FORAGING BEHAVIOR OF *ANOPHELES* MOSQUITOES IN NCHELENGE DISTRICT, ZAMBIA

By

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Abstract

Approximately 3.2 billion people worldwide are at risk of contracting malaria. In 2013, there were 200 million cases and close to 600,000 deaths, most of which occurred in sub-Saharan Africa and affected mainly children, denoting the disease as a major public health problem. In Nchelenge District, Zambia, there is holoendemic transmission of Plasmodium falciparum, vectored by *Anopheles funestus* sensu stricto and *An. gambiae* s.s. mosquitoes. Since 2006, the Zambian government has provided LLINs, providing approximately one net per person, and has implemented IRS campaigns in Nchelenge. Despite the use of vector control, the prevalence of malaria has not reduced, making it necessary to define and characterize the *Anopheles* mosquitoes involved in transmission to better develop strategies for control. Therefore, the specific aims of this study were: 1) to identify and characterize the anopheline mosquitoes and their respective roles in *P. falciparum* transmission both temporally and spatially, 2) to determine the extent of underestimation of *Anopheles* foraging behavior by standard field methods across the three southern Africa ICEMR field sites, and 3) to investigate multiple blood feeding behavior and human gender preference of human fed mosquitoes, and *P. falciparum* complexity of infection (COI) in infected anophelines in Nchelenge District.

CDC LT and PSC trap methods were performed over three consecutive wet and dry seasons in three villages lakeside and two villages streamside, revealing *An. funestus* and *An. gambiae* as the dominant vectors. Both vectors were also highly anthropophilic. Temporally, *An. funestus* is the primary vector year round, whereas *An. gambiae* is more abundant during the wet season compared to the dry season.

Spatially, during the wet season, *An. funestus* is the predominant vector inland and along the stream, whereas *An. gambiae* is the predominant vector along Lake Mweru. Additionally, the abundances of both *An. funestus* and *An. gambiae* streamside are higher than lakeside. Throughout all three collections, *An. funestus* had the highest transmission intensity, indicating its important role in maintaining transmission year round. When investigating the foraging behavior of malaria vectors across the three southern Africa ICEMR sites, it was found that by not including visually "unfed" mosquitoes in host blood meal PCR assays, the overall proportion of fed vectors is underestimated by up to 18.7%, and that this can have potential effects on the human blood index (HBI), human biting rate (HBR), and entomological inoculation rate (EIR). Finally, *Anopheles* mosquito foraging behavior in Nchelenge was further defined through multiple blood feeding behavior, human gender preference, and COI studies. Both *An. funestus* and *An. gambiae* displayed multiple blood feeding rates of 23.2% and 25.7% respectively, rates that are among the highest recorded in sub-Saharan Africa and lead to an underestimation of the EIR. There was a trend towards both vectors feeding on human males more so than females. Furthermore, the *P. falciparum* COI in infected mosquitoes was 6.4, suggesting a high transmission setting and supports the inoculation of multiple clones in a single mosquito bite in Nchelenge District. The studies described in this dissertation have provided a foundation for future entomological studies on mosquito vectors in Nchelenge District, Zambia, as well as vector control evaluation and development of effective intervention programs.

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Chapter I

Introduction

Malaria in Sub-Saharan Africa is a major public health problem, affecting an estimated 198 million people and killing approximately 584,000 each year, primarily children under the age of five years [1]. In 2010, it was shown that at the country-level in Africa, an estimated 80% of malaria fatalities occur in just 14 countries and 80% of malaria cases occur in 17 countries [2]. Globally, the Democratic Republic of Congo and Nigeria together account for 40% of malaria deaths [2]. The burden of malaria also significantly affects the political and economic climate of endemic countries. The loss of productivity due to illness and death caused by the parasite has been implicated in an estimated 1.3% decrease of Gross Domestic Product (GDP) [2]. The long-term effect will continue to be a substantial increase in the economic disparity between countries with and without malaria. Therefore, it is necessary to conduct malaria research studies in endemic areas to inform intervention strategy for effective control.

Renewed interest and increased funding has directed an aggressive malaria control scale-up campaign in Africa. The 2005-2015 Roll Back Malaria (RBM) Partnership has committed to achieving 80% coverage of preventative interventions in malaria at-risk populations, and a 50% decrease in malaria cases worldwide [3]. Specifically in Zambia, efforts to increase malaria control interventions have been particularly successful. During the period 2003-2008, the Zambia Ministry of Health distributed approximately 5.9 million insecticide-treated bed nets (ITNs), most of which were long-lasting insecticide-treated nets (LLINs) [4]. Additionally, increased

microscopy training, rapid diagnostic test (RDT) use, and availability of artemesinin-based combination therapy (ACT) in all health clinics were implemented [4]. By 2008, ITN coverage according to national surveys was 68%, of which 62.3% owned more than one ITN and 30.9% owned more than two ITNs [4]. Furthermore, indoor residual spray (IRS) coverage increased 66% between 2006 and 2008 in targeted districts [4]. During this time period, the malaria prevalence decreased by 53% in children under the age of five years, but it is unclear if the reduction is entirely the result of the malaria control scale-up initiative [4].

A second analysis of malaria epidemiology in Zambia showed that while there was a significant impact on malaria prevalence from 2000-2008, resurgence did occur in 2009-2010 [5]. The resurgence lead to the classification of three epidemiological zones based on malaria parasite prevalence in children under five years old [5]: Zone I is low transmission (<1% prevalence; Lusaka Province), Zone II is moderate transmission (2-4% prevalence; Northwestern, Copperbelt, Central, Western, and Southern Provinces), and Zone III is high transmission (>15% prevalence; Luapula, Northern, Muchinga, and Eastern Provinces). In particular, Zone III experienced a large-scale increase in malaria cases [5]. The reasons behind the increase remain undefined, but it has been suggested that factors such as insufficient LLIN and IRS coverage, damaged and ineffective LLINs, insecticide resistance in mosquito vectors, and anti-malarial immunity due to decreasing transmission may be involved [5]. These factors highlight the importance of performing studies that can direct resources for implementation of successful interventions.

Since 2012, the Johns Hopkins Southern Africa International Centers for Excellence in Malaria Research (ICEMR) project has been investigating the high malaria

burden in one of the areas in Zone III, Nchelenge District in Luapula Province. The Southern Africa ICEMR is a seven-year project in three distinct epidemiological settings in Zambia and Zimbabwe (Figure 1.3). In Zambia, Macha and Nchelenge represent successful and unsuccessful malaria control respectively. Mutasa in Zimbabwe represents resurgent malaria. The ICEMR project utilizes a multidisciplinary approach that includes understanding local malaria epidemiology, vector biology, and parasite genomics. One of the goals of the project is to develop recommendations for effective and sustainable malaria control strategies.

Nchelenge District

The studies that comprise this dissertation were done in association with the Southern Africa ICEMR project to further the entomological research component in Nchelenge District, Luapula Province, Zambia. Nchelenge experiences holoendemic *Plasmodium falciparum* malaria transmission. Although there have not been any formal entomological studies in Nchelenge, preliminary collections in 2011 have suggested that *An. funestus* s.s. and *An. gambiae* s.s. are the major malaria vectors (Personal communication with Mbanga Muleba, Tropical Disease Research Centre, Ndola, Zambia). The current control measures include treatment of malaria cases with artemether-lumefantrine, as well as IRS and LLIN distribution campaigns. In Nchelenge, LLIN ownership was less than 10% in 2001 [6]; LLIN distribution began in 2006 and coverage increased dramatically to 65%, and then decreased to 56% in 2010, which was one of the lowest rates in Zambia [6]. From 2006 to 2012, 429,753 nets were distributed resulting in approximately one net per two people in 2007, which then increased to 1.24 LLIN per person by 2012 [7]. An upcoming LLIN distribution campaign is scheduled for

2015 (Personal communication with Dr. Mike Chaponda, Tropical Disease Research Centre, Ndola, Zambia). In 2008, IRS in Nchelenge formally began and reached 49% of households in 2010 [6]. Between 2006 and 2012, IRS was implemented in 109,095 houses, or approximately 80% coverage of eligible targeted structures [7]. IRS insecticides that have been used since 2008 include pyrethroids (alphacypermethrin, lambdacyhalothrin) and a carbamate (bendiocarb) [8]. Another IRS campaign began in October 2014 and utilized an organophosphate (pyrimiphos-methyl) (Personal communication with Dr. Douglas Norris, JHSPH). Despite these interventions, Nchelenge District continues to have high parasite prevalence of approximately 64% (Personal communication with Dr. Jessie Pinchoff, JHSPH). According to the District Health Information System (DHIS) data from 11 health facilities, prevalence of malaria increased from 38% in 2006 to 53% in 2012 and continued to increase in 2013 [7]. Clearly, the burden of malaria in Nchelenge is a major public health problem despite the use of vector control. It will therefore be essential to characterize the mosquito vectors in this area to understand why vector control is ineffective and to use that knowledge to develop successful control strategies.

Study Area

Nchelenge District is located in Luapula Province, Zambia (9° 19.115'S, 28° 45.070'E) (Figure 1.1). The area lies along the eastern perimeter of Lake Mweru, which separates the southwestern part of the Democratic Republic of Congo (DRC) from the northeastern part of Zambia. There is also a large stream known as Kenani Stream that flows roughly south to north into Lake Mweru. The mean altitude is 807 meters above sea level and the area has a marsh ecotype. There is a single rainy season from November

to May, followed by a cool dry season from May to August and a hot dry season from August to November (Figure 1.2). Rainfall follows a seasonal pattern, with a peak of 2700 mm during the rainy months and close to 0 mm during the dry months (Figure 1.2).

As in the rest of Luapula Province, Nchelenge is populated primarily by the Bemba-speaking peoples. In these villages, autonomous households are loosely organized under a headman, who is mainly seen as a mediator or ceremonial leader. A regional chief provides leadership to headmen and is consulted for important decisions within the geographical area. A household consists of a husband, wife, and children. A typical household has one hut, which is constructed with mud brick and a thatch or tin sheet roof. The most common means of livelihood for people in Nchelenge are fishing and agriculture. A majority of people engage in fishing in Lake Mweru and then move inland to participate in agricultural endeavors when fishing is seasonally (annually) not permitted, often for several months. The most important crop in this area is maize, but other crops such as peanuts, sugarcane, and sweet potatoes are also farmed. Interestingly, this part of Zambia is a cattle-free zone, as it is illegal to have cattle due to the presence of tsetse flies and risk of trypanosome transmission. Other animals kept in or associated with the household may include cats, dogs, goats, chickens, and guinea pigs.

Specific Aims

There is little information about the mosquito vectors and their role in human malaria transmission in Luapula Province. Over the last decade, a dozen entomological studies have been published in Zambia [4, 9-20], but none have investigated the vectors in Nchelenge District, where the prevalence of malaria is currently among the highest in

the country. The specific aims of this dissertation were to conduct mosquito collections in Nchelenge that would (1) define the dynamics and bionomics of malaria vectors temporally, and characterize the micro-spatial heterogeneity of *An. funestus* sensu stricto and *An. gambiae* s.s. during the wet season, (2) assess the disparity in visual identification and polymerase chain reaction (PCR) identification of fed and unfed mosquitoes across the three ICEMR field sites, and (3) characterize the multiple blood feeding behavior by malaria vectors during a single gonotrophic cycle and *P. falciparum* complexity of infection in infected mosquitoes to more accurately define transmission intensity.

Hypotheses

For the first aim, it is predicted that *An. funestus* sensu stricto and *An. gambiae* s.s. are the primary and secondary vectors of malaria transmission in Nchelenge District. Temporally, *An. funestus* will dominate throughout the year, whereas *An. gambiae* will proliferate in the wet season and declines in the dry season. Furthermore, there will be spatial heterogeneity of both proportions of mosquitoes and their entomological inoculation rates (EIRs). The hypothesis of the second aim is that both visually fed and unfed mosquitoes contain host blood meals when tested by PCR at all three ICEMR field sites in southern Africa. The proportion of blooded mosquitoes and the human blood index (HBI) will change when accounting for the visually "unfed" mosquitoes that are actually fed by PCR. The third hypothesis is that *An. funestus* and *An. gambiae* take multiple blood meals during a single gonotrophic cycle at high rates, resulting in an underestimation of the EIR. Additionally, infected *Anopheles* mosquitoes will harbor

multiple clones of *P. falciparum* that are transmitted with each bite, further characterizing foraging behavior in the context of multiple blood feeding and intensity of transmission.

