as mean number of mosquitoes per household with their standard errors.

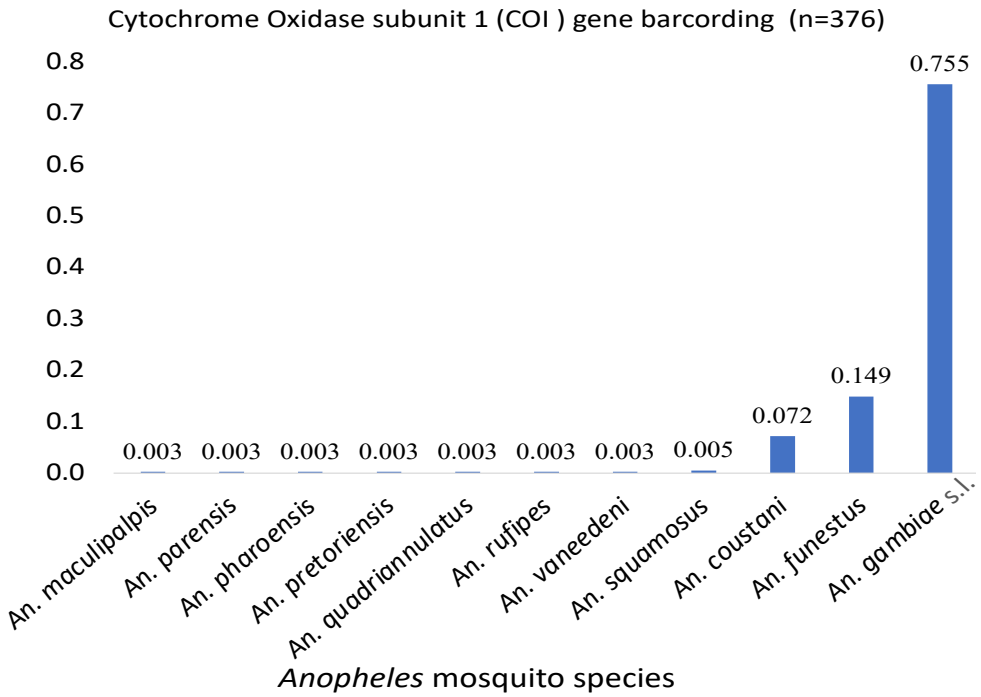

Figure 2.3. *Anopheles* mosquito species proportions detected by COI gene bar coding from a subset of the non- PCR detected samples indicating presence of other *Anopheles* species.

2.4.2. Mosquito processed in head-thorax, for blood fed status and species abundance

Of the 6,763 mosquitoes analyzed, 827 (12%) were visibly engorged blood fed. There were significant differences in mosquito feeding status across species between the two sites ($X^2 = 121.1$, $P < 0.001$) (Table 2.2.). However, blood fed status across species showed *An. arabiensis*, *An. gambiae* s.s. and *An. funestus* s.s. to have higher proportion of blood fed than *An. parensis*, *An. vaneedeni* and *Anopheles spp*. Significantly more blood fed mosquitoes were collected in Namanolo (17%; n=542) than Ntaja (11%; n=285) ($X^2 = 37$, $P < 0.001$) (Table 2.2).

Table 2.2. Blood-fed status of *Anopheles* mosquitoes processed in the head-thorax section.

Number of mosquitoes based on blood fed status (fed and unfed) of all processed head sections (n (%))				
	Namanolo (n=3,766)		Ntaja (n=2,997)	
Species	**fed (n=542)**	**unfed (n=3224)**	**fed (=285)**	**unfed (n=2712)**
An. arabiensis	244 (0.45)	257 (0.08)	39 (0.14)	52 (0.02)
An. funestus	165 (0.31)	564 (0.17)	138 (0.48)	826 (0.30)
An. gambiae s.s.	32 (0.06)	18 (0.01)	67 (0.24)	81 (0.03)
An. parensis	1 (0.002)	5 (0.00)	1 (0.00)	1 (0.00)
An. vaneedeni	1 (0.002)	0	0	0
Anopheles spp.	99 (0.18)	2,380 (0.74)	40 (0.14)	1,752 (0.65)

Note: percentage in parenthesis

2.4.3. *Plasmodium falciparum* infection status per body part of blood fed and unfed mosquitoes combined.

Plasmodium falciparum infection detections in the head-thorax and abdominal sections of the mosquitoes revealed significantly higher infection rates in the abdominal sections (16%; n = 221) of the mosquitoes compared to the head-thorax sections (2%; n = 149) in all the two sites combined ($X^2 = 422$, $P < 0.001$) (Table 2.3). Overall, there was no significant difference in head-thorax or abdomen infection between the two sites.

Table 2.3. Number of *Plasmodium falciparum* infected mosquitoes per body section

	Head-Thorax section			Abdomen section		
***P. falciparum* status**	**Overall (n=6763)**	**Namanolo (n=3766)**	**Ntaja (n=2997)**	**Overall (n=1386)**	**Namanolo (n=855)**	**Ntaja (n=531)**
positive	149 (0.02)	95 (0.03)	54 (0.02)	221 (0.16)	130 (0.15)	90 (0.17)
negative	6614 (0.98)	3671 (0.97)	2943 (0.98)	1165 (0.84)	725 (0.85)	441 (0.83)

2.4.4. Mosquito blood-fed status and *Plasmodium falciparum* infection per body part

There was an observable difference in infection rates between blood-fed status as well as body parts. The blood-fed mosquitoes had higher infection rates than non-blood feds and abdominal sections than heads (Table 2.4). There were no significant differences in infections between the study sites per mosquito body part. Significant differences were observed in the overall infection of the head-thorax section of fed (14%) vs unfed (0.006%), and overall abdominal infections of fed (26%) vs unfed (0.009%).

Table 2.4. Mosquito blood-fed status and *Plasmodium falciparum* infection per body part and study site.

Number of infected mosquitoes according to feeding status (n (%))							
Head-Thorax section				Abdomen section			
Blood fed status	**Overall (6763)**	**Namanolo (n=3766)**	**Ntaja (n=2997)**	**Blood fed status**	**Overall (n=1386)**	**Namanolo (n=855)**	**Ntaja (n=531)**
Fed (n=827)	112 (0.14)	69 (0.13)	43 (0.15)	Fed (n=827)	215 (0.26)	126 (0.23)	89 (0.31)
unfed (5936)	37 (0.006)	26 (0.008)	11 (0.004)	unfed (559)	5 (0.009)	4 (0.013)	1 (0.004)

Plasmodium falciparum infection across *Anopheles* species per body part and study site blood fed and unfed mosquitoes combined.

The infection proportions varied greatly across the main malaria vector species and study sites ranging from 1 to 12% in head-thorax section and 5-39% in abdominal section. The overall infection proportion in both mosquito head and abdominal sections in Namanolo when compare across the mosquito species, *An. arabiensis* had number of high infections, followed by *An. funestus* and lastly *An. gambiae*. In Ntaja however, the highest number of infections were observed in *An. funestus* followed by *An. gambiae* and lastly *An. arabiensis* (Table 2.5) All species showed

no significant difference in infection within body parts across study sites except for *An. gambiae* which had significantly higher abdominal infection in Ntaja (48%; n = 32) than Namanolo (21%; n = 7) ($X^2 = 30.4$, $P < 0.001$).

Table 2.5. *Anopheles* species *Plasmodium falciparum* infection proportion per body part and study site.

Number of infected mosquito species per body section (n (%))						
	Heads-Thorax			Abdomen		
Species	overall (n=6763)	Namanolo (n=3766)	Ntaja (n=2997)	overall (n=1386)	Namanolo (n=855)	Ntaja (n=531)
An. arabiensis	51 (0.09)	45 (0.09)	6 (0.07)	79 (0.25)	72 (0.25)	8 (0.20)
An. funestus	39 (0.02)	20 (0.03)	19 (0.02)	77 (0.20)	36 (0.18)	41 (0.21)
An. gambiae s.s.	24 (0.12)	6 (0.12)	18 (0.12)	39 (0.39)	7 (0.21)	32 (0.48)
An. parensis	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
An. vaneedeni	0 (0.00)	0. (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Anopheles spp.	35 (0.01)	24 (0.01)	11 (0.01)	26 (0.05)	15 (0.05)	9 (0.04)

2.4.5. Mosquito species abundance and *Plasmodium falciparum* infection of blood fed mosquitoes analyzed in the abdomen section

A total of 827 female blood fed *Anopheles* mosquitoes were collected in the two study sites. The overall species composition of blood-fed mosquitoes included *An. arabiensis* (n=283; 34%), *An. funestus* s.s. (n=303; 37%) and *An. gambiae* s.s. (n=99; 12%), *An. parensis* (n=2; 0.002%)), *An. vaneedeni* (n=1; 0.001%) and other *Anopheles* spp. (n=139; 17%). The most abundant species in general was *An. funestus* s.s., however, per site *An. arabiensis* (n=244) was most abundant in Namanolo followed by *An. funestus* s.s. (n=165) and lastly *An. gambiae* s.s. (n=32). In Ntaja the most abundant species was *An. funestus* s.s. (n=138) followed by *An. gambiae* s.s. (n=67) and lastly *An. arabiensis* (n=39). *An. parensis* (n=2) and *An. vaneedeni* (n=1) were the least abundant species. There were no significant differences in overall blood fed mosquito abundance between the two sites ($X^2 = 3.3$, $P = 0.07$) although there were significant differences across species

abundance between the sites ($X^2 = 121.1$, $P < 0.001$) (Table 2.6). Figure 2.4 shows abundance and distribution of mosquito analyzed in the abdomen section of blood fed samples.

Table 2.6. Number of mosquitoes showing distribution and abundance of blood fed mosquitoes analyzed in the abdomen section (n (%).

Anopheles species blood feeding status of all processed **Abdomens** (n=1386)					
Namanolo (n=855)			Ntaja (n=531)		
Species	fed (n=542)	unfed (n=313)	Species	fed (n=285)	unfed (n=246)
An. arabiensis	244 (0.45)	37 (0.11)	*An. arabiensis*	39 (0.14)	1 (0.004)
An. funestus s.s.	165 (0.31)	36 (0.11)	*An. funestus* s.s.	138 (0.48)	57 (0.23)
An. gambiae s.s.	32 (0.06)	3 (0.01)	*An. gambiae* s.s.	67 (0.25)	0
An. parensis	1 (0.002)	4 (0.01)	*An. parensis*	1 (0.004)	0
An. vaneedeni	1 (0.002)	0	*An. vaneedeni*	0	0
Anopheles spp.	99 (0.18)	233 (0.74)	*Anopheles spp.*	40 (0.14)	188 (0.76)

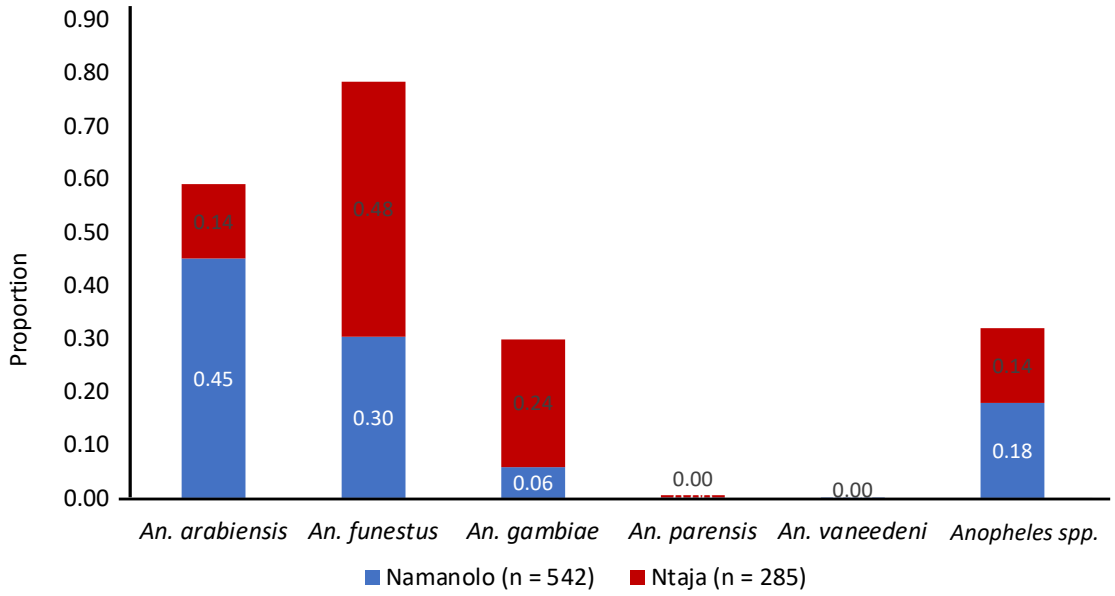

Figure 2.4. Composition, abundance, and distribution of mosquitoes from blood fed abdominal sections only, per site expressed as a proportion.

2.4.6. *Plasmodium falciparum* infection of blood fed *Anopheles* species

We looked at the infection in the mosquitoes by testing the presence of *Plasmodium falciparum* DNA of the head-thorax and abdominal section of the blood fed mosquitoes in the two sites. The overall infection was 14% in head-thorax and 27% in abdomen section. There was no statistical difference in head-thorax infection (13% Namanolo, 15% Ntaja; $X^2 = 0.9$, $P = 0.34$), but significant difference was observed in abdominal infection (23% Namanolo, 31% Ntaja; $X^2 = 49$, $P < 0.001$) between the sites (Figure 2.5).

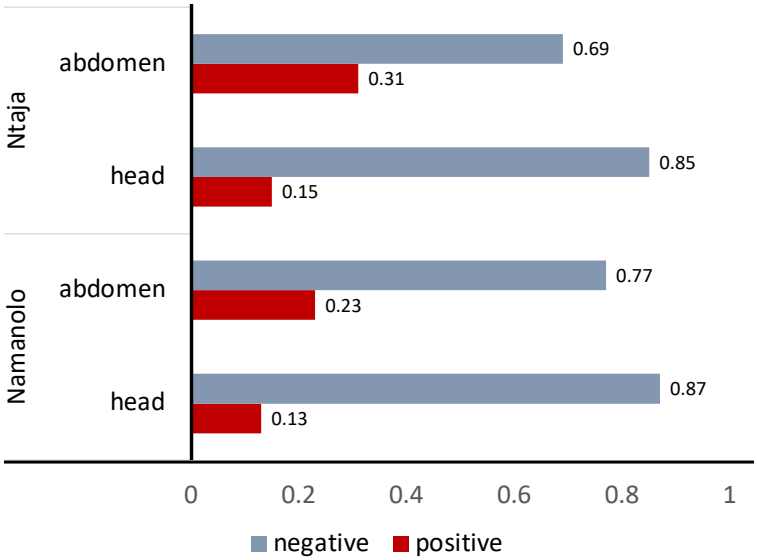

Figure 2.5. Blood fed mosquito *Plasmodium falciparum* (Pf) infection status in head-thorax and abdomen section expressed as a proportion. Positive infection rate ranging from 13 to 31%.

In all species, there were significant differences in infection among them in the head-thorax section of the mosquitoes ($X^2 = 33.1$, $P < 0.001$) with some species having higher infections than others and significant difference in the abdominal section ($X^2 = 64.6$, $P < 0.001$) across all species in the in all sites.

P. falciparum infections in the three main malaria vectors were higher in the abdominal section

than the head-thorax across all species in all sites. The overall infection rates ranged from 6 to 25% in the head-thorax, and from 13 to 48% in the abdomen.

In Namanolo, in the three main malaria vector mosquitoes, the abdominal infection rate ranged from 21 to 30% with the highest infection observed in *An. arabiensis* (30%) followed by *An. gambiae* s.s. (23%) and lastly *An. funestus* s.s. (21%) (X^2= 4.2, $P < 0.12$). The head-thorax infection rate (sporozoites) ranged from 8 to18% with the highest infection observed in *An. arabiensis* (18%) followed by *An. gambiae* s.s. (16%) and lastly *An. funestus* s.s. (8%) (X^2= 7.3, $P < 0.02$) (Figure 2.5).

In Ntaja, in the three main malaria vector mosquitoes, the abdominal infection rate ranged from 21 to 48% with the highest infection observed in *An. gambiae* s.s. (48%) followed by *An. funestus* s.s. (30%) and lastly *An. arabiensis* (21%) (X^2= 9.9, $P < 0.007$). The head-thorax infection (sporozoites) rate ranged from 12 to 25% with highest infection rates observed in *An. gambiae* s.s. (25%), followed by *An. arabiensis* (15%) and lastly *An. funestus* s.s. (12%). (X^2= 9.9, $P < 0.007$). Overall infection was higher in Ntaja than Namanolo in all body sections. (Figure 2.6).

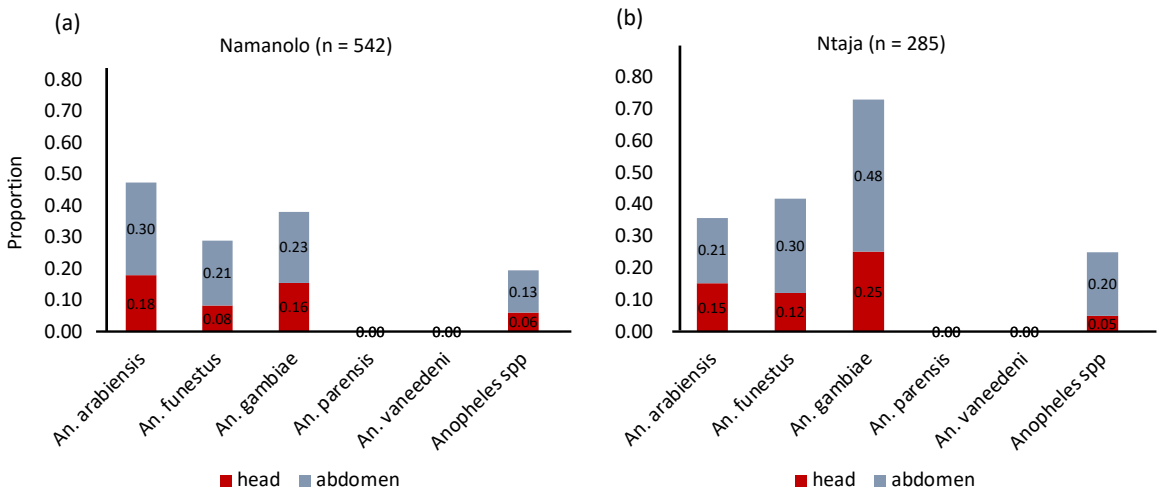

Figure 2.6. Mosquito *Plasmodium falciparum* infection proportion across the five *Anopheles* species in head-thorax vs. abdomen in Namanolo and Ntaja.

2.5 Discussion

The study results from indoor house collections show large numbers of unidentified species (62.1%), low percentage of blood fed mosquitoes (12%), high infection rates in abdominal sections and blood fed mosquitoes, and varied infection rates across species and sites.

Several reasons could explain the high number of non-identified samples, including failure of the PCR (possible but very unlikely because these are standard methods in our lab) or availability of other *Anopheles* species outside the main known malaria vectors in Malawi. Other studies in Malawi have identified *An. pretoriensis*, *An. maculupalpis* and *An. coustani*, the same species morphologically identified in the current study (Maekawa et al., 2021). This observation could suggest the existence of potential secondary vector of malaria as evidenced by 18% blood fed mosquitoes and 1 to 5% infection rate for *P. falciparum* and calls for more research in the unidentified *Anopheles* species, or to a lesser extent, this could be due to indoor resting behavior of other *Anopheles* species. The molecular identification on a subset these mosquitoes by using COI gene barcoding revealed the presence of other *Anopheles* mosquito species with the majority belonging to the *An. gambiae* complex. These observation are consistent with other studies recently conducted in Malawi (Maekawa et al., 2021) using DNA barcoding where other *Anopheles* species were identified. However, COI gene barcoding need to be used with caution or in combination with other approaches like using ITS2 gene to resolve closely related species (Beebe, 2018).

Prior studies have shown that dominant and epidemiologically important *Anopheles* species in Malawi are *An. funestus* s.s*., An. gambiae* s.s. and *An. arabiensis.* The other two species *An. parensis* (n=2) and *An. vaneedeni* (n=1) are in low abundance, rarely reported and not very important in malaria transmission in Malawi (Gillies & Coetze, 1987; Hunt et al., 2010;

Mzilahowa et al., 2012; Riveron et al., 2015; Vezenegho et al., 2013). However, the presence of *An. parensis* and *An. vaneedeni* resting in human dwellings is an interesting observation because both of these have been demonstrated to be potential malaria vectors (Burke et al., 2019; Mouatcho et al., 2018). The same can be said about the PCR non-identified species of which some were by morphological and DNA barcoding identified as *An. pretoriensis*, *An. maculipalpis* and *An. coustani* and others. However, their role in malaria transmission in Malawi has not been fully explored largely due to their low abundance. This observation emphasizes the importance of accurate identification of mosquitoes in malaria studies (Dahan-Moss et al., 2020).

Observed difference in species composition between the two sites was not significant despite differences in environmental conditions of the two sites where Namanolo is lower in elevation and flatter, as opposed to Ntaja which is drier and more elevated.

The proportion of blood fed mosquitoes (12%) could be considered low because elsewhere *Anopheles* blood feeding is much higher ranging from 58 to 94% (Adugna et al., 2021, 2021; Escobar et al., 2020). The lower number of blood fed mosquitoes could be attributed to the widespread use of malaria control intervention in the area or that the mosquitoes are resting somewhere. In the study area it was very difficult to find blood fed mosquitoes. The use of LLINs and IRS initiatives could be responsible for the reduction of human mosquito contact and hence reduced accessibility to blood meals (CDC, 2016; Killeen et al., 2000; Mathanga et al., 2012). The proportions of blood fed mosquitoes were high in Nanamolo than Ntaja probably due to the presence LLINs containing pyrethroid with the synergist piperonyl butoxide (PBO) in Ntaja which is more effective against pyrethroid resistant mosquito species available in Malawi (Mzilahowa et al., 2016; Ogola et al., 2017; WHO, 2018a).

We looked at the infection in the mosquitoes by testing the presence of *Plasmodium falciparum* DNA of the head-thorax and abdominal section of the mosquitoes in the two sites. The results showed statistical difference in head-thorax infection between the sites analyzing all samples (fed and unfed, head-thorax sections) and differences in abdominal infections of blood fed mosquitoes only or all samples. This observation could mean that the infection prevalence in the population of the two districts is different with Ntaja having a higher *plasmodium* infection in the head-thorax region of the blood-fed mosquitoes than Namanolo. This is in line what literature shows where Ntaja (Machinga) is an area with high Malaria transmission than Namanolo (Balaka) (Bennett et al., 2013; Townes et al., 2013). The abdominal infections on the other hand, showed statistical differences in the two sites especially in the blood fed mosquitoes. This shows that the rate of acquisition of infection from human to mosquitoes during blood feeding is different in the two sites. One reason to explain this could be the difference in the mosquito species abundance in the two sites. As shown in this study, although species composition were similar, Namanolo was dominated by *An. arabiensis* and *An. funestus* s.s. while Ntaja was dominated by *An. funestus* s.s. and *An. gambiae* s.s. in significantly different proportions. Several studies have shown that these species exhibit differences in their host seeking and resting behavior, where *An. funestus* s.s. is highly anthropophilic and endophilic while *An. gambiae s.l.* is more zoophilic and exophilic (Sinka et al., 2010). This phenomenon adds to the explanation of the differences in the abdominal infection in the two sites where Ntaja has a higher positive infection rate (probably due to *An. gambiae* s.s.) and Namanolo having more blood feds (probably due to *An. arabiensis*).

The infection results comparing the two mosquito body parts in the two sites showed that infection was higher in the abdominal section than the head-thorax across all species in all sites. This

observation is as expected. The infections in the abdomen could first and foremost be due to the gametocytes and other parasite asexual stages obtained from infected humans during blood feeding. However, remnants of previous feeding may also be detected in the mosquito midgut (oocytes) as the infection cycle progresses in the mosquito (cdc.gov, 2020; Pimenta et al., 1994). The combination of these two as detected by the standard *Plasmodium* PCR may explain the elevated levels of infection in the abdomen. As such not all infections in the abdomen could be attributed to the infected human population. However, the comparison with the head-thorax infection is one step toward understanding *Plasmodium* infection rates in the human population. In all cases the infection rates were high in Ntaja than Namanolo as explained above.

The differences in infection among species in the head-thorax section and in the abdominal section across all species underscores the fact that different mosquito species have different efficiencies in acquiring *plasmodium* infection and becoming infectious (Medica & Sinnis, 2005; Stresman et al., 2010). This is a phenomenon referred to related concepts of vector competency and vectorial capacity (Ceccato et al., 2012; Garrett-Jones & Grab, 1964; Sallum et al., 2019). Some mosquitoes can acquire the parasites upon blood feeding activity, but they may not become infectious, that is, the infection not detected in the salivary glands (head-thorax) due to failure in the complex developmental process to infect the salivary glands (Ndoen et al, 2012). This may explain the lower infection rates observed in the head-thorax region of all species. The non-significant difference in the abdominal infection among the mosquito species maybe due to the mosquitoes obtaining the blood meal from the same infected populations and hence the rate of *plasmodium* acquisition may not vary greatly. The relatively lower infection rates in *An. arabiensis* could be attributed to its zoophilic and exophagic tendencies (Sinka et al., 2010). Considered at the district

level, the three major vector of malaria in the study sites maybe equally responsible in the acquisition of malaria parasite infection (abdomen infection) and transmission (head-thorax infection) from human to human respectively (Medica & Sinnis, 2005; Stresman et al., 2010).

The rate of mosquito infection in the head-thorax is high indicating a presence of infectious mosquitoes which is crucial in malaria transmission (Ghosh et al., 2000; Kariu et al., 2002; Medica & Sinnis, 2005; Pimenta et al., 1994). The high rate of mosquito abdomen infections brings to light the fact that malaria maybe high in the human population where the mosquitoes obtain the blood meal from the human reservoir (Adhikari et al., 2018; Cheaveau et al., 2019; Githeko et al., 1992; Karl et al., 2011; Stresman et al., 2010). Also, high infection in blood fed mosquitoes could suggest that access to blood feeding increases chances of mosquitoes acquiring malaria parasites.

These observations have serious implication on malaria transmission. First is the challenge posed by multiple vectors in malaria control strategies. This is even made worse due to the emergent of insecticide resistance that is observed in these species in many African countries including Malawi (Hunt et al., 2010; Mzilahowa et al., 2016; Ranson et al., 2011; Riveron et al., 2015). Second, the study has revealed that the three main malaria vector species are actively acquiring malaria infection from humans and becoming infectious. The availability of malaria reservoirs in the study area as demonstrated by the higher number of infected abdomen (blood meals) mean that there is perpetuation of residual malaria transmission. Unless these people are identified and targeted with control intervention, the basic reproductive number (R_o) remains high and transmission continues (Karl et al., 2011; Smith et al., 2007). And lastly, there exist potential secondary malaria vectors in the area which needs further investigation.

2.6 Conclusion

The study has shown that there are five blood feeding *Anopheles* mosquito species present in the study sites with low percentage of blood fed mosquitoes. Of these five, three *An. arabiensis*, *An. funestus* s.s. and *An. gambiae* s.s. continue to pose threat of malaria transmission. The infection rates in head-thorax and abdomen of mosquitoes point to a situation where perpetuation of residual malaria transmission exists. Potential secondary vectors maybe present in the study area and needs more investigation. Understanding malaria species vector composition, infection rate in both head-thorax and abdominal section of the mosquito is key in determining effective malaria control and prevention strategies. We therefore recommend mass drug administration to reduce infection in the human population, increased and continued LLINs coverage and use to reduce contact between mosquitoes and humans as well as strategies aimed at further reducing mosquito populations.

APPENDIX

NUMBER OF MOSQUITOES SAMPLED IN 2019 AND 2020

Table 2.7. Total number of *Anopheles* mosquitoes collected per month of sampling in 2019 and 2020 in the two sites.