Entomological Studies in Zambia

Before the 21st century, there was a scarcity of knowledge about malaria vector biology and distribution of vectors in Zambia. The flight ranges of *An. gambiae* sensu lato and *An. funestus* s.l. were studied in the copper mining town N'kana in northern Zambia and bordering the Democratic Republic of Congo [21]. However, the exact species of *Anopheles* was not determined as those respective complexes had not yet been defined [21]. Breeding site surveys from 1944-1946 were performed in northern Rhodesia (Zambia) and showed the presence of *An. gambiae* s.l. and *An. funestus* s.l., but the various locations were not well documented [22]. Further studies between 1962 and 1964 described the coexistence and potential hybridization of members A, B, and C of the *An. gambiae* species complex in Chirundu, Northern Rhodesia [18]. The *An. gambiae* species complex is made up of six members and at the time, the distinctness of members A, B, and C were defined by examination of male mosquitoes and sex-ratios in offspring of wild-mated female mosquitoes collected from households in areas where it was thought that all three members exist sympatrically [18]. A decade later, polytene chromosome analysis showed that *An. gambiae* sensu stricto (A) was not in the aforementioned locale, but instead *An. arabiensis* (B) and the non-vector species *An. quadriannulatus* (C) [19]. It is important to note that the complex was under selection pressure in Chirundu during the aforementioned studies as this may have affected the species composition over time (Personal communication with Dr. Clive Shiff, 2014). Many of the entomological studies during this time took place in southern Zambia, and

the identification of malaria vectors and their behavior in the northern part was not well documented.

Over the last decade, extensive entomological studies have been performed in Zambia to inform vector control strategies [12, 15, 23, 24]. Many of these studies were focused in Choma District, Southern Province (2004-Present) and were among the first to use molecular tools to investigate the entomological parameters of malaria transmission. An initial collection spanning from 2004-2006 identified *An. arabiensis* by both morphology and molecular techniques as the primary malaria vector at this southern Zambia site [13]. The role of *An. arabiensis* in an area with hypoendemic malaria transmission were also investigated, and revealed that *An. arabiensis* is highly anthropophilic, and in later studies, remained anthrophophilic despite a large ITN distribution campaign in 2007 [11, 13]. Additionally, it was observed that *An. arabiensis* had a multiple blood-feeding rate of 18.9% pre-ITN distribution, and then reduced to 9% after the introduction of ITNs, suggesting a strong heterogeneity in biting behavior by this vector and that ITNs had a significant effect on mosquito foraging behavior [15]. Although insecticide resistance was not detected in *An. arabiensis* in this study area, the East allele for the knock down resistant (*kdr*) gene mutation and upregulation of metabolic detoxification enzymes were observed in sympatric *Culex quinquefasciatus*, an arbovirus and filarial vector [16]. The knock down resistance gene, *kdr*, which affects the sodium voltage gated channel in insects, is a genetic resistance mechanism that confers cross resistance to pyrethroids and DDT. The *kdr* gene has two alleles, East and West, based on mutations that result in a leucine to serine or phenylalanine amino acid change respectively [25]. The detection of *kdr* in *Cx. quinquefasciatus* suggests that selection

pressure for insecticide resistance was present, but that the *An. arabiensis* population was apparently escaping this selection. The population genetic structure of *An. arabiensis* was also investigated after a severe drought in 2004-2005 in Choma District, Southern Province, Zambia; the results showed very little evidence of genetic structuring and no significant changes in allele frequency distributions or observed heterozygosity [23]. Despite these findings, it is crucial to understand the genetic population structure and barriers to gene flow of potential vectors in order to strengthen our understanding of malaria epidemiology and predict the flow of genes that could confer insecticide resistance or phenotypic changes within and between vector populations [26, 27].

More recently, an entomological study over a wider geographic scale took place at 18 sentinel sites in Zambia from 2008-2010, and helped define the main anopheline species found within the country [28]. These species included members of the *An. funestus* s.l. and *An. gambiae* s.l. complexes; Specifically, *An. gambiae* s.s., *An. arabiensis*, and *An. quadrannulatus* in the *An. gambiae* s.l. complex, and *An. funestus* s.s., *An. parensis*, *An. rivulorum*, *An. leesoni*, *An. vaneedeni, An. longipalpis, An. funestus-like* in the *An. funestus* s.l. complex, as well as *An. squamosus, An. coustani*, and *An. nili* s.s. [12, 13, 28]. In many of those surveyed areas with endemic malaria, *An. gambiae*, *An. arabiensis*, and/or *An. funestus* were found to be highly anthropophilic, endophilic, and harboring *Plasmodium falciparum* sporozoites [10, 11, 24, 28]. Such information is relevant for identifying the vectors and determining their role, if any, in human malaria transmission.

In addition to identifying major malaria vectors in Zambia, other countrywide studies have focused on insecticide resistance surveillance in areas where IRS and LLIN

distribution campaigns have occurred [28-30]. Insecticide resistance threatens to undermine vector control efforts by becoming ineffective against the malaria vectors and in some cases, causing an increase in human malaria rates [24]. The selection pressure resulting from widespread use of DDT previously, and more recently, pyrethroids and carabamates have resulted in the development of phenotypic, genetic, and metabolic resistances by *An. gambiae* s.s. and *An. funestus* s.s. in Zambia [28, 30]. The WHO bottle assay, which exposes mosquito samples to a series of different insecticides at standard doses to test for mortality (susceptibility) [31], performed in several parts of Zambia have suggested partial or complete phenotypic resistance of *An. gambiae* to deltamethrin, lambdacyhalothrin, alphacypermethrin, DDT, and carbamates and of *An. funestus* to the same pyrethroids and carbamates [28]. The genetic mutation, *kdr*-West, which confers resistance to pyrethroids and DDT, has only been observed in *An. gambiae* in Nchelenge, as well as the eastern areas of Zambia [8, 28]. Furthermore, throughout Zambia, increases in metabolic enzyme activity such as cytochrome P450s and glutathione s-transferases (GSTs), which metabolize insecticides such as pyrethroids, organochlorines, and carbamates, have been detected in *An. funestus* [30]. Due to these findings in Nchelenge District, there has been a shift to the organophosphate pirimiphos methyl (ACTELLIC 300 CS, Syngenta Limited) for IRS because both *An. funestus* and *An. gambiae* remain susceptible to it [32] (Personal correspondence with Mbanga Muleba, Tropical Disease Research Centre, Ndola, Zambia). However, to date, the primary vectors of malaria and their role in transmission have not been well defined in Nchelenge District despite LLIN campaigns since 2007, and IRS with alphacypermethrin (2008-2009), lambdacy halothrin (2010-2011), and carbamate (2012-2013) (Personal

correspondence with Mbanga Muleba, Tropical Disease Research Centre, Ndola, Zambia). It is therefore necessary for the malaria vector dynamics and bionomics in Nchelenge District to be characterized so that future control efforts are effective and can be evaluated.

Entomological Inoculation Rate (EIR) as a measure of malaria transmission intensity

The importance of vectors that drive transmission in a defined area can be determined by comparing their relative contribution to the intensity of *Plasmodium* transmission in a given area. The measure of human exposure to *Plasmodium* parasites via mosquito bites is known as the entomological inoculation rate (EIR), which is defined as the number of infectious bites per person per unit of time [33]. The utility of the EIR is that it can indicate which *Anopheles* species are important and unimportant vectors, and which vector has the greatest contribution to transmission in a particular locale during a time period. The EIR is conceptually straightforward and is measured by multiplying the sporozoite infection rate in the vector population by the nightly human biting pressure (human biting rate) for each mosquito species under investigation. Many researchers have noted variability and challenges in methods used to determine values for infection and biting rates [34-42]. Therefore, the EIR should only be considered an estimated measure of malaria parasite transmission. Additionally, the EIR does not take into account the infectivity success rate, or the number of infectious bites required to result in human infection [43]; In a laboratory setting, *An. stephensi* with *P. falciparum* sporozoites successfully transmitted the malaria parasite to only 5 out of 10 volunteers that received 1-2 infectious bites [43]. The infectivity success rate has been known to

range from 5% to 26% in endemic areas and is negatively associated with the EIR due to higher protective immunity, exposure to multiple infections, and longer occurrences of parasitemia [44, 45]. In Africa, transmission intensity as measured by EIR can vary from <1 to >1,000 infective bites per year. The EIRs can also fluctuate based on the geographical region, anopheline vectors, human behavior, and length of the transmission season [46-49]. It has been shown that even EIR levels of <5 infectious bites per person per year can sustain an excess of 40% *P. falciparum* prevalence [50]. At any level of transmission, malaria endemic areas are associated with heavy disease burden [50-54]. Models of transmission in highly endemic areas suggest that EIR can be dramatically reduced by 30- to 50- fold if bed nets and IRS are used, and can be further impacted by larviciding or a reduction in breeding sites, and by a decrease in the disease prevalence in humans [55]. Undoubtedly, significant reductions in transmission intensity are required to negate the public health consequences of endemic malaria in Sub-Saharan Africa [55, 56].

An assumption of the EIR is that mosquitoes take only one blood meal/bite per gonotrophic cycle. However, due to natural disturbances in the environment or the need for more blood to finish oogenesis, mosquitoes actually take multiple blood meals [57]. Multiple blood feeding behavior varies by species, and has been observed throughout Africa and even in other parts of the world. *Aedes aegypti* is well known for its high rate of multiple blood feeding and has been observed to have taken a second blood meal 45% and 18% of the time in south central Thailand and Puerto Rico respectively [58, 59]. In Western Kenya, the multiple blood feeding rates for the two dominant vectors *An. gambiae* s.s. and *An. funestus* s.s. was 11% and 14% respectively [57]. Infection status of

mosquitoes can also affect the number of blood meals taken during a gonotrophic cycle. *An. gambiae* s.s. has been reported to take more than one blood meal when infected due to *P. falciparum* mediated decrease in salivary gland apyrase activity, an anti-coagulant, at the bite site [60]. In southern Zambia, it was observed that the multiple blood feeding decreased from 18.9% to 9% pre- and post-ITN campaigns respectively [15]. Overall, multiple blood feeding behavior has serious implications for EIR estimates and mathematical models of malaria transmission because it not only increases the likelihood of a vector becoming infected, but may also increased the number of individuals exposed to the parasite during a single gonotrophic cycle. Thus, entomological studies that characterize local mosquito vectors foraging behaviors and account for multiple blood feeding behavior are key for understanding transmission intensity of *Plasmodium* by each vector.

In many parts of Africa where both *An. gambiae* sensu stricto and *An. funestus* s.s. are present, such as in the case of Nchelenge, the EIRs of both mosquito species indicate the primary and/or secondary roles in *Plasmodium* transmission [41, 61-70]. In Kenya, mosquito collections were performed in three epidemiologically and ecologically distinct districts. Collections in Malindi District, Kenya revealed that both *An. gambiae* s.s. and *An. funestus* s.s. equally contributed to transmission with daily EIRs of 0.48 and 0.50 [63]. Whereas in Kilifi District (urban), Kenya, *An. funestus* had a slightly higher daily EIR of 0.50 than *An. gambiae*, which had a daily EIR of 0.40. In the more rural district of Kwale, Kenya, *An. funestus* predominated with a daily EIR of 0.75 and *An. gambiae* had a daily EIR of 0.42 [63]. Studies in other areas of Kenya have revealed the important role of spatial-temporal dynamics in malaria vector composition and human malaria

intensity [66]. In the Kenyan highland site, Iguhu, *An. gambiae* s.s. was the dominant vector and *An. funestus* s.s. was the secondary vector with annual EIRs of 13.1 and 3.5 respectively. In the lowland site, however, *An. funestus* was the primary vector and *An. gambiae* was a minor vector with annual EIRs of 21 and 10.1 respectively [66]. In Uganda, *An. gambiae* s.s. was the dominant vector and very few *An. funestus* s.l. were caught at three sites (two urban and one rural sites) [64]. As a result, only the EIRs of *An. gambiae* were measurable and compared among collection methods. Although the EIR differed among all three sites and also within each site, the range of EIRs was larger for the rural area with 70-95 infectious bites per person per year than the urban areas with an of EIR of 73 infectious bites per person per year [64]. At all three sites, the EIRs fluctuated with season, but it was only in the rural site where seasonality of transmission was significant [64]. The relative roles of multiple vectors in each locale can also fluctuate due to environmental factors such as precipitation, temperature, specific humidity, vegetation, as well as elevation [63]. Although many endemic areas with sympatric *An. gambiae* s.l. and *An. funestus* s.l. may predict that *An. gambiae* s.s. is the dominant vector with some secondary contribution from *An. funestus* s.s., it has actually been observed that *An. funestus* most often predominates in rural areas of Sub-Saharan Africa [63]. Therefore, in an area like Nchelenge, where holoendemic malaria is maintained by both *An. gambiae* s.s. and *An. funestus* s.s., it is crucial to identify the extent to which each vector contributes to *Plasmodium* transmission and if there are any spatial and/or temporal dynamics in transmission intensity.