Namanolo										
	2019					2020				
month	May	June	August	October	December	January	February	March	April	Total
An. arabiensis	0	0	0	2	27	82	278	105	8	502
An. gambiae	0	1	0	0	3	12	34	1	1	52
An. funestus	10	15	0	0	2	61	434	317	107	946
An. parensis	0	4	0	0	1	0	0	1	0	6
An. vaneedeni	0	0	0	0	0	0	0	1	0	1
Anopheles spp.	1	10	68	1	24	412	1309	290	144	2259
Total	11	30	68	3	57	567	2055	715	260	3766
Ntaja										
	2019					2020				
month	May	June	October	November	December	January	February	March	April	Total
An. arabiensis	0	0	0	1	0	4	36	46	8	95
An. gambiae	0	0	0	0	2	8	98	44	2	154
An. funestus	16	62	3	1	40	129	353	302	60	966
An. parensis	0	0	0	0	0	1	0	1	0	2
An. vaneedeni	0	0	0	0	0	0	0	0	0	0
Anopheles spp.	4	4	0	0	17	152	820	725	58	1780
Total	20	66	3	2	59	294	1307	1118	128	2997

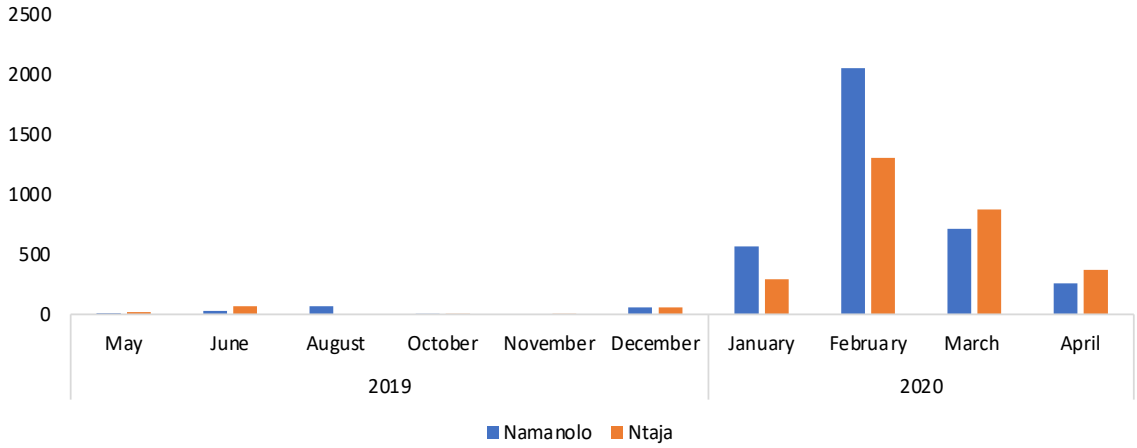

Figure 2.7. Number of mosquitoes collected per month of sampling

Table 2.8. Blood fed and unfed Anopheles mosquitoes sampled in 2019 and 2020 showing month of sampling and numbers per site.

Namanolo																			
	2019										2020								
	May		June		August		October		December		January		February		March		April		
	Fed	Unfed	Fed	Unfed	Fed	Unfed	Fed	Unfed	Fed	Unfed	Fed	Unfed	Fed	Unfed	Fed	Unfed	Fed	Unfed	total
An. arabiensis	0	0	0	0	0	0	2	0	15	12	50	32	124	154	44	61	6	2	502
An. gambiae	0	0	1	0	0	0	0	0	3	0	6	6	22	12	0	1	1	0	52
An. funestus	0	10	2	13	0	0	0	0	1	1	11	50	57	377	83	234	10	97	946
An. parensis	0	0	1	4	0	0	0	0	0	0	0	0	0	0	0	1	0	0	6
An. vaneedeni	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
Anopheles spp.	0	1	3	7	0	68	0	1	16	8	28	384	43	1266	12	278	3	141	2259
Total	0	11	7	24	0	68	2	1	35	21	95	472	246	1809	140	575	20	240	3766

Ntaja																			
	2019										2020								
	May		June		August		October		December		January		February		March		April		
	Fed	Unfed	Fed	Unfed	Fed	Unfed	Fed	Unfed	Fed	Unfed	Fed	Unfed	Fed	Unfed	Fed	Unfed	Fed	Unfed	total
An. arabiensis	0	0	0	0	0	0	1	0	0	0	2	2	4	32	30	16	2	6	95
An. gambiae	0	0	0	0	0	0	0	0	2	0	6	2	29	69	32	12	1	1	154
An. funestus	0	16	5	57	1	2	0	1	2	38	25	104	50	303	45	257	11	49	966
An. parensis	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	2
An. vaneedeni	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Anopheles spp.	0	4	0	4	0	0	0	0	1	16	3	149	16	801	19	709	0	58	1780
Total	0	20	5	61	1	2	1	1	5	54	36	258	99	1205	127	994	14	114	2997

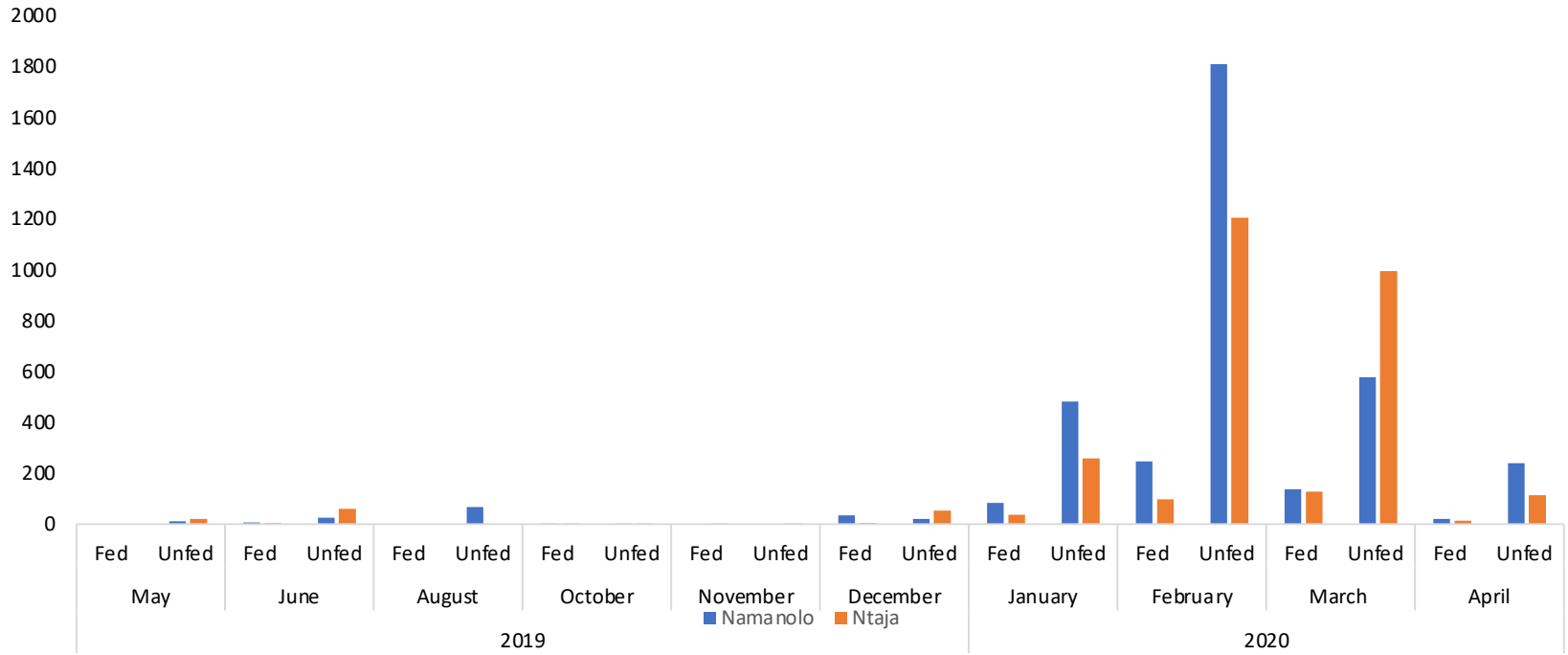

Figure 2.8. Number of blood-fed and unfed mosquitoes collected and month of sampling per site.

CHAPTER 3 : BLOOD MEAL SOURCES OF *ANOPHELES* VECTORS OF HUMAN MALARIA IN MALAWI: IMPLICATIONS FOR MALARIA TRANSMISSION AND EFFECTIVENESS OF INTERVENTIONS

Manuscript submitted, Malaria journal

Rex B. Mbewe, John B. Keven, Themba Mzilahowa, Don P. Mathanga, Mark L. Wilson, Lauren M. Cohee, Miriam K. Laufer, Edward D. Walker

3.1. Abstract

Background. Selection of blood meal hosts by mosquitoes is a key variable in the vectorial capacity of *Anopheles* mosquitoes for human malaria. Blood feeding on humans is likely to be modulated by use of different types of long-lasting insecticidal nets (LLINs) and the effectiveness of LLINs is impacted by the relative intensity of insecticide resistance. The aim of this study was to test the hypothesis that LLINs containing pyrethroid and the synergist piperonyl butoxide (PBO) would lead to a reduction of human host utilization than LLINs containing only pyrethroid and that blood feeding patterns of *Anopheles* in Malawi compromise malaria interventions.

Methods. Female *Anopheles* mosquitoes were sampled indoors from May 2019 through April 2020 by aspiration, pyrethrum spray catch, and CDC light trap in rural villages of Namanolo (conventional nets) and Ntaja (PBO nets) in Balaka and Machinga districts respectively. *Anopheles* species, blood meal sources, and infection with *Plasmodium falciparum* in the head-thorax of individual mosquitoes were determined by PCR. Human blood index (HBI) and Entomological inoculation rate (EIR) were also estimated.

Results. Of a total of 6,585 *Anopheles* females sampled indoors in 203 houses, 633 (9.6%) were blood-fed and consisted of *An. arabiensis* (44.1% (n=279)), *An. gambiae* s.s (16.2% (n=103)), *An. funestus* s.s (33.5% (n=212)), *An. parensis* 0.3% (n=2), and unidentified *Anopheles* spp (5.8% (n=37)). Of the 541 mosquitoes (85.5%) successfully identified blood meals, 436 (81.0%) were solely human, 28 (5.2%) goat, 11 (2.0%) dog, 60 (11.1%) mixed goat-human, 5 (0.9%) dog-human, and 1 dog-goat. Human blood index and EIR were higher in Namanolo than Ntaja (0.96 vs 0.89 ($p = 0.001$) and 0.11 vs 0.06 infective bites per person per year respectively) despite high net ownership (92%) and nightly use (75%) rates. Relative to host availability, non-human hosts were over selected in the two sites.

Conclusion. The use of PBO nets was associated with lower HBI and EIR, however, the wide availability of LLINs was still associated with extensive successful human blood meals by the main malaria vectors in Malawi. The presence of a small fraction of mixed blood meals indicates constrained plasticity of *Anopheles* vectors to switch to non-human hosts and circumvent malaria control interventions.

3.2. Introduction

Malaria is endemic in Malawi with transmission occurring throughout the country, having generally greater transmission in the south than the north (*Malawi Malaria Indicatior Survey*, 2017; Mathanga et al., 2012; Wilson et al., 2012). The nation-wide prevalence of infection for *Plasmodium falciparum* among 2 to10 year old individuals, sampled from 2010 to 2017, declined from 29.4% in 2010 to 15.2% in 2017 but this change was uneven across the country's 28 jurisdictional districts (Chipeta et al., 2019). Despite these successes, which are largely attributed to reduction in transmission owing to the implementation of long-lasting insecticidal nets (LLINs) distributed throughout the country, Malawi remains a high malaria burden country with meso-endemic transmission (Chipeta et al., 2019). In 2017, the country-wide incidence of malaria was 247 per 1,000, with an estimated 7,077 deaths (WHO, 2018b).

One of the drivers of persistent *Plasmodium* transmission is access to human blood by host-seeking female *Anopheles* mosquitoes. Selection of blood meal hosts by *Anopheles* mosquitoes is a key variable in their vectorial capacity - a measure of transmission - for human malaria because vectorial capacity increases with the square of the rate of human biting (Garrett-Jones & Grab, 1964). Further, host selection is likely to be modulated by use of different types of LLINs whose effectiveness is impacted by the extent of insecticide resistance in the *Anopheles* populations, and the relative availability of hosts (Keven et al., 2017; Toe et al., 2018). These factors will enhance or limit mosquitoes' access to humans relative to non-human hosts. Emergence of pyrethroid resistance could severely compromise malaria control efforts by reducing the effectiveness of LLINs. Newer LLINs which combine pyrethroids with a synergist, piperonyl butoxide (PBO), have shown to be effective at restoring pyrethroid susceptibility (Protopopoff et al., 2018; Staedke et al., 2020; WHO, 2018a).

Vector incrimination studies in Malawi have identified members of the *Anopheles gambiae* sensu lato (s.l.) and *Anopheles funestus* species assemblage as the most important malaria vectors and pyrethroid insecticide resistance have been reported (Hunt et al., 2010; Mzilahowa et al., 2008, 2016; Spiers et al., 2002). However, only a single study has analyzed mosquito host selection and estimated the human blood index ((HBI) the proportion of blood meals obtained from humans by mosquito vectors) of these vectors in the southern region of the country before LLINs were available (Mzilahowa et al., 2012). Accordingly, whether human host selection has changed after LLINs availability is unknown.

The objective of this study was therefore twofold. Firstly, we aimed to expand on knowledge of blood feeding patterns of malaria vectors, a key feature of malaria risk, by conducting host selection analyses and estimation of HBI and Entomological inoculation rate ((EIR) a standard matrix for malaria transmission intensity by anopheline vectors, defined as the number of infectious bites per person per time period) (Das et al., 2017; Mwesigwa et al., 2017).

Secondly, we aimed to test the prediction that blood feeding on human hosts is reduced where LLINs containing pyrethroid and the synergist PBO (i.e., Olyset Net Plus, Sumitomo Corporation, Tokyo, Japan) compared to where standard, pyrethroid-only LLINs (i.e., Olyset Net, Sumitomo) have been distributed. Our hypothesis was that mosquito feeding is host specific with regard to human and non-human hosts available in the study area.

3.3. Methodology

3.3.1. Study area

This study was conducted in two malaria-endemic districts of Malawi (Figure 3.1), Balaka (14° 58’ 45” S; 34° 57’ 20” E) and Machinga (15° 10’ 6” S; 35° 18’ 0” E). These districts, like the whole of Malawi, have distinct wet and dry seasons where malaria proliferates especially in the rainy wet season (Mathanga et al., 2012). In 2018, residents of Machinga received LLINs with PBO (Olyset Plus net, Sumitono Corporation, Tokyo, Japan), whilst residents of Balaka received standard LLINs only (Olyset net) (Government of Malawi, National Malaria Control Programme, 2017) (PMI, 2018). Households in rural areas located within the catchment areas of Ntaja (Machinga) and Namanolo (Balaka) health centers were enrolled in the study. Household surveys were conducted to determine the extent of LLINs ownership and utilization by householders and to quantify the number of occupants.

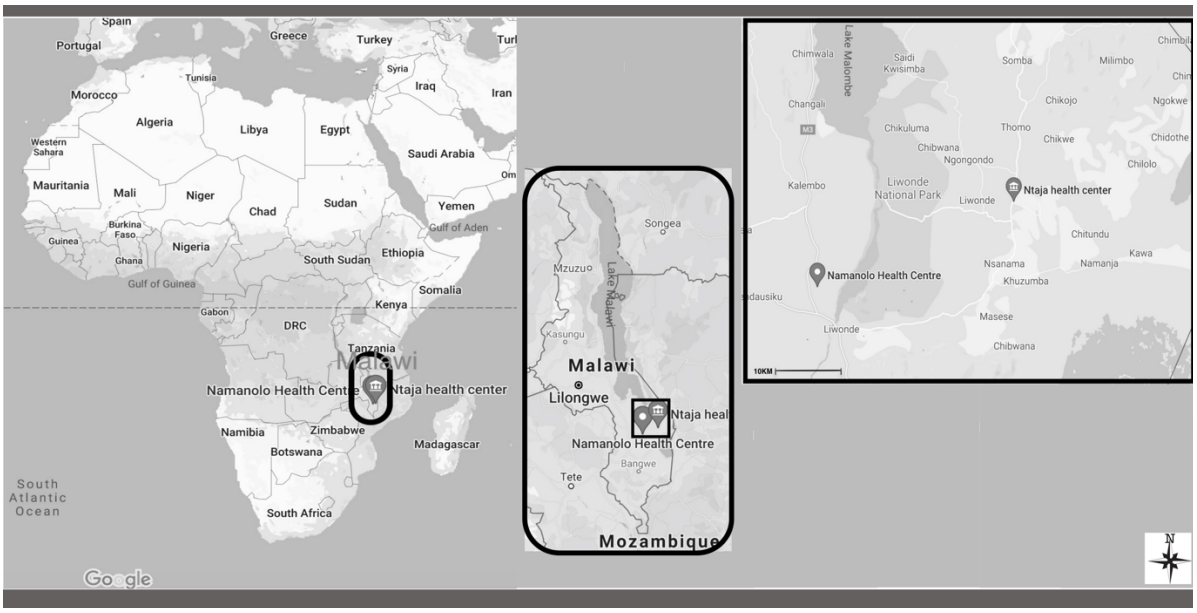

Figure 3.1. Map of Africa showing the study sites in Namanolo and Ntaja in Malawi.

3.3.2. Mosquito sampling and analysis

Blood-fed *Anopheles* mosquitoes were sampled in houses, selected by the randomized cluster sampling method. All households were sampled three times each period from May-June 2019, October-November 2019, and December 2019-January 2020. Beginning in February to April of 2020, only a subset of households in each site were sampled once every two weeks. The mosquito samples for each visit were accumulated. The number of humans and goats in the households were also counted. Dogs were too mobile to be counted reliably. Cattle were rare in the study area and scored as absent. Mosquito sampling tools included mouth and battery-powered aspirators, standard miniature CDC light traps (Model 512; John W. Hock Company, Gainesville, Florida, USA) and pyrethrum spray catches (PSCs) (Mzilahowa et al., 2012, 2016). Mosquitoes were morphologically identified (Gillies & Coetze, 1987) into *An. gambiae* sensu lato, *An. funestus* sensu lato, or other *Anopheles* species, stored in tubes with silica gel, and kept at laboratory temperatures.

The abdomen was separated from the head-thorax of each mosquito using sterile, cross-contamination-proof technique. For each mosquito, genomic DNA was extracted from the abdomen and head-thorax separately using the Qiagen extraction kit (DNeasy® Blood & Tissue Kit; Cat. No. 69506) following the manufacturer's standard protocol.

3.3.3. PCR identification of mosquitoes

Mosquitoes of the *An. gambiae* (s.l.) complex were identified to one of two species, *An. gambiae* sensu stricto (s.s.) or *An. arabiensis*, using a published multiplex quantitative PCR (qPCR) method (Walker et al., 2007). Mosquitoes of the *An. funestus* (s.l.) species assemblage were identified to one of three species (*An. funestus* (s.s.), *Anopheles parensis* and *Anopheles vaneedeni*) using

another multiplex qPCR as follows. A universal primer pair (forward: 5'-AGA ACACTA TGG CGA GCA GC-3', reverse: 5'-TTA CGA CGG ATA CGG TCA ACG-3') that amplifies a region of the internal transcribed spacer region 2 of rRNA gene of members of the *An. funestus* (s.l.) species assemblage was designed along with two oligonucleotide probes specific to *An. funestus* (s.s.) (5'-FAM-CAT GGG GAA ATT CAA TCG AAA ACC TCT-QSY-3') and *An. parensis* (ABY-TGG CGT GCT CGG AAC CTA GC-QSY). The probe specific to *An. vaneedeni* (5'-VIC-CGT TGT GAA AAA TGG AGA TTC ATT TGA AAA CC-QSY-3') was obtained from a published source (Vezenegho et al., 2009). After performing optimization tests involving 10-fold dilution series of positive DNA control of the three species, the optimum PCR mixture (10 µL reaction volume) consisted of 1x TaqMan Universal Master Mix (Cat. No. 4304437; Thermo Fisher Scientific, Waltham, MA, USA), 0.6 µM of each primer, 0.4 µM of each probe, and 2 µL of mosquito DNA. The reactions were performed on QuantStudio 7 Flex PCR system (Applied Biosystems, Foster City, CA, USA) using the following cycling conditions: one cycle of 50 °C for 2 minutes and 95 °C for 10 minutes, 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. PCR sensitivity was one target gene copy/µL sample and efficiency was > 90%. Positive genomic DNA controls for *An. arabiensis An. gambiae*, *An. funestus* obtained from beiresources.org (MRA-495, MRA-142 and MRA-1027 respectively) and negative controls as blank were included in each experiment. The PCR results were visualized with QuantStudio software (version 1.3).

3.3.4. Blood meal analysis

Individual mosquito abdominal DNA was first tested for human blood meal using a uniplex qPCR method that involved amplification of a region of intron 1 of the nuclear tyrosine hydroxylase gene with primers and probes (forward: 5'-GGC CTG TTC CTC CCT TAT TT-3′, reverse: 5'- TAC

ACA GGG CTT CCG AGT-3′, probe: FAM-ATG GAG TCT GTG TTC CCT GTG ACC -QSY) as described in Keven et al., (2020). Samples that did not react with the human probe in the qPCR were subjected to a standard PCR to amplify the vertebrate mitochondrial cytochrome B gene using a generic primer pair (forward: 5′-CCC CTC AGA ATG ATA TTT GTC CTC A-3′, reverse: 5′-CCA TCC AAC ATC TCA GCA TGA TGA AA-3′) (Boakye et al., 1999; Goldberg et al., 2009; Lee et al., 2002; Meece et al., 2005; Molaei et al., 2006). The PCR reaction mixture (25 µL volume) consisted of 10 mM Tris at pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 1.0 mM dNTP, 0.5 units of Taq polymerase, 50 pmol of each primer, and approximately 20 ng of DNA template. The reaction condition included one cycle of 95 °C for 5 minutes (initial denaturation) followed by 35 cycles of 95 °C for 1 minute (denaturization), 57 °C for 1 minute (annealing) and 68 °C for 1 minute (extension), followed by one cycle of 68 °C for 5 minutes (final extension). The PCR products were visualized with 2% agarose gel electrophoresis and amplicons of positive samples were purified using QIAquick PCR purification kit (Cat. No. 2810; Qiagen) following the manufacturer's protocol. The nucleotide sequence of amplicons was determined by direct sequencing and the sequences were subjected to BLAST (Basic Local Alignment Search Tool) search for matches to the available vertebrate host cytochrome B gene sequences in GenBank (*https://blast.ncbi.nlm.nih.gov/Blast.cgi*). A sequence similarity of 97% or higher was used as the cut-off for an acceptable match, based on literature (Goldberg et al., 2009; Kent, 2009; Nagaki et al., 2021; Stevenson et al., 2012).

The different hosts identified in the mosquito blood meals based on the results of BLAST searches aided the development of new qPCR primers and probes specific to those host species; humans, dogs and goats were the only hosts identified by BLAST searches. Primers and probes (Table 3.1) for humans and dogs were adopted from literature (Keven et al., 2020) but those for goats were

designed and validated in this study using the same procedure for humans and dogs (Keven et al., 2020). Another set of qPCR procedures was performed on all the blood meal samples. The purpose of this second qPCR was to detect presence of mixed blood meals as well as to confirm the results of the previous uniplex qPCR. The qPCR mixture (10 µL reaction volume) consisted of 1X TaqMan Universal Master Mix (Cat. No. 4304437; Thermo Fisher Scientific), 0.5 µM of each primer, 0.25 µM of each probe, and 2 µL of DNA. PCR cycling condition (QuantStudio 7 Flex PCR System) was the same as described for *An. funestus* (s.l.) above positive and negative DNA controls were included in each experiment.

Table 3.1. Blood meal host primers and probes used in the qPCR reactions

Species		Forward	Reverse	probe
human	*Homo sapiens*	5'-GGCCTGTTCCTCCCT TAT TT-3'	5'-TACACAGGGCTTCCGAGT -3'	5'-FAM-ATGGAGTCTGTGT T CCCTGTGACC -QSY- 3'
goat	*Capra hircus*	5'-TAGGCGCCATGCTAC TAATTC-3'	5'-GAGTGGATTTGCTGGGAT ATAG-3'	5'-ABY-ATTCACACCCGAC CTACTCGGAGA-QSY-3'
dog	*Canis lupus familiaris*	5'-TGGACAAAGCAACC CTAACA-3'	5'-CCGGTTTCGTGTAGAAAT AGGA-3'	5'-ABY-TCATCCTCCCTTTC ATCATCGCAGC-QSY-3'

*Note: goat primers and probes were developed in this study, human and dog were from Keven et al., (2020).

3.3.5. Molecular identification of *Plasmodium falciparum*

DNA from both the abdomen and head-thorax of each mosquito was tested for presence of *P. falciparum* using a multiplex qPCR containing two fluorescent-labelled TaqMan probes targeting the 18S rRNA gene of *P. falciparum* (forward primer: 5'-ATT GCT TTT GAG AGG TTT TGT

TAC TTT-3′; reverse primer: 5’-GCT GTA GTA TTC AAA CAC AAT GAA CTC AA-3′; probe: 5’-FAM-CAT AAC AGA CGG GTA GTC AT- MGBNFQ-3′). These primers and probes were developed and tested for specificity to the target organism and gene locus using published methods (Kamau et al., 2013). The PCR reaction (10 µL volume) consisted of 1X TaqMan Multiplex Master Mix (Cat. No. 4461882; Thermo Fisher Scientific), 0.6 µM of each primer, 0.4 µM of each probe and 2 µL of DNA. PCR amplification (QuantStudio 7 Flex PCR System) was performed using the same cycling condition as with *An. funestus* (s.l.) described above. Positive genomic DNA control for *P. falciparum* was obtained from beiresources.org (MRA-506).

3.3.6. Data analysis

Mosquitoes that had fed on one species of host, as determined by blood meal analysis as described above, were classified as single host blood meals. If the blood meal analysis revealed two or more species of vertebrate hosts, then the blood meals were classified as mixed blood meals. The HBI of an *Anopheles* population (i.e., mosquitoes of a particular species from a particular village) were calculated as the proportion of blood-fed mosquitoes that fed on human hosts. Human-fed mosquitoes included both single human blood meals as well as human-nonhuman mixed blood meals. (Escobar et al., 2020; Garrett-Jones, 1964; Keven et al., 2020; Overgaard et al., 2011).

Differences in host utilization by district were analyzed by contingency table analysis. To test for variation in the propensity of the three main malaria vector species to feed on humans, non-humans and human-nonhuman mix blood meal types, a 3 x 3 contingency table analysis was carried out, and the percentage deviations of observed from expected frequencies were calculated. Host selection of a vector population was quantified using the theta statistic ($\theta = \pi_1/\pi_2$), which tested whether the ratio (θ) of the proportion of a host species in mosquito blood meals (π_1) and

proportion of the host in the village (π_2) is different from unity (Aho & Bowyer, 2015). A host species was considered over-selected by the vector population if theta was significantly greater than 1.0 or under-selected if theta was significantly less than 1.0. A host species was considered to be fed on by the mosquitoes in proportion to its relative availability in the village if theta was not significantly different from 1.0. The theta statistical analysis was performed using the *ci.prat.ak* function of *asbio* package in Rstudio Version 1.1.456.

The sporozoite rate (SR) was estimated as the proportion of mosquito heads-thoraces that tested positive for *Plasmodium*. The entomological inoculation rate (EIR) can be estimated indirectly from samples obtained by indoor resting mosquitoes, and also directly from samples obtained by the human landing catch (HLC) method (Kilama et al., 2014). In our study, samples from indoor resting collections were used to indirectly calculate the EIR using the formula: **EIR = (M*SR*HBI)/N**, where M is the mean number of blood fed mosquitoes per house, SR is Sporozoite rate, HBI is Human blood index and N is the mean number of human occupants per house per night. Annual EIR was estimated by multiplying the EIR by 365 days.

3.4. Results

3.4.1. Species composition

Mosquitoes were collected in 203 household yielding 6,585 female *Anopheles* mosquitoes including 633 (9.6%) blood-fed. The blood-fed mosquitoes consisted of the following species: *An. arabiensis* (Overall: 44.1%, n=280; Namanolo: 59.2%, n = 243; Ntaja: 13.0%, n = 37), *An. funestus* (s.s.) (Overall: 33.5%, n=213; Namanolo: 26.7%, n = 110; Ntaja: 46.2%, n = 103), *An. gambiae* (s.s.) (Overall: 16.2%, n=103; Namanolo: 7.8%, n = 32; Ntaja: 30.0%, n = 70), and *Anopheles parensis* (0.3%, n=2, one each in Namanolo and Ntaja). Thirty-seven (5.8%) were not identified to species by morphological or molecular means.

3.4.2. Host selection and human blood index (HBI)

Of the blood-fed *Anopheles* mosquitoes (n = 633), the blood meal host of 541 (85.5%) were successfully identified either by qPCR or direct sequencing (Figure 3.2 and Table 3.2). The remaining 92 (15%) were either non-reactors in PCR reactions (n=42), or amplicons generated by standard PCR failed to match any feasible host (n=50). Of the 541 mosquitoes whose blood meal host was successfully identified, 436 (81.0%) were solely human blood meals, 28 (5.2%) were solely goat blood meals, 11 (2.0%) were solely dog blood meals, and mixed blood meals were: 1 (0.2%) dog-goat, 5 (0.9%) dog-human, and 60 (11%) goat-human (Figure 3.2 and Table 3.2).