Plasmodium falciparum complexity of infection (COI) and *Anopheles* mosquitoes

The *Plasmodium falciparum* sporozoite infection rate (SIR), a major component of the EIR, is the proportion of mosquitoes found to harbor sporozoites in the salivary gland and therefore infectious to human hosts. The *Plasmodium* sporozoites themselves can be composed of multiple clones in an endemic area, defined as the complexity of infection (COI). The COI may influence clinical outcomes, and can be used to to determine if genetic diversity is associated with high or low malaria prevalence in a particular locale [71-74]. In chronic asymptomatic infections, which often occur in high transmission settings, large numbers of clones persist as asexual forms and gametocytes [75]. On the other hand, a smaller number of parasite clones are found in low transmission settings where symptomatic infections are often observed [75]. One of the fundamental characteristics of the malaria parasite's multiple clones in natural infections is its ability to produce gametocytes with subsequent recombination in the mosquito midgut, a process that produces novel parasite clones and contributes to the parasite's overall evolutionary success [76]. The complexity of *P. falciparum* clones has been correlated to the frequency of crossing when a mosquito acquires an infectious human blood meal [77-80]. The level of transmission intensity directly affects the number of infected hosts and the number of parasite clones per individual [81]. This in turn, depending on the endemic setting, influences parasite population genetic structure, potentially resulting in the development of virulence, drug resistance, and changes in the parasite's ability to evade the human host immune system [81]. In Tanzania, the Gambia, and Sudan, where the EIRs vary from high to low respectively, the average number of clones was 3.3, 2.3, and 1.3 respectively, suggesting that there is equal transmissibility of

the clones with each bite [82]. However, equal transmissibility of parasite clones has not been supported in cases where the observed rate of crossing was lower than expected based on transmission intensity, suggesting that only a proportion of transmitted clones are able to reach the erythrocytic stage in humans [76, 78, 83]. When parasite genotypes were compared between infected anopheline vectors and humans in the Gambia, a variety of clones were found in the mosquito, but not in infected human blood upon which the mosquitoes had fed on [76]. The authors suggested that the levels of particular clones in human blood decrease to undetectable levels due to the host immune response, but upon entering the mosquito midgut, the clones are able to cross-mate and produce many clones [76]. Additionally, some clones detected in the mosquito did not reach the human blood-stage [84]. Clearly, the interplay of *P. falciparum* infection complexity in mosquitoes, transmission intensity, and subsequent development in the human blood stage are not well defined.

P. falciparum COI and *Anopheles* Multiple Blood Feeding Behavior

In the context of multiple blood feeding behavior, which results in an underestimation of the EIR, multiple clones of the parasite are transmitted to more than one person by a single mosquito during a gonotrotrophic cycle. These multiple clones may harbor genetic changes that allow for its survival, and are constantly accumulating mutations that allow the parasite to adapt to new conditions during recombination events in the mosquito midgut. Using the mosquito to detect multiple clones may be an additional tool for evaluation of vector control measures, whereby a decrease in the transmission intensity is also associated with a decrease in the COI, indicative of a lower transmission setting. This may be especially useful in endemic areas where there may not

be reliable health center reporting, limited resources for malaria testing, and/or unwillingness by individuals to give blood samples. The COI of malaria in infected mosquitoes may also be a useful surveillance system for identifying and predicting potential parasite clones that may successfully circulate through the human population and association novel clones with clinical malaria cases. While multiple blood feeding behavior affects the EIR estimates, it is equally important to understand how anopheline vector transmission intensity affects the COI of the malaria parasite. It is therefore crucial to molecularly identify *P. falciparum* clones transmitted by an infectious mosquito to further our understanding of *Anopheles* foraging behavior in an endemic area.

Anopheles funestus **and** ***An. gambiae*** **Host-Seeking Behavior**

Host choice by mosquitoes is often mediated by olfactory signals, as well as physical indicators including temperature and humidity [42]. Carbon dioxide, 1-octen-3-ol [85], L-lactic acid [86, 87], acetone [85], and carboxylic acids [88] have been identified as a strong olfactory stimuli for mosquitoes, and have been detected in human and animal sweat, skin, and breath [89]. The attraction to these olfactory signals by mosquitoes differs by species. For example, it has been shown in laboratory conditions that *An. gambiae* s.s. is more attracted to acetone and CO2 in human breath, whereas *An. stephensi*, which is more zoophilic, responded strongly to CO2 and 1-octenol-3-ol from cattle [85]. Other factors that may mediate mosquito biting behavior include host size and even infection status; previous studies have suggested that *An. gambiae* s.s. is more attracted to adults than infants, men than women [90], and individuals with gametocytes are more likely to be bitten by malaria vectors than those who are not infected or only

have blood stage malaria only [91]. Other factors yet to be identified are also likely playing a role in mosquito host choice.

The feeding preferences of *Anopheles* mosquitoes are especially important to define because they offer valuable insight into *P. falciparum* transmission intensity in a given location. The proportion of fed vectors with human blood meals, also known as the human biting index (HBI), is measured to better understand host preference and vectorial capacity. The efficiency of transmission, also known as the vectorial capacity, as well as the reproductive ratio Ro, varies as the square of the HBI [92, 93]. If a vector changes its feeding preference to non-human hosts, then transmission efficiency to humans is reduced. Many studies have observed a decrease in HBI as a response to vector control interventions [94-96]. As a result, HBI is useful in evaluating malaria control programs. In Sub-Saharan Africa, entomological studies have identified *Anopheles gambiae* s.s. and *An. funestus* s.s. as the most dangerous and medically relevant vectors of malaria [70, 97, 98]. Both vectors present a threat to malaria control efforts because of their expansive distribution throughout the African continent, highly anthropophilic and endophilic behavior, and ability to develop both genetic and behavioral resistances to commonly used insecticides [16, 28, 99-101]. Furthermore, local socio-economic and environmental factors contribute to the extent of disease transmission by anopheline mosquitoes [13, 99, 102].

The HBI can also indicate heterogeneity, or inequality in risk, in foraging behaviors, which substantially affect EIR estimates and ultimately malaria risk. For example, some people may be bitten more than others due to house construction, sleeping patterns, use of vector control such as LLINs or IRS, or proximity to larval breeding sites

[15, 103]. When heterogeneity has been included in malaria models, the results in Zambia have indicated that approximately 20% of people receive 80% of infectious mosquito bites [104]. As a result, the individuals who are bitten more often are suspected to contribute more to infecting the vector population and also receive more infectious mosquito bites [104]. These individuals may also serve as the reservoirs of malaria parasite during the dry season, which then allows infected vectors to continue transmission into the wet season. In addition, other models have suggested that in areas with high EIRs (>100), biting heterogeneity increases the basic reproductive rate (Ro), or the number of infected cases originating from one infectious individual, whereas in localities with low or moderate EIRs, biting heterogeneity is predicted to lead to lower transmission rates [105]. In fact, heterogeneity in biting can alter the Ro approximately 2-4 times [106, 107]. Regardless of transmission intensity, accounting for heterogeneity is crucial when developing and implementing control efforts because resources can be targeted towards high-risk individuals and populations with the highest disease burden. This strategy is not only cost-effective, but can also be the difference between failure and success of malaria control efforts [105]. On the other hand, a disproportionate impact on a community may be observed in the presence of targeted control efforts because infected vectors may shift to nearby areas that did not receive any interventions [104].

In Kenya, it was observed that between 1990 and 2000, the *An. gambiae* and *An. funestus* densities increased [108]. However, almost a decade later and with implementation of vector control, both *An. gambiae* and *An. funestus* populations declined dramatically, almost to undetectable levels at times [108]. Similarly, the HBI of *An. gambiae* and *An. funestus* in 1990 was 99% and 100% respectively, but then

decreased to 16% and 3% respectively between 1997 and 2008 [108]. These once highly anthropophilic vectors shifted to being zoophilic, feeding on mainly bovine, as well as goat, donkey, and chicken hosts [108]. While the primary vectors were declining between 1997 and 2008, an upward trend was observed in *An. arabiensis*, which became the dominant malaria vector in late 2000, and *An. merus*, and *An. quadriannulatus* also increased in relevance [108]. The changes in vector dynamics over a period of twenty years has been attributed to many factors such as vector control, housing construction, and man-made ecological disturbances [108, 109]. This is a primary example of the importance of vector identification because control measures often lead to the emergence of other *Anopheles* species that may contribute to malaria transmission, as well as discerning shifts in biting behavior of the predominant vectors [110]. Another study in western Kenya showed that both *An. gambiae* s.s. and *An. funestus* s.s. had similar HBIs of 60-70% across three villages and were endophilic, but *An. funestus* was the predominant malaria vector because it was 6.6-8.2 times as likely to bite a person indoors than *An. gambiae* [111]. Furthermore, the effect of permethrin-impregnated sisal curtains placed under eaves of houses led to *An. gambiae* to display anthropophily and exophily [111]. Surprisingly, *An. funestus* switched its preferred host to cattle [111]. When exposed to vector control, there were shifts in both vectors' behaviors that are relevant for surveillance and implementation of future interventions. Spatial and temporal variables, malaria control interventions, ecological changes, and human behavior and patterns across Africa influence the variability of *An. gambiae* and *An. funestus* foraging behavior in various locales. By understanding the occurrence of *An. gambiae* and *An.*

funestus in a malaria endemic area and their feeding behavior, prevention tools that reduce human-vector contact can be better designed and implemented.

Summary

This dissertation represents some of the first vector studies in Nchelenge District, Zambia that address the role of anopheline mosquitoes in an area with holoendemic malaria. Data are presented on the spatial and temporal dynamics of malaria vectors, transmission intensity, and blood feeding behavior. Additionally, the field and molecular techniques used to score unfed and fed *Anopheles* species are investigated to assess more accurate measurements of the human blood indices across three epidemiologically distinct sites in Zambia and Zimbabwe. Lastly, heterogeneity in foraging behavior by both *An. gambiae* sensu stricto and *An. funestus* s.s. are explored by assessing the spatial and temporal changes in multiple blood feeding behavior, human gender preference, and determining the complexity of infection in infected mosquitoes. Collectively, these studies have succeeded in initiating advanced entomological research in northern Zambia, contributing to the training of local personnel in both entomology and field techniques, and generating awareness about entomology and its crucial role in malaria transmission in Nchelenge.