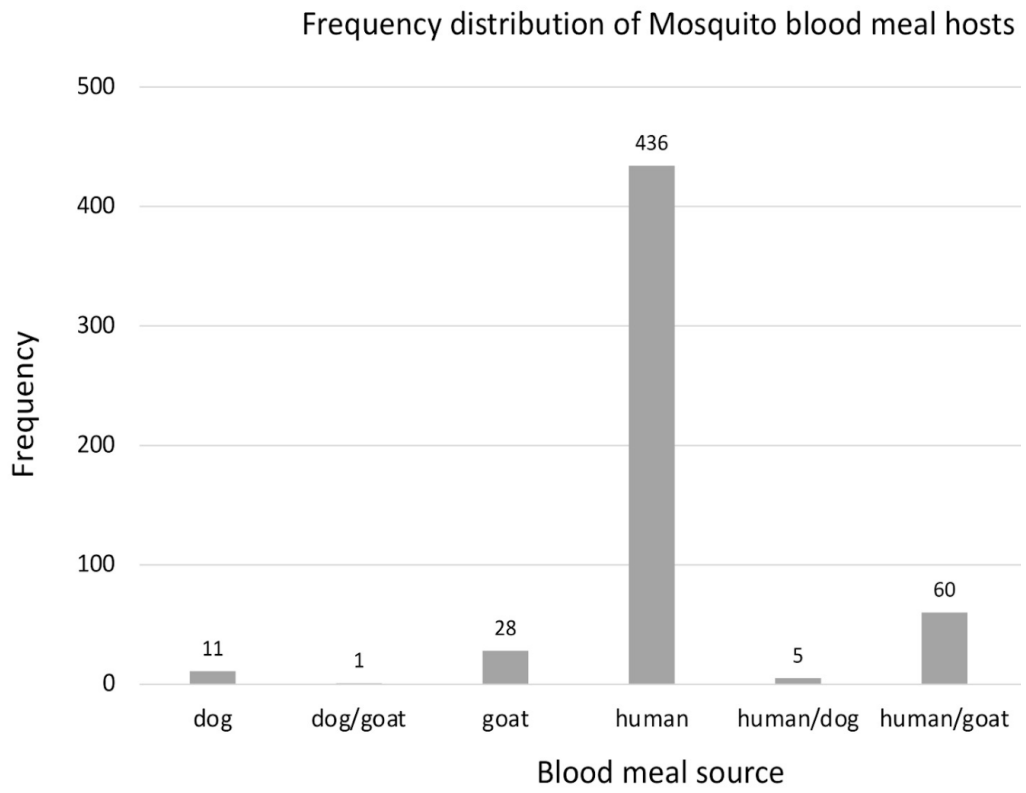

Figure 3.2. Blood meal identification to vertebrate host, including mixed meals, for *Anopheles* mosquitoes two sites combined.

Humans were the most frequently identified blood-meal host for all three of the most abundant *Anopheles* species (*An. arabiensis*, *An. funestus* s.s. and *An. gambiae* s.s.), at approximately 70% of blood meal samples for each species (Table 3.2). Mixed blood meals comprising human and goat were present for all three mosquito species and ranged from 7.1 to 11.7% among species. Blood meals identified solely from goats ranged from 1.4 to 5.8% and were also found in all three species. Dog-only, and mixed dog-human or dog-goat blood meals, were present but uncommon. Differences in human or non-human (goat and dog) and mixed (human-nonhuman) host feeding by these three species, and the percentage deviations of observed from expected frequencies are shown in Table 3.3. Although the chi-square test was not significant (X^2 =6.4, p=0.17), the percentage deviation values were suggestive that *An. arabiensis* tended to feed on nonhuman hosts more so than did *An. gambiae* (s.s.) and *An. funestus* (s.s.), whereas *An. funestus* s.s. tended to

underutilize nonhuman hosts compared to *An. gambiae* (s.s.) and *An. arabiensis*. Additionally, *An. funestus* (s.s.) tended to have fewer human-nonhuman mixed blood meals compared to the other species (Table 3.3).

Table 3.2. Blood meal sources identified from *Anopheles* mosquitoes of Namanolo and Ntaja.

Mosquito Species	**Blood meal source**							
	No. tested	**Human n (%)**	**Dog n (%)**	**Goat n (%)**	**Human/Dog n (%)**	**Human/Goat n (%)**	**Dog/Goat n (%)**	**Other n (%)**
An. arabiensis	280	195(69.6)	8(2.9)	11(3.9)	4(1.4)	30(10.7)	1(0.4)	31(11.1)
An. gambiae	103	75(72.8)	0	6(5.8)	0	12(11.7)	0	10(9.7)
An. funestus	212	152(71.8)	3(1.4)	3(1.4)	0	15(7.1)	0	39(18.4)
An. parensis	2	0	0	1(50)	0	1(50)	0	0
An. spp	36	14(38.9)	0	7(19.4)	1(2.8)	2(5.6)	0	12(33.3)
Total	**633**	**436(68.9)**	**11(1.7)**	**28(4.4)**	**5(0.8)**	**60(9.5)**	**1(0.2)**	**92(14.5)**

Table 3.3. Percent deviation of observed blood meal frequencies from those expected by chi-square analysis, two sites combined.

Species	Human	Non-human	Human-nonhuman mix
An. arabiensis	- 4.2	+27	+15.5
An. gambiae	-1.8	+7	+8.7
An. funestus	+7.0	-42.5	-26.9

The results for analysis of host selection by the theta statistic are shown in Figure 3.3 In Ntaja, *An. arabiensis* and *An. gambiae* (s.s.) overselected goats and under-selected humans, whilst *An. funestus* (s.s.) selected these two host species in proportion to their relative abundance in the village. In Namanolo, by contrast, all three vector species selected both hosts in proportion to their relative abundance, although there was a nonsignificant tendency for over-selection of goats compared to humans.

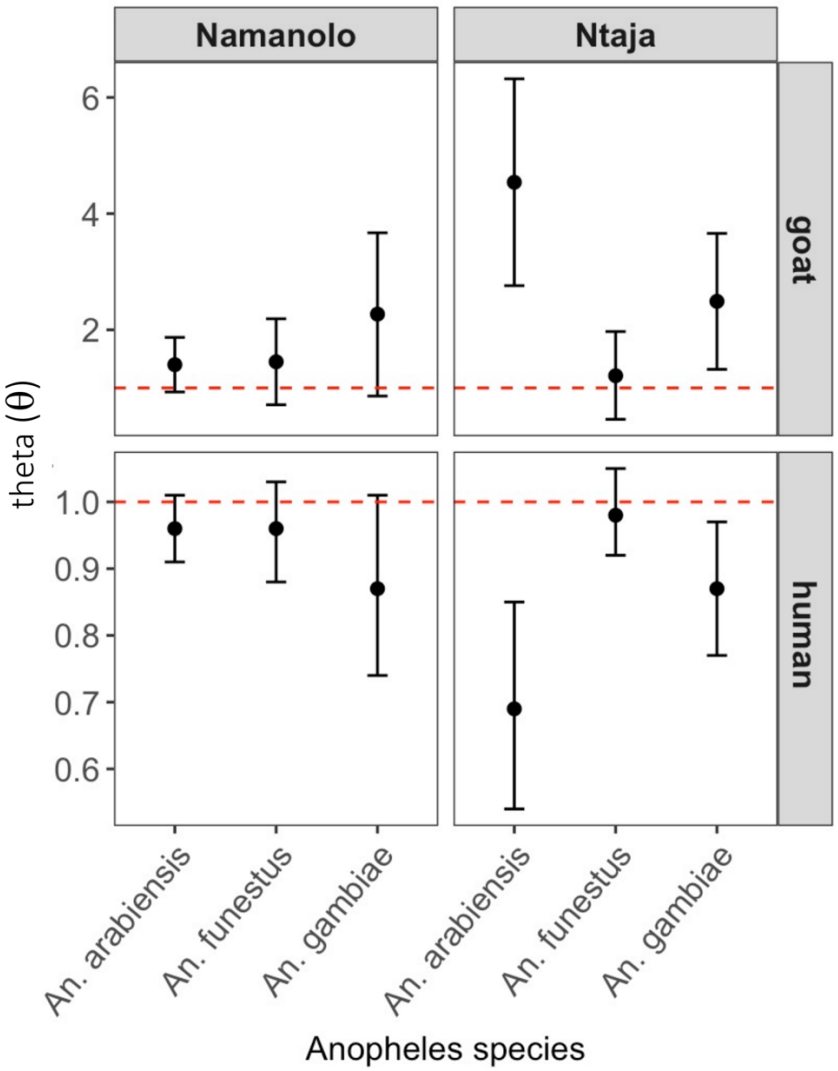

Figure 3.3. *Anopheles* species blood meal host selection in relation to number of available hosts. Top panels; goat selection in relation to human, bottom panel; human selection in relation to goats. The black shaded circles are theta values with 95% CI bars. Red dotted line at 1.0 represents random selection in relation to availability of both hosts. The 95% CI bars represent deviation from random selection pointing to over-selection (theta significantly > 1.0) or under-selection (theta significantly < 1.0).

The three abundant mosquito species (data from both villages combined) had similar HBI: *An. funestus* (s.s.) (96.5%); *An. gambiae* (s.s.) (93.5%); *An. arabiensis* (92.3%) (X^2 =0.11, p=0.946).

Their combined HBI (i.e., regardless of species) was significantly higher in Namanolo with conventional nets (96.4%) compared to Ntaja with PBO nets (88.9%) (z =3.32, p = 0.001).

3.4.3. Entomological inoculation rate (EIR)

Results of PCR analysis for *P. falciparum* infection showed that 15.6% (99/633) of the *Anopheles* mosquitoes (regardless of species) were positive in the head-thorax. The infection prevalence according to mosquito species was as follows: *An. arabiensis* 17.7% (50/283), *An. funestus* (s.s.) 12.3% (26/212), *An. gambiae* (s.s.) 22.2% (22/99), *An. parensis* 0% (0/2), and unidentified *Anopheles* spp. 2.6% (1/39).

To estimate EIR, results from 203 houses with 1,106 occupants (97 houses, 488 occupants in Namanolo, 106 houses, 618 occupants in Ntaja) were used. EIR for both sites combined was equal to (633/203 * 16% * 94.0%)/(1106/203) or 0.09 infectious bites per person per night. For Namanolo, the estimated EIR was equal to (410/97 * 14.3% * 96.4%)/(488/97), or 0.11 infectious bites per person per night. For Ntaja, the estimated EIR was equal to (223/106 * 18% * 88.9%)/(618/106) or 0.06 infectious bites per person per night. Annualized EIR was 40 and 22 infectious bites/person/year in Namanolo and Ntja respectively with combined annual EIR of 0.09 translating into ~33 infectious bites/person/year.

Household surveys revealed similar and high ownership rates for LLINs in the two sites (Namanolo: 92%, Ntaja: 90%, and nightly use rates (Namanolo: 75%, n = 109 households; Ntaja: 74%, n = 158 households).

3.5. Discussion

Indoor mosquito sampling of rural houses in Namanolo and Ntaja in Balaka and Machinga Districts, respectively, of southeastern Malawi revealed three major malaria vector species. This finding was consistent with past studies in Malawi and southern Africa (Hunt et al., 2010; Mzilahowa et al., 2008; Spiers et al., 2002). The results here provide key malariologic transmission indices (HBI, EIR) that demonstrate the vulnerability of humans to bites of vector *Anopheles* mosquitoes, despite the presence and use of LLINs as the primary anti-malaria intervention. Although no species was numerically dominant, *An. funestus* (s.s.) and *An. gambiae* (s.s.) were relatively more common in Ntaja and *An. arabiensis* was more common in Namanolo. By contrast, indoor collections of mosquitoes at other locations close to both Namanolo and Ntaja, Lindblade et al., (2015) found *An. funestus* (s.s.) to be dominant, while *An. arabiensis* was next in abundance and *An. gambiae* (s.s.) was uncommon. These populations exhibited resistance to the synthetic pyrethroid deltamethrin, with 38% mortality in WHO bioassays for *An. funestus* (s.l.) and 53% mortality for *An. gambiae* s.l. (probably, *An. arabiensis*) (Lindblade et al. 2015). Despite these variations in mosquito species abundances between sites, *An. funestus* (s.s.) and *An. gambiae* (s.s.) are generally considered epidemiologically more important than *An. arabiensis* due to their well-documented anthropophilic and endophilic behaviors (Killeen et al., 2001; Orsborne et al., 2019, 2020; Takken & Verhulst, 2013). This study found non-host specific blood feeding of mosquitoes although high rates of feeding on human blood was observed by all three species, regardless of their variable phenotypes.

These findings are more evident in the fact that human blood comprised most blood meals in unmixed conditions, regardless of species or study site, and that blood meals from other potential

sources (goats, dogs) were secondary, with goat blood meal being more frequent than dog. This observation is not surprising because it was commonly observed that people kept goats indoors in special rooms at night, probably for protection against theft, while dogs were left outside as guard dogs. This may explain the higher number of goat blood meals compared to dog blood meals. Killeen et al., (2001), in modeling mosquito populations of Tanzania and Kenya, demonstrated that there is a relationship between host availability and the amount of time that vectors spend seeking blood meals; by inference, hosts that require less time to locate will be fed upon more frequently. Orsborne et al., (2020), studying mosquito populations in Ghana, reached a similar conclusion, emphasizing that local host availability even for known anthropophilic malaria vectors, is a powerful driver for host selection. In Malawi, there have been no previous studies that consistently quantified relative availability of potential blood meal hosts. The high prevalence of human host blood-feeding by *Anopheles* species observed here is consistent with 2002 findings from southern Malawi, in which blood meals were nearly entirely from humans and secondarily from bovines (Mzilahowa et al., 2012). In northern and southern Zambia, similar high human host selection (>90%) and comparatively lower goat selection (<5%) by *An. gambiae* and *An. funestus* (s.s.) were observed (Das et al., 2017; Mharakurwa et al., 2007). In contrast, the dominant blood meal of malaria vectors around Lake Victoria in western Kenya was humans for *An. gambiae* (s.s.) and *An. funestus* (s.s.), but for *An. arabiensis* was predominantly bovine (Fritz et al., 2013) or equally bovine and human (Hamel et al., 2014). Through application of the "ratio of ratios" method of host selection, this study approached the problem of variation in host selection semi-quantitatively in order to assess host selection tendencies of these often behaviorally stereotyped species.

The high frequency of human blood meals detected in this study can be attributed to several factors, in particular bed net use practices. The higher HBI and EIR in Namanolo compared to Ntaja (96.4% vs. 88.9% and 0.11 vs. 0.06, respectively) could be due to widespread use of PBO-containing Olyset Net Plus in Ntaja, which have been shown to be more effective than convention LLINs against pyrethroid-resistant *Anopheles* populations (Mzilahowa et al., 2016; WHO, 2018) Lindsay et al., (2021) have suggested that the underlying mechanism of PBO-containing LLINs may simply be that they are more toxic, rather than overcoming insecticide resistance. Regardless, other randomized field trials in Tanzania and Uganda have shown significantly lower human infection prevalence where LLINs with PBO were distributed (Protopopoff et al., 2018; Staedke et al., 2020). Although the entomological mediators of these reductions were not investigated, they are likely due to reduced transmission intensity. In the present study, the lower HBI of *An. arabiensis* and *An. gambiae* (s.s.) in Ntaja than Namanolo could also be explained by the use of LLINs with PBO in Ntaja, which apparently increases their susceptibility to the insecticide as explained above (Mzilahowa et al., 2016; Ogola et al., 2017).

In the only other study analyzing blood meals of *Anopheles* vectors in Malawi, conducted in Chikwawa district (southern Malawi) during 2002 prior to any mass distribution of insecticide-treated nets, most blood meals were from humans, with relatively few coming from bovine or mixed human-bovine feeding (Mzilahowa et al., 2012). The 2002 HBI estimates for the three dominant malaria vector species were similar to what was found in the present 2019-2020 study, despite there now being a long history of malaria control and LLIN use. The species-specific HBI estimates for 2002 vs. 2019-2020 were: *An. arabiensis*, 85.0% vs. 92.3%; *Anopheles gambiae* (s.s.), 99.2% vs. 93.5%; and *An. funestus* (s.s.), 99.2% vs. 96.5%. However, the estimated EIR in the present study (33 infectious bites per person per year) was lower than that reported by

Mzilahowa et al., (2012) (183 infectious bites per person per year). Both studies used PCR-based detection of sporozoite infection in the head-thorax of individual mosquitoes, although prevalence was lower in the 2002 investigation (4.9%) compared to the present study (16.0%). However, indoor mosquito density was lower at the present study sites, thereby reducing the EIR. Another more recent study in Chikwawa, done during the implementation of a community-based control program, showed that 4 of 91 *Anopheles* (4.4%) tested by PCR were positive for *P. falciparum* infection during the rainy season, with an estimated EIR of 13.5 infectious bites per person per year (Mburu et al., 2019), suggesting a reduced EIR in that region.

Molecular-based approaches to blood meal analysis to detect vertebrate host feeding have advanced since the review of this topic (Fritz et al., 2013; Kent, 2009; Keven et al., 2020; Logue et al., 2016)). At the forefront of this advance has been development of qPCR methods using host-specific probes by either TaqMan or SYBR green detection (Keven et al., 2020; Tajadini et al., 2014). However, host species-specific probes in multiplex qPCR targeting *Anopheles* blood-meal hosts were developed only recently (Keven et al., 2020). The use of species-specific probes, designed within a qPCR format here, favored the detection of single and multiple (i.e., mixed) blood meals in this study. By screening all blood meals for human blood, and then analyzing by PCR, amplicon sequencing, and BLAST search matching those blood meals not reacting to the human probe, it was possible to reveal the narrow breadth of dominant hosts being utilized by the *Anopheles* community, and then using qPCR to reveal the extent of multiple feeding. This combinatorial approach indicated that ~12% of the blood meals were mixed feeding of human and goat (10.8%), human and dog (1%) or goat and dog (0.2%).

The host-selection analysis showed that, in both sites, two of the *Anopheles* species fed more often on goats than humans in proportion to availability of these hosts. *An. arabiensis* and *An. gambiae* (s.s.) over-selected goats and under-selected humans, while *An. funestus* (s.s.) selected the two hosts about equally (i.e., randomly) in proportion to their availability. These results are not surprising, although *An. arabiensis* is reportedly more zoophilic, while *An. funestus* (s.s.) is more anthropophilic (Sinka et al., 2010) plasticity and or opportunistic tendencies have been observed in various *Anopheles* species (Keven et al., 2017; Killeen et al., 2001; Orsborne et al., 2019, 2020; Takken & Verhulst, 2013). The explanation to the relatively higher goat feeding in indoor mosquito samples is consistent with goats being kept indoors at night, and malaria control interventions in the area (LLINs) (PMI, 2018). These two activities provide easy accessibility to goat blood meal and makes it more difficult to access human blood meal due increased mosquito-goat contact and reduced mosquito-human contact (Killeen et al., 2001). The switch in host utilization is indicated by the reduced access to human blood meal and increase in the utilization of the non-human hosts. The comparable increase in mixed blood meal may also suggest disruption in feeding either due to LLINs' activity or otherwise, pushing the mosquito to get a full blood meal from other hosts. Either way this is one of the few studies in Malawi to report blood meal analysis including detection of mixed blood meals. This study will prompt more research in blood meal studies in Malawi to document the range of blood meal hosts, especially those involving goat blood meal which is rarely reported in literature.

The findings of this investigation suggest important implications for *Plasmodium* transmission and malaria control. Multiple host feeding by some *Anopheles* females might allow for increased survival and reproduction (Logue et al., 2016; Stone & Gross, 2018). The presence of multiple malaria vector species that successfully obtain human blood meals could lead to an increased

Plasmodium transmission by increasing the basic reproductive number *(R_o)* (Das et al., 2017; Kilpatrick et al., 2006). More widespread use of LLINs, particularly with PBO, could help reduce transmission, but this intervention alone is unlikely to reduce malaria incidence in this meso-endemic setting to acceptable levels where elimination can be contemplated. Residual *Plasmodium* transmission and weakened intervention efforts (Cohen et al., 2012) are likely to persist into the future.

3.6. Conclusion

This study has shown that, in southern Malawi, human blood comprises the bulk of the blood meals of the three species of *Anopheles* vectors, yet dog and goat blood meals are also present and commonly mixed with that of humans. Host selection analysis revealed that goats were over-selected compared to humans in proportion to host availability. This could be a direct result of LLIN usage. The presence of mixed-blood meals showed the adaptability of these vectors to switch hosts to obtain a full blood meal, possibly reducing effectiveness of malaria control interventions. The frequent use of humans as a blood meal source elevates the human blood index and consequently the entomological inoculation rate, sustaining malaria incidence. Some evidence suggests that pyrethroid-based LLINs containing PBO reduced mosquito-human contact, as the HBI was significantly lower at the site using these nets. Nonetheless, estimates of EIR remain high, indicating that transmission is well sustained despite the use of PBO-containing LLINs. Other factors, such as net durability and use may be reducing effectiveness of interventions.

CHAPTER 4 : GENOTYPING OF *ANOPHELES* MOSQUITO BLOOD MEALS REVEALS NONRANDOM HUMAN HOST SELECTION: IMPLICATIONS FOR *PLASMODIUM FALCIPARUM* TRANSMISSION, MALAWI

Manuscript

Rex B. Mbewe, John B. Keven, Charles Mangani, Mark L. Wilson, Themba Mzilahowa, Don Mathanga, Clarissa Valim, Miriam K. Laufer, Edward D. Walker, Lauren M. Cohee.

4.1. Abstract

Analysis of mosquito blood meals to determine the extent of human host utilization relative to animal hosts extends to genotyping of human blood meals to assess patterns of vector-human contact and characteristics of individual human hosts. This approach has improved the understanding of parasite transmission in several vector-borne diseases systems including malaria. This study used a microsatellite-based human-genotyping method to implicate within the human population, demographic groups that are the main drivers of malaria parasite transmission from human to mosquitoes.

The study was conducted in two districts of moderate to high malaria transmission in southeastern Malawi. Household surveys garnered human demographic information. Blood samples obtained by finger prick from consented participants were absorbed onto filter paper. Indoor resting, blood fed female *Anopheles* mosquitoes were sampled from these same houses. Genomic DNA from human blood spots and mosquito blood meals was subjected to genotyping methods to generate genetic profiles from each individual sample. Using a genotype matching algorithm written in R, the profiles from human and mosquitoes were matched to identify the unique human sources of blood meals. Polymerase chain reactions (PCR) were used to detect *Plasmodium falciparum* DNA

in human and mosquito samples. The results were used to identify the human demographic grouping which was most frequently bitten and had highest malaria infection prevalence.

Anopheles females selected human hosts non-randomly and fed on more than one human in 10% of the blood meals. Few individuals contributed most of the blood meals to the *Anopheles* vector population, and more males than females were selected, even though males were a smaller fraction of the population. Children ≤ 5 years and adults ≥16 were under-selected for blood meals compared to school children (6 to 15 years old). Mosquitoes that had fed upon males of the school age group had the highest *P. falciparum* infection rate in their abdomens.

These results show that humans of the 6-15 years age group, particularly males, are drivers of *Plasmodium falciparum* infection from humans to mosquitoes because of the blood host selection outcome. This observation is relevant to community-based malaria control and prevention, as it indicates the need for deliberate efforts targeting males of the school age group.

4.2. Introduction

Patterns of mosquito utilization of individual humans for blood meals, such as biases towards feeding on particular demographic groups in the human population, can have profound impacts on malaria epidemiology by allowing transmission to persist even in the presence of active control measures such as widespread distribution and utilization of long-lasting insecticidal nets (LLINs) (Dye & Hasibeder, 1986; Hasibeder & Dye, 1988; Smith et al., 2007; Woolhouse et al., 1997; Keven et al., 2021). One approach to understanding human host utilization patterns is through mosquito blood meal analysis (Borland & Kading, 2021). Mosquito blood meal analysis has been used in many studies for various reasons, including investigations of blood meal sources (especially to determine the human blood index, or fraction of the hosts that are humans) as well as determination of proportions of mosquito infections originating from different demographic groups in the human population (Gonçalves et al., 2017; Escobar et al., 2020).

In Malawi, school-age children are frequently infected with *Plasmodium falciparum* malaria and carry gametocytes, the gametic parasite stage required for infective human-to-mosquito-transmission, suggesting that this age group is an important reservoir of infection even when children are asymptomatic (Ali et al., 2015; Walldorf et al., 2015; Coalson et al., 2016, 2018; Cohee et al., 2021). This observation makes it imperative to conduct studies in Malawi that assess the contribution of certain demographic groups to the transmission of malaria parasites from humans to mosquitoes. Genetic profiling of mosquito blood meals has been applied elsewhere in three important ways of understanding malaria transmission that are relevant to this pressing issue: (1) to identify the most frequently bitten population group, (2) to assess the frequency distribution of mosquito biting on individuals, and (3) to estimate the proportions of multiple blood meals on

different human hosts (Keven et al. 2021). This genetic profiling method utilizes microsatellite genetic markers, or short tandem repeats (STR) of nucleotides, which occur at thousands of Mendelian loci in the human genome with multiple alleles operating at each locus (Willems et al., 2014). The resultant genetic variation provided by the combination of numerous loci and multiple alleles provides for numerous applications in research such as studies of population genetic structure (Rosenberg et al., 2002) and in forensic sciences (Mirghani et al., 2010; Smith et al., 2017).

Human accessibility and vector preferences are factors that determine which human hosts are most frequently bitten. We hypothesized that school-age children in Malawi are the predominant sources of *Anopheles* blood meals. Previous studies have suggested that a greater proportion of blood meals are taken from adults and older children than young children and infants (Carnevale, Frézil, Bosseno, Le Pont, & Lancien, 1978; Muirhead-Thomson, 1951) partially because of differences in body surface area. Studies have also shown differential use of LLINs by age, with school-age children being least likely to sleep under nets in Malawi (Buchwald et al., 2016; Walldorf et al., 2015). Thus, school-age children are likely to be bitten more frequently based on body surface area and limited LLIN use. In addition, previous studies reported that *Plasmodium*-infected individuals are more attractive to vectors than uninfected individuals (De Moraes et al., 2014; Lacroix et al., 2005).

In this study, we aim to characterize the human sub-populations responsible for driving malaria parasites into the mosquito populations thereby propagating malaria transmission in the community. This was achieved by matching human genetic profiles analyzed from mosquito blood meals to those analyzed from human blood samples.

We hypothesize that (1) Mosquito feeding on individual humans is nonrandom, and (2) Mosquito feeding is preferential on certain human population groups (males and 6-15 years old), classified by age and gender in the study area and these groups drive malaria transmission.

4.3. Methods

4.3.1. Study site

The study was conducted in Machinga and Balaka districts in the southeastern part of Malawi. The catchment area in the two districts was a 10-kilometer radius around Ntaja health center in Machinga and Namanolo health center in Balaka. These districts were chosen because they have moderate to high malaria transmission throughout the year (Mbewe et al. 2021). The climate is characterized by high temperatures and seasonal rainfall. The residents of the area are mostly subsistence farmers whilst others ply trade in market centers. Malaria transmission is highest in areas of high temperature and seasonal rainfall, from October through April (Government of Malawi, 2015).

4.3.2. Sample collection and processing

Household surveys. Monthly surveys of a household-based cohort were conducted to collect demographic and malariometric data. A total of 130 households participated, 73 in Namanolo and 57 in Ntaja. The head of each household was consented to participate in a questionnaire designed to gather information on number of people per household, name, sex and date-of-birth of each household member. Blood droplets were collected by finger prick from all consented household members, dried on Whatman filter paper, and stored under cool laboratory conditions.

Mosquito sampling. Indoor resting, female *Anopheles* mosquitoes were sampled from the consented households. Households were sampled three times each period from May-June 2019, October-November 2019, and December 2019-January 2020. Beginning in February to April of 2020, only a subset of households in each site were sampled once every two weeks. Blood fed mosquitoes in the households were sampled until none were present using battery powered

aspirators (Model 1419 John W. Hock Company, Gainesville, Florida, USA) and breath-operated aspirators. In 2020, in addition to aspiration methods, mosquitoes were also sampled by standard miniature CDC light traps (Model 512; John W. Hock Company, Gainesville, Florida, USA), and pyrethrum spray catch (Odiere et al., 2007).

DNA Extraction. Each blood fed *Anopheles* mosquito was bisected into head attached to thorax (hereafter, "head-thorax"), and abdomen, using sterile technique. Genomic DNA was extracted from the mosquito abdomens and mosquito head-thorax separately, and from human blood spots using the DNeasy® Blood & Tissue Kit (Cat. No. 69582; Qiagen, Valencia, CA, USA).

***Anopheles* species identification**. Mosquitoes morphologically identified as members of the *An. gambiae* sensu lato (s.l.) or *An. funestus* species complex were subjected to a polymerase chain reaction (PCR) following the method of Walker et al., (2007) to identify the species, following modifications in Mbewe et al., (2021).

***Plasmodium falciparum* detection.** Presence of *Plasmodium falciparum* parasites in the human blood samples and the mosquitoes' heads-thoraces and abdomens was tested using the molecular based method described in Mbewe et al., (2021).

Identification of human blood meals. DNA obtained from blood-fed mosquito abdomens were subjected to a qPCR method containing primers (forward 5′-GGCCTGTTCCTCCCTTATTT-3′, reverse 5′-TACACAGGGCTTCCGAGT-3′) and probes (FAM-ATGGAGTCTGTGTTCCCTGTGACC-QSY) that detects human DNA (Keven et al., 2020).

4.3.3. Microsatellite genotyping and profile matching

Human-derived mosquito blood meals and human blood samples were subjected to microsatellite genotyping method (Keven et al. 2021). The following 24 well-characterized, tetranucleotide microsatellite loci were used for genotyping: D3S1358, D1S1656, D2S441, D10S1248, D13S317, D16S539, D18S51, D2S1338, CSF1PO, TH01, vWA, D21S11, D7S820, D5S818, TPOX, D8S1179, D12S391, D19S433, FGA, D22S1045, Penta E, Penta D, and DYS391, plus an X-linked amelogenin sex-determining marker (Oostdik et al., 2014). Amplification of these markers was performed using a commercially available and validated multiplex PCR kit from Promega (PowerPlex Fusion System; catalog no. DC2402) that contained locus-specific fluorescent-labelled primers (Oostdik et al., 2014). Each locus had two alleles, one from each parent. Alleles were analyzed by capillary electrophoresis (ABI 3730 Genetic Analyzer, Applied Biosystems, Foster City, CA) with LIZ 500 (Applied Biosystems, Foster City, CA) as the internal size standard. GeneMapper software version 4.1 (Applied Biosystems) was used to visualize the allelic sizes of all the loci in each sample, creating the genetic profile of the sample (Appendix 5.1). For each sample (i.e., human-fed mosquito or human blood), all observed allele sizes at each locus were listed and together served as the genetic profile ("fingerprint") of the person. While different mosquitoes that fed on the same individual had the same genetic profile, each study participant who donated a blood sample had a unique genetic profile. The genetic profile of a mosquito blood meal from a house was compared to the genetic profile of all members of the household and the entire human survey, to determine which (if any) person was fed on by the mosquito. An example of a genetic profile generated by this method is shown in Appendix 4.2.

4.3.4. Data analysis

Data analysis was performed using RStudio (Version 1.1.456; https://www.R-project.org/). An R code for matching genetic profiles was developed and applied in the analysis. Briefly the R script involves loop functions and conditional statements to compare the genotype of each locus in a query sequence (i.e., a genetic profile) to its corresponding locus in a subject sequence (i.e., another genetic profile). The similarity of the two genetic profiles was expressed as the proportion of identical loci (i.e., same alleles) times 100. For example, 19 identical loci divided by 24 total loci multiplied by 100 gives 79% profile similarity.

In principle, genetic profiles from two blood-meal specimens that originated from a same human source should have 100% profile match. Therefore, it could be expected that match values < 100% should indicate blood meals from different individuals. However, since some blood meal specimens could yield insufficient human DNA (e.g., due to very small mosquito blood meal volume) or false mismatches due to "allele dropout" (a failure of the alleles in a locus to be detected) (Findlay et al., 1998) the ideal 100% match is not always attained. To account for these errors and to minimize their effects in obtaining a reliable comparison or match of genotypes, a value less than 100% has to be used as the criterion for establishing a match. To determine this value, pairwise percent profile match analysis was performed on the genetic profiles of individual humans in the study sites. This generated n(n-1)/2 profile match results or values, where n is the total number of humans sampled on a site. A value higher than the highest value in the pairwise match output (excluding match results of monozygotic twins which would be 100%) was the criterion below which two genetic profiles were considered different (Keven et al., 2021).

The number of different human individuals in the blood-meal sample represented the number of different human individuals in the sample. The frequency of occurrence of each unique genetic

profile in the blood-meal sample represented the number of mosquitoes that fed on each individual human. These data were used to construct frequency distribution histograms that relate the number of different human individuals (y-axis) to the number of blood-meals they provided (x-axis). The observed frequency distributions were fitted to zero-truncated Poisson and zero-truncated negative binomial frequency distribution models using the functions *zerotrunc* and *rootogram* of the package countreg in RStudio (Kleiber & Zeileis, 2016). The fit of the two distributions was compared by chi-squared test to determine if selection of humans by mosquitos was random (Poisson) or aggregated (negative binomial).

The study participants who donated their blood sample to analyze their genetic profile were classified into 3 age groups, namely those ≤ 5 years of age, those between 6-15 years of age, and those ≥16 years of age. This age classification is commonly practiced in malaria epidemiological studies to assess risk of infection among age groups (Waldforf et al. 2015). Genetic profiles of human-fed mosquitoes were compared to the genetic profiles of human individuals to identify the person who was bitten by the mosquito. The sex of a person in the blood meal could be determined using the sex-specific genetic marker but identification of the person's age could not be done based on a genetic marker. The age of the person in the blood meal was determined by matching the blood meal profile to the profiles of human individuals (whose age were recorded). The proportion of each sex and age group observed in the mosquito blood meals was compared with its expected proportion (the proportion of individuals of that group in the study site) using two-tailed binomial tests.

4.4. Results

4.4.1. Human genetic profiling

Genetic profiles of 243 sampled human individuals (99 from Namanolo and 144 from Ntaja) were generated. A comparison of each human genetic profile with all other profiles to determine the degree of matching, represented as the percent profile match, resulted in 29,161 pairwise profile match values. There were 20 unique percent match values ranging from 0-79%, each with a varying frequency of occurrence (Table 4.1). The lowest value of percent match was 0% with 0.09 probability of occurrence. This result means that 9% of all the pairwise comparisons were between individuals with no identical genotype at any locus. The highest percentage match value was 79% (i.e 19/24 matched loci) with a 10^{-5} probability of occurrence (Table 4.1); there were only two such matches. Because it was highly unlikely (probability < 0.00007) for two individuals in the study to have matches greater than 79%, this value was used as a criterion for deciding whether two genetic profiles in blood meals originated from the same person or not. Hence matching values greater than 79% was indicative of the human source of the blood meal

Table 4.1. Pairwise testing, percent match and probability of having similar genetic makeup.

% Match	0	4	8	12	17	21	25	29	33	38	42	46	50	54	58	62	67	71	75	79
Frequency of occurrance	2612	5161	7397	6470	3813	1989	832	376	208	133	69	47	14	12	14	8	2	1	1	2
Probability of occurance	0.090	0.177	0.254	0.222	0.131	0.068	0.029	0.013	0.007	0.005	0.002	0.00161	0.00048	0.00041	0.00048	0.00027	0.00007	0.00003	0.00003	0.00007

4.4.2. Genotyping human blood meals

Of 501 *Anopheles* spp. blood meals found to contain human blood, 480 (95.8%) yielded human genetic profiles. Of these, 436 (91%) were found to contain a single human genetic profile. The remaining 44 were found to contain profiles from two or more humans. When the single-human blood meal genetic profiles were compared with the profiles of human residents (n = 243 individuals), a total of 183 blood meals matched profiles of household residents whilst 253 successfully genotyped blood meals did not match any profiles of the residents represented in the sample. The blood meals with multiple human sources could not be matched to any human profile owing to the presence of more than two alleles at many loci.

There were 633 female *Anopheles* mosquitoes identified to mosquito species: *Anopheles arabiensis* (279, 44.1%), *Anopheles gambiae* s.s. (103, 16.2%), *Anopheles funestus* (212, 33.5%), *Anopheles parensis* (2, 0.3%), and unidentified *Anopheles* species (37, 5.8%). Our analysis of mosquito behavior focused on the *Anopheles* genus rather than species due to great variations in species representation, where other species of *Anopheles* mosquitoes had their blood meal profiles not matching the human profiles.

4.4.3. Frequency distribution of human blood meals

The unique genetic profiles identified in the blood meal sample represented different human individuals that the mosquitoes in the sample fed upon. The frequency distribution of blood meals taken from these individual humans as reflected solely by genotyped but nonmatched blood meals, showed that some individuals were more frequently bitten than others, such that there were few blood meals on most individuals, and many blood meals on few individuals (Figure 4.1). The number of blood meals per individual person ranged from 1 to 51. Although this pattern was

evident for males and females, the three highest number of blood meals were from male individuals.

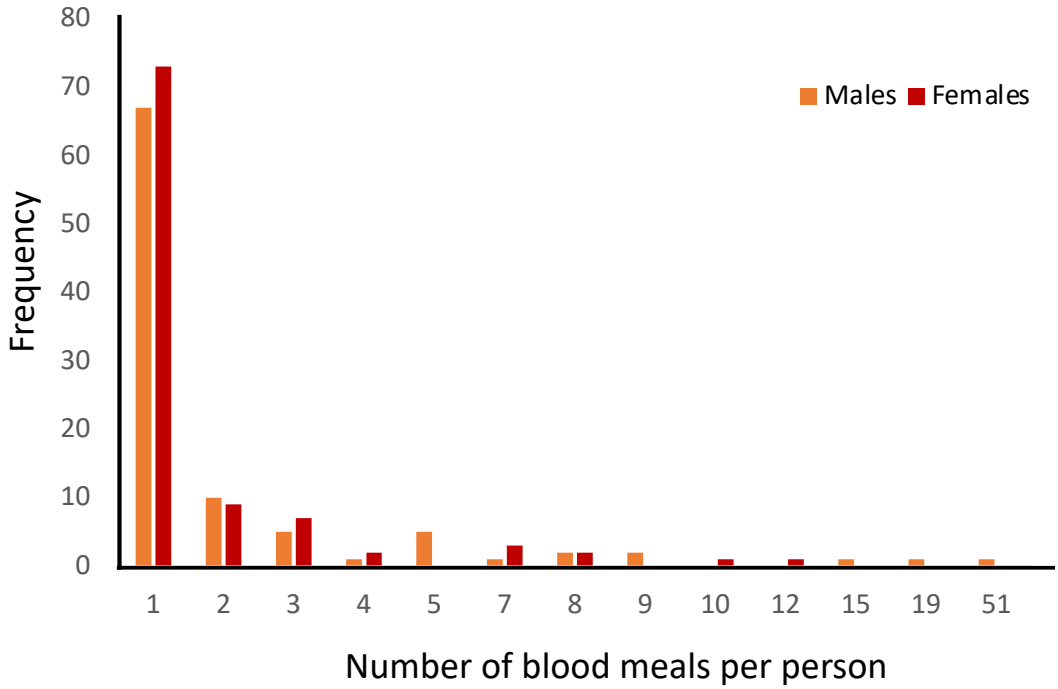

Figure 4.1. Frequency distribution of number of blood meals on individual male and female humans (n=436) taken by *Anopheles* mosquitoes sampled in Ntaja and Namanolo communities of southeastern Malawi.

The frequency distribution did not fit the Poisson distribution, which would indicate a random pattern of individual human host selection; rather, it fit well the negative binomial distribution, which indicates a nonrandom and aggregated (or clumped) pattern of human host selection (Figure 4.2).

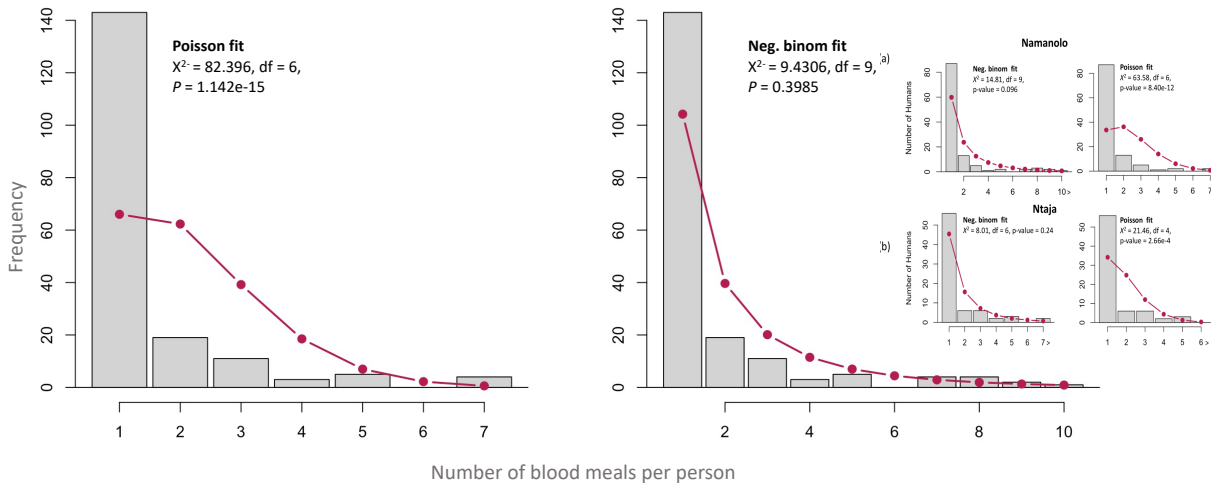

Figure 4.2. Fits of the frequency distribution of number of human blood meals taken per person from *Anopheles* mosquitoes sampled in houses using all data (Insert: distributions in Ntaja and Namanolo showing similar trend) in communities, southeastern Malawi.

4.4.4. Mosquito human host selection by sex and age category

When the proportion of male and female humans in the mosquito blood meals was compared with their relative proportion in the village (expected proportion), the results revealed that males contributed a significantly higher proportion of blood meals and their female counterpart contributed a significantly lower proportion of blood meals than expected. Without considering their relative proportion in the village, and focusing only on the blood meal proportion, there were significantly more male-fed than female-fed blood meals (Figure 4.3). Comparison by binomial tests of the proportion of blood meals obtained from the three age groups to their expected proportion (i.e., their relative proportion in the village) showed that there were fewer blood meals from individuals ≤ 5 and ≥16 years old. In contrast, the proportion of blood meals obtained from individuals in the 6-15 years old group was higher than expected but this difference was not statistically significant (Figure 4.4a). Comparison (Chi-square tests) of the relative proportion of the age groups in mosquito blood meals revealed that humans in the age group 6-15 years old

contributed a significantly high proportion of blood meals than did the younger and older age groups (Figure 4.4b).

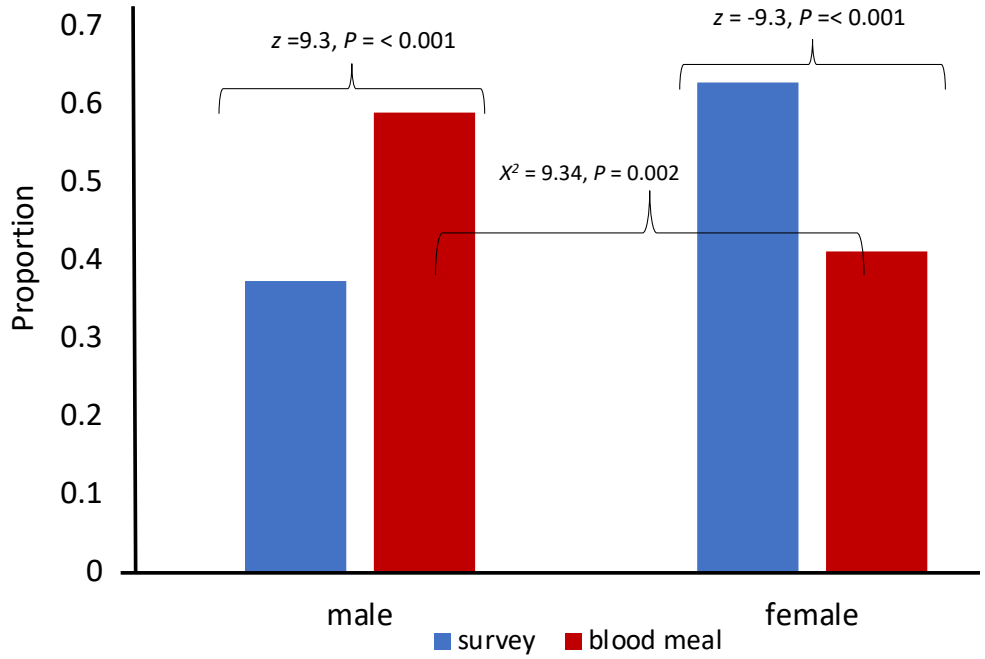

Figure 4.3. Proportion of *Anopheles* blood meals (red) taken from males and females humans compared to the proportion of the two human sexes in the household survey (blue). Results of Chi-square tests comparing the proportion of males and females in the blood meals to their proportion in the household survey, and of male versus female blood meals are shown inside the plot. Note, data from both sites (Namanolo and Ntaja) were combined in this analysis.

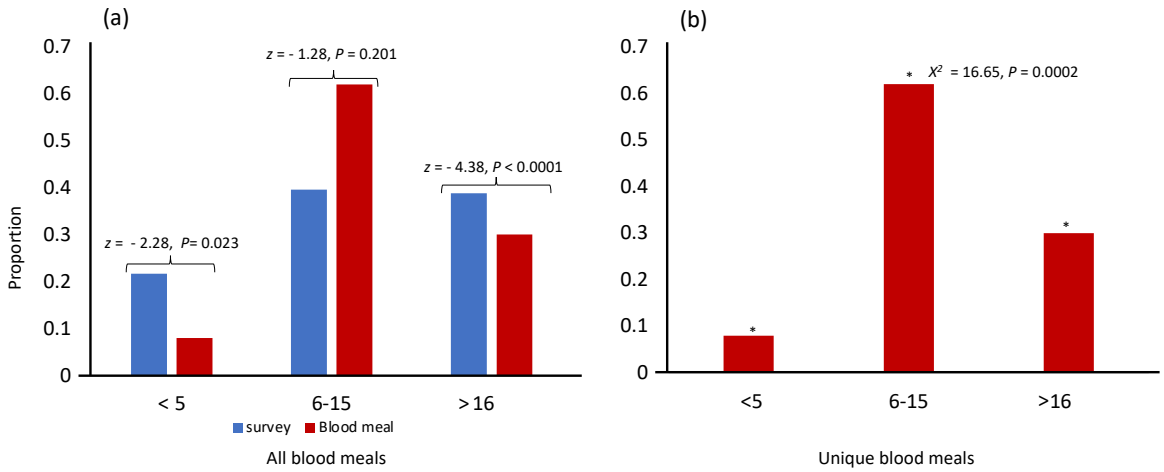

Figure 4.4. (a) Comparisons of the proportion of blood meals taken from humans in three age groups in relationship to the number of humans in those age groups as determined by household survey. (b) Comparison of the proportion of blood meals taken from the three human age groups along with Chi-square results of test of homogeneity of blood-meal proportion. Data from both sites (Namanolo and Ntaja) were combined in this analysis.

4.4.5. *P. falciparum* infection in mosquito blood meals and the human population

Plasmodium falciparum DNA was detected by PCR in abdomens of blood fed *Anopheles* (total, N = 160, 37% positive), head-thorax of blood fed *Anopheles* (total, N = 85, 19 % positive), and in human blood spots (total, N = 27,11 % positive). The proportion of abdomens and head-thorax of *Anopheles* mosquitoes positive for *P. falciparum* was not significantly different between sexes of the human host as determined by genotyping the blood meals present in the abdomen (Figure 4.5a). The proportion of human blood spot samples positive for *P. falciparum* was not different by sex of the human source of the blood (Figure 4.5b). Interestingly, the proportions of infections were higher in the mosquitoes than in the human blood spot regardless of mosquito body part (Figure 4.6).

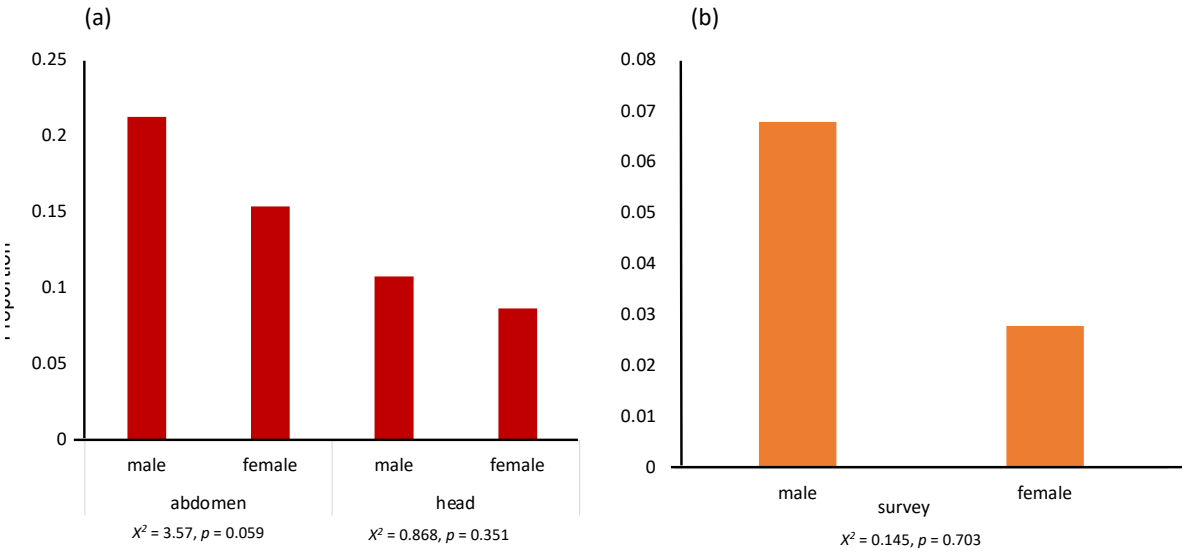

Figure 4.5. Proportion of abdomens and head-thorax of *Anopheles* mosquitoes positive for *P. falciparum* classified by whether the sex of the human host in the blood meal was male or female. (b) Proportion of human blood spot samples positive for *P. falciparum* classified by sex of the human source of the blood. Chi-square tests of homogeneity of proportion of infections between the two sexes are shown in the plots. Data from both sites (Namanolo and Ntaja) were combined in this analysis.

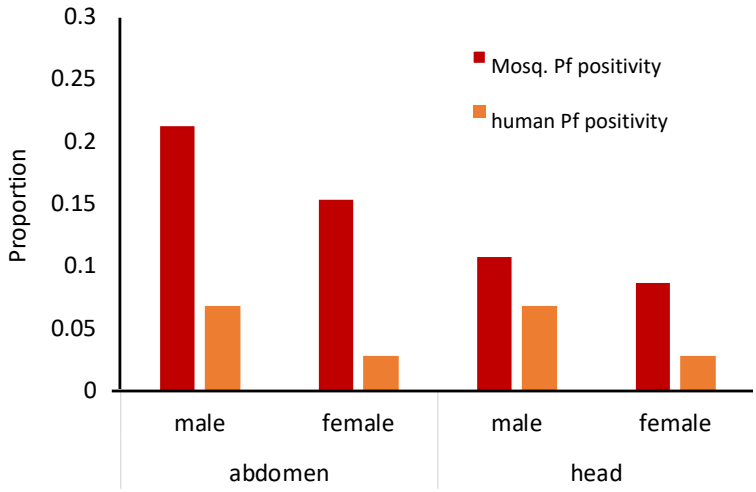

Figure 4.6. The proportion of *Anopheles* mosquito abdomens and head-thorax body sections in relation to human blood samples positive for *P. falciparum* for all samples regardless of human genotype, classified by male or female. Namanolo and Ntaja communities, southeastern Malawi.

For all the three age groups, the proportion of *Anopheles* abdomens positive for *P. falciparum* were not significantly different from the proportion of human blood that were positive (Figure 4.7a). However, the proportions of the head-thorax positive for *P. falciparum* were significantly higher than were human blood for the two older age classes (Figure 4.7a). Comparisons of the proportion of *Anopheles* mosquito body part (abdomen with blood; head-thorax) positive for *P. falciparum* across human age group of the genotyped blood meal showed higher infection proportions in the 6-15 years old group compared to the ≤ 5 years old and ≥16 years old groups (Figure 4.7b).

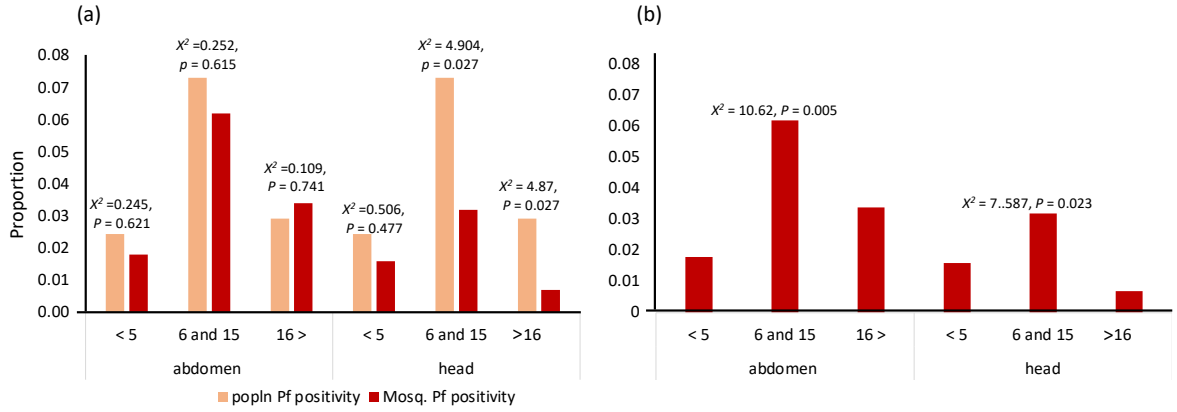

Figure 4.7. Comparisons of the proportion of *Anopheles* mosquito body part (abdomen with blood; head-thorax) positive for *P. falciparum* classified by age category and compared to the proportion of the human population positive by age category. (b) Comparisons of the proportion of *Anopheles* mosquito body part (abdomen with blood; head-thorax) positive for *P. falciparum* classified by human age group of the genotyped blood meal.

4.5. Discussion

The results from the study have shown nonrandom pattern of mosquito feeding on individual humans and that the mosquitoes were feeding more on certain human population groups that is, males and children aged 6-15 years old.

Genetic profiling of mosquito blood meals to investigate patterns of contact between human hosts and mosquito vectors of diseases, including malaria vectors, has been applied in numerous contexts (Chow-Shaffer et al., 2000; Edman et al., 2003; Guelbéogo et al., 2018; Paul et al., 2001; Scott et al., 2006; Soremekun et al., 2004; Gonçalves et al., 2017). Most of the previous studies used 10 or fewer loci in the construction of the genetic profiles and did not consider the impact of this limitation on error rates in matching genotypes. The primary limitation in using fewer number of loci is the reduction in the power to discriminate related individuals. The current study is the second to use a greater number of loci (24) in the construction of genetic profiles. The first known study to apply this method of investigation with 24 microsatellite loci to *Anopheles* vectors of malaria was conducted in PNG (Keven et al., 2021). The use of more microsatellite loci increases power to discriminate between genetically related individuals, and decreases the possibility of error when allele dropouts occur (Keven et al., 2021). The assessment used here is robust for these reasons.

In our study we were able to identify human sources of blood meals by matching human genetic profiles to mosquito blood meal profiles, and by matching profiles from mosquito blood meals to each other. More than half of the mosquito blood meals did not match any human profile provided by blood spots in the human survey. This could possibly be due to indoor resting behavior of the mosquitoes that might have taken blood meals outdoors from non-household members or blood meals from non-participating household members. Harrington et al., (2014) found that the

majority of blood meals of *Aedes aegypti* in villages in Thailand came from nonhousehold members, a finding attributed to peddlers, visitors, and passersby in the community during the daytime hours, when *Aedes aegypti* tends to seek out blood hosts. We did not assess the volume of traffic in households in the households in Ntaja and Namanolo at night, in order to determine the number and diversity of people who might have been in or near the houses at night and into early morning hours when *Anopheles* mosquitoes seek hosts, but the blood meal genotyping data suggest that such human movement was considerable.

The observation of a non-random and aggregated pattern of human host utilization emerged in other studies using a blood meal genetic profiling approach in different mosquito-borne disease systems such as those involving *Anopheles*, *Culex*, and *Aedes* species (Edman et al., 2003; Guelbéogo et al., 2018; Harrington et al., 2014; Paul et al., 2001). The mosquito populations in this study exhibited markedly non-random human utilization for blood meal source, apparent at both the individual and household level, meaning certain individuals and households were at greater risk to infectious *Anopheles* bites than were others. This finding was most likely due to variation in accessibility of the mosquitoes to individuals; and variation in mosquito access to houses due to such factors as distance from larval habitat, presence of insecticide-treated bed nets in houses, cooking inside or outside of houses, and presence of open or blocked eaves which provide or block entry of mosquitoes (McCann et al., 2017). Differences in human behavior such as bed-net usage and late-night activity may be the cause of differences in the accessibility of individuals to the mosquitoes (Buchwald et al., 2016; Walldorf et al., 2015; Bayoh et al., 2014). Also, humans are most likely to encounter female *Anopheles* where the mosquitoes themselves aggregate. This is because humans likely undertake particular activities during *Anopheles* biting

times, and often these activities are related to their age and sex (Rodríguez-Rodríguez et al., 2021; Smith et al., 2004).