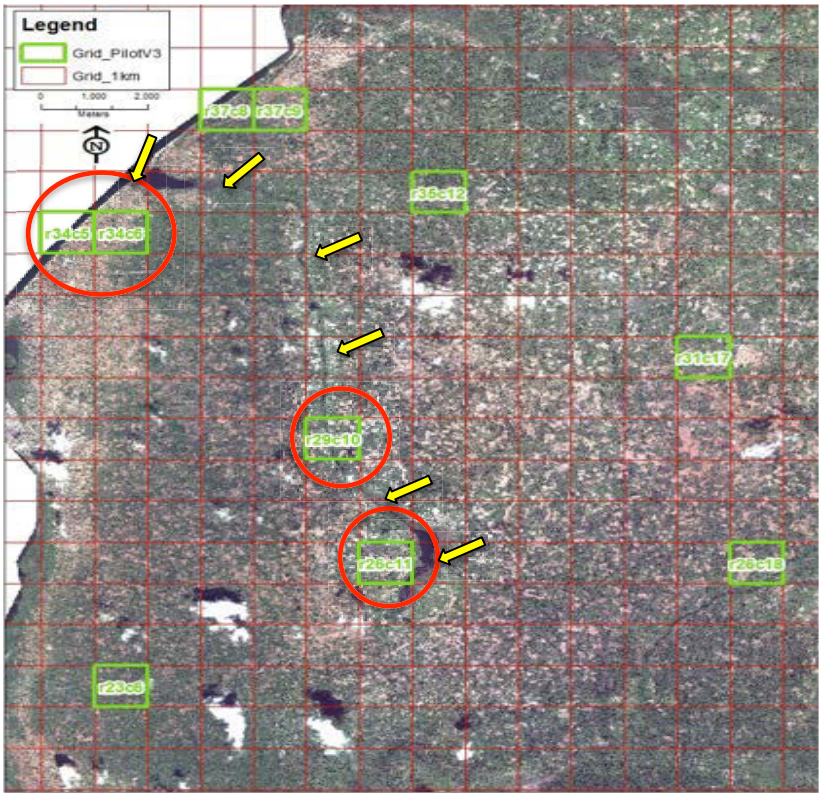

Figure 1.1. Satellite image of the study area in Nchelenge District. The initial 1-km^2 grids for ICEMR epidemiological and entomological surveys are highlighted in green. The white areas on the left side of the image represent Lake Mweru. The yellow arrows point to Kenani Stream that flows into Lake Mweru. The red circles denote the grids where mosquito collections were performed for thesis research: Katuna, Yenga, and Malulu villages are located in grids r34c5 and r34c6, Kapande B village is located in grid r29c10, and Kapande B village is located in grid r26c11.

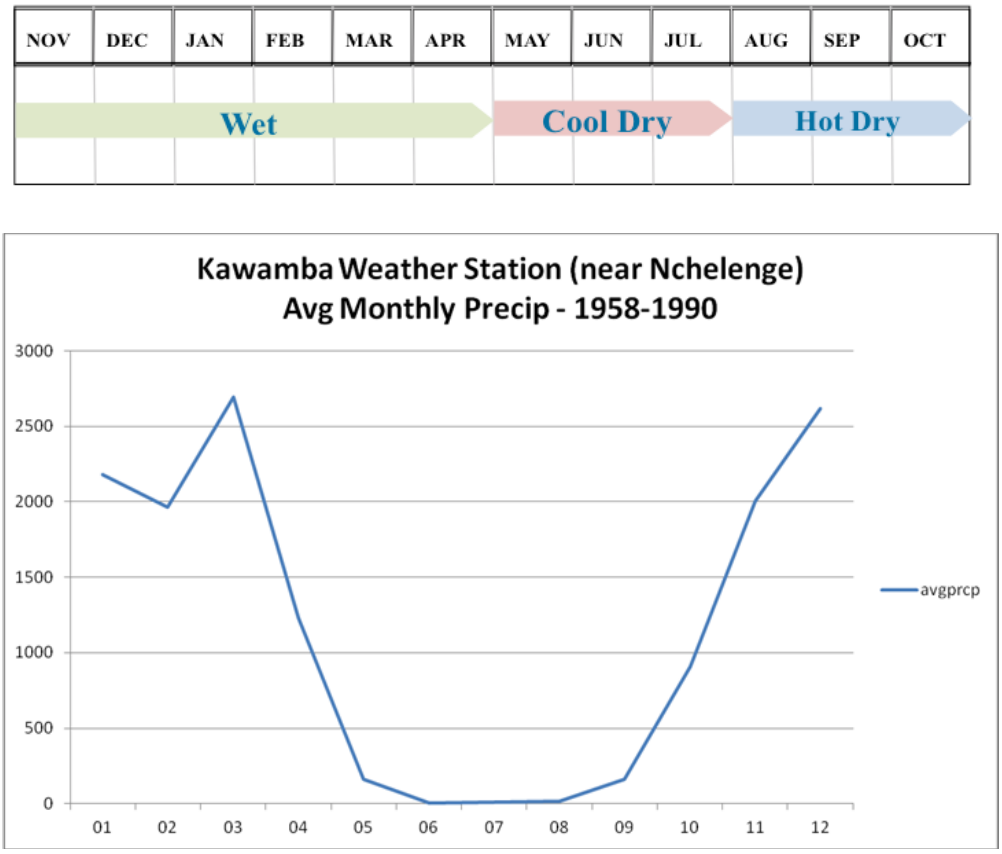

Figure 1.2. Seasons and Average Monthly Rainfall in Nchelenge District. The wet season is from November to May, the cool dry season is from May to August, and the hot dry season is from August to November. The rainfall also follows a seasonal pattern in Nchelenge.

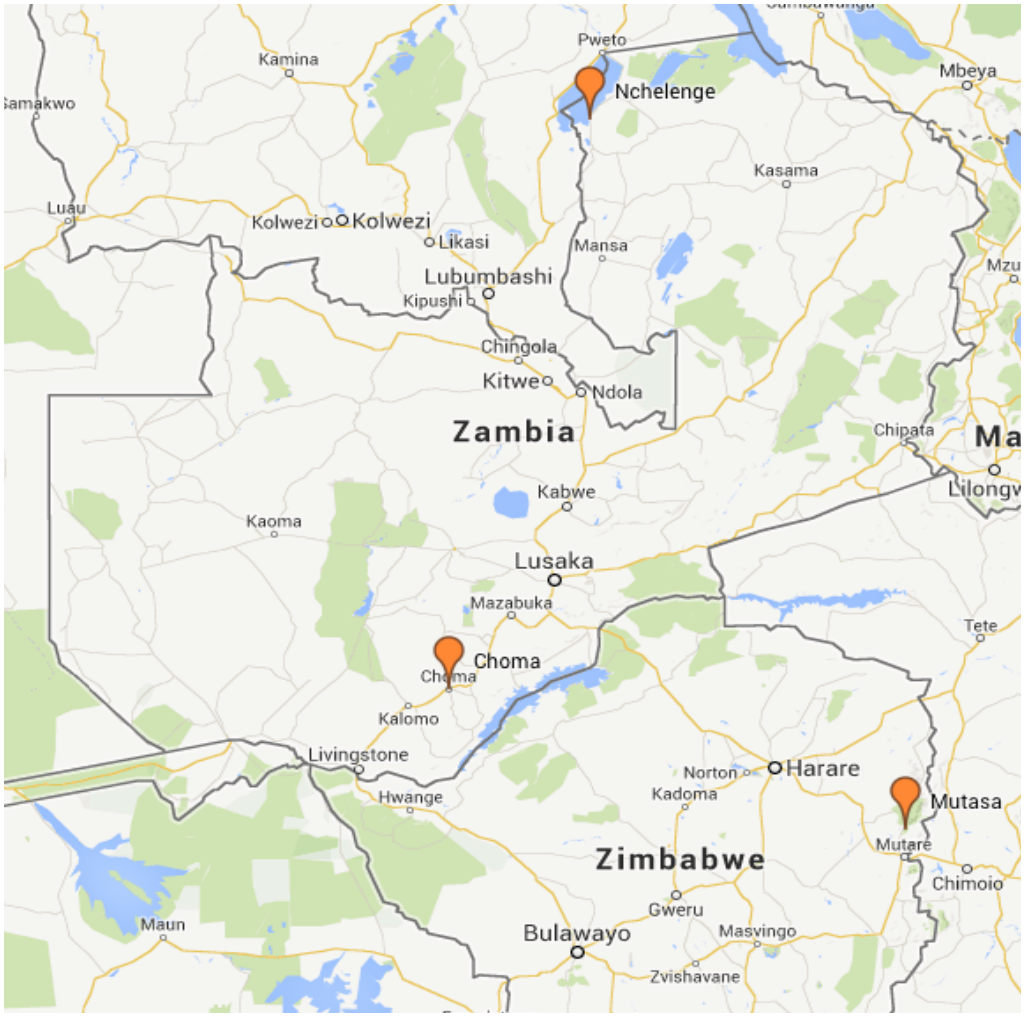

Figure 1.3. The southern Africa ICEMR field sites. Nchelenge District is in northern Zambia and represents unsuccessful malaria control, Choma District is in southern Zambia and represents successful malaria control, and Mutasa District is in eastern Zimbabwe and represents resurgent malaria.

Chapter II

Spatial and Temporal Dynamics of Malaria Vectors in Nchelenge District, Zambia

Abstract. Located along Lake Mweru and bordering the Democratic Republic of Congo (DRC) in Luapula Province, northern Zambia, Nchelenge District experiences holoendemic malaria transmission despite the implementation of control measures such as long lasting insecticide treated bed nets (LLINs) and indoor residual spraying (IRS). The dominant malaria vectors that contribute to *Plasmodium falciparum* transmission in this area had not previously been defined. This study identified the malaria vectors, their feeding behavior, and entomological inoculation rates (EIR) both spatially and temporally. Three collections were performed using both Center for Disease Control light traps (CDC LTs) and pyrethroid spray catches (PSCs): March-April 2012 (wet season), August-September 2012 (dry season), and March-April 2013 (wet season) at three villages along Lake Mweru and two villages along Kenani Stream, a large stream that flows from south to north into Lake Mweru. The collections revealed that *Anopheles funestus* sensu stricto and *An. gambiae* s.s. are the main malaria vectors, are highly anthropophilic, and *An. funestus* has the higher EIR year-round. Temporal and spatial changes in vector dynamics and other entomological parameters were observed within Nchelenge District. During both wet season collections, it was observed that *An. gambiae* was the predominant vector in villages along Lake Mweru, whereas *An. funestus* was the predominant vector inland and along Kenani Stream. During the dry season collection, *An. gambiae* population decreases dramatically and *An. funestus* is the primary vector in all areas. Additionally, the abundances of *Anopheles* vectors were higher along Kenani

Stream than Lake Mweru. Together, the vector data in Nchelenge present unique opportunities to further our understanding of malaria transmission and the implications for malaria control in high-risk areas.

Introduction

Anopheles funestus sensu stricto and *An. gambiae* s.s. are the most notorious vectors of *Plasmodium falciparum* malaria transmission in sub-Saharan Africa. Each of these species' distributions, their abilities to transmit the malaria parasite, and other demographic and environmental factors are heterogeneous and influence the intensity of malaria transmission [63]. The relative composition of the vectors in areas where both *An. funestus* and *An. gambiae* are sympatric and their respective feeding behaviors vary from region to region in Africa. When both *An. funestus* and *An. gambiae* are present in a community, disease transmission can be exceedingly high. This often occurs because both vectors exploit different breeding habitats and may appear at different times, which can prolong the transmission season [63]. For example, *An. gambiae* tends to oviposit in temporary breeding sites such as puddles and animal foot prints, which are abundant during the rainy season [26, 97, 112]. On the other hand, *An. funestus*, which prefers more permanent breeding sites, peaks at the end of the rainy season and the beginning of the dry season [26, 97, 112]. Both vectors have been shown to be highly anthropophilic, expressing a tendency to feed on humans. In addition, *An. funestus* and *An. gambiae* are considered to be endophagic and endophilic, feeding and resting indoors respectively [26, 112].

However, there may be variability in these foraging behaviors for both vectors. Field sampling in Uganda revealed that *An. gambiae* s.s. had a greater tendency to bite humans outside versus *An. funestus* s.s. [67]. Conversely, after the implementation of long-lasting insecticide-treated nets (LLINs) in Benin, there was a shift in *An. funestus* s.s. peak biting period to early morning, which is alarming because in rural Africa,

villagers often awake pre-dawn to work and are therefore no longer protected by bed nets [65]. In communities where both vector species contribute to transmission, there may be differences in the dominant and secondary vectors depending on the season and the geography. In western Kenya, it was found that *An. gambiae* s.s. was the major vector species driving transmission in the lowlands and highlands, but in one of the lowland villages which had experienced recent larval habitat changes, *An. funestus* s.s. was found to be the major vector species [66]. There was pronounced spatial heterogeneity in vector composition and transmission rates in the lowland areas [66]. Differences in vector composition, biting behaviors, and transmission intensities among study sites that also vary temporally and spatially suggest unequal risk of malaria transmission within an area.