The study showed that males were proportionately over-utilized for blood meal compared to females. Further, the youngest and oldest age groups were under-selected, and the school age group was selected in higher proportion than these two groups. These results suggest that the males of school age group (6-15 years old) were the most frequently bitten population group in the study area. The disproportionate representation of this demographic group in the study area aligns with results of studies conducted elsewhere; for example, in Papua New Guinea, the *Anopheles* mosquitoes obtained more blood meals from males and individuals of the 15–30 years age group compared to females or other age groups. In that study, an interesting observation was the under-representation of the youngest age group (< 15 years old), which constituted a large proportion of the village censuses (52%) in the blood meals (Keven et al., 2021).

In Tanzania, males and individuals ≥ 20 years old were fed upon more than expected by *An. funestus* and *An. gambiae* s.s., whereas females and those < 20 years old were bitten less frequently in a village with bed nets, while in a village without bed nets, such variations were not observed (Soremekun et al., 2004). In a Kenyan study, individuals < 20 years old received more bites than expected compared to those between 20 – 50 years old, but no variation was observed between sexes (Scott et al., 2006). Gonçales et al., (2017) conducting blood meal analysis and genotyping study in areas of Burkina Faso and Kenya, found that children aged 5-15 years old were more often bitten than those of the other age groups. These variations amongst studies suggest that local conditions and study design will influence outcomes and resultant conclusions. The present study is internally consistent and compares well with recent epidemiologic studies of age-specific malaria in Malawi (Mangani et al., 2021). The common agreement in all these studies to the current

one, is that different population groups received proportionately different mosquito bites as reflected by blood meal analysis. In the current study the age group providing most of the blood meals was the 6 -15 years old group.

The presence of non-random distribution of vector blood meal has strong epidemiological implications. Several studies on mathematical modeling of malaria transmission show that where non-random distribution of vector bites among human hosts exists, the result is an increase in the Basic Reproductive number (R_o), causing the disease to persist (Dye & Hasibeder, 1986; Woolhouse et al., 1997; T R Burkot, 1988) even in the presence of control intervention programs such as bed nets (Smith et al., 2007). Therefore, targeted interventions focusing on those human demographic groups biasedly over-represented/selected by vector blood meals may help greatly in reducing malaria transmission and infection rates.

A considerable number of the blood meals in mosquitoes from multiple individuals were also observed. The presence of multiple feeding in the mosquitoes could be due to interrupted mosquito feeding which is caused by several factors including human defensive behavior, bed net availability, bed net usage and sleeping times. The presence and use of bed nets may cause irritation or excitation to the mosquitoes due to the action of the insecticide or its repellant effect and the individual human's intolerance to mosquito bite. Hence the mosquito may not be in a position to stay still for the duration of blood feeding. Blood meals obtained while the individuals are awake are likely to be disrupted than those obtained late at night when individuals are fast asleep. Individuals that are more intolerant of mosquito bites are more likely to disrupt the mosquito blood feeding process (Anderson & Brust, 1997; Boreham & Garrett-Jones, 1973). In

the current study, the proportion of multiple feeding by *Anopheles* mosquito species were similar to other studies. In Papua New Guinea, Burkina Faso, and Kenya the proportions of multiple blood meals ranged respectively from 6-15%, 11-18% and 14.5% (Keven et al., 2021; Burkot et al., 1988; Gonçales et al., 2017). In similar studies done in *An. funestus* and *An. gambiae* in two Kenyan villages, the proportions ranged from 2-14%, (Scott et al., 2006) and 10% in a Tanzanian village (Soremekun et al., 2004). Other studies reported similar percentages in *Culex* and *Aedes* populations, (Edman et al., 2003; Harrington et al., 2014; Paul et al., 2001). The one study that showed an exception was done for an *Aedes aegypti* population in Thailand where a much higher proportion about 45% was observed (Chow-Shaffer et al., 2000). In the latter case, one might expect much more interrupted feeding of a daytime biting mosquito when people are active and awake, compared to the nighttime biting *Anopheles* when people are inactive and sleeping. Multiple feeding has several implications in malaria transmission. In an arthropod vector of a disease affecting man, the taking of cryptic multiple meals may be expected to increase the man-biting habit, and thereby the vectorial capacity of the population. The chances of acquiring and of transmitting the disease agent would both be increased as a consequence (Boreham & Garrett-Jones, 1973). This applies where the feeding involves a number of different hosts, human or animal. But looking at human hosts alone, the number of bites per gonotrophic cycle has the pontential to raise the transmission potential, both R_o and vectorial capacity (VC) of the mosquito population above those expected without multiple feeding (Tedrow et al., 2019). According to Keven et al., (2021), *Anopheles* mosquitoes that feed on two human hosts per gonotrophic cycle increase both R_0 and VC by two-fold or greater (Tedrow et al., 2019). This is possible because those vectors can infect more than one human if they are sporozoite-positive, and also have more

than one chance of obtaining an infectious (i.e., gametocytemic) blood meal in a single gonotrophic cycle.

Our results have shown that the school age group was the most frequently bitten group, and that the mosquitoes biting this group had higher rate of infection in the abdomen and the head. This entails that this group was proportionately driving higher infection into the mosquitoes since a higher proportion of mosquitoes biting this age group had higher *P. falciparum* positives detected in their blood meal. The higher *P. falciparum* positivity in the abdomen of the mosquito biting this age group may suggest this group to be responsible for driving the infection into the mosquito populations. An assessment of the rate of infection in this age group as revealed in this study and in literature shows that this group has higher *Plasmodium* infection rate in the population (Walldorf et al., 2015; Coalson, 2015; Gonçalves et al., 2017). The higher *P. falciparum* positivity in the head (sporozoite) of mosquitoes biting this age group may suggest that the mosquitoes were infectious, and they could be transmitting the infection back to the population hence maintaining the transmission cycle. This is an interesting observation because it relates the point that some groups in the population are responsible for the transmission of infection to the mosquitoes while others bear the burden on the infection. This observation agrees with other studies which showed that children below 15 years of age were more likely to infect mosquitoes than adults (Goncalves et al., 2017).

Thus, our study incriminates males of the school age group (6-15 years old) as drivers of infection from humans to mosquitoes and as reservoirs of *Plasmodium falciparum* infection in Malawi. Males of this age category can be considered "super-spreaders", when it comes to infecting mosquitoes. These individuals who contribute most of the blood meals are more likely to infect

and be infected by the mosquitoes and serve as reservoirs of the parasite to which later are spread to the community population (Burkot, 1988; Cooper et al., 2019; Smith et al., 2004).

The limitation of the study was the low participation from household members in the survey, which increased the rate of non-matching of genetic profiles generated from human and mosquito blood meal. Hence, the non-matching profiles were excluded from the analysis. Also, the timing of blood sample collection was not the same as the mosquito sample collection with a lag period of about a few days in between, as such it was difficult to directly relate the infections in the humans to the mosquitos. However, this limitation was overcome by considering the infection in the blood meals rather than the humans to draw conclusions. However, the *P. falciparum* infection proportions in the abdomen of blood fed mosquitoes (blood meal) were not significantly different from the *P. falciparum* infection proportions (prevalence) in the human population. Hence, we propose further research in using mosquitoes (abdomen or blood meal) in malaria studies as a tool for sampling human population for *P. falciparum* infection detection (prevalence) as proposed for human and animal Virus surveillance (Yang et al., 2015). The advantage of using mosquitoes as blood sampling tool include the reduction in cost associated with current sampling methods and addressing issues to do with ethical implications of using human subjects in research.

A conceptual model of the results emerges from the findings of this study and allows inferences about the relative transmission potentials among sex and age groups. All observations point to age group 6-15 as being the mostly frequently bitten (contributing most blood meals), frequently bitten by infected mosquito (mosquito head infection) and most infected (high mosquito abdomen infection and human population infection). The flow of blood meals and *P. falciparum* infection from these observations is proposed below (Figure 4.8).

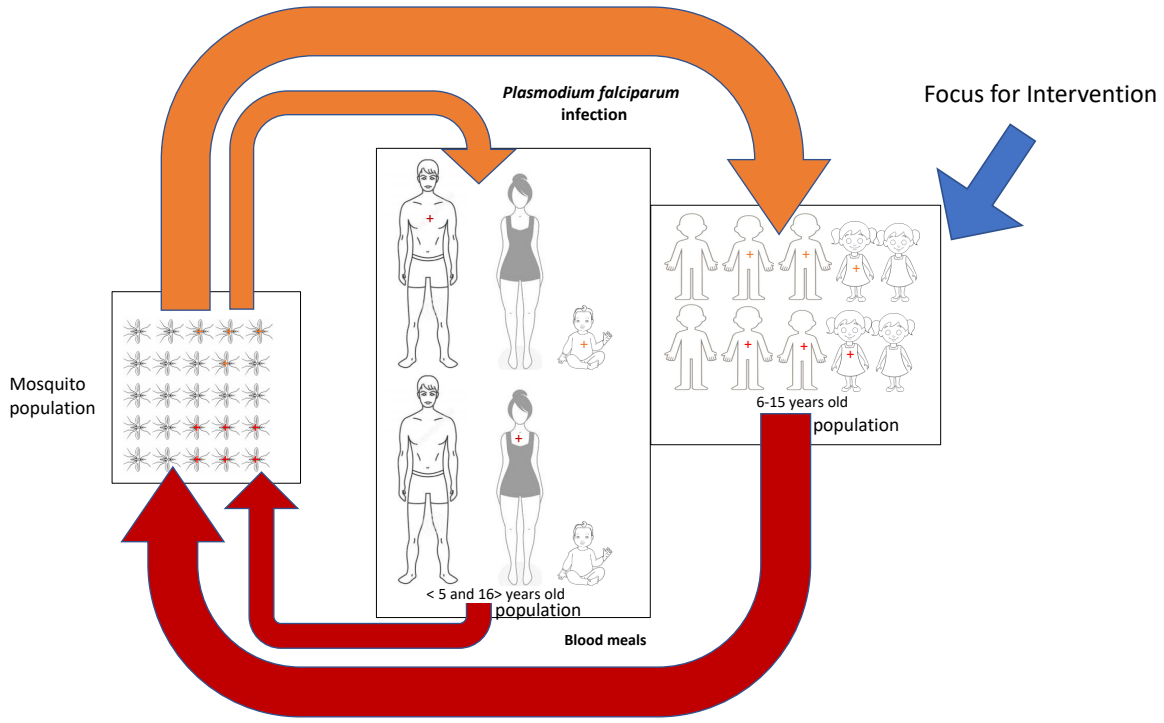

Figure 4.8. Proposed model of flow of blood meals and *P. falciparum* infection between human and mosquito populations. Age 6-15 as reservoir and contributors of *P. falciparum* to mosquitoes.

Age group 6-15 years (mostly males) contribute more blood meal and *P. falciparum* infections to the mosquitoes, they also receive mosquito bites and *P. falciparum* infections from mosquitoes. They are both reservoirs of infection and drivers of infection into the mosquitoes. Age group ≤ 5 and ≥16years old contributes less blood meals and few infections to the mosquitoes, while receiving fewer bites and *P. falciparum* infection from mosquitoes. This situation could lead to persistent residual malaria affecting even the vulnerable populations (young children and pregnant mothers). An increase in interventions focusing and incorporating more of school age children will as a result drastically reduce transmission in the vulnerable population.

4.6. Conclusion

Mosquito population in the study area exhibit non-random human selection where school age (6-15 years old) children (largely males) were more frequently bitten than other population groups. The availability of multiple blood meals across ages and sex increases the transmission potential from human to mosquitoes and vice versa. Why some household receive more mosquito bites than others is an observation that needs further exploration. Biases in biting different population groups based on age and sex by mosquito species was not firmly established in the study because the outcome variable was blood meal analysis, not empirically measured biting rates. Whether biting rate equates to blood meal analysis cannot be readily confirmed; for example, one could not ethically conduct mosquito biting studies on children or malaria-infected people.

The probabilities of being bitten according to age and sex was high in school age children, with high prevalence of infection. Males of this age group (5-16 years old) have been incriminated as the drivers of *Plasmodium* infection from humans to mosquitoes. This conclusion is crucial in malaria control and prevention that deliberately target males of this age group. The current control strategies seem to be leaving out this crucial population group. We therefore propose deliberate effort to increase bed net access and usage in this group as a starting point in reducing the human to mosquito malaria transmission.

APPENDICES

APPENDIX 4.1. OUTPUT OF ALLELIC SIZE VISUALIZATION.

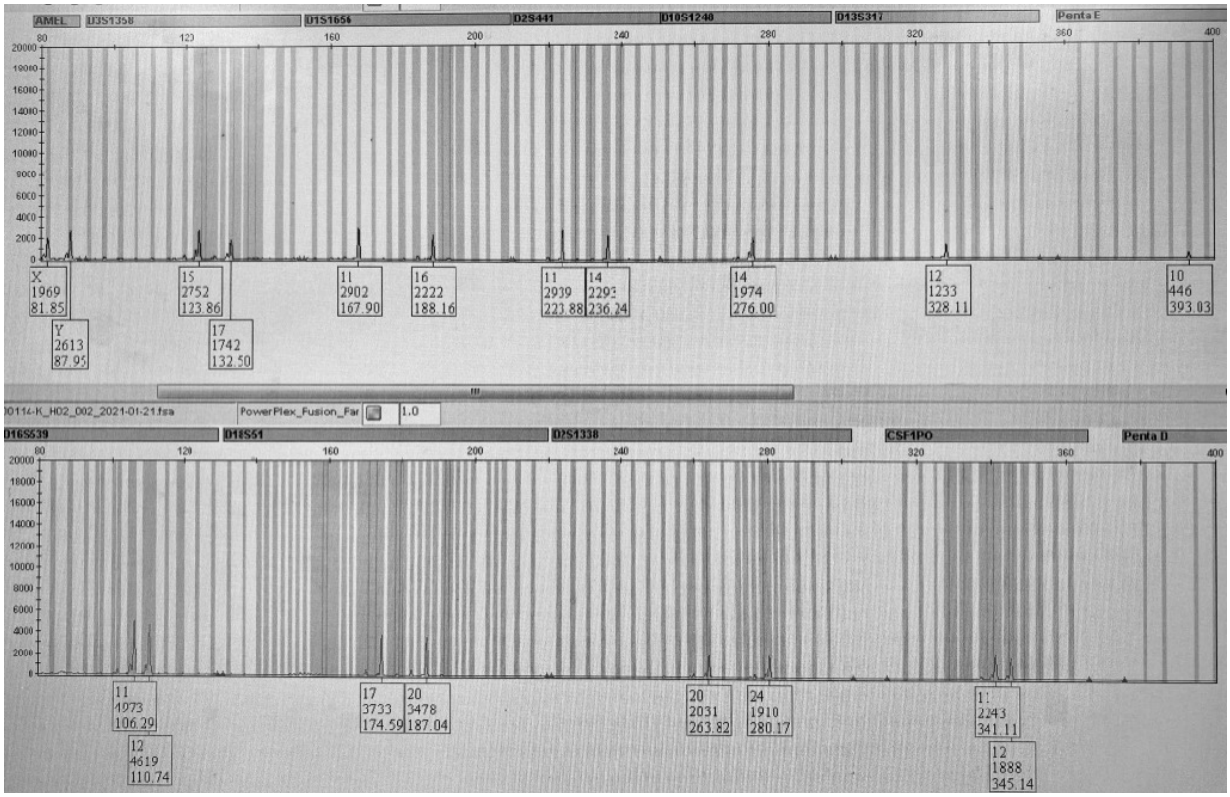

Figure 4.9. Example of output for visualization of allelic sizes on each loci for creating genetic profiles of each individual sample.

APPENDEX 4.2. EXAMPLE OF GENETIC PROFILES GENERATED FROM SAMPLES

Table 4.2. Example of profiles generated from human blood spot samples.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE
1	site	Individual_ID	DOB	AGE	Relationship	human_blood	P_falciparum	SEX	D3S1358	D1S1656	D2S441	D10S1248	D13S317	Penta E	D16S539	D18S51	D2S1338	CSF1PO	Penta D	TH01	vWA	D21S11	D7S820	D5S818	TPOX	DYS391	D8S1179	D12S391	D19S433	FGA	D22S104
48	Namanolo	MW1HC1022002	7/28/01	18	Son/Daughter	positive	positive	F	16,17	13,16	11,14	13,15	11,12	0,0	11,11	17,17	21,22	10,11	12,12	6,6	15,17	30,30	8,10	9,12	9,11	10,10	16,16	16,18	13,14	21,24	11,12,14
49	Namanolo	MW1HC1022005	1/9/08	12	Son/Daughter	positive	negative	F	16,17	13,16	11,14	13,14	11,12	10,13	11,12	16,17	20,21	10,12	10,12	7,7	15,17	29,30	10,10	9,12	9,11	0,0	14,16	18,18	11,12.2	23,24	14,17
50	Namanolo	MW1HC1022006	6/19/10	9	Son/Daughter	positive	positive	M	16,17	13,16	10,12	13,14	11,11	8,10	11,12	17,17	21,22	10,11	7,7	7,7	15,17	28,30	8,10	9,12	9,9	10,10	14,14	16,18	12.2,13	23,24	11,11
51	Namanolo	MW1HC1022008	5/18/14	5	Son/Daughter	positive	negative	F	16,16	14,16	10,12	13,15	11,11	10,10	11,12	16,16	21,22	10,11	10,12	7,7	15,16	28,30	8,10	9,12	9,9	0,0	14,16	18,24	11,12.2	21,24	14,17
52	Namanolo	MW1HC1022011	7/1/06	13	Other Relative	positive	negative	M	15,16	14,15	9,11.3	15,15	0,0	0,0	10,13	16,20	16,21	12,12	10,12	7,7	14,18	28,28	11,11	9,13	6,9	0,0	14,14	18,18	12,14.2	24,24	17,17
53	Namanolo	NAAGK1010001	4/19/76	43	Head	positive	negative	M	16,16	12,12	12,13	12,16	11,12	0,0	8,11	17,21.2	16,18	12,12	2.2,10	7,7	14,15	27,28	11,12	11,15	6,10	10,10	13,15	18,19	13,13.2	23,25	17,17
54	Namanolo	NAAGK1010002	3/17/83	37	Spouse	positive	negative	F	14,16	12,13	14,14	14,14	11,12	13,13	9,11	14,17	18,19	12,12	2.2,8	6,8	14,18	31.2,31.	9,12	12,13	9,10	0,0	12,15	18,20	11,13	19,24	17,17
55	Namanolo	NAAGK1010004	3/3/01	19	Son/Daughter	positive	negative	M	14,16	12,12	13,14	14,16	11,12	12,13	8,11	14,17	16,19	12,12	2.2,2.2	6,7	15,18	28,31.2	12,12	12,15	9,10	10,10	12,15	18,20	13,13	23,24	17,17
56	Namanolo	NAAGK1010005	3/7/07	13	Son/Daughter	positive	negative	F	16,16	12,13	12,14	14,16	11,12	13,15	8,11	14,17	16,19	12,12	8,10	7,8	14,18	28,31.2	11,12	11,13	6,9	0,0	15,15	19,20	13,13	23,24	17,17
57	Namanolo	NAAGK1010006	3/27/10	10	Son/Daughter	positive	negative	F	14,16	12,13	13,14	12,14	11,12	12,13	8,11	17,17	18,18	12,12	2.2,10	7,8	14,18	27,31.2	9,12	11,12	9,10	0,0	15,15	19,20	11,13.2	23,24	17,17
58	Namanolo	NAATC1013004	6/15/04	15	Son/Daughter	positive	negative	M	16,16	11,14	11,14	14,14	12,13	5,8	11,12	13,18	16,22	12,14	9,9	7,9	14,15	27,28	10,11	12,12	9,11	11,11	14,16	17,19	13,14	21,23	16,17
59	Namanolo	NAATC1013005	6/15/04	15	Son/Daughter	positive	positive	M	16,16	11,15	12,14	11,14	12,13	0,0	10,13	17,18,19	22,24	7,7	11,11	7,9	14,18	28,31.2	8,10	11,13	8,8	10,10	11,12	15,18	14,15	23,25	16,16
60	Namanolo	NAATC1013006	6/15/05	14	Son/Daughter	positive	negative	F	16,16	12,16	11,14	14,14	12,12	0,0	11,11	18,18	22,22	0,0	0,0	0,0	0,0	27,28	0,0	11,11	9,9	0,0	0,0	19,19	13,13	21,21	0,0
61	Namanolo	NAATC1013008	6/15/10	9	Son/Daughter	positive	negative	F	16,16	12,16	11,14	14,15	12,13	5,8	11,12	18,18	16,22	12,14	9,10	7,9	14,15	27,28	9,10	10,11	9,11	0,0	11,16	17,19	14,15	21,23	16,17
62	Ntaja	KAMCM5011003	10/26/10	9	Grandchild	positive	negative	M	15,15	12,14	11,14	13,15	11,11	8,14	9,10	17,21	18,23	11,11	9,11	7,8	16,19	31,31.2	10,12	11,12	11,11	10,10	11,15	16,18	13,14	22,24	10,10
63	Ntaja	KAMCM5011003	10/26/10	9	Grandchild	positive	negative	M	15,15	12,14	11,14	13,15	11,11	8,14	9,10	17,21	18,23	11,11	9,11	7,8	16,19	31,31.2	12,12	11,12	11,11	10,10	11,15	16,18	13,14	22,24	10,10
64	Ntaja	KAMCM5013001	1/15/75	45	Head	positive	negative	M	15,16	12,14	12,14	13,13	11,11	7,7	9,11	15,21.2	19,21	11,12	11,15	8,8	15,18	28,28	9,10	12,13	11,11	10,10	12,14	16,21	11,14	19,25	17,17
65	Ntaja	KAMCM5013002	12/7/80	39	Spouse	positive	negative	F	15,17	16,16	11,13	13,14	10,12	12,15	10,12	17,17	23,24	12,12	9,10	6,8	15,15	27,29	9,10	11,12	8,9	0,0	14,14	16,20	12.2,14.2	21,26	15,15
66	Ntaja	KAMCM5013003	1/11/97	23	Son/Daughter	positive	negative	M	15,16	13,16	11,12	14,14	10,12	8,15	11,12	15,17	17,23	11,12	9,10	6,7	15,18	29,34	9,9	11,11	9,10	10,10	14,14	18,20	13,14.2	24,26	15,15
67	Ntaja	KAMCM5013004	6/12/02	17	Son/Daughter	positive	negative	F	15,16	16,16.3	11,13	14,14	11,12	5,12	10,11	17,21.2	22,23	11,12	2.2,9	8,11	15,16	28,29	9,10	11,11	6,9	0,0	14,14	20,24	12.2,14.2	21,25	11,15
68	Ntaja	KAMCM5013005	11/8/04	15	Son/Daughter	positive	negative	F	16,17	16,16	11,12	14,16	10,12	13,15	11,12	12,17	24,26	11,12	9,9	6,7	15,18	27,28	10,10	10,12	8,8	0,0	14,15	19,20	12.2,13	23,26	15,17
69	Ntaja	KAMCM5013006	10/8/04	15	Son/Daughter	positive	negative	F	16,17	14,16	11,12	14,14	10,11	7,15	10,11	15,17	21,23	11,12	9,11	6,8	15,15	27,28	9,10	11,12	8,11	0,0	12,14	20,21	11,12.2	19,26	15,17
70	Ntaja	KAMCM5013007	11/7/11	8	Son/Daughter	positive	negative	M	15,15	15,16	11,11	13,16	10,11	8,15	9,10	17,18	19,21,24,2	11,12	12,12	6,8	15,15	27,28	9,11	12,12	8,9	10,10	14,15	16,19	13,14.2	23,26	15,16
71	Ntaja	KAMHE1017002	8/11/90	29	Spouse	positive	negative	F	15,16	14,14	13,14	12,14	12,12	9,9	9,11	15,18	19,21	10,10	0,0	7,7	15,15	27,31.2	9,9	11,11	8,11	0,0	13,14	17,20	11,14	21,25	11,11
72	Ntaja	KAMHE1017003	2/3/10	10	Son/Daughter	positive	positive	F	14,14	12,14	11,11	12,13	12,12	16,16	10,11	17,19	19,23	10,12	11,12	6,9	14,14	30,31.2	8,10	10,13	8,8	0,0	13,14	18,18	14,15	23,23	14,17
73	Ntaja	KAMHE1017004	10/10/10	9	Son/Daughter	positive	positive	M	15,16	12,12	11,13	12,12	13,13	7,7	9,9	17,18	19,21	11,12	10,11	7,8	14,18	27,28	8,9	10,11	11,11	0,0	13,14	19,20	14,14	21,26	10,15
74	Ntaja	KAMHE1017007	3/11/18	2	Son/Daughter	positive	negative	M	16,16	14,14	14,14	12,14	0,0	8,8	11,12	16,16	19,19	10,11	13,13	9,9	14,18	31.2,32.	8,8	11,11	8,9	10,10	15,15	18,20	13,14	24,24	10,15
75	Ntaja	KAMHE1018002	1/1/50	70	Spouse	positive	positive	F	15,18	14,15	13,14	16,16	10,12	14,16	11,13	17,18	19,25	12,12	2.2,13	8,8	16,18	31.2,32.	9,13	13,13	8,11	0,0	12,13	17,18	14.2,18.2	23,26	11,17
76	Ntaja	KAMHE1018003	8/1/94	25	Son/Daughter	positive	negative	M	18,18	14,15	11,11	15,16	11,12	0,0	12,13	15,17	19,25	12,12	0,0	9,9	15,16	0,0	9,9	11,13	9,11	10,10	13,15	17,19	13.2,18.2	22,23	17,17
77	Ntaja	KAMHE1018004	8/6/00	19	Son/Daughter	positive	negative	F	15,18	14,15	11,14	15,16	11,12	8,16	12,13	16,17	19,25	12,12	10,13	8,8	16,16	30,32.2	10,13	10,13	8,9	0,0	12,15	18,18	14.2,14.2	22,26	11,17
78	Ntaja	KAMRI1014001	3/13/78	42	Head	positive	negative	F	15,17	11,11	11,14	13,14	11,11	12,12	10,11	19,20	19,25	0,0	0,0	7,7	11,11	27,27	8,8	12,12	8,8	9,9	12,16	17,18	13.2,17.2	30.2,30.2	10,10
79	Ntaja	KAMRI1014002	2/3/85	35	Spouse	positive	negative	F	15,15	15,16	11,13	14,14	10,12	7,14	12,12	15,17	19,24	8,11	9,10	6,8	15,15	27,29	10,11	11,12	8,8	0,0	14,15	16,20	14,14.2	21,26	15,16
80	Ntaja	KAMRI1014004	7/7/10	9	Son/Daughter	positive	negative	F	15,17	16,16	11,11	0,0	12,12	12,14	11,12	17,17	19,25	8,12	10,11	7,7	11,11	27,29	8,11	12,12	8,8	0,0	12,12	18,18	13.2,14.2	26,30.2	10,10
81	Ntaja	KAMJJ1010006	7/20/14	5	Son/Daughter	positive	negative	F	15,15	13,14	12,14	14,15	10,11	0,0	11,11	16,16	19,19	10,12	9,10	7,9	15,18	28,29	10,10	9,9	8,11	0,0	14,15	18,19	12,13	23,23	0,0
82	Ntaja	KAMJJ1010007	2/20/18	2	Son/Daughter	positive	negative	F	16,16	11,18.3	14,14	12,13	11,11	8,8	11,11	16,17	19,24	10,12	9,9	8,9	15,18	29,29	8,12	8,8	8,8	10,10	13,14	18,18	12,13	24,24	10,10
83	Ntaja	KAMJJ1011002	12/2/99	20	Son/Daughter	positive	negative	F	15,16	11,15	12,14	13,13	11,13	12,12	9,13	15,16	19,26	12,13	2.2,10	7,8	14,18	29,36	9,11	11,12	8,8	0,0	12,13	18,19	13,13	19,21	15,16
84	Ntaja	KAMJJ1011003	1/15/01	19	Son/Daughter	positive	negative	F	15,16	11,15	11,12	13,14	12,13	12,12	9,12	13,15	19,26	12,12	2.2,2.2	7,8	14,16	30,35	9,11	12,13	8,11	0,0	13,14	18,19	12,13	20,22	15,16
85	Ntaja	KAMJJ1011004	8/7/15	4	Grandchild	positive	negative	M	15,16	15,15	0,0	14,14	11,11	12,12	11,11	15,15	0,0	11,11	5,10	7,9	17,17	27,27	12,12	0,0	0,0	11,11	13,13	18,19	13,13	21,26	15,15
86	Ntaja	KAMJJ1013002	6/15/00	19	Spouse	positive	negative	F	15,16	14,16	11,14	11,14	13,14	0,0	11,13	17,21	19,22	11,12	11,12.2	7,8	15,16	28,30	9,10	12,12	8,8	0,0	14,14	15,18	14,14	22,23	11,17
87	Ntaja	KAMJJ1013003	9/24/17	2	Son/Daughter	positive	negative	F	15,15	14,16.3	12,14	11,14	12,13	10,10	10,11	16,21	21,22	10,12	9,12.2	8,8	15,16	27,30	10,12	12,12	9,11	0,0	14,14	15,18	13,14	22,22	11,11
88	Ntaja	KAMMG1016002	6/24/83	36	Spouse	positive	negative	F	17,17	16,16	0,0	13,13	0,0	0,0	0,0	15,19	24,24	10,10	2.2,2.2	6,7	15,16	28,29	9,11	11,11	8,9	0,0	12,12	22,22	13,14	19,22	17,17