Heterogeneity in malaria risk have implications for developing and targeting interventions because a single vector control measure such as indoor residual spraying (IRS) may successfully interrupt transmission in a low transmission setting, whereas multiple vector control measures such as IRS, LLINs, larviciding, and/or larval habitat source reduction may be needed to have an impact in high transmission settings [55, 102, 113-117]. A recent study by Coetzee et al. (2014) demonstrated that both *An. funestus* s.s. and *An. gambiae* s.s. showed a high level of resistance to pyrethroid and carbamate insecticides in northern Zambia and eastern Zimbabwe [8] (Personal communication with Dr. Maureen Coetzee). In those same locations, *An. gambiae* also displayed resistance to DDT, while *An. funestus* remained susceptible [8] (Personal communication with Dr. Maureen Coetzee). The mechanisms of resistance in both of these vectors have been investigated; resistance by *An. funestus* appears to be mediated by up regulation of

detoxification enzymes, while *An. gambiae* has the *kdr*-west mutation that confers cross-resistance to pyrethroid and DDT insecticides [8]. Both vectors remain susceptible to the organophosphate class of insecticide, prompting the recent IRS campaign with Actellic 300 CS (Syngenta Limited). Therefore, it is necessary to identify the vectors, characterize their feeding behaviors, and determine their roles in malaria transmission in each locale using traditional field sample techniques, and ultimately use the findings to inform successful control strategies.

Nchelenge District in Luapula Province, northern Zambia experiences holoendemic transmission of *Plasmodium falciparum* [7]. Despite clinical evidence of high transmission rates, the mosquito vectors and their characteristics that influence transmission have not been well characterized in Nchelenge. Recent studies over the last decade have investigated *An. arabiensis* extensively in Southern Province, as well as *An. funestus* s.s. and *An. gambiae* s.s. mainly in the context of country-wide vector control and insecticide resistance surveillance and management, but none of the country-wide study sites were in Nchelenge District [11, 13, 17, 23, 29, 30, 32]. Preliminary mosquito collections by the southern Africa ICEMR in-country partner Tropical Disease Research Centre (Ndola, Zambia) suggested that *An. funestus* s.s. and *An. gambiae* s.s. are the primary and secondary vectors respectively and together, contribute to the maintenance of year-round transmission in Nchelenge.

The specific aims of this study were to investigate the spatial and temporal compositions of the malaria vectors *An. funestus* s.s. and *An. gambiae* s.s. in two ecologically distinct habitats within Nchelenge District. We also sought to characterize the blood feeding behavior by determining the human blood index (HBI). each vector's

contribution to transmission intensity through estimation of the entomological inoculation rate (EIR) was determined. This study provides fundamental information on both the spatial and seasonal heterogeneities in vector dynamics and transmission intensities of *An. funestus* s.s. and *An. gambiae* s.s. populations in Nchelenge District.

Materials and Methods

Study Area.

This study was carried out in association with the Johns Hopkins Southern Africa International Centers of Excellence for Malaria Research (ICEMR) project that is being carried out at three field sites: Choma District, southern Zambia (16.39292°S, 26.79061°E), Nchelenge District, northern Zambia (9° 19.115'S, 28° 45.070'E), and Mutasa District, eastern Zimbabwe (18° 23.161'S, 32° 59.946'E) (See Figure 2.1). The focus of the research reported here is Nchelenge District, Luapula Province, in Zambia at an elevation of approximately 807 meters above sea level and in a marsh ecotype (Figure 2.2). The district lies along the eastern perimeter of Lake Mweru, which serves as the border between the southwestern part of the Democratic Republic of Congo (DRC) and the northeastern part of Zambia. Flowing from south to north into Lake Mweru, Kenani Stream is also found in the area. There is a single rainy season from November to May, followed by a cool dry season from May to August and a hot dry season from August to November (Figure 2.3). Rainfall follows a seasonal pattern, with a peak of 2700 mm during the rainy months and close to 0 mm during the dry months (Figure 2.3). Passive surveillance of health centers in the district have revealed that there is some seasonality in confirmed malaria cases, but the overall rates throughout the year remain high (Figure

2.4). Longitudinal and cross-sectional households that were already enrolled in the ICEMR program, easily accessible, and were also located within two defined 1-km^2 grids along both Lake Mweru and Kenani Stream were chosen for mosquito sampling. Mosquito collections were conducted at three villages in grids r34c5 and r34c6 (Katuna, Yenga, and Malulu) along Lake Mweru and two villages in grids r29c10 and r26c11 (Kapande B and Mutepuka) near Kenani Stream (Figure 2.2). These villages are representative of the local demography and landscape, reflect the migration of local people from fishing to farming livelihoods (seasonally) annually, have a high malaria incidence, accessible and willing to participate in the study. Both long-lasting insecticide treated nets (LLINs) and IRS programs have been implemented in Nchelenge District starting in 2006 and 2007 respectively. The LLIN distribution campaign in Nchelenge took place in 2012 and resulted in an average 1.24 nets per person [7]. Indoor residual spraying was performed primarily in areas along a major road near Lake Mweru in 2013 using a carbamate insecticide (Personal communication with Dr. Mike Chaponda, TDRC, 2014) (Figure 2.5).

Mosquito Collection and Handling.

Mosquitoes were collected by Center for Disease Control light trap (CDC LT) and pyrethroid spray catch (PSC) in three villages (Katuna, Yenga, and Malulu) near Lake Mweru and two villages (Kapande B and Mutepuka) inland and near Kenani Stream during the periods March 24-April 10, 2012 (wet), August 27-September 9, 2012 (dry), and March 5-April 25, 2013 (wet). Additionally, aspiration collections were performed in the March-April 2012 and August-September 2012, and were only included for determining vector abundances. Collection methods were approved by the Johns Hopkins

Bloomberg School of Public Health IRB (#00003467) and in Zambia (TDRC/ERC/2010/14/11). During the March-April 2012 (wet) and August-September 2012 (dry) collections, lakeside and streamside villages were sampled on alternate days each week. For March-April 2012 collection, sampling took place at 11 households near the lake and 11 households along the stream. In the August-September 2012, collections took place at 21 households lakeside and 15 households streamside. From March-April 2013, 39 lakeside households and 38 streamside households were sampled on alternate weeks. During each collection week, CDC LTs were performed on Monday, Wednesday, and Friday, and PSCs on Tuesday, Thursday, and Saturday. CDC LTs were hung indoors next to sleeping persons, approximately 1 m above the floor, and would typically run from 6:00pm to 6:00am. PSCs were performed in the morning (6:00am-10:00am) in selected households, where white sheets were placed over all surfaces and a 100% synthetic aerosol pyrethroid was applied towards the ceiling, eaves, walls, and the home closed. After approximately 15 minutes, the sheets were taken out of each household and knocked down mosquitoes were collected. All field-caught mosquitoes were killed immediately by freezing. The female anopheline mosquitoes were then morphologically identified to species using a dichotomous key [97, 118] and dissecting microscope (both vectors and non-vectors). Up to three mosquitoes were placed in each labeled 0.6 mL microcentrifuge tube containing silica gel desiccant and cotton wool, and stored either at room temperature or frozen at -20°C until laboratory processing, which took place at Johns Hopkins University Bloomberg School of Public Health in Baltimore, Maryland.

DNA Preparation and PCR.

For all collected anophelines, the head and thoraces were separated from the abdomen, and DNA extraction of the abdomen was performed using a modified salt extraction [119]. The morphological identification of anopheline mosquitoes was confirmed using a polymerase chain reaction (PCR) specific for members of the *An. gambiae* or *An. funestus* complexes [120-123]. If a mosquito sample could not be identified by the *An. gambiae* complex and *An. funestus* complex-specific PCRs, then an ITS2 PCR was used [121]. The ITS2 PCR utilized amplifies the intergenic spacer region 2 of the ribosomal DNA, and has a range of base pair sizes that are specific to other anopheline species [121]. The mosquitoes that were molecularly confirmed as *An. gambiae* s.s. were also tested for molecular M- (also known as *An. coluzzii*) or S- form using the Favia et al. 2001 protocol. Briefly, the 5' end of the intergenic rDNA spacer region is amplified to produce a 727 base pair band for M-form, 475 base pair band for S-form, and both bands for hybrid specimens [124].

All specimens, regardless of being visually fed or unfed, were then tested for host animal by PCR using the Kent et al. (2005) multiplex PCR, which amplifies the cytochrome b gene of the mitochrondrial genome producing a range of mammalian host specific bands from 132 to 680 base pairs [13]. A modification to the Kent et al. PCR was made to better detect a human host blood meal; the original Kent et al. forward primer to detect human blood meal was replaced with another forward and reverse primer set that amplifies a 193 base pair region of the cytochrome B gene. The forward primer is FOR16068: 5'- GAC TCA CCC ATC AAC AAC CG -3' and the reverse primer is REV16260: 5'- GGC TTT GGA GTT GCA GTT GA -3'.

Samples that did not amplify a band(s) for host blood meal were then tested with a more sensitive PCR [11], which amplifies a 98 base pair region of the cytochrome b gene of the mitochondrial genome of the mammalian host, followed by a restriction fragment length polymorphism (RFLP) assay specific to an animal host of interest [11].

The abdominal DNA for each anopheline was then tested for the presence of the *P. falciparum* parasite. The Norris et al. PCR, amplifies a small portion of the cytochrome b gene of *P. falciparum,* and is more sensitive than the commonly used Snounou et al. (1993) PCR, and has an expected size of 183 base pairs [125].

Enzyme-linked Immunosorbent Assay (CSP-ELISA) for Plasmodium falciparum Detection

The CSP-ELISA method as described by the Malaria Research and Reference Reagent Resource Center (MR4) was used to detect *P. falciparum* circumsporozoite protein (CSP) in the mosquito head and thorax. Briefly, a 96-well U-bottom plate is incubated overnight with *P. falciparum* CSP capture antibody. Then, each mosquito head and thorax is homogenized using a pestle, and added to the plate (38 mosquitoes per plate) with CSP capture monoclonal antibody. Following a two-hour incubation, the plate is washed 7 times, and then the CSP monoclonal antibody with a conjugated peroxidase tag is added to the plate and incubated for one hour. The plate is then washed 7 times, and ABTS solution is added to visualize the presence of CSP in the mosquito head and thorax for one hour. If CSP protein is present in the mosquito head and thorax, then the well containing an individual mosquito will turn green and suggests that the mosquito was infectious. The plate is placed in a spectrophotometer set at 400-nanometer wavelength for analysis. The values associated with each mosquito that are

two times the average of the negative controls on the plate are considered to be CSP or *P. falciparum* positive. CSP-ELISA was performed on all *Anopheles* mosquitoes in the March-April 2012 and August-September 2012. However, due to the large number of anophelines caught during the March-April 2013 collection, a representative subsample form all collection households was used for the assay.

Spatial and Temporal Mapping of Malaria Vectors

In order to map the presence of malaria vectors and their proportions within each household in Nchelenge District both spatially and temporally, Geographic Information Systems (GIS) methods were utilized. Specifically, ArcMap 2.0 (ESRI ArcGIS) software was used to create a layer with a satellite image of Nchelenge District. Individual households with unique GPS coordinates where collections took place were added to the map, and then the vector species and their abundances relative to each household were added as pie charts. This was done for the Nchelenge March-April 2012 (wet), August-September 2012 (dry), and March-April 2013 (wet) collections to make observations about changes in vector composition and abundances spatially and temporally.

Human biting rate (HBR) and Entomological Inoculation Rate (EIR)

The average number of bites per person per night was calculated based on two collection methods. First, the number of foraging *An. funestus* and *An. gambiae* vectors from CDC LTs were divided by the number of people sleeping the prior night in the household. Second, the number of human fed *An. funestus* and *An. gambiae* from PSCs were divided by the number of inhabitants in each household. Because CDC LT and PSC collections attempt to identify foraging or fed and resting mosquitoes respectively, and

were performed at households unsystematically and not always repeated, the two methods and their estimates of HBR were not compared statistically.

The annual EIR for each vector species per household was calculated as the product of the human biting rate, sporozoite infection rate, and 180 days (6 months for each season), as collections were conducted in either the wet or dry seasons. Both the HBR and EIR were estimated using CDC LT only, CDC LT and PSC, and PSC only collections to observe variation in measurements. The average of all household EIRs was the overall EIR estimated for Nchelenge District. For the March-April 2013 collection, lakeside and streamside villages further defined the overall EIR calculations.

Statistical Analysis

The abundances, SIR, and EIR of *An. funestus* sensu stricto and *An. gambiae* s.s. within and among the March-April 2012 (wet), August-September 2012 (dry), and March-April 2013 (wet) collections were compared using STATA version 11. For comparison of vector abundances and EIRs, the negative binomial regression model for over-dispersed data was used. Logistic regression model was used to compare SIRs. A *P* value less than 0.05 was considered statistically significant. Using an Excel spreadsheet, the kappa statistic was calculated to classify the agreement between CSP-ELISA and PCR method results as "poor" (< 0.40), "fair" (0.40-0.70), and "excellent" (> 0.70).

Results

Seasonal and Spatial Variation

From March 24-April 10, 2012 (wet season), a total of 411 *Anopheles* mosquitoes were collected using CDC LTs, PSCs and aspirations, and was composed of 83.5% (n =

343) *An. funestus* s.s., 8.8% (n = 36) *An. gambiae* s.s., and 7.7% (n = 32) *An. leesoni.* Within the collection, there was an overall and significantly higher number of *An. funestus* caught per household, 9.5 times that of *An. gambiae* (Ratio= 9.5, 95% CI: 4.8, 19.1, P= 0.00).

The August 27-September 9, 2012 (dry season) collection from CDC LTs, PSCs, and aspirations was composed of 1324 anophelines, of which 99.3% (n = 1315) were *An. funestus* s.s. and 0.6% (n = 8) were *An. gambiae* s.s., and 0.1% (n = 1) *An. leesoni*. There were 161 times more *An. funestus* caught per household per trap night or morning than *An. gambiae* (Ratio= 161, 95% CI: 66.8, 387.9, P= 0.00).

A total of 2989 *Anopheles* mosquitoes were caught from March 5-April 25, 2013 (wet season) using CDC LTs and PSCs. The majority of the collection was made up of *An. funestus* s.s. (80.9%, n = 2417), followed by *An. gambiae* s.s. (18.9%, n = 564) and *An. leesoni* (0.2%, n = 8). Within the collection, the number of collected *An. funestus* was 4.3 times that of *An. gambiae* (Ratio= 4.3, 95% CI: 3.1, 6.0, P= 0.00).

Compared to the March-April 2013 collection, there were 0.53 and 2.6 times the number of *An. funestus* collected per household per trap night or morning in the March-April 2012 (Ratio= 0.53, 95% CI: 0.30, 0.92, P=0.023) and August-September 2012 collections (Ratio= 2.6, 95% CI: 1.4, 4.8, P= 0.002). The August-September 2012 collection had 5.1 times the number of *An. funestus* caught per household in the March-April 2012 collection, and was also statistically significant. (Ratio= 5.1, 95% CI: 2.9, 9.0, P= 0.00). There were 3.2 and 13.8 times the number of collected *An. gambiae* in the March-April 2012 (Ratio= 3.2, 95% CI: 1.3, 8.3, P= 0.013) and March-April 2013 (Ratio= 13.8, 95% CI: 6.1, 31.5, P= 0.00) collections respectively compared to August-

September 2012. The March-April 2012 collection had 3.2 times the number of *An. gambiae* caught in the August-September 2012 collection (Ratio= 3.2, 95% CI: 1.1, 10.1, P= 0.038).

Furthermore, the March-April 2013 (wet) collection also revealed spatial differences in vector composition between villages near Lake Mweru and more inland villages along Kenani Stream (Figure 2.8). Collections at the lakeside villages (Katuna, Yenga, and Malulu) resulted in a total of 133 anophelines caught, of which the majority was *An. gambiae* (85.7%, n = 114), with the remainder *An. funestus* (14.3%, n = 19). In contrast, in roughly the same number of collections, 2847 anophelines were collected at the streamside villages (Kapande B, Mutepuka), which were composed of 2397 *An. funestus* (84.2%) and 450 *An. gambiae* (15.8%). The streamside collection revealed significantly higher numbers of *An. funestus* caught per household per trap night or morning in the streamside collection, 59.5 times more than the lakeside collection (Ratio= 59.5, 95% CI: 33.3, 106.4, P= 0.00). Similarly, the streamside had 1.9 times collected *An. gambiae* compared to the lakeside collections (Ratio= 1.9, 95% CI: 1.27, 2.84, P= 0.002).

Additionally, in the March-April 2013 collection, there were statistically significant differences in the abundance of *An. funestus* and *An. gambiae* caught within the lakeside and streamside areas. In the lakeside collection, 5.9 times *An. gambiae* were caught than *An. funestus* (Ratio= 5.9, 95% CI: 3.3, 10.5, P= 0.00). Conversely, in the streamside villages, the number of *An. funestus* caught per household per trap night or morning was 5.3 times that of *An. gambiae* (Ratio= 5.3, 95% CI: 3.9, 7.3, P= 0.00).

Although both malaria vector species appear to be abundant and contribute to transmission during the wet season, they differ in composition and abundance spatially. Along Lake Mweru, overall vector numbers are very low and *An. gambiae* is the most abundant malaria vector during the wet season. However, in areas near Kenani Stream, overall vector numbers are very large with *An. funestus* serving as the most abundant vector and *An. gambiae* is secondary in abundance.

The majority of the collected *An. gambiae* were identified as S-form (527/564; 93.4%). The unidentified molecular forms of *An. gambiae* were due to failed PCRs that were repeated twice. Assays for detection of fixed nucleotide differences on the X chromosome using molecular form diagnostic SNPs (MFDS) could potentially better detect the molecular forms or lack thereof in the unknown samples, but were not performed in this study [126].

The collections revealed that during the wet season, both *An. funestus* and *An. gambiae* are important vectors of *P. falciparum* transmission, whereas during the dry season, *An. funestus* is the primary vector and likely continues transmission into the following wet season (See Figures 2.6-2.8).

Blood feeding behavior

For all collections, regardless of being classified as morphologically fed, all mosquitoes were tested using the Kent et al. (2005) PCR and the Fornadel et al. (2010) PCRs to test for mammalian host blood meal. This was performed due to the possibility that there may be mosquitoes that appear "unfed", but are actually fed as determined by molecular assays. Additionally, the identification of the mammalian host in a blood meal was used to calculate the human blood index (HBI), which is the proportion of human

blood meals relative to the total blood meals, for each vector species. For mixed blood meals, they were counted as blood meals for each host animal.

Of the visually fed mosquitoes from the March-April, 2012 (wet) collection, 111/343 *An. funestus* (32.4%), 9/36 *An. gambiae* (25%), and 6/32 *An. leesoni* (18.8%) that were visually fed. All blood meals for *An. funestus* and *An. gambiae* were identified as human, whereas 5 of the *An. leesoni* fed on humans and 1 *An. leesoni* had fed on goat. Of those anophelines that were visually "unfed", 5 were found to have human blood meals: 2/232 *An. funestus* (0.9%), 1/27 *An. gambiae*, (3.7%) and 2/26 *An. leesoni* (7.7%). No other mammalian hosts were detected in the visually "unfed" mosquitoes. The HBI for both *An. funestus* and *An. gambiae* was 1.0, whereas the HBI for *An. leesoni* was 0.88.

The August-September 2012 (dry) revealed 215/1315 *An. funestus* (16.5%), 2/8 *An. gambiae* (25%), and 0/1 *An. leesoni* (0%) were visually fed. With the exception of two *An. funestus* blood meals belonging to goat, all fed mosquitoes had taken human blood meals. Of those anophelines that were visually "unfed", 45/1098 (4.1%) were identified as *An. funestus* and were human fed. No blood meals from other hosts were identified. The HBIs for *An. funestus* and *An. gambiae* were 0.99 and 1.0 respectively.

The March-April 2013 (wet) collection had 544/2989 (18.2%) visually fed *Anopheles* mosquitoes. Of the collected *An. funestus* and *An. gambiae* mosquitoes, 444/2417 (18.4%) and 100/564 (17.7%) were visually fed respectively and were identified as human and/or goat. No visually fed *An. leesoni* were observed. A majority of the "fed" *An. funestus* and *An. gambiae* had taken blood meals from human hosts, but mixed human and goat blood meals were detected in 24 *An. funestus* and 6 *An. gambiae*.

One *An. funestus* mosquito fed on a goat exclusively. Of the anophelines that were classified as "unfed", 164/2437 (6.7%) fed on human and/or goat hosts: 153 *An. funestus* and 11 *An. gambiae*. 146/153 (95.4%) and 8/11 (72.7%) of *An. funestus* and *An. gambiae* respectively were human fed. 7 blood meals taken by *An. funestus* and 3 blood meals taken by *An. gambiae* were mixed blood meals of human and goat. The HBIs for *An. funestus* and *An. gambiae* were 0.95 and 0.93 respectively.

CSP-ELISA versus PCR Detection of P. falciparum

The CSP-ELISA method of *P. falciparum* detection was compared to the PCR method by calculating the kappa value for each collection. The kappa values for the March-April 2012 (wet), August-September 2012 (dry), and March-April 2013 (wet) collections are 0.0, 0.23 (SE = 0.073; 95% CI = 0.092-0.380), and 0.20 (SE = 0.067; 95% CI = 0.071-0.334) respectively, suggesting poor agreement between the two methods for all field collections.

EIR by Collection Method

The sporozoite infection rate (SIR) and seasonal EIR within and among collections varied by trapping method (See Tables 2.10-2.14). Because CDC LTs have been shown previously in Zambia to be a good alternative for estimating the human biting rate when human landing catches (HLC) cannot be performed, EIR calculations from CDC LT collections only were regarded as the best measurement of transmission intensity by each species [34].

CDC LT Collections only

When the SIR and EIR were measured for the March-April 2012 (wet) based on CDC LT collections only, *An. funestus* had a SIR of 1.8% and a seasonal EIR of 3.7 ib/p/6mo

(Table 2.10). No infectious *An. gambiae* by CSP-ELISA were detected and the EIR was 0.0 ib/p/6mo. There was no statistical significance between both vector's SIRs and EIRs (P> 0.05).

The August-September 2012 (dry) collection revealed that *An. funestus* had an SIR of 2.4% and an increased seasonal EIR relative to the previous wet season collection at 41.5 ib/p/6mo (Table 2.11). During this collection, only 6 *An. gambiae* were caught by CDC LT and both the SIR and seasonal EIR were 0.0 ib/p/6mo. There was no statistical significance between both vector's SIRs and EIRs (P> 0.05).

In the March-April 2013 (wet) collection, both *An. funestus* and *An. gambiae* had SIRs of 3.0% and 2.5% respectively (Table 2.12). The resulting EIR for *An. funestus* was 39.6 ib/p/6mo and for *An. gambiae* was 5.9 ib/p/6mo. The comparison of each vector's SIR revealed that there is a decreased risk of collecting infectious *An. gambiae* in a household per trap night than *An. funestus* (OR= 0.29, 95% CI: 0.09- 0.92, P= 0.035). In contrast, there was no statistical significance observed between each vector's EIR (P> 0.05).

The SIRs for each vector were compared among collections. There were significant differences in the SIRs among all collections. The was a lower risk of collecting infectious *An. funestus* in the March-April 2012 collection than in August-September 2012 (OR= 0.16, 95% CI: 0.03-0.85, P= 0.031) Similarly, there was a lower risk of collecting infectious *An. funestus* in March-April 2013 than in the August-September 2012 collection (OR= 0.16, 95% CI: 0.04-0.63, P= 0.009). There was no statistical significance in the SIRs of *An. funestus* between the March-April 2012 and

March-April 2013 collections (P> 0.05) and also *An. gambiae* SIRs among all collections (P> 0.05).

The EIRs among collections were also examined and compared for any significant changes in transmission intensity. There was no statistical significance in EIRs among collections for both *An. funestus* and *An. gambiae* (P> 0.05), but there is a trend that *An. funestus* has a greater EIR than *An. gambiae* year round.

Interestingly, the seasonal EIRs for both species varied during the 2013 (wet) collection based on geographical location: Lake Mweru villages versus Kenani Stream villages (Tables 2.13-2.14). Along the lakeside, *P. falciparum* was detected by CSP-ELISA in only *An. gambiae* s.s., resulting in an SIR of 1.5% and an EIR of 0.60 ib/p/6mo (Table 2.13). In contrast, both *An. funestus* s.s. and *An. gambiae* s.s. near the stream were found to be infectious and at much higher rates than the anopheline vectors found along the lakeside: 3.9% and 3.4% respectively (Table 2.14). The seasonal EIRs in the streamside collections were also higher than the lakeside collections for *An. funestus* and *An. gambiae*, 51.2 ib/p/6mo and 10.1 ib/p/6mo respectively. There were no statistical significance between lakeside and streamside areas for *An. funestus* and *An. gambiae* SIRs and EIRs (P> 0.05). In the lakeside collection, there was also no statistical significance between the *An. funestus* and *An. gambiae* SIRs and EIRs (P> 0.05). In the streamside, there was a lower risk of collecting infectious *An. gambiae* per trap night than *An. funestus* (OR=0.32, 95% CI: 0.12-0.83, P= 0.019). There was statistically significant difference in EIRs between the two vectors in the streamside collections (Ratio=0.11, 95% CI: 0.014, 0.79, P= 0.028).