Table 4.3. Example of profiles generated from mosquito blood meal samples.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD
1	Site	mosquito_tube_id	Blood_meal_status	*Pf*_Head	*Pf*_Abdomen	SpeciesID	SEX	D3S1358	D1S1656	D2S441	D10S1248	D13S317	Penta E	D16S539	D18S51	D2S1338	CSF1PO	Penta D	TH01	vWA	D21S11	D7S820	D5S818	TPOX	DYS391	D8S1179	D12S391	D19S433	FGA	D22S104
91	Namanolo	MT205090-1	human	negative	negative	other	XY	16,17	16,16	11,12	12,15	11,12	10,15	12,13	17,18	21,25	11,12	7,11	7,7	18,19	35,36,37	10,12	12,13	8,11	10,10	13,15	17,22	12,13	22,24	16,17
92	Namanolo	MT204822-Y	human	negative	negative	An. funestus	XY	16,16	16,16.3	11,14	13,14	11,12	0,0	13,13	12,18	19,21	9,11,12	8,11	8,9.3	15,18	31.2,31.2	9,10	12,14	9,11	0,0	14,15	18,20	12,15	24,24	17,17
93	Namanolo	MT204893-F	human	negative	negative	other	XY	16,17	16,16	11,12	12,15	11,12	10,15	12,13	17,18	21,25	11,12	7,11	7,7	18,19	35,37	10,12	12,13	8,11	10,10	13,15	17,22	12,13	22,24	16,17
94	Namanolo	MT204892-D	human	negative	negative	An. arabiensis	XY	16,17	16,16	11,12	12,15	11,12	10,15	12,13	17,18	21,25	11,12	7,11	7,7	18,19	35,37	10,12	12,13	8,11	10,10	13,15	17,22	12,13	22,24	16,17
95	Namanolo	MT204890-5	human	negative	negative	An. arabiensis	XY	16,16	16,16.3	11,14	13,14	11,12	9,12	13,13	12,18	19,21	11,12	8,11	8,9.3	15,18	31.2,31.2	9,10	12,14	9,11	10,10	14,15	18,20	12,15	24,24	15,17
96	Namanolo	MT204891-I	human	negative	negative	An. arabiensis	XY	16,17	16,16	11,12	12,15	11,12	10,15	12,13	17,18	21,25	11,12	7,11	7,7	18,19	35,37	10,12	12,13	8,11	10,10	13,15	17,22	12,13	22,24	16,17
97	Namanolo	MT209991-J	human	negative	negative	An. arabiensis	XY	16,16	16,16.3	11,14	13,14	11,12	9,12	13,13	12,18	19,21	11,12	8,11	8,9.3	15,18	31.2,31.2	9,10	12,14	9,11	10,10	14,15	18,20	12,15	24,24	15,17
98	Namanolo	MT209990-0	human	negative	negative	An. arabiensis	XX	15,16	15,16	11,13	13,14	11,12,13	7,9	9,11,12	14,16,17	19,19	9,11,12	3.2,9	7,9	15,17,19	28,32	8,10,11	8,12	6,10,11	0,0	12,16	15,17	13,15	22,24,26	11,15
99	Namanolo	MT206202-P	human/goat	negative	negative	An. funestus	XX	15,15	16,16	11,12	14,15	11,12	8,12	10,13	12,18	19,21	10,12	11,13	6,7	18,20	27,31.2	9,10	10,12	8,10,11	0,0	12,13	19,22	13.2,14.2	19,24	17,17
100	Namanolo	MT201028-J	human	negative	negative	An. gambiae	XX	14,15	16,16.3	11,12	13,14	11,12	5,9	9,12	15,16	21,24	10,12	8,11	7,8	16,18	31.2,34	11,11	13,14	8,11	0,0	15,15	15,22	15,15.2	22,24	15,17
101	Namanolo	MT201324-5	human	negative	negative	An. arabiensis	XY	14,17	14,16.3	10,12	13,13	11,11	9,11	9,12	16,17	23,24	10,10	3.2,8	7,8	16,18	28,31.2	11,12	12,13	8,11	10,10	14,15	18,22	13,15.2	22,24	11,17
102	Namanolo	MT201776-8	human	negative	negative	An. funestus	XY	15,17	15,16	10,11	13,13	11,11	0,0	11,12	15,16,17	24,24	9,10	8,8	7,7	16,18	27,27	11,11	13,13	11,11	0,0	14,16	18,22	13,15.2	24,24	0,0
103	Namanolo	MT204832-5	human	negative	negative	An. funestus	XY	15,15	11,14	11,13	0,0	11,11	0,0	10,11	16,17	20,20	9,9	0,0	6,8	16,16	28,30.2	11,11	12,12	8,8	0,0	13,13	16,23	12,12.2	19,25	0,0
104	Namanolo	MT206227-8	human	negative	negative	An. arabiensis	XY	16,17	16,18	11,11	12,15	11,12	9,12	9,13	18,19,20	21,25	10,11	11,11	7,8	15,18	31,31.2	10,12	11,12	10,10	10,10	14,15	16,22	12,12	23,25	11,11
105	Namanolo	MT206225-U	human	negative	positive	An. arabiensis	XX	15,16	16,16.3	11,14	14,15	11,12	9,10	12,13	16,18	21,24	10,11	2.2,11	7,9.3	16,18	31.2,37	11,12	12,13	10,11	0,0	14,15	17,22	12,13	24,25	15,16
106	Namanolo	MT206226-F	human	negative	negative	An. arabiensis	XX	15,16	16,16.3	11,14	14,15	11,12	9,10	12,13	16,18	21,24	10,11	2.2,11	7,9.3	16,18	31.2,37	11,12	12,13	10,11	0,0	14,15	17,22	12,13	24,25	15,16
107	Ntaja	MT200101-R	human	negative	negative	An. funestus	XX	15,16,17	13,14,16	11,12	14,14	10,11	7,8	10,11	15,17,20	17,21,23	9.1,11,12	9,11	6,7,8	15,15	27,28	9,10	11,12	8,9,10,11	10,10	11,12,14	20,21	11,12.2	19,26	15,17
108	Ntaja	MT200143-Y	human	negative	negative	An. gambiae	XX	9,9	16,16	11,13	0,0	0,0	0,0	4,10	17,17	23,24	0,0	0,0	6,8	15,15	29,29	0,0	0,0	8,8	0,0	14,14	16,20	12.2,14.2	26,26	0,0
109	Ntaja	MT202913-M	human	negative	negative	An. funestus	XY	16,18	11,15	10,13	14,14	13,13	0,0	8,11	16,20	19,26	9,10	2.2,5	7,9	15,19	30,31	10,10	10,13	6,11	10,10	13,15	18,20	13,14	23,26	0,0
110	Ntaja	MT200891-O	human	negative	negative	An. funestus	XX	16,17	14,16	11,12	14,14	10,11	7,7	10,11	15,17	21,23	11,12	9,11	6,8	15,15	27,28	9,10	11,12	8,11	0,0	12,14	20,21	11,12.2	19,26	17,17
111	Ntaja	MT203009-A	non	negative	negative	An. funestus	XY	15,16	13,16	11,12	14,14	0,0	0,0	11,12	15,17	17,23	11,11	9,10	6,7	15,18	29,34	9,9	11,11	10,10	10,10	14,14	17,18,20,2	13,14.2	24,26	15,15
112	Ntaja	MT200109-C	human	negative	positive	other	XX	12,16	14,14	11,13	12,14	11,12	0,0	9,9	10.2,18	16,17	12,12	2.2,11	7,7	16,18	27,28	8,11	8,11	11,11	0,0	14,15	17,20	11,11	24,25	15,15
113	Ntaja	MT200237-P	human	negative	negative	An. gambiae	XX	12,16	14,14	11,13	12,14	11,12	12,16	9,9	10.2,18	16,17	12,12	2.2,11	7,7	16,18	27,28	8,11	8,11	11,11	0,0	14,15	17,20	11,11	24,25	15,15
114	Ntaja	MT200238-9	human	negative	negative	An. funestus	XX	12,14,17	12,12	11,11	13,13	12,12	0,0	9,10,11	17,19	0,0	12,12	11,12	6,9	14,14	30,31.2	8,8	0,0	8,8	0,0	13,14	18,18	14,15	0,0	17,17
115	Ntaja	MT200239-L	human	negative	positive	An. funestus	XX	12,15,16	14,14	11,13	12,14	11,12,13	9,12,16	9,9	11,12,16	9,9	10.2,18	2.2,10,11	7,7	14,16,18	27,28	8,11	8,10,11	11,11	10,10	13,14,15	17,19,20	11,11	21,24,25	15,15
116	Ntaja	MT200241-R	human	negative	negative	An. funestus	XX	12,16	14,14	11,13	12,14	11,12	12,16	9,9	10.2,18	16,17	12,12	2.2,11	7,7	16,18	27,28	8,11	8,11	11,11	0,0	14,15	17,20	11,11	24,25	15,15
117	Ntaja	MT200242-I	human	negative	positive	other	XX	12,16	14,14	11,13	12,14	11,2	12,16	9,9	10.2,18	16,17	12,12	2.2,11	7,7	16,18	27,28	8,11	8,11	11,11	0,0	14,15	17,20	11,11	24,25	15,15
118	Ntaja	MT200332-M	human	negative	negative	An. funestus	XX	15,16	14,14	14,14	12,12	11,12	0,0	11,12	16,18	16,21	12,12	7,11	6,7	15,21	27,27	8,9	11,11	8,11	0,0	13,15	17,20	11,14	21,21	15,15
119	Ntaja	MT202957-2	human	negative	negative	An. funestus	XY	15,16	14,16	13,14	13,17	10,13	10,16	9,13	17,19	22,24	7,10	2.2,11	9,9	15,17	28,32	10,10	12,12	9,9	11,11	13,13	16,18	13,14	24,26	10,15
120	Ntaja	MT203002-F	human	negative	positive	An. funestus	XY	15,16	12,14	11,13	12,14	12,13	7,9	9,11	17,18	19,21	11,12	10,11	7,8	14,18	27,28	8,9	10,11	9,11	10,10	13,14	19,20	14,15	21,26	10,15
121	Ntaja	MT203001-G	human	positive	positive	An. gambiae	XY	15,16	12,14	11,13	12,14	12,13	7,9	9,11	17,18	19,21	11,12	10,11	7,8	14,18	27,28	8,9	10,11	9,11	10,10	13,14	19,20	14,15	21,26	10,15
122	Ntaja	MT203000-T	human	positive	positive	An. gambiae	XX	12,16	14,14	11,13	12,14	11,12	12,16	9,9	10.2,18	16,17	12,12	2.2,11	7,7	16,18	27,28	8,11	8,11	11,11	0,0	14,15	17,20	11,11	24,25	15,15
123	Ntaja	MT202999-6	human	negative	positive	An. funestus	XY	15,16	12,14	11,13	12,14	12,13	7,9	9,11	17,18	19,21	11,12	10,11	7,8	14,18	27,28	8,9	10,11	9,11	10,10	13,14	19,20	11,14,15	21,26	10,15
124	Ntaja	MT202998-2	human	negative	positive	An. gambiae	XX	12,15,16	12,14	11,13	12,14	11,12,13	12,12	9,11	10.2,,17,18	16,17,19,2	11,13	2.2,10,11	7,7	14,16,18	27,28	8,9,11	8,10,11	9,11	0,0	13,14,15	17,19,20	11,15	21,24,25,2	11,15
125	Ntaja	MT202997-G	human	negative	positive	An. funestus	XY	14,15,16,1	12,14	11,13	12,13,14	12,12	7,9,16	9,10,11	17,18,19	19,21	10,11,12	10,11,12	6,7,8,9	14,18	27,28,30,3	8,9,10	10,11,13	8,9,11	10,10	13,14	18,19,20	14,15	21,23,25,2	10,14,1
126	Ntaja	MT202996-E	human	negative	positive	An. funestus	XY	15,16	12,14	11,13	12,14	12,13	7,9	9,11	17,18	19,21	11,12	10,11	7,8	14,18	27,28	8,9	10,11	9,11	0,0	13,14	19,20	14,15	21,26	10,15
127	Ntaja	MT202995-V	human	positive	positive	An. arabiensis	XY	15,16	12,14	12,13	12,14	12,13	7,9	9,11	17,18	19,21	11,12	10,11	7,8	14,18	27,28	8,9	10,11	9,11	10,10	13,14	19,20	14,15	21,26	10,15
128	Ntaja	MT202994-A	human	negative	positive	An. funestus	XY	15,16	12,14	11,13	12,14	12,13	7,9	9,11	17,18	19,21	11,12	10,11	7,8	14,18	27,28	8,9	10,11	9,11	10,10	13,14	19,20	14,15	21,26	10,15
129	Ntaja	MT202993-R	human/goat	positive	positive	An. funestus	XY	15,16	12,14	11,13	12,14	12,13	7,9	9,11	17,18	19,21	10,12	10,11	7,8	14,18	27,28	8,9	10,11	9,11	10,10	13,14	19,20	14,15	21,26	10,15
130	Ntaja	MT202992-2	human	positive	positive	An. funestus	XY	15,16	12,14	11,13	12,14	12,13	7,9	9,11	17,18	19,21	11,12	10,11	7,8	14,18	27,28	8,9	10,11	9,11	10,10	13,14	19,20	14,15	21,26	10,15

Multiple feeding

Allele dropout

CHAPTER 5 : GENERAL CONCLUSION

Chapters 2 to 4 make several conclusions which were arrived at with inferences made after detailed evaluation of the data. In this final chapter the focus is on summarizing the main conclusions to cohesively address the overarching hypothesis of this dissertation. The hypothesis was that biases in mosquito human feeding could be driving malaria infection transmission in Malawi and that certain population groups are drivers of malaria infection into the mosquito population. In Chapter 2, the results showed *Anopheles* species composition, distribution and abundance in the study areas and their role in malaria transmission. Five *anopheles* mosquito species identified were *An. arabiensis, An. funestus*, *An. gambiae, An. parensis* and *An. vaneedeni* but *An. parensis* and *An. vaneedeni* were least in abundance. The infection rates in *An. arabiensis*, *An. funestus* and *An. gambiae* showed that these three species continue to pose threat of malaria transmission as main malaria vectors. The high infection rates in head-thorax and abdomen of mosquitoes point to a potential situation of residual malaria transmission. Potential secondary vectors maybe present in the study area and needs more investigation.

In chapter 3 the results showed that three malaria vector mosquito species were utilizing blood meals from both human and non-human hosts and multiple hosts utilization was detected. The majority of the blood meals were mostly from humans, resulting in a high human blood index. Both HBI and EIR were considerably high suggesting active malaria transmissions. The availability of mixed blood meals shows the adaptability of the vectors to switch hosts, obtaining a full bloodmeal and potentially circumventing malaria control interventions. Finally, Chapter 4 revealed that mosquito feeding exhibited non-random human selection for blood meal source and multiple human sources (10%) of blood meal in a single mosquito exists. There were more

mosquito bites in certain individuals than others with males of the school going age group (6-15 years old) being the most frequently bitten (av. 47%) and had highest malaria infection. The results suggest that males of 6-15 years old are the main drivers of malaria infection from human to mosquitoes.

In summary, of the five *anopheles* species identified in the study, *An. arabiensis*, *An. funestus* and *An. gambiae* continues to be dominant species posing threat of malaria transmission in Malawi. Presence of other *Anopheles* species in indoor collections points to potential existence of secondary malaria vectors which needs further investigation.

In the human population, 6-15-years old are the most malaria infected group with males showing highest infection rates. This conclusion was confirmed by the blood meal and genotyping analysis approaches which found that mosquitoes utilize blood meals from both human and non-human hosts. Of the human population, age group 6-15 years old was the most frequently bitten category providing mosquito blood meal. Since the same age group was found to be having higher malaria infection rates, it stands to reason that this particular age group could be potential main drivers of malaria infection from human to mosquitoes. This study dissertation, therefore, incriminates males of 6-15-year-old population group as the main drivers of *Plasmodium* infection from humans to mosquitoes.

These conclusions have several implications on malaria transmission in Malawi. One, for success to be realized in malaria control and prevention it is critical to put in place deliberate efforts targeting males of this particular age group (6-15 years old). Second, the availability of mixed blood meal shows the adaptability of the vectors to switch hosts for obtaining bloodmeal and therefore circumvent malaria control interventions. Multiple feeding in human populations has the potential of increasing malaria transmission and reducing the effectiveness of on doing

interventions. As way forward we propose a study intervention that targets 5-16-year-olds by giving them prophylactic drugs to reduce *Plasmodium* prevalence and providing more LLINs coverage to reduce mosquito-human contact. The outcome of the intervention would be measured by the reduction in mosquito blood meal and infection prevalence in this group after six months to a year.

APPENDIX

RECORD OF DEPOSITION OF VOUCHER SPECIMENS

The specimens listed below have been deposited in the named museum as samples of those species or other taxa, which were used in this research. Voucher recognition labels bearing the voucher number have been attached or included in fluid preserved specimens.

Voucher Number: 2021-04

Author and Title of thesis:

Rex Barnett Marregah Mbewe
Blood-meal host selection, malaria infection, and genotyping to delineate human to mosquito *plasmodium* transmission: Implication for malaria parasite infections in Malawi
Museum(s) where deposited:
Albert J. Cook Arthropod Research Collection, Michigan State University (MSU)

Specimens:

Specimens:

Family	Genus-Species	Life Stage	Quantity	Preservation
Culicidae	*Anopheles coustani*	adult	10	pinned
Culicidae	*Anopheles funestus*	adult	10	pinned
Culicidae	*Anopheles gambiae*	adult	10	pinned
Culicidae	*Anopheles maculipulpis*	adult	10	pinned
Culicidae	*Anopheles pretoriensis*	adult	10	pinned

REFERENCES

REFERENCES

Adhikari, B., Phommasone, K., Pongvongsa, T., Soundala, X., Koummarasy, P., Henriques, G., Peto, T. J., Seidlein, L. von, White, N. J., Day, N. P. J., Dondorp, A. M., Newton, P. N., Cheah, P. Y., Mayxay, M., & Pell, C. (2018). Perceptions of asymptomatic malaria infection and their implications for malaria control and elimination in Laos. *PLOS ONE*, *13*(12), e0208912. https://doi.org/10.1371/journal.pone.0208912

Adugna, T., Yewhelew, D., & Getu, E. (2021). Bloodmeal sources and feeding behavior of anopheline mosquitoes in Bure district, northwestern Ethiopia. *Parasites & Vectors*, *14*(1), 166. https://doi.org/10.1186/s13071-021-04669-7

Aho, K., & Bowyer, R. T. (2015). Confidence intervals for ratios of proportions implications for selection ratios. *Methods in Ecology and Evolution*, *6*, 121–132. https://doi.org/10.1111/2041-210X.12304

Ali, D., Brooker, S. J., Roschnik, N., Witek-McManus, S., Verney, A., Halliday, K. E., Mathanga, D. P., Jawati, M., Sande, J., Jones, R., & Bauleni, A. (2015). The High Burden of Malaria in Primary School Children in Southern Malawi. *The American Journal of Tropical Medicine and Hygiene*, *93*(4), 779–789. https://doi.org/10.4269/ajtmh.14-0618

Almeida, A. P. G., Galão, R. P., Sousa, C. A., Novo, M. T., Parreira, R., Pinto, J., Piedade, J., & Esteves, A. (2008). Potential mosquito vectors of arboviruses in Portugal: Species, distribution, abundance and West Nile infection. *Transactions of The Royal Society of Tropical Medicine and Hygiene*, *102*(8), 823–832. https://doi.org/10.1016/j.trstmh.2008.03.011

Anderson, R. A., & Brust, R. A. (1997). Interrupted Blood Feeding by Culex (Diptera: Culicidae) in Relation to Individual Host Tolerance to Mosquito Attack. *Journal of Medical Entomology*, *34*(2), 95–101. https://doi.org/10.1093/jmedent/34.2.95

Antinori, S., Galimberti, L., Milazzo, L., & Corbellino, M. (2012). Biology of Human Malaria Plasmodia including Plasmodium knowlesi. *Mediterranean Journal of Hematology and Infectious Diseases*, *4*(1), e2012013. https://doi.org/10.4084/mjhid.2012.013

Bass, C., Nikou, D., Blagborough, A. M., Vontas, J., Sinden, R. E., Williamson, M. S., & Field, L. M. (2008). PCR-based detection of Plasmodium in Anopheles mosquitoes: A comparison of a new high-throughput assay with existing methods. *Malaria Journal*, *7*, 177. https://doi.org/10.1186/1475-2875-7-177

Bayoh, M. N., Walker, E. D., Kosgei, J., Ombok, M., Olang, G. B., Githeko, A. K., Killeen, G. F., Otieno, P., Desai, M., Lobo, N. F., Vulule, J. M., Hamel, M. J., Kariuki, S., & Gimnig, J. E. (2014). Persistently high estimates of late night, indoor exposure to malaria

vectors despite high coverage of insecticide treated nets. *Parasites & Vectors*, *7*(1), 380. https://doi.org/10.1186/1756-3305-7-380

Beebe, N. W. (2018). DNA barcoding mosquitoes: Advice for potential prospectors. *Parasitology*, *145*(5), 622–633. Cambridge Core. https://doi.org/10.1017/S0031182018000343

Bennett, A., Kazembe, L., Mathanga, D. P., Kinyoki, D., Ali, D., Snow, R. W., & Noor, A. M. (2013). Mapping Malaria Transmission Intensity in Malawi, 2000–2010. *The American Journal of Tropical Medicine and Hygiene*, *89*(5), 840–849. https://doi.org/10.4269/ajtmh.13-0028

Besansky, N. (2008). *Genome Analysis Of Vectorial Capacity In Major Anopheles Vectors Of Malaria Parasites*. https://www.genome.gov/pages/research/sequencing/seqproposals/anophelesgenomesproposal_aug3.pdf

Boakye, D. A., Tang, J., Truc, P., Merriweather, A., & Unnasch, T. R. (1999). Identification of bloodmeals in haematophagous Diptera by cytochrome B heteroduplex analysis. *Medical and Veterinary Entomology*, *13*(3), 282–287. https://doi.org/10.1046/j.1365-2915.1999.00193.x

Boreham, P. F. L., & Garrett-Jones, C. (1973). *Prevalence of mixed blood meals and double feeding in a malaria vector (Anopheles sacharovi Favre)*. 10.

Borland, E. M., & Kading, R. C. (2021). Modernizing the Toolkit for Arthropod Bloodmeal Identification. *Insects*, *12*(1), 37. https://doi.org/10.3390/insects12010037

Boyd, M. F. (Mark F., 1889-1968. (1949). *Malariology; a comprehensive survey of all aspects of this group of disease from a global standpoint*. BASE. http://ezproxy.msu.edu/login?url=https://search.ebscohost.com/login.aspx?direct=true&AuthType=ip,uid,cookie&db=edsbas&AN=edsbas.4E4E08C7&site=eds-live

Brady, O. J., Godfray, H. C. J., Tatem, A. J., Gething, P. W., Cohen, J. M., McKenzie, F. E., Perkins, T. A., Reiner, R. C., Tusting, L. S., Sinka, M. E., Moyes, C. L., Eckhoff, P. A., Scott, T. W., Lindsay, S. W., Hay, S. I., & Smith, D. L. (2016). Vectorial capacity and vector control: Reconsidering sensitivity to parameters for malaria elimination. *Transactions of The Royal Society of Tropical Medicine and Hygiene*, *110*(2), 107–117. https://doi.org/10.1093/trstmh/trv113

Briggs, J., Teyssier, N., Nankabirwa, J. I., Rek, J., Jagannathan, P., Arinaitwe, E., Bousema, T., Drakeley, C., Murray, M., Crawford, E., Hathaway, N., Staedke, S. G., Smith, D., Rosenthal, P. J., Kamya, M., Dorsey, G., Rodriguez-Barraquer, I., & Greenhouse, B. (2020). Sex-based differences in clearance of chronic Plasmodium falciparum infection. *ELife*, *9*, e59872. https://doi.org/10.7554/eLife.59872

Buchwald, A. G., Coalson, J. E., Cohee, L. M., Walldorf, J. A., Chimbiya, N., Bauleni, A., Nkanaunena, K., Ngwira, A., Sorkin, J. D., Mathanga, D. P., Taylor, T. E., & Laufer, M. K. (2017). Insecticide-treated net effectiveness at preventing Plasmodium falciparum infection varies by age and season. *Malaria Journal*, *16*. https://doi.org/10.1186/s12936-017-1686-2

Buchwald, A. G., Sixpence, A., Chimenya, M., Damson, M., Sorkin, J. D., Wilson, M. L., Seydel, K., Hochman, S., Mathanga, D. P., Taylor, T. E., & Laufer, M. K. (2018). Clinical Implications of Asymptomatic Plasmodium falciparum Infections in Malawi. *Clinical Infectious Diseases*. https://doi.org/10.1093/cid/ciy427

Buchwald, A. G., Walldorf, J. A., Cohee, L. M., Coalson, J. E., Chimbiya, N., Bauleni, A., Nkanaunena, K., Ngwira, A., Kapito-Tembo, A., Mathanga, D. P., Taylor, T. E., & Laufer, M. K. (2016). Bed net use among school-aged children after a universal bed net campaign in Malawi. *Malaria Journal*, *15*(1), 127. https://doi.org/10.1186/s12936-016-1178-9

Burke, A., Dahan-Moss, Y., Duncan, F., Qwabe, B., Coetzee, M., Koekemoer, L., & Brooke, B. (2019). Anopheles parensis contributes to residual malaria transmission in South Africa. *Malaria Journal*, *18*(1), 257. https://doi.org/10.1186/s12936-019-2889-5

Burkot, T. R. (1988). Non-random host selection by anopheline mosquitoes. *Parasitol. Today*, *4*(6), 156–162. https://doi.org/10.1016/0169-4758(88)90151-2

Burkot, T. R., Graves, P. M., Paru, R., & Lagog, M. (1988). Mixed Blood Feeding by the Malaria Vectors in the Anopheles punctulatus Complex (Diptera: Culicidae). *Journal of Medical Entomology*, *25*(4), 205–213. https://doi.org/10.1093/jmedent/25.4.205

Carnevale, P., Frézil, J. L., Bosseno, M. F., Le Pont, F., & Lancien, J. (1978). [The aggressiveness of Anopheles gambiae A in relation to the age and sex of the human subjects]. *Bulletin of the World Health Organization*, *56*(1), 147–154.

Catano-Lopez, A., Rojas-Diaz, D., Laniado, H., Arboleda-Sánchez, S., Puerta-Yepes, M. E., & Lizarralde-Bejarano, D. P. (2019). An alternative model to explain the vectorial capacity using as example Aedes aegypti case in dengue transmission. *Heliyon*, *5*(10), e02577. https://doi.org/10.1016/j.heliyon.2019.e02577

CDC. (2016). *Evaluation of the Impact of Malaria Control Interventions on All-Cause Mortality in Children under Five Years of Age in Malawi* (Malawi Malaria Impact Evaluation Group). https://reliefweb.int/sites/reliefweb.int/files/resources/Malawi%20-%20Malaria%20Impact%20Evaluation%20Report.pdf

cdc.gov. (2020, July 16). *CDC - Malaria—About Malaria—Biology*. https://www.cdc.gov/malaria/about/biology/index.html

Ceccato, P., Vancutsem, C., Klaver, R., Rowland, J., & Connor, S. J. (2012). A Vectorial Capacity Product to Monitor Changing Malaria Transmission Potential in Epidemic Regions of Africa. *Journal of Tropical Medicine*, *2012*, 1–6. https://doi.org/10.1155/2012/595948

Chanda, E., Mzilahowa, T., Chipwanya, J., Mulenga, S., Ali, D., Troell, P., Dodoli, W., Govere, J. M., & Gimnig, J. (2015). Preventing malaria transmission by indoor residual spraying in Malawi: Grappling with the challenge of uncertain sustainability. *Malaria Journal*, *14*, 254. https://doi.org/10.1186/s12936-015-0759-3

Cheaveau, J., Mogollon, D. C., Mohon, M. A. N., Golassa, L., Yewhalaw, D., & Pillai, D. R. (2019). Asymptomatic malaria in the clinical and public health context. *Expert Review of Anti-Infective Therapy*, *17*(12), 997–1010. https://doi.org/10.1080/14787210.2019.1693259

Chilanga, E., Collin-Vézina, D., MacIntosh, H., Mitchell, C., & Cherney, K. (2020). Prevalence and determinants of malaria infection among children of local farmers in Central Malawi. *Malaria Journal*, *19*(1), 308. https://doi.org/10.1186/s12936-020-03382-7

Chima, R. I., Goodman, C. A., & Mills, A. (2003). The economic impact of malaria in Africa: A critical review of the evidence. *Health Policy*, *63*(1), 17–36. https://doi.org/10.1016/S0168-8510(02)00036-2

Chipeta, M. G., Giorgi, E., Mategula, D., Macharia, P. M., Ligomba, C., Munyenyembe, A., Chirombo, J., Gumbo, A., Terlouw, D. J., Snow, R. W., & Kayange, M. (2019). Geostatistical analysis of Malawi's changing malaria transmission from 2010 to 2017. *Wellcome Open Research*, *4*, 57. https://doi.org/10.12688/wellcomeopenres.15193.2

Chow-Shaffer, E., Sina, B., Hawley, W. A., De Benedictis, J., & Scott, T. W. (2000). Laboratory and Field Evaluation of Polymerase Chain Reaction-Based Forensic DNA Profiling for Use in Identification of Human Blood Meal Sources of *Aedes aegypti* (Diptera: Culicidae). *Journal of Medical Entomology*, *37*(4), 492–502. https://doi.org/10.1603/0022-2585-37.4.492

Cibulskis, R. E., Alonso, P., Aponte, J., Aregawi, M., Barrette, A., Bergeron, L., Fergus, C. A., Knox, T., Lynch, M., Patouillard, E., Schwarte, S., Stewart, S., & Williams, R. (2016). Malaria: Global progress 2000 – 2015 and future challenges. *Infectious Diseases of Poverty*, *5*(1), 61, s40249-016-0151–0158. https://doi.org/10.1186/s40249-016-0151-8

Coalson, J. (2015). *"Silent" malaria: Defining the infectious reservoirs of Plasmodium falciparum in communities of Southern Malawi*. University of Michigan.

Coalson, J. E., Cohee, L. M., Buchwald, A. G., Nyambalo, A., Kubale, J., Seydel, K. B., Mathanga, D., Taylor, T. E., Laufer, M. K., & Wilson, M. L. (2018). Simulation models predict that school-age children are responsible for most human-to-mosquito Plasmodium

falciparum transmission in southern Malawi. *Malaria Journal*, *17*. https://doi.org/10.1186/s12936-018-2295-4

Coalson, J. E., Walldorf, J. A., Cohee, L. M., Ismail, M. D., Mathanga, D., Cordy, R. J., Marti, M., Taylor, T. E., Seydel, K. B., Laufer, M. K., & Wilson, M. L. (2016). High prevalence of Plasmodium falciparum gametocyte infections in school-age children using molecular detection: Patterns and predictors of risk from a cross-sectional study in southern Malawi. *Malaria Journal*, *15*. https://doi.org/10.1186/s12936-016-1587-9

Cohee, L. M., Ingrid, P., Jenna, C. E., Valim, C., Chilombe, M., Ngwira, A., Bauleni, A., Schaffer-DeRoo, S., Wilson, M., Taylor, T. E., Mathanga, D. P., & Laufer, M. K. (2021). Impact of school-based malaria screening and treatment on P. falciparum infection and anemia prevalence in two transmission settings in Malawi. *Preprint*. https://doi.org/10.1101/2021.04.22.21253119

Cohen, J. M., Smith, D. L., Cotter, C., Ward, A., Yamey, G., Sabot, O. J., & Moonen, B. (2012). Malaria resurgence: A systematic review and assessment of its causes. *Malaria Journal*, *11*(1), 122. https://doi.org/10.1186/1475-2875-11-122

Collins, F. H., Brogdon, W. G., & Scott, J. A. (1993). Identification of Single Specimens of the Anopheles Gambiae Complex by the Polymerase Chain Reaction. *The American Journal of Tropical Medicine and Hygiene*, *49*(4), 520–529. https://doi.org/10.4269/ajtmh.1993.49.520

Collins, F. H., & Paskewitz, S. M. (1995). Malaria: Current and Future Prospects for Control. *Annual Review of Entomology*, *40*(1), 195–219. https://doi.org/10.1146/annurev.en.40.010195.001211

Collins, F. H., Sakai, R. K., Vernick, K. D., & Paskewitz, S. (1986). *Genetic selection of a Plasmodium-refractory strain of the malaria vector Anopheles gambiae. - PubMed—NCBI*. https://www.ncbi.nlm.nih.gov/pubmed/3532325

Cooper, L., Kang, S. Y., Bisanzio, D., Maxwell, K., Rodriguez-Barraquer, I., Greenhouse, B., Drakeley, C., Arinaitwe, E., G. Staedke, S., Gething, P. W., Eckhoff, P., Reiner, R. C., Hay, S. I., Dorsey, G., Kamya, M. R., Lindsay, S. W., Grenfell, B. T., & Smith, D. L. (2019). Pareto rules for malaria super-spreaders and super-spreading. *Nature Communications*, *10*(1), 3939. https://doi.org/10.1038/s41467-019-11861-y

Czeher, C., Labbo, R., Arzika, I., & Duchemin, J.-B. (2008). Evidence of increasing Leu-Phe knockdown resistance mutation in Anopheles gambiae from Niger following a nationwide long-lasting insecticide-treated nets implementation. *Malaria Journal*, *7*, 189. https://doi.org/10.1186/1475-2875-7-189

Dahan-Moss, Y., Hendershot, A., Dhoogra, M., Julius, H., Zawada, J., Kaiser, M., Lobo, N. F., Brooke, B. D., & Koekemoer, L. L. (2020). Member species of the Anopheles gambiae

complex can be misidentified as Anopheles leesoni. *Malaria Journal*, *19*(1), 89–89. PubMed. https://doi.org/10.1186/s12936-020-03168-x

Das, S., Muleba, M., Stevenson, J. C., Pringle, J. C., & Norris, D. E. (2017). Beyond the entomological inoculation rate: Characterizing multiple blood feeding behavior and Plasmodium falciparum multiplicity of infection in Anopheles mosquitoes in northern Zambia. *Parasites & Vectors*, *10*(1), 45. https://doi.org/10.1186/s13071-017-1993-z

De Moraes, C. M., Stanczyk, N. M., Betz, H. S., Pulido, H., Sim, D. G., Read, A. F., & Mescher, M. C. (2014). Malaria-induced changes in host odors enhance mosquito attraction. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(30), 11079–11084. https://doi.org/10.1073/pnas.1405617111

Dear, N. F., Kadangwe, C., Mzilahowa, T., Bauleni, A., Mathanga, D. P., Duster, C., Walker, E. D., & Wilson, M. L. (2018). Household-level and surrounding peri-domestic environmental characteristics associated with malaria vectors Anopheles arabiensis and Anopheles funestus along an urban–rural continuum in Blantyre, Malawi. *Malaria Journal*, *17*(1), 229. https://doi.org/10.1186/s12936-018-2375-5

Dechavanne, C., Sadissou, I., Bouraima, A., Ahouangninou, C., Amoussa, R., Milet, J., Moutairou, K., Massougbodji, A., Theisen, M., Remarque, E. J., Courtin, D., Nuel, G., Migot-Nabias, F., & Garcia, A. (2016). Acquisition of natural humoral immunity to P. falciparum in early life in Benin: Impact of clinical, environmental and host factors. *Scientific Reports*, *6*, 33961–33961. PubMed. https://doi.org/10.1038/srep33961

Desai, M., ter Kuile, F. O., Nosten, F., McGready, R., Asamoa, K., Brabin, B., & Newman, R. D. (2007). Epidemiology and burden of malaria in pregnancy. *The Lancet Infectious Diseases*, *7*(2), 93–104. https://doi.org/10.1016/S1473-3099(07)70021-X

Djouaka, R. F., Bakare, A. A., Coulibaly, O. N., Akogbeto, M. C., Ranson, H., Hemingway, J., & Strode, C. (2008). Expression of the cytochrome P450s, CYP6P3 and CYP6M2 are significantly elevated in multiple pyrethroid resistant populations of Anopheles gambiae s.s. From Southern Benin and Nigeria. *BMC Genomics*, *9*, 538. https://doi.org/10.1186/1471-2164-9-538

Dye, C., & Hasibeder, G. (1986). Population dynamics of mosquito-borne disease: Effects of flies which bite some people more frequently than others. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *80*(1), 69–77. https://doi.org/10.1016/0035-9203(86)90199-9

Eba, K., Habtewold, T., Yewhalaw, D., Christophides, G. K., & Duchateau, L. (2021). Anopheles arabiensis hotspots along intermittent rivers drive malaria dynamics in semi-arid areas of Central Ethiopia. *Malaria Journal*, *20*(1), 154. https://doi.org/10.1186/s12936-021-03697-z

Edalat, H., Moosa-Kazemi, S. H., Abolghasemi, E., & Khairandish, S. (2015). Vectorial capacity and Age determination of Anopheles Stephens Liston (Diptera: Culicidae), during the malaria transmission in Southern Iran. *Journal of Entomology and Zoology Studies*, 8.

Edman, J. D., Chow-Shaffer, E., Clark, G. G., De Benedictis, J., Costero, A., & Scott, T. W. (2003). Identification of the people from whom engorged Aedes aegypti took blood meals in Florida, Puerto Rico, using polymerase chain reaction-based DNA profiling. *The American Journal of Tropical Medicine and Hygiene*, *68*(4), 437–446. https://doi.org/10.4269/ajtmh.2003.68.437

Escobar, D., Ascencio, K., Ortiz, A., Palma, A., Sánchez, A., & Fontecha, G. (2020). Blood Meal Sources of Anopheles spp. In Malaria Endemic Areas of Honduras. *Insects*, *11*(7), 450. https://doi.org/10.3390/insects11070450

Feachem, R. G., Phillips, A. A., Hwang, J., Cotter, C., Wielgosz, B., Greenwood, B. M., Sabot, O., Rodriguez, M. H., Abeyasinghe, R. R., Ghebreyesus, T. A., & Snow, R. W. (2010). Shrinking the malaria map: Progress and prospects. *The Lancet*, *376*(9752), 1566–1578. https://doi.org/10.1016/S0140-6736(10)61270-6

Findlay, I., Matthews, P., & Quirke, P. (1998). Multiple genetic diagnoses from single cells using multiplex PCR: reliability and allele dropout. *Prenatal Diagnosis*, *18*(13), 1413–1421. https://doi.org/1https://doi.org/10.1002/(SICI)1097-0223(199812)18:13<1413::AID-PD496>3.0.CO;2-1

Fontenile, D., & Lochouarn, L. (1999). The compex of the malaria vectorial system in Africa. *Parassitologia*, *41*, 267–271.

Friedheim, S. (2016). *Comparison of Species Identification Methods DNA Barcoding versus Morphological Taxonomy*. http://manoa.hawaii.edu/undergrad/horizons/wp-content/uploads/2016/10/Friedheim-Sophie.pdf

Fritz, M. L., Miller, J. R., Bayoh, M. N., Vulule, J. M., Landgraf, J. R., & Walker, E. D. (2013). Application of a reverse dot blot DNA-DNA hydridization method to quantify host-feeding tendencies of two sibling species in the *Anopheles gambiae* complex. *Medical and Veterinary Entomology*, *27*(4), 398–407. https://doi.org/10.1111/j.1365-2915.2012.01063.x

Garrett-Jones, C. (1964). The Human Blood Index of Malaria Vectors in Relation to Epidemiological Assessment. *Bull. Wld Hlth Org.*, *30*, 241–261. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2554803/pdf/bullwho00287-0092.pdf

Garrett-Jones, C., & Grab, B. (1964). The Assessment of Insecticidal Impact on the Malaria Mosquito's Vectorial Capacity, from Data on the Proportion of Parous Females. *Bull. Wld Hlth Org.*, 16.

Ghosh, A., Jacobs-Lorena, M., & Edwards, M. J. (2000). The Journey of the Malaria Parasitein the Mosquito:Hopes for the New Century. *Parasitology Today*, *16*(5), 196–201.

Gillies, H. (1993). The malaria parasite. In *Gilles HM, Warrel DA, editors. Bruce-Chwatt's essential malariology.* (3rd ed., pp. 12–34). Edward Arnold.

Gillies, M. T., & Coetze, M. (1987). A supplement to the Anophelinae of Africa south of the Sahara (Afrotropical region). *South African institute for medical research*, 146.

Githeko, A. K., Brandling-Bennett, A. D., Beier, M., Atieli, F., Owanga, M., & Collins, F. H. (1992). The reservoir of Plasmodium Falciparum malaria in a holoendemic area of western Kenya. *Transactions of The Royal Society of Tropical Medicine and Hygiene*, *86*, 355–358.

Goldberg, T. L., Ruiz, M. O., Hamer, G. L., Brawn, J. D., Kitron, U. D., Hayes, D. B., Loss, S. R., & Walker, E. D. (2009). Host Selection by Culex pipiens Mosquitoes and West Nile Virus Amplification. *The American Journal of Tropical Medicine and Hygiene*, *80*(2), 268–278. https://doi.org/10.4269/ajtmh.2009.80.268

Gonçalves, B. P., Kapulu, M. C., Sawa, P., Guelbéogo, W. M., Tiono, A. B., Grignard, L., Stone, W., Hellewell, J., Lanke, K., Bastiaens, G. J. H., Bradley, J., Nébié, I., Ngoi, J. M., Oriango, R., Mkabili, D., Nyaurah, M., Midega, J., Wirth, D. F., Marsh, K., … Bousema, T. (2017). Examining the human infectious reservoir for Plasmodium falciparum malaria in areas of differing transmission intensity. *Nature Communications*, *8*(1), 1133. https://doi.org/10.1038/s41467-017-01270-4

Government of Malawi. (2015). *Malawi Malaria Indicator Survey 2014*. https://dhsprogram.com/pubs/pdf/MIS18/MIS18.pdf

Government of Malawi. (2018). *2018 Mass Distribution Campaign of long Lasting Insecticidal Treated Mosquito Nets in Malawi*. Government of Malawi, Ministry of Health, National Malaria Control Programme. https://files.givewell.org/files/DWDA%202009/AMF/AMF_Malawi2018_Distribution_Plan_verJan18.pdf

Griffin, J. T., Ferguson, N. M., & Ghani, A. C. (2014). Estimates of the changing age-burden of Plasmodium falciparum malaria disease in sub-Saharan Africa. *Nature Communications*, *5*(1), 3136. https://doi.org/10.1038/ncomms4136

Guelbéogo, W. M., Gonçalves, B. P., Grignard, L., Bradley, J., Serme, S. S., Hellewell, J., Lanke, K., Zongo, S., Sepúlveda, N., Soulama, I., Wangrawa, D. W., Yakob, L., Sagnon, N., Bousema, T., & Drakeley, C. (2018). Variation in natural exposure to anopheles mosquitoes and its effects on malaria transmission. *ELife*, *7*, e32625. https://doi.org/10.7554/eLife.32625

Guerra, C. A., Snow, R. W., & Hay, S. I. (2006). Mapping the global extent of malaria in 2005. *Trends in Parasitology*, *22*(8), 353–358. https://doi.org/10.1016/j.pt.2006.06.006

Hamel, M. J., Hawley, W. A., Walker, E. D., McCann, R. S., Ochomo, E., Bayoh, M. N., Vulule, J. M., & Gimnig, J. E. (2014). Reemergence of Anopheles funestus as a Vector of Plasmodium falciparum in Western Kenya after Long-Term Implementation of Insecticide-Treated Bed Nets. *The American Journal of Tropical Medicine and Hygiene*, *90*(4), 597–604. https://doi.org/10.4269/ajtmh.13-0614

Hargreaves, K., Koekemoer, L. L., Brooke, B. D., Hunt, R. H., Mthembu, J., & Coetzee, M. (2000). Anopheles funestus resistant to pyrethroid insecticides in South Africa. *Medical and Veterinary Entomology*, *14*(2), 181–189. https://doi.org/10.1046/j.1365-2915.2000.00234.x

Harrington, L. C., Fleisher, A., Ruiz-Moreno, D., Vermeylen, F., Wa, C. V., Poulson, R. L., Edman, J. D., Clark, J. M., Jones, J. W., Kitthawee, S., & Scott, T. W. (2014). Heterogeneous Feeding Patterns of the Dengue Vector, Aedes aegypti, on Individual Human Hosts in Rural Thailand. *PLoS Neglected Tropical Diseases*, *8*(8), e3048. https://doi.org/10.1371/journal.pntd.0003048

Hasibeder, G., & Dye, C. (1988). Population dynamics of mosquito-borne disease: Persistence in a completely heterogeneous environment. *Theoretical Population Biology*, *33*(1), 31–53. https://doi.org/10.1016/0040-5809(88)90003-2

Hay, S. I., Guerra, C. A., Tatem, A. J., Noor, A. M., & Snow, R. W. (2004). The global distribution and population at risk of malaria: Past, present, and future. *The Lancet Infectious Diseases*, *4*(6), 327–336. https://doi.org/10.1016/S1473-3099(04)01043-6

Https://blast.ncbi.nlm.nih.gov/Blast.cgi. (n.d.). NIH U.S. National Library of Medicine National Centre for Biotechnology Information. https://blast.ncbi.nlm.nih.gov/Blast.cgi

Http://www.healthdata.org. (2013, September 4). The Global Burden of Disease: Generating Evidence, Guiding Policy. http://www.healthdata.org/sites/default/files/files/data_for_download/2013/WorldBank_SubSaharanAfrica/IHME_GBD_WorldBank_SubSaharanAfrica_FullReport.pdf

Hunt, R., Edwardes, M., & Coetzee, M. (2010). Pyrethroid resistance in southern African Anopheles funestus extends to Likoma Island in Lake Malawi. *Parasites & Vectors*, *3*(1), 122. https://doi.org/10.1186/1756-3305-3-122

Idro, R., Marsh, K., John, C. C., & Newton, C. R. J. (2010). Cerebral malaria: Mechanisms of brain injury and strategies for improved neurocognitive outcome. *Pediatric Research*, *68*(4), 267–274. PubMed. https://doi.org/10.1203/PDR.0b013e3181eee738

Kamau, E., Alemayehu, S., Feghali, K. C., Saunders, D., & Ockenhouse, C. F. (2013). Multiplex qPCR for Detection and Absolute Quantification of Malaria. *PLoS ONE*, *8*(8), e71539. https://doi.org/10.1371/journal.pone.0071539

Kamau, L., Coetzee, M., Hunt, R. H., & Koekemoer, L. L. (2002). A cocktail polymerase chain reaction assay to identify members of the Anopheles funestus (Diptera: Culicidae) group. *The American Journal of Tropical Medicine and Hygiene*, *66*(6), 804–811. https://doi.org/10.4269/ajtmh.2002.66.804

Kariu, T., Yuda, M., Yano, K., & Chinzei, Y. (2002). MAEBL Is Essential for Malarial Sporozoite Infection of the Mosquito Salivary Gland. *Journal of Experimental Medicine*, *195*(10), 1317–1323. https://doi.org/10.1084/jem.20011876

Karl, S., Gurarie, D., Zimmerman, P. A., King, C. H., St. Pierre, T. G., & Davis, T. M. E. (2011). A Sub-Microscopic Gametocyte Reservoir Can Sustain Malaria Transmission. *PLoS ONE*, *6*(6), e20805. https://doi.org/10.1371/journal.pone.0020805

Kent, R. J. (2009). Molecular methods for arthropod bloodmeal identification and applications to ecological and vector-borne disease studies. *Molecular Ecology Resources*, *9*(1), 4–18. https://doi.org/10.1111/j.1755-0998.2008.02469.x

Keven, J. B., Artzberger, G., Gillies, M. L., Mbewe, R. B., & Walker, E. D. (2020). Probe-based multiplex qPCR identifies blood-meal hosts in Anopheles mosquitoes from Papua New Guinea. *Parasites & Vectors*, *13*(1), 111. https://doi.org/10.1186/s13071-020-3986-6

Keven, J. B., Katusele, M., Vinit, R., Rodr, D., Robinson, L. J., Laman, M., Karl, S., Foran, D. R., & Walker, E. D. (2021). Nonrandom Selection and Multiple Blood Feeding of Human Hosts by Anoph_ eles Vectors: Implications for Malaria Transmission in Papua New Guinea. *Am. J. Trop. Med. Hyg.*, *in Pres.*

Keven, J. B., Reimer, L., Katusele, M., Koimbu, G., Vinit, R., Vincent, N., Thomsen, E., Foran, D. R., Zimmerman, P. A., & Walker, E. D. (2017). Plasticity of host selection by malaria vectors of Papua New Guinea., Plasticity of host selection by malaria vectors of Papua New Guinea. *Parasites & Vectors, Parasites & Vectors*, *10, 10*(1), 95–95. https://doi.org/10.1186/s13071-017-2038-3, 10.1186/s13071-017-2038-3

Keven, J. B., Walker, E. D., & Venta, P. J. (2019). A Microsatellite Multiplex Assay for Profiling Pig DNA in Mosquito Bloodmeals. *Journal of Medical Entomology*, *56*(4), 907–914. https://doi.org/10.1093/jme/tjz013

Kilama, M., Smith, D. L., Hutchinson, R., Kigozi, R., Yeka, A., Lavoy, G., Kamya, M. R., Staedke, S. G., Donnelly, M. J., Drakeley, C., Greenhouse, B., Dorsey, G., & Lindsay, S. W. (2014). Estimating the annual entomological inoculation rate for Plasmodium falciparum transmitted by Anopheles gambiae s.l. Using three sampling methods in three sites in Uganda. *Malaria Journal*, *13*(1), 111. https://doi.org/10.1186/1475-2875-13-111

Killeen, G. F., McKenzie, F. E., Foy, B. D., Bøgh, C., & Beier, J. C. (2001). The availability of potential hosts as a determinant of feeding behaviours and malaria transmission by African mosquito populations. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *95*(5), 469–476. https://doi.org/10.1016/S0035-9203(01)90005-7

Killeen, G. F., McKenzie, F. E., Foy, B. D., Schieffelin, C., Billingsley, P. F., & Beier, J. C. (2000). A simplified model for predicting malaria entomologic inoculation rates based on entomologic and parasitologic parameters relevant to control. *The American Journal of Tropical Medicine and Hygiene*, *62*(5), 535–544.

Kilpatrick, A. M., Daszak, P., Jones, M. J., Marra, P. P., & Kramer, L. D. (2006). Host heterogeneity dominates West Nile virus transmission. *Proceedings of the Royal Society B: Biological Sciences*, *273*(1599), 2327–2333. https://doi.org/10.1098/rspb.2006.3575

Kitzmiller, J. B. (1982). *Anopheline names: their derivations and histories*. The Thomas Say Foundation.

Kleiber, C., & Zeileis, A. (2016). Visualizing Count Data Regressions Using Rootograms. *The American Statistician*, *70*(3), 296–303. https://doi.org/10.1080/00031305.2016.1173590

Klootwijk, L., Chirwa, A. E., Kabaghe, A. N., & van Vugt, M. (2019). Challenges affecting prompt access to adequate uncomplicated malaria case management in children in rural primary health facilities in Chikhwawa Malawi. *BMC Health Services Research*, *19*(1), 735. https://doi.org/10.1186/s12913-019-4544-9

Kramer, L. D., & Ciota, A. T. (2015). Dissecting vectorial capacity for mosquito-borne viruses. *Current Opinion in Virology*, *15*, 112–118. https://doi.org/10.1016/j.coviro.2015.10.003

Lacroix, R., Mukabana, W. R., Gouagna, L. C., & Koella, J. C. (2005). Malaria infection increases attractiveness of humans to mosquitoes. *PLoS Biology*, *3*(9), e298. https://doi.org/10.1371/journal.pbio.0030298

Lee, J. H., Godsey, M. S., Higazi, T. B., Cupp, E. W., Unnasch, T. R., Hassan, H., Hill, G., & Mitchell, C. J. (2002). Identification of mosquito avian-derived blood meals by polymerase chain reaction-heteroduplex analysis. *The American Journal of Tropical Medicine and Hygiene*, *66*(5), 599–604. https://doi.org/10.4269/ajtmh.2002.66.599

Lindblade, K. A., Mwandama, D., Mzilahowa, T., Steinhardt, L., Gimnig, J., Shah, M., Bauleni, A., Wong, J., Wiegand, R., Howell, P., Zoya, J., Chiphwanya, J., & Mathanga, D. P. (2015). A cohort study of the effectiveness of insecticide-treated bed nets to prevent malaria in an area of moderate pyrethroid resistance, Malawi. *Malaria Journal*, *14*, 31. https://doi.org/10.1186/s12936-015-0554-1

Lindsay, S. W., Thomas, M. B., & Kleinschmidt, I. (2021). Threats to the effectiveness of insecticide-treated bednets for malaria control: Thinking beyond insecticide resistance.

The Lancet Global Health, S2214109X21002163. https://doi.org/10.1016/S2214-109X(21)00216-3

Logue, K., Keven, J. B., Cannon, M. V., Reimer, L., Siba, P., Walker, E. D., Zimmerman, P. A., & Serre, D. (2016). Unbiased Characterization of Anopheles Mosquito Blood Meals by Targeted High-Throughput Sequencing. *PLOS Neglected Tropical Diseases*, *10*(3), e0004512. https://doi.org/10.1371/journal.pntd.0004512

Ludwig, J. A., & Reynolds, J. F. (1988). *Statistical Ecology: A Primer on Methods and Computing.* John Wiley & Sons, Inc.

Lysenko, A. J., & Semashko, I. N. (1968). Geography of Malaria. In *Medical Geography*. Academy of Sciences.

Macdonald. G. (1957). *The Epidemiology and Control of Malaria.* Oxford University Press; CABDirect.

Maekawa, Y., Pemba, D., Kumala, J., Gowelo, S., Higa, Y., Futami, K., Sawabe, K., & Tsuda, Y. (2021). DNA barcoding of mosquitoes collected through a nationwide survey in 2011 and 2012 in Malawi, Southeast Africa. *Acta Tropica*, *213*, 105742. https://doi.org/10.1016/j.actatropica.2020.105742

Malawi Malaria Indicatior Survey. (2017). ational Malaria Control Programme (NMCP) and ICF. 2018. https://dhsprogram.com/pubs/pdf/MIS28/MIS28.pdf

Mangani, C., Frake, A., Chipula, G., Mkwaila, W., Kakota, T., Mambo, I., Chigonda, J., Mathanga, D. P., Mzilahowa, T., & Walker, E. D. (2021). Proximity of human residence to irrigation determines malaria risk and Anopheles mosquito abundance at an irrigated agro-ecosystem in Malawi. *In Pres*.

Mangold, K. A., Manson, R. U., Koay, E. S. C., Stephens, L., Regner, M., Thomson, R. B., Peterson, L. R., & Kaul, K. L. (2005). Real-Time PCR for Detection and Identification of Plasmodium spp. *Journal of Clinical Microbiology*, *43*(5), 2435–2440. https://doi.org/10.1128/JCM.43.5.2435-2440.2005

Martinez, J., Showering, A., Oke, C., Jones, R. T., & Logan, J. G. (2021). Differential attraction in mosquito–human interactions and implications for disease control. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *376*(1818), 20190811. https://doi.org/10.1098/rstb.2019.0811

Martinez-Torres, D., Chandre, F., Williamson, M. S., Darriet, F., Bergé, J. B., Devonshire, A. L., Guillet, P., Pasteur, N., & Pauron, D. (1998). Molecular characterization of pyrethroid knockdown resistance (kdr) in the major malaria vector Anopheles gambiae s.s. *Insect Molecular Biology*, *7*(2), 179–184. https://doi.org/10.1046/j.1365-2583.1998.72062.x

Mathanga, D. P., Walker, E. D., Wilson, M. L., Ali, D., Taylor, T. E., & Laufer, M. K. (2012). Malaria control in Malawi: Current status and directions for the future. *Acta Tropica*, *121*(3), 212–217. https://doi.org/10.1016/j.actatropica.2011.06.017

Mbewe, R. B., Keven, J., Mzilahowa, T., Cohee, L., Laufer, M. K., Mathanga, D., Wilson, M., & Walker, E. D. (2021). *Blood meal sources of Anopheles vectors of human malaria in Malawi: Implications for malaria transmission and effectiveness of interventions* [Preprint]. In Review. https://doi.org/10.21203/rs.3.rs-841591/v1

Mbokazi, F., Coetzee, M., Brooke, B., Govere, J., Reid, A., Owiti, P., Kosgei, R., Zhou, S., Magagula, R., Kok, G., Namboze, J., Tweya, H., & Mabuza, A. (2018). Changing distribution and abundance of the malaria vector Anopheles merus in Mpumalanga Province, South Africa. *Public Health Action*, *8*(Suppl 1), S39–S43. https://doi.org/10.5588/pha.17.0034

Mburu, M. M., Mzilahowa, T., Amoah, B., Chifundo, D., Phiri, K. S., van den Berg, H., Takken, W., & McCann, R. S. (2019). Biting patterns of malaria vectors of the lower Shire valley, southern Malawi. *Acta Tropica*, *197*, 105059. https://doi.org/10.1016/j.actatropica.2019.105059

McCann, R. S., Messina, J. P., MacFarlane, D. W., Bayoh, M. N., Gimnig, J. E., Giorgi, E., & Walker, E. D. (2017). Explaining variation in adult Anopheles indoor resting abundance: The relative effects of larval habitat proximity and insecticide-treated bed net use. *Malaria Journal*, *16*(1), 288. https://doi.org/10.1186/s12936-017-1938-1

Medica, D. L., & Sinnis, P. (2005). Quantitative Dynamics of Plasmodium yoelii Sporozoite Transmission by Infected Anopheline Mosquitoes. *Infection and Immunity*, *73*(7), 4363–4369. https://doi.org/10.1128/IAI.73.7.4363-4369.2005

Meece, J. K., Reynolds, C. E., Stockwell, P. J., Jenson, T. A., Christensen, J. E., & Reed, K. D. (2005). Identification of Mosquito Bloodmeal Source by Terminal Restriction Fragment Length Polymorphism Profile Analysis of the Cytochrome B Gene. *Journal of Medical Entomology*, *42*(4), 11.