CDC LT and PSC Collections

When PSC collections were combined with CDC LT collections, the SIRs for *An. funestus* and *An. gambiae* in the March-April 2012 (wet) collection were 1.3% and 0% respectively. The seasonal EIR for *An. funestus* was 5.9 ib/p/6mo (See Table 2.10). There was no statistically significance between the SIRs and EIRs of *An. funestus* and *An. gambiae* (P>0.05).

During the August-September 2012 (dry) collection, the SIR and EIR of *An. funestus* were 2.4% and 41.3 ib/p/6mo (Table 2.11). None of the *An. gambiae* mosquitoes were found to harbor *P. falciparum*. There was no statistical significance in the SIR and EIR measurements between *An. funestus* and *An. gambiae* (P> 0.05).

In the March-April 2013 (wet) collection, the SIRs for *An. funestus* and *An. gambiae* were 3.0% and 2.5% respectively and the EIRs were 39.6 ib/p/6mo and 5.9 ib/p/6mo respectively (Table 2.12). In this collection, households per trap night or morning were 0.27 times more likely to have infectious *Anopheles gambiae* than *An. funestus* (95% CI: 0.11- 0.66, P= 0.004). The EIR of *An. funestus* was 6.6 times that of *An. gambiae* per household, and was close to significance (Ratio= 6.6, 95% CI: 0.94, 47.1, P= 0.058)

The SIRs calculated per household for *An. funestus* and *An. gambiae* among the three collections were explored, and there was no statistical significance among collections (P> 0.05).

The seasonal EIRs among collections were also calculated and compared for differences temporally. For *An. funestus*, a significant difference in seasonal EIRs was detected between the March-April 2012 and March-April 2013 collections only, where

the March-April 2012 collection had an EIR incidence rate of 0.16 times that of the March-April 2013 collection (Ratio= 0.16, 95% CI: 0.03, 0.88, P= 0.035). There was no statistical significance in seasonal EIRs of *An. funestus* between the March-April 2012 and August-September 2012 collections and the August-September 2012 and March-April 2013 collections (P> 0.05). There was also no statistically significance in *An. gambiae* seasonal EIRs per household per trap night or morning across collections (P> 0.05).

Differences in SIR and EIR between the lakeside and streamside villages during the March-April 2013 (wet) were investigated (Tables 2.13-2.14). The SIR and EIR of lakeside *An. funestus* were 0.0% and 0.0 ib/p/6mo respectively. The *An. gambiae* SIR was 1.2% and the EIR was higher at 2.8 ib/p/6mo. The streamside *An. funestus* and *An. gambiae* had SIRs of 3.8% and 3.4% respectively. Additionally, *An. funestus* and *An. gambiae* collected at the streamside villages had EIRs of 54.2 ib/p/6mo and 10.8 ib/p/6mo respectively. No statistical significance found in both *An. funestus* and *An. gambiae* SIRs and seasonal EIRs between the lake and streamside collections (P> 0.05). Within the lakeside collection, there were also no statistical significance between *An. funestus* and *An. gambiae* SIRs and EIRs (P> 0.05). In the streamside collection, there was a statistically significant difference in EIRs between *An. gambiae* and *An. funestus* (Ratio= 0.11, 95% CI: 0.014, 0.079, P= 0.028). Additionally, there was a lower risk of trapping infectious *An. gambiae* than *An. funestus* (OR=0.32, 95% CI: 0.12- 0.83, P= 0.019)

PSC Collections Only

For the majority of the collections, the SIRs and seasonal EIRs for both anopheline vectors in the PSC only collections were higher than both the CDC LT only and CDC LT and PSC collections. In the March-April 2012 (wet) collection, the SIRs for *An. funestus* and *An. gambiae* were 0.8% and 0.0% and the EIRs were 10.6 ib/p/6mo and 0.0 ib/p/6mo respectively (Table 2.10). There was no statistical significance in the SIRs and EIRs of both vectors (P> 0.05).

Similarly, the August-September 2012 (dry) collection revealed the SIR and seasonal EIR for *An. funestus* as 2.4% and 41.1 ib/p/6mo respectively (Table 2.11). The SIR and EIR for *An. gambiae* were 0.0% and 0.0 ib/p/6mo respectively. When the SIRs and EIRs of the anopheline vectors were compared, there was no statistical significance (P> 0.05).

In the March-April 2013 (wet) collection, the SIR and seasonal EIR were 3.7% and 58.5 ib/p/6mo for *An. funestus*. Similarly, when measurements were performed based on PSC only collections, the SIR and EIR for *An. gambiae* were slightly higher than the other methods, 2.6% and 8.5 ib/p/6mo respectively. The risk of trapping infectious *An. gambiae* was 0.17 times that of *An. funestus* (OR=0.17, 95% CI: 0.04-0.75, P= 0.02). However, the EIRs of both vectors were not statistically significant (P> 0.05).

The SIRs of *An. funestus* and *An. gambiae* were compared among seasons to observe any temporal patterns. The March-April 2013 and March-April 2012 collection had a lower risk of collecting infectious *An. funestus* in a trap morning compared to *An. funestus* in the August-September 2012 collection (OR=0.13, 95% CI: 0.02-0.81, P= 0.029). There was no statistical significance in the *An. funestus* SIRs between the March-

April 2012 and March-April 2013 collections (P> 0.05). Statistical significance in *An. gambiae* SIRs among seasons was not observed (P> 0.05). The seasonal EIRs for both anopheline vectors were also compared among collections. For both vectors, no significance in EIRs was observed (P> 0.05).

The lakeside villages where sampling occurred from March-April 2013 (wet) revealed that there were no *P. falciparum* infected vectors leading to 0.0% SIR, and the transmission intensity at the lakeside was 0.0 ib/p/6mo for both *An. funestus* and *An. gambiae* (Table 2.13). This differs from the other collection methods, where *An. gambiae* was found to contribute to transmission near the lake. In the streamside villages, the SIR of *An. funestus* was high at 4.4% and the EIR of *An. funestus* was 58.5 ib/p/6mo (Table 2.14). The SIR was 3.6% and the EIR was 11.9 ib/p/6mo for *An. gambiae* collected along Kenani Stream. Comparisons of the SIRs and EIRs of each vector species in the lakeside and streamside areas did not yield any statistically significant differences (P>0.05). Within the lakeside area, there was no statistical significance in the SIR of *An. funestus* and *An. gambiae* (P> 0.05). In contrast, in villages along the stream, there is a decreased risk in finding infectious *An. gambiae* in a trap morning than *An. funestus* (OR=0.20, 95% CI: 0.04-0.96, P= 0.044). There was no statistical significance in the EIRs of *An. funestus* and *An. gambiae* (P> 0.05).

CDC LT only vs. CDC LT and PSC vs. PSC only collections

The SIRs and EIRs measured according to three collection methods (CDC LT only, CDC LT and PSC, and PSC only) for both *An. funestus* and *An. gambiae* were compared to observe any differences. In the March-April 2012, August-September 2012, and March-April 2013 collections, the *An. funestus* and *An. gambiae* SIRs (*An. funestus*;

CDC LT: 1.8%, CDC LT and PSC: 1.3%, PSC only: 0.8%, *An. gambiae*; CDC LT only: 0%, CDC LT and PSC: 0%, and PSC only: 0%) were not statistically significant among methods (P> 0.05) (See Table 2.10-2.12). Similarly, there was no statistical significance in the *An. funestus* and *An. gambiae* EIRs (*An. funestus*; CDC LT only: 3.7 ib/p/6mo, CDC LT and PSC: 7.3 ib/p/6mo, PSC only: 10.6 ib/p/6mo, *An. gambiae*; CDC LT only: 0 ib/p/6mo, CDC LT and PSC: 0 ib/p/6mo, PSC only: 0 ib/p/6mo) among trapping methods (P> 0.05) (See Table 2.10-2.12).

Discussion

In this study, we characterize the malaria vector transmission dynamics in Nchelenge District, Zambia over three consecutive seasons: March-April 2012 (wet), August-September 2012 (dry), and March-April 2013 (wet). The preliminary collection in March-April 2012 (wet) suggested that *An. funestus* is the primary vector and *An. gambiae* is the secondary vector, and the subsequent collections through the August-September 2012 (dry) and March-April 2012 (wet) confirmed those vector roles. *An. leesoni* mosquitoes were also identified among the three collections, but too few were caught to draw any conclusions regarding the role of *An. leesoni* in malaria transmission. Additional indoor and outdoor collections will be needed to obtain enough *An. leesoni* to detect malaria parasite and better assess their foraging behavior in Nchelenge.

The densities of *An. funestus* and *An. gambiae* varied seasonally and all collections revealed micro-spatial dynamics of *An. funestus* and *An. gambiae* relative to villages located along Lake Mweru and Kenani Stream. During the wet seasons, *An. gambiae* was the primary vector found in lakeside villages, while *An. funestus* and *An.*

gambiae were the primary and secondary vectors respectively in interior streamside villages. The dry season collection revealed that *An. funestus* is the dominant vector of transmission at both the lakeside and streamside villages, while only a few *An. gambiae* were caught in those same areas. It should also be noted that lakeside mosquito densities were a fraction of inland and streamside densities, regardless of season. The relative abundance of *An. funestus* was significantly larger in the August-September 2012 collection compared to both wet season collections, suggesting that the *An. funestus* population increases dramatically during the dry season [97, 127]. The abundance of *An. funestus* per trap night or morning was also significantly higher in the March-April 2013 collection compared to the March-April 2012 collection, but this is likely due to a smaller collection period and that only one village near the streamside was sampled in the March-April 2012 collection. In contrast, the abundance of *An. gambiae* in the March-April 2012 and March-April 2013 collections was significantly higher when compared to the August 2012 collection. The trend of the *An. gambiae* population proliferating at the beginning of the wet season, while the *An. funestus* population increases towards the end of the wet season and continues malaria transmission through the dry season, has been observed in many places in sub-Saharan Africa [108, 128].

The increases in *An. gambiae* mosquitoes throughout the collection sites during the wet seasons are likely due to a wide range of temporary sunlit breeding habitats found throughout Nchelenge District such as animal footprints, puddles, and ground depressions formed due to heavy rains [97, 129, 130]. In villages near Lake Mweru, *An. gambiae* larvae and pupae in oviposition surveys have been collected in puddles and boats onshore along the lake, as well as in ditches along the main road (Personal observation and

personal communication with Drs. Douglas Norris, JHU, and Jenny Stevenson, MRT). In contrast, *An. funestus* typically prefer vegetated, semi-permanent to permanent breeding sites such as swamps and marshes, which may be found along large streams [97, 129, 131]. Overall, *An. funestus* was found throughout all collection seasons at high densities, especially near Kenani Stream, which is likely the breeding site area that is stable with viable breeding sites such as permanent marsh and swamps along the stream through the dry season.

The foraging behavior of both *An. funestus* and *An. gambiae* was determined. Both species were found to be highly anthropophilic, although a few mixed human/goat and goat only blood meals were identified in both vectors. In Nchelenge District, goats are occasionally kept indoors at night, and may serve as an incidental host to both *An. funestus* and *An. gambiae,* but should be explored further (personal observation). *An. funestus* and *An. gambiae* are widely known for their anthropophilic, as well as endophilic and endophagic behavior. It is not surprising that in Nchelenge District, both vectors have a tendency to feed almost exclusively on humans. However, it should be noted that inhabitants often do not sleep at dusk, but rather stay up for social gatherings into the late evening. As a result, we cannot overlook the possibility of outdoor biting behavior by the two vectors that may contribute to malaria transmission by the major *Anopheles* species at this field site. A major limitation of this study is that we were unable to perform outdoor collections to be able to observe if *An. funestus* and *An. gambiae*, as well as other *Anopheles* species, participate in outdoor biting.