Mendis, K., Rietveld, A., Warsame, M., Bosman, A., Greenwood, B., & Wernsdorfer, W. H. (2009). From malaria control to eradication: The WHO perspective. *Tropical Medicine & International Health*, *14*(7), 802–809. https://doi.org/10.1111/j.1365-3156.2009.02287.x

Mharakurwa, S., Norris, D. E., Kent, R. J., & Thuma, P. E. (2007). Seasonality, blood feeding behavior, and transmission of *Plasmodium falciparum* by *Anopheles arabiensis* after an extended drought in southern Zambia. *The American Journal of Tropical Medicine and Hygiene*, *76*(2), 267–274. https://doi.org/10.4269/ajtmh.2007.76.267

Mirghani, S. E., Nour, B. Y., Bushra, S. M., Elhassan, I. M., Snow, R. W., & Noor, A. M. (2010). *RTehseaerchspatial-temporal clustering of Plasmodium falciparum infection over eleven years in Gezira State, The Sudan*. 9.

Molaei, G., Andreadis, T. G., Armstrong, P. M., Anderson, J. F., & Vossbrinck, C. R. (2006). Host Feeding Patterns of *Culex* Mosquitoes and West Nile Virus Transmission, Northeastern United States. *Emerging Infectious Diseases*, *12*(3), 468–474. https://doi.org/10.3201/eid1203.051004

Molineaux, L., & Gramiccia, G. (1980). *The Garki project: Research on the epidemiology and control of malaria in the Sudan savanna of West Africa*.

Mouatcho, J., Cornel, A. J., Dahan-Moss, Y., Koekemoer, L. L., Coetzee, M., & Braack, L. (2018). Detection of Anopheles rivulorum-like, a member of the Anopheles funestus group, in South Africa. *Malaria Journal*, *17*(1), 195. https://doi.org/10.1186/s12936-018-2353-y

Muirhead-Thomson, R. C. (1951). The distribution of anopheline mosquito bites among different age groups; a new factor in malaria epidemiology. *British Medical Journal*, *1*(4715), 1114–1117.

Mullen, G., & Durden, L. (2009). *Medical and Veterinary Entomology—2nd Edition* (2nd ed.). Elsevier. https://www.elsevier.com/books/medical-and-veterinary-entomology/mullen/978-0-12-372500-4

Mwesigwa, J., Achan, J., Di Tanna, G. L., Affara, M., Jawara, M., Worwui, A., Hamid-Adiamoh, M., Kanuteh, F., Ceesay, S., Bousema, T., Drakeley, C., Grietens, K. P., Lindsay, S. W., Van geertruyden, J.-P., & D'Alessandro, U. (2017). Residual malaria transmission dynamics varies across The Gambia despite high coverage of control interventions. *PLOS ONE*, *12*(11), e0187059. https://doi.org/10.1371/journal.pone.0187059

Mzilahowa, T., Ball, A. J., Bass, C., Morgan, J. C., Nyoni, B., Steen, K., Donnelly, M. J., & Wilding, C. S. (2008). Reduced susceptibility to DDT in field populations of *Anopheles quadriannulatus* and *Anopheles arabiensis* in Malawi: Evidence for larval selection. *Medical and Veterinary Entomology*, *22*(3), 258–263. https://doi.org/10.1111/j.1365-2915.2008.00736.x

Mzilahowa, T., Chiumia, M., Mbewe, R. B., Uzalili, V. T., Luka-Banda, M., Kutengule, A., Mathanga, D. P., Ali, D., Chiphwanya, J., Zoya, J., Mulenga, S., Dodoli, W., Bergeson-Lockwood, J., Troell, P., Oyugi, J., Lindblade, K., & Gimnig, J. E. (2016). Increasing insecticide resistance in Anopheles funestus and Anopheles arabiensis in Malawi, 2011–2015. *Malaria Journal*, *15*, 563. https://doi.org/10.1186/s12936-016-1610-1

Mzilahowa, T., Hastings, I. M., Molyneux, M. E., & McCall, P. J. (2012).
Entomological indices of malaria transmission in Chikhwawa district, Southern Malawi. *Malaria Journal*, *11*(1), 380. https://doi.org/10.1186/1475-2875-11-380

Nagaki, S. S., Chaves, L. S. M., López, R. V. M., Bergo, E. S., Laporta, G. Z., Conn, J. E., & Sallum, M. A. M. (2021). Host feeding patterns of Nyssorhynchus darlingi (Diptera:

Culicidae) in the Brazilian Amazon. *Acta Tropica*, *213*, 105751. https://doi.org/10.1016/j.actatropica.2020.105751

Nankabirwa, J., Brooker, S. J., Clarke, S. E., Fernando, D., Gitonga, C. W., Schellenberg, D., & Greenwood, B. (2014). Malaria in school-age children in A frica: An increasingly important challenge. *Tropical Medicine & International Health*, *19*(11), 1294–1309. https://doi.org/10.1111/tmi.12374

Ndoen, E., Wild, C., Dale, P., Sipe, N., & Dale, M. (2012). *Mosquito Longevity, Vector Capacity, and Malaria Incidence in West Timor and Central Java, Indonesia* [Research article]. International Scholarly Research Notices. https://doi.org/10.5402/2012/143863

NSO. (2019a). *2018 Malawi Population and Housing Census Main Report*. Government of Malwi, National Statisical Office. http://www.nsomalawi.mw/images/stories/data_on_line/demography/census_2018/2018%20Malawi%20Population%20and%20Housing%20Census%20Main%20Report.pdf

NSO. (2019b). *2018 Malawi Population and Housing Census Main Report.pdf*. Government of Malawi, National Statistics Office. http://www.nsomalawi.mw/images/stories/data_on_line/demography/census_2018/2018%20Malawi%20Population%20and%20Housing%20Census%20Main%20Report.pdf

Odiere, M., Bayoh, M. N., Gimnig, J., Vulule, J., Irungu, L., & Walker, E. (2007). *Sampling Outdoor, Resting Anopheles gambiae and Other Mosquitoes (Diptera: Culicidae) in Western Kenya with Clay Pots*. *44*(1), 14–22.

Ogola, E., Villinger, J., Mabuka, D., Omondi, D., Orindi, B., Mutunga, J., Owino, V., & Masiga, D. K. (2017). Composition of Anopheles mosquitoes, their blood-meal hosts, and Plasmodium falciparum infection rates in three islands with disparate bed net coverage in Lake Victoria, Kenya. *Malaria Journal*, *16*(1), 360. https://doi.org/10.1186/s12936-017-2015-5

Oostdik, K., Lenz, K., Nye, J., Schelling, K., Yet, D., Bruski, S., Strong, J., Buchanan, C., Sutton, J., Linner, J., Frazier, N., Young, H., Matthies, L., Sage, A., Hahn, J., Wells, R., Williams, N., Price, M., Koehler, J., … Storts, D. R. (2014). Developmental validation of the PowerPlex® Fusion System for analysis of casework and reference samples: A 24-locus multiplex for new database standards. *Forensic Science International: Genetics*, *12*, 69–76. https://doi.org/10.1016/j.fsigen.2014.04.013

Orsborne, J., Furuya-Kanamori, L., Jeffries, C. L., Kristan, M., Mohammed, A. R., Afrane, Y. A., O'Reilly, K., Massad, E., Drakeley, C., Walker, T., & Yakob, L. (2019). Investigating the blood-host plasticity and dispersal of Anopheles coluzzii using a novel field-based methodology. *Parasites & Vectors*, *12*(1), 143. https://doi.org/10.1186/s13071-019-3401-3

Orsborne, J., Mohammed, A. R., Jeffries, C. L., Kristan, M., Afrane, Y. A., Walker, T., & Yakob, L. (2020). Evidence of extrinsic factors dominating intrinsic blood host preferences of major African malaria vectors. *Scientific Reports*, *10*(1), 741. https://doi.org/10.1038/s41598-020-57732-1

Overgaard, H. J., Abaga, S., Pappa, V., Reddy, M., & Caccone, A. (2011). Estimation of the Human Blood Index in Malaria Mosquito Vectors in Equatorial Guinea after Indoor Antivector Interventions. *The American Journal of Tropical Medicine and Hygiene*, *84*(2), 298–301. https://doi.org/10.4269/ajtmh.2011.10-0463

Paul, M. R., Grenfell, B. T., Hoti, S. L., Ramaiah, K. D., Bundy, D. A., Das, P. K., Yuvaraj, J., Barker, G., & Michael, E. (2001). Quantifying mosquito biting patterns on humans by DNA fingerprinting of bloodmeals. *The American Journal of Tropical Medicine and Hygiene*, *65*(6), 722–728. https://doi.org/10.4269/ajtmh.2001.65.722

Pimenta, P. F., Touray, M., & Miller, L. (1994). The Journey of Malaria Sporozoites in the Mosquito Salivary Gland. *The Journal of Eukaryotic Microbiology*, *41*(6), 608–624. https://doi.org/10.1111/j.1550-7408.1994.tb01523.x

PMI. (2018). *Malawi Malaria Operational Plan FY 2018*. President's malaria initiative Malawi. https://www.pmi.gov/docs/default-source/default-document-library/malaria-operational-plans/fy-2018/fy-2018-malawi-malaria-operational-plan.pdf?sfvrsn=5

Postels, D. G., & Birbeck, G. L. (2013). Cerebral malaria. In *Handbook of Clinical Neurology* (Vol. 114). Neuroparasitology and Tropical Neurology.

Protopopoff, N., Mosha, J. F., Lukole, E., Charlwood, J. D., Wright, A., Mwalimu, C. D., Manjurano, A., Mosha, F. W., Kisinza, W., Kleinschmidt, I., & Rowland, M. (2018). Effectiveness of a long-lasting piperonyl butoxide-treated insecticidal net and indoor residual spray interventions, separately and together, against malaria transmitted by pyrethroid-resistant mosquitoes: A cluster, randomised controlled, two-by-two factorial design trial. *The Lancet*. https://doi.org/10.1016/S0140-6736(18)30427-6

Ranson, H., Abdallah, H., Badolo, A., Guelbeogo, W. M., Kerah-Hinzoumbé, C., Yangalbé-Kalnoné, E., Sagnon, N., Simard, F., & Coetzee, M. (2009). Insecticide resistance in Anopheles gambiae: Data from the first year of a multi-country study highlight the extent of the problem. *Malaria Journal*, *8*, 299. https://doi.org/10.1186/1475-2875-8-299

Ranson, H., N'Guessan, R., Lines, J., Moiroux, N., Nkuni, Z., & Corbel, V. (2011). Pyrethroid resistance in African anopheline mosquitoes: What are the implications for malaria control? *Trends in Parasitology*, *27*(2), 91–98. https://doi.org/10.1016/j.pt.2010.08.004

Riveron, J. M., Chiumia, M., Menze, B. D., Barnes, K. G., Irving, H., Ibrahim, S. S., Weedall, G. D., Mzilahowa, T., & Wondji, C. S. (2015a). Rise of multiple insecticide resistance in Anopheles funestus in Malawi: A major concern for malaria vector control. *Malaria Journal*, *14*, 344. https://doi.org/10.1186/s12936-015-0877-y

Roberts, D. R., & Hsi, B. P. (1979). An Index of Species Abundance for Use with Mosquito Surveillance Data. *Environmental Entomology*, *8*(6), 1007–1013. https://doi.org/10.1093/ee/8.6.1007

Rodríguez-Rodríguez, D., Katusele, M., Auwun, A., Marem, M., Robinson, L. J., Laman, M., Hetzel, M. W., & Pulford, J. (2021). Human Behavior, Livelihood, and Malaria Transmission in Two Sites of Papua New Guinea. *The Journal of Infectious Diseases*, *223*(Supplement_2), S171–S186. https://doi.org/10.1093/infdis/jiaa402

Rosanas-Urgell, A., Mueller, D., Betuela, I., Barnadas, C., Iga, J., Zimmerman, P. A., del Portillo, H. A., Siba, P., Mueller, I., & Felger, I. (2010). Comparison of diagnostic methods for the detection and quantification of the four sympatric Plasmodium species in field samples from Papua New Guinea. *Malaria Journal*, *9*(1), 361. https://doi.org/10.1186/1475-2875-9-361

Rosenberg, N. A., Pritchard, J. K., Weber, J. L., Cann, H. M., Kidd, K. K., Zhivotovsky, L. A., & Feldman, M. W. (2002). Genetic structure of human populations. *Science (New York, N.Y.)*, *298*(5602), 2381–2385. https://doi.org/10.1126/science.1078311

Sachs, S. E., Mellinger, A., Malaney, P., Sachs, J., Kiszewski, A., & Spielman, A. (2004). A global index representing the stability of malaria transmission. *The American Journal of Tropical Medicine and Hygiene*, *70*(5), 486–498. https://doi.org/10.4269/ajtmh.2004.70.486

Sallum, M. A. M., Conn, J. E., Bergo, E. S., Laporta, G. Z., Chaves, L. S. M., Bickersmith, S. A., de Oliveira, T. M. P., Figueira, E. A. G., Moresco, G., Olívêr, L., Struchiner, C. J., Yakob, L., & Massad, E. (2019). Vector competence, vectorial capacity of Nyssorhynchus darlingi and the basic reproduction number of Plasmodium vivax in agricultural settlements in the Amazonian Region of Brazil. *Malaria Journal*, *18*(1), 117. https://doi.org/10.1186/s12936-019-2753-7

Schaber, K. L., Perkins, T. A., Lloyd, A. L., Waller, L. A., Kitron, U., Paz-Soldan, V. A., Elder, J. P., Rothman, A. L., Civitello, D. J., Elson, W. H., Morrison, A. C., Scott, T. W., & Vazquez-Prokopec, G. M. (2021). Disease-driven reduction in human mobility influences human-mosquito contacts and dengue transmission dynamics. *PLOS Computational Biology*, *17*(1), e1008627. https://doi.org/10.1371/journal.pcbi.1008627

Schlagenhauf, P. (2004). Malaria: From prehistory to present. *Infect Dis Clin North Am*, *18*, 189–205.

Scott, T. W., Harrington, L. C., Yan, G., Githeko, A. K., & Fleisher, A. (2006). DNA profiling of human blood in anophelines from lowland and highland sites in western Kenya. *The American Journal of Tropical Medicine and Hygiene*, *75*(2), 231–237. https://doi.org/10.4269/ajtmh.2006.75.231

Sinka, M. E., Bangs, M. J., Manguin, S., Coetzee, M., Mbogo, C. M., Hemingway, J., Patil, A. P., Temperley, W. H., Gething, P. W., Kabaria, C. W., Okara, R. M., Van Boeckel, T., Godfray, H. C. J., Harbach, R. E., & Hay, S. I. (2010). The dominant Anopheles vectors of human malaria in Africa, Europe and the Middle East: Occurrence data, distribution maps and bionomic précis. *Parasites & Vectors*, *3*(1), 117. https://doi.org/10.1186/1756-3305-3-117

Sinka, M. E., Bangs, M. J., Manguin, S., Rubio-Palis, Y., Chareonviriyaphap, T., Coetzee, M., Mbogo, C. M., Hemingway, J., Patil, A. P., Temperley, W. H., Gething, P. W., Kabaria, C. W., Burkot, T. R., Harbach, R. E., & Hay, S. I. (2012). A global map of dominant malaria vectors. *Parasites & Vectors*, *5*(1), 69. https://doi.org/10.1186/1756-3305-5-69

Smith, D. L., Dushoff, J., & McKenzie, F. E. (2004). The Risk of a Mosquito-Borne Infectionin a Heterogeneous Environment. *PLoS Biology*, *2*(11), e368. https://doi.org/10.1371/journal.pbio.0020368

Smith, D. L., McKenzie, F. E., Snow, R. W., & Hay, S. I. (2007). Revisiting the Basic Reproductive Number for Malaria and Its Implications for Malaria Control. *PLoS Biology*, *5*(3), e42. https://doi.org/10.1371/journal.pbio.0050042

Smith, J. L., Auala, J., Tambo, M., Haindongo, E., Katokele, S., Uusiku, P., Gosling, R., Kleinschmidt, I., Mumbengegwi, D., & Sturrock, H. J. W. (2017). Spatial clustering of patent and sub-patent malaria infections in northern Namibia: Implications for surveillance and response strategies for elimination. *PLOS ONE*, *12*(8), e0180845. https://doi.org/10.1371/journal.pone.0180845

Snow, R. W., Ali, D., Kazembe, L., Bennett, A., Kinyoki, D., Mathanga, D. P., & Noor, A. M. (2013). Mapping Malaria Transmission Intensity in Malawi, 2000–2010. *The American Journal of Tropical Medicine and Hygiene*, *89*(5), 840–849. https://doi.org/10.4269/ajtmh.13-0028

Soremekun, S., Maxwell, C., Zuwakuu, M., Chen, C., Michael, E., & Curtis, C. (2004). Measuring the efficacy of insecticide treated bednets: The use of DNA fingerprinting to increase the accuracy of personal protection estimates in Tanzania. *Tropical Medicine and International Health*, *9*(6), 664–672. https://doi.org/10.1111/j.1365-3156.2004.01250.x

Southwood, T.R.E. (1978). *Ecological methods, with particular reference to the study of insect populations.* Chapman and Hall.

Spiers, A., Mzilahowa, T., Atkinson, D., & McCall, P. (2002). The malaria vectors of the Lower Shire valley, Malawi. *Malawi Medical Journal : The Journal of Medical Association of Malawi*, *14*(1), 4–7. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3345416/

Staedke, S. G., Gonahasa, S., Dorsey, G., Kamya, M. R., Maiteki-Sebuguzi, C., Lynd, A., Katureebe, A., Kyohere, M., Mutungi, P., Kigozi, S. P., Opigo, J., Hemingway, J., &

Donnelly, M. J. (2020). Effect of long-lasting insecticidal nets with and without piperonyl butoxide on malaria indicators in Uganda (LLINEUP): A pragmatic, cluster-randomised trial embedded in a national LLIN distribution campaign. *The Lancet*, *395*(10232), 1292–1303. https://doi.org/10.1016/S0140-6736(20)30214-2

Stevenson, J., Stewart, B. St., Lobo, N. F., Cooke, M. K., Kahindi, S. C., Oriango, R. M., Harbach, R. E., Cox, J., & Drakeley, C. (2012). Novel Vectors of Malaria Parasites in the Western Highlands of Kenya. *Emerging Infectious Diseases*, *18*(9), 1547–1549. http://dx.doi.org/10.3201/eid1809.120283

Stone, C., & Gross, K. (2018). Evolution of host preference in anthropophilic mosquitoes. *Malaria Journal*, *17*, 257. https://doi.org/10.1186/s12936-018-2407-1

Stresman, G. H., Kamanga, A., Moono, P., Hamapumbu, H., Mharakurwa, S., Kobayashi, T., Moss, W. J., & Shiff, C. (2010). A method of active case detection to target reservoirs of asymptomatic malaria and gametocyte carriers in a rural area in Southern Province, Zambia. *Malaria Journal*, *9*(1), 265. https://doi.org/10.1186/1475-2875-9-265

Tajadini, M., Panjehpour, M., & Javanmard, S. (2014). Comparison of SYBR Green and TaqMan methods in quantitative real-time polymerase chain reaction analysis of four adenosine receptor subtypes. *Advanced Biomedical Research*, *3*(1), 85. https://doi.org/10.4103/2277-9175.127998

Takken, W., & Verhulst, N. O. (2013). Host Preferences of Blood-Feeding Mosquitoes. *Annual Review of Entomology*, *58*(1), 433–453. https://doi.org/10.1146/annurev-ento-120811-153618

Tedrow, Riley. E., Zimmerman, P. A., & Abbott, K. C. (2019). Multiple BloodFeeding: A ForceMultiplier for Transmission. *Trends in Parasitology*, *35*(10), 741–742. https://doi.org/10.1016/j.pt.2019.07.011

Thongsripong, P., Qu, Z., Yukich, J. O., Hyman, J. M., & Wesson, D. M. (2019). *Quantification of human-mosquito contact rate using surveys and its application in determining dengue viral transmission risk* [Preprint]. Ecology. https://doi.org/10.1101/821819

Thongsripong, P., Qu, Z., Yukich, J. O., Hyman, J. M., & Wesson, D. M. (2020). An Investigation of Human–Mosquito Contact Using Surveys and Its Application in Assessing Dengue Viral Transmission Risk. *Journal of Medical Entomology*, *57*(6), 13.

Toe, K. H., Müller, P., Badolo, A., Traore, A., Sagnon, N., Dabiré, R. K., & Ranson, H. (2018). Do bednets including piperonyl butoxide offer additional protection against populations of Anopheles gambiae s.l. that are highly resistant to pyrethroids? An experimental hut evaluation in Burkina Fasov. *Medical and Veterinary Entomology*, *0*(0). https://doi.org/10.1111/mve.12316

Townes, L. R., Mwandama, D., Mathanga, D. P., & Wilson, M. L. (2013). Elevated dry-season malaria prevalence associated with fine-scale spatial patterns of environmental risk: A case–control study of children in rural Malawi. *Malaria Journal*, *12*(1), 407. https://doi.org/10.1186/1475-2875-12-407

Tukei, B. B., Beke, A., & Lamadrid-Figueroa, H. (2017). Assessing the effect of indoor residual spraying (IRS) on malaria morbidity in Northern Uganda: A before and after study. *Malaria Journal*, *16*(1). https://doi.org/10.1186/s12936-016-1652-4

USAID. (2017). *Combating Malaria*. U.S. President's Malaria Initiative. https://www.usaid.gov/sites/default/files/documents/1860/Malaria_Fact_Sheet_9.26.18.pdf

Vernick, K. D., Oduol, F., Lazzaro, B. P., Glazebrook, J., Xu, J., Riehle, M., & Li, J. (2005). Molecular genetics of mosquito resistance to malaria parasites. *Current Topics in Microbiology and Immunology*, *295*, 383–415.

Vezenegho, S. B., Bass, C., Puinean, M., Williamson, M. S., Field, L. M., Coetzee, M., & Koekemoer, L. L. (2009). Development of multiplex real-time PCR assays for identification of members of the Anopheles funestus species group. *Malaria Journal*, *8*(1), 282. https://doi.org/10.1186/1475-2875-8-282

Vezenegho, S. B., Chiphwanya, J., Hunt, R. H., Coetzee, M., Bass, C., & Koekemoer, L. L. (2013). Characterization of the Anopheles funestus group, including Anopheles funestus-like, from northern Malawi. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *107*(12), 753–762. https://doi.org/10.1093/trstmh/trt089

Vinit, R., Timinao, L., Bubun, N., Katusele, M., Robinson, L. J., Kaman, P., Sakur, M., Makita, L., Reimer, L., Schofield, L., Pomat, W., Mueller, I., Laman, M., Freeman, T., & Karl, S. (2020). Decreased bioefficacy of long-lasting insecticidal nets and the resurgence of malaria in Papua New Guinea. *Nature Communications*, *11*(1), 3646. https://doi.org/10.1038/s41467-020-17456-2

Walker, E. D., Thibault, A. R., Thelen, A. P., Bullard, B. A., Huang, J., Odiere, M. R., Bayoh, N. M., Wilkins, E. E., & Vulule, J. M. (2007). Identification of field caught Anopheles gambiae s.s. And Anopheles arabiensis by TaqMan single nucleotide polymorphism genotyping. *Malaria Journal*, *6*(1), 23. https://doi.org/10.1186/1475-2875-6-23

Walldorf, J. A., Cohee, L. M., Coalson, J. E., Bauleni, A., Nkanaunena, K., Kapito-Tembo, A., Seydel, K. B., Ali, D., Mathanga, D., Taylor, T. E., Valim, C., & Laufer, M. K. (2015a). School-Age Children Are a Reservoir of Malaria Infection in Malawi. *PloS One*, *10*(7), e0134061. https://doi.org/10.1371/journal.pone.0134061

Walldorf, J. A., Cohee, L. M., Coalson, J. E., Bauleni, A., Nkanaunena, K., Kapito-Tembo, A., Seydel, K. B., Ali, D., Mathanga, D., Taylor, T. E., Valim, C., & Laufer, M. K. (2015b). School-Age Children Are a Reservoir of Malaria Infection in Malawi. *PLOS ONE*, *10*(7), e0134061. https://doi.org/10.1371/journal.pone.0134061

Warrell, D. A., & Gilles, H. M. (2017). *Essential Malariology, 4Ed* (4th ed.). CRC Press. https://doi.org/10.1201/9780203756621

Wernsdorfer, W. H., & McGregor, I. (1988). *Malaria: Principles and practice of malariology*. Churchill Livingstone; /z-wcorg/.

WHO. (1995). *Manual on practical entomology in malaria / prepared by the WHO Division of Malaria and Other Parasitic Diseases*. WHO IRIS. https://apps.who.int/iris/handle/10665/42481

WHO. (2006). *Global Malaria Programme, Indoor Residual Spraying. Use of indoor residual spraying for scaling up global malaria control and elimination*. https://apps.who.int/iris/bitstream/handle/10665/69386/WHO_HTM_MAL_2006.1112_eng.pdf

WHO. (2014). *WHO World Malaria Report*. https://www.who.int/malaria/publications/world_malaria_report_2014/en/

WHO. (2018a). *WHO Pyrethroid-PBO nets appear to provide improved protection against malaria in areas with high resistance*. https://www.who.int/malaria/news/2018/pbo-nets-lancet/en/

WHO. (2018b). *World Malaria report 2018*. World Health Organization, Global Malaria Programme. http://apps.who.int/iris/bitstream/handle/10665/275867/9789241565653-eng.pdf?ua=1

WHO. (2021a). *Monitoring progress on universal health coverage and the health-related Sustainable Development Goals in the WHO South-East Asia Region: 2021 update*. World Health Organization. Regional Office for South-East Asia. https://apps.who.int/iris/handle/10665/344764

WHO. (2021b). *World Malaria Report 2020* (2021 update). World Health Organization. https://apps.who.int/iris/handle/10665/342995

Willems, T., Gymrek, M., Highnam, G., Mittelman, D., & Erlich, Y. (2014). The landscape of human STR variation. *Genome Research*, *24*(11), 1894–1904. https://doi.org/10.1101/gr.177774.114

Wilson, M. L., Walker, E. D., Mzilahowa, T., Mathanga, D. P., & Taylor, T. E. (2012). Malaria elimination in Malawi: Research needs in highly endemic, poverty-stricken contexts. *Acta Tropica*, *121*(3), 218–226. https://doi.org/10.1016/j.actatropica.2011.11.002

Woolhouse, M. E. J., Dye, C., Etard, J.-F., Smith, T., Charlwood, J. D., Garnett, G. P., Hagan, P., Hii, J. L. K., Ndhlovu, P. D., Quinnell, R. J., Watts, C. H., Chandiwana, S. K., & Anderson, R. M. (1997). Heterogeneities in the transmission of infectious agents: Implications for the design of control programs. *Proceedings of the National Academy of Sciences*, *94*(1), 338–342. https://doi.org/10.1073/pnas.94.1.338

Yang, Y., Garver, L. S., Bingham, K. M., Hang, J., Jochim, R. C., Davidson, S. A., Richardson, J. H., & Jarman, R. G. (2015). Feasibility of Using the Mosquito Blood Meal for Rapid and Efficient Human and Animal Virus Surveillance and Discovery. *The American Journal of Tropical Medicine and Hygiene*, *93*(6), 1377–1382. PubMed. https://doi.org/10.4269/ajtmh.15-0440