The *Plasmodium falciparum* infection rate, or the sporozoite infection rate (SIR), of *Anopheles* mosquitoes is a critical measurement because it provides evidence of vector

involvement in malaria parasite transmission. Moreover, the SIR is required to calculate the entomological inoculation rate (EIR), a valuable measurement of vector transmission intensity in an affected area. The SIR was determined for both *An. funestus* and *An. gambiae* using the CSP-ELISA and PCR methods for each collection, and the agreement between the two methods was considered "poor" for all collections. There have been several studies that have reported false CSP-ELISA positive results compared to microscopy and PCR [40, 132-135]. The discrepancy may be attributed to the advantages and disadvantages of both methods. The main advantage of CSP-ELISA is that it is faster and more efficient than dissection, especially because mosquitoes can be stored and processed later [132]. However, a limitation of CSP-ELISA is that it is not as sensitive as dissection when there are low numbers of sporozoites in the salivary gland, resulting in false negatives [132]. Additionally, the CSP-ELISA may not only detect CSP in the salivary gland, but also in mosquito tissues, which may result in an overestimation of the SIR [132]. Another method to detection *Plasmodium* spp. in mosquitoes is polymerase chain reaction (PCR), which can detect as little as 10 sporozoites, while CSP-ELISA requires at least 100 sporozoites for detection [132]. A major disadvantage of PCR is that it detects all stages of *Plasmodium* spp., making CSP-ELISA the more preferred method as it detects only the infectious stage of the parasite. The PCR performed in this study was done on the mosquito abdomen only because there is currently no effective method of extracting DNA from ELISA homogenate of mosquito head and thorax. It was assumed that a positive result by PCR implied salivary gland infection. However, this may not be the case, and could lead to overestimation of the SIR. Other studies have been unable to identify the cross-reacting antigens suspected of

leading to false CSP-ELISA results, but have shown that the unknown antigen is heat-unstable and can be removed by heating the CSP-ELISA homogenate to 100°C for 10 minutes [8, 132]. Because this method of heating CSP-ELISA homogenate was not performed for this study, it is essential that future research confirm initial CSP-ELISA results with a second heated CSP-ELISA.

The SIRs and EIRs reported in this study are based on the CSP-ELISA because the assay detects only infectious sporozoites that that are ultimately transmitted and cause human disease. The CSP-ELISA was performed on both CDC LT and PSC collections, and various combinations of CDC LT and PSC results were examined for differences in SIRs and EIRs based on trapping method. The SIR based on CDC LT only collections revealed that the *An. funestus* SIR was significantly higher during the August-September 2012 collection compared to the March-April 2012 and March-April 2013 collections. This suggests that a higher number of infected *An. funestus* are found within a household during the dry season than the wet season, which may be due to stable breeding habitats and low rainfall that support survival of the *An. funestus* population. The increased longevity of *An. funestus* during the dry season is particularly important because the malaria parasite can successfully develop, invade the salivary gland, and propagate transmission into the wet season. During the March-April 2013 (wet) collection, only infected *An. gambiae* were found in the lakeside relative to *An. funestus*, whereas both *An. funestus* and *An. gambiae* had higher SIRs near the streamside. This is likely due to the relatively fewer numbers of *An. funestus* and low densities of mosquitoes in general along Lake Mweru, and the much higher densities of *An. funestus* and *An. gambiae* inland near Kenani Stream. In the streamside villages, the *An. funestus* SIR was higher

than that of *An. gambiae*. The large numbers of both *An. funestus* and *An. gambiae* in the streamside collections helped define patterns in SIRs and EIRs, whereas differences in the smaller lakeside collection were not significant, but there are trends that suggest predominately infected *An. gambiae* near the lake. Furthermore, the EIR of *An. funestus* was higher in the March-April 2013 collection than the March-April 2012 collection, which was likely due to a shorter collection period in 2012. In the streamside villages, the *An. funestus* EIR was higher than *An. gambiae*.

When *An. funestus* and *An. gambiae* SIR and EIR measurements were investigated for CDC LT and PSC and PSC only collections, a variety of differences within and among collection periods was observed and was distinct from CDC LT only measurements. However, when each method, CDC LT only, CDC LT and PSC, and PSC only, was statistically compared among each other, the SIRs and EIRs of both *An. funestus* and *An. gambiae* were not statistically different. This preliminary data suggests that the method used does not affect the SIR and EIR outcomes, but further studies with larger numbers of infectious *Anopheles* vectors need to be performed to support this view.

General trends for *An. funestus* and *An. gambiae* in each collection period show that the EIR of *An. funestus* is highest during the dry season when *An. gambiae* is generally absent. Throughout the wet seasons, *An. funestus* remains the dominant vector of transmission, but at a lower intensity than in the dry season. On the other hand, *An. gambiae* contributes to almost no transmission during the dry season, and then serves as a secondary vector with an EIR similar to *An. funestus* in the wet season. As a result, malaria transmission is maintained year-round, likely by *An. funestus*. Spatially,

transmission of malaria primarily occurs in the inland streamside villages at high EIRs by both *An. funestus* and *An. gambiae*. In the lakeside villages, *An. gambiae* is the dominant malaria vector, while no infectious *An. funestus* were detected, which may be an artifact of the small sample size.

One of the limitations of the crude EIR calculations is the use of CDC LT collections to estimate the HBR. In theory, the CDC LT collection method attracts foraging mosquitoes, and thus should be an adequate alternative to the human landing catch (HLC) collection method because it is more cost-effective, exposure-free, and can be performed at any field site [34]. By nature, HLCs, although often considered the "gold standard", are unreliable because they are non-standardized due to variability in host attractiveness, the collection method is labor intensive, and there is an ethical dilemma about exposing individuals to infectious mosquitoes, especially in light of the possibility of emergent drug resistant parasite strains [34, 35, 37, 39, 136]. However, studies comparing CDC LT and HLC collections to estimate HBR and eventually EIR have been ambiguous and produce varying sampling efficiencies. CDC LTs produced poor estimates of human biting rate compared to HLCs in Dar es Salaam [36], which was attributed to an illuminated and urban environment that reduces the attractiveness of the light trap. When comparisons between HLC and CDC LT methods were performed in Bioko Island, Papua New Guinea, it was observed that the efficiencies derived from various and appropriate statistical tests produced variable and inconsistent results [137]. As a result, it was decided that CDC LTs could not be calibrated to give HBR estimates reliably. However, in Macha, Southern Province, Zambia, it was shown that in an area of low vector density and high bednet use, the CDC LT captured an average of two times

the number of *An. arabiensis* caught per night by HLC collections, was not density dependent, and deltamethrin-treated bednets compared to untreated nets did not affect the number of vectors caught [34]. *An. funestus* and *An. gambiae* vectors caught in CDC LTs collections were found to be directly proportional to HLC collections regardless of mosquito densities in Lwanda, western Kenya. Additionally, there were no differences in SIR and parity rates of collected vectors in Lwanda [38]. In western Kenya, six trapping methods were performed to compare with HLC collections for HBR estimation [138]. CDC LT collections were found to be an effective method for large-scale vector sampling, especially in areas with high vector densities of *An. funestus* and *An. gambiae* [138]. In summary, the sampling efficiency of CDC LT collections compared to HLCs appears to be largely dependent on the study site, and may underestimate, be about the same, or overestimate the annual EIR for each vector relative to HLC-derived EIRs. In Nchelenge District, where there are high vector densities of *An. funestus* and *An. gambiae*, CDC LTs were used to determine the HBR as estimated in Zambia, Kenya, and Tanzania, where CDC LTs were confirmed to produce reliable HBRs. Further studies using indoor and outdoor HLCs should be performed in Nchelenge to further evaluate measurements of vector EIRs as compared to other collection methods.

Together, the EIRs of *An. funestus* and *An. gambiae* strongly suggest that both vectors play major roles in malaria transmission during the wet season. During the dry season, however, *An. funestus* appears to maintain transmission; no *P. falciparum* infected *An. gambiae* were detected. Moreover, the villages found along Kenani Stream are much more vulnerable to infectious bites by *An. funestus* and *An. gambiae* than the areas along Lake Mweru, where the primary vector is *An. gambiae.* The spatial and

temporal differences in vector composition during both the wet and dry seasons have been observed elsewhere in sub-Saharan Africa [139-144], data that has been used in the development of recommendations for vector control strategies that will account for these heterogeneities. Similarly, the microspatial heterogeneity in vector composition and transmission intensity in Nchelenge is especially concerning because of high year-round human infection rates, disparate IRS and LLINs coverage, and insecticide resistance in both vectors. Moreover, inhabitants transit between the lakeside and streamside area throughout both seasons, which further complicates vector control programs when infected individuals travel back and forth between these transmission zones. It will also be relevant to identify if one or both vectors take multiple blood meals and if there is any preference for a particular host gender or age-group. If so, this behavior would underestimate the crude EIRs reported in this study [15]. In conclusion, our findings suggest that malaria vector control interventions such as LLIN distribution and IRS using effective insecticides should be implemented at high year-round coverage throughout Nchelenge District, especially in inland villages where access by spray teams may be limited, in order to reduce vector populations and transmission intensity.

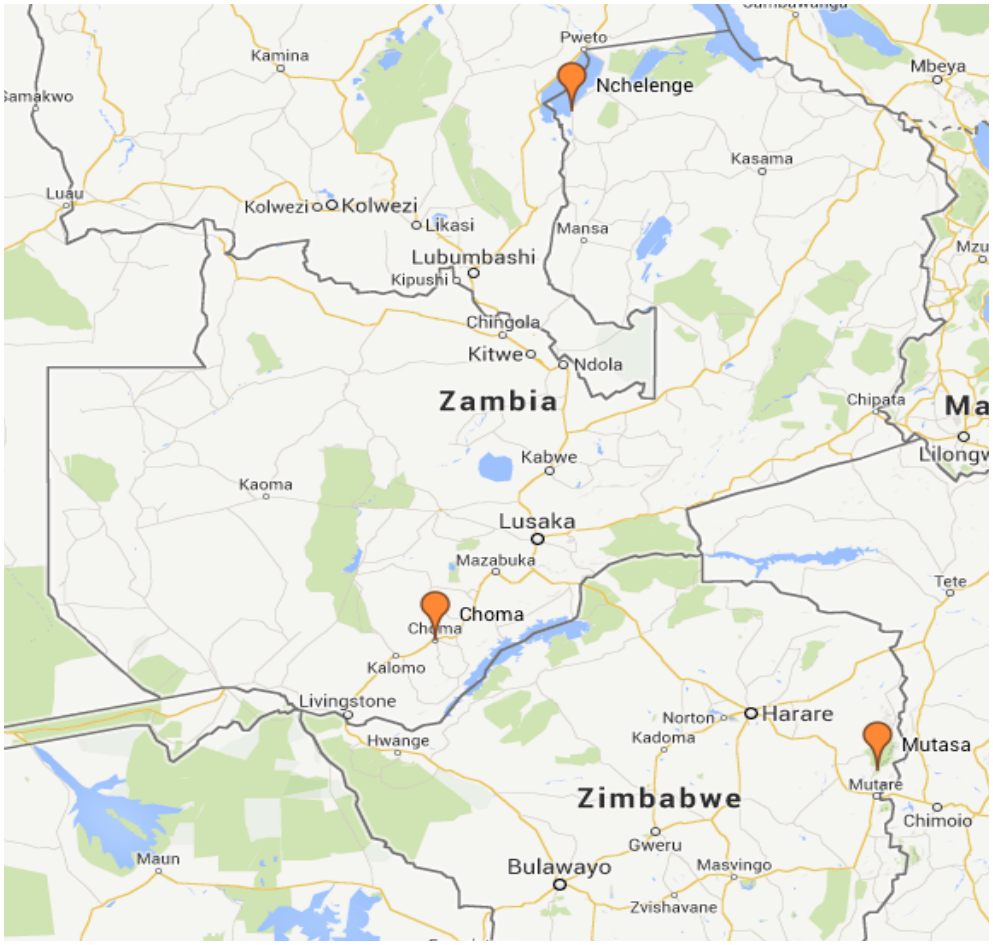

Figure 2.1. The southern Africa ICEMR field sites. Nchelenge District is in northern Zambia and represents a site with unsuccessful malaria control, Choma District in southern Zambia represents successful malaria control, and Mutasa District in eastern Zimbabwe represents resurgent malaria.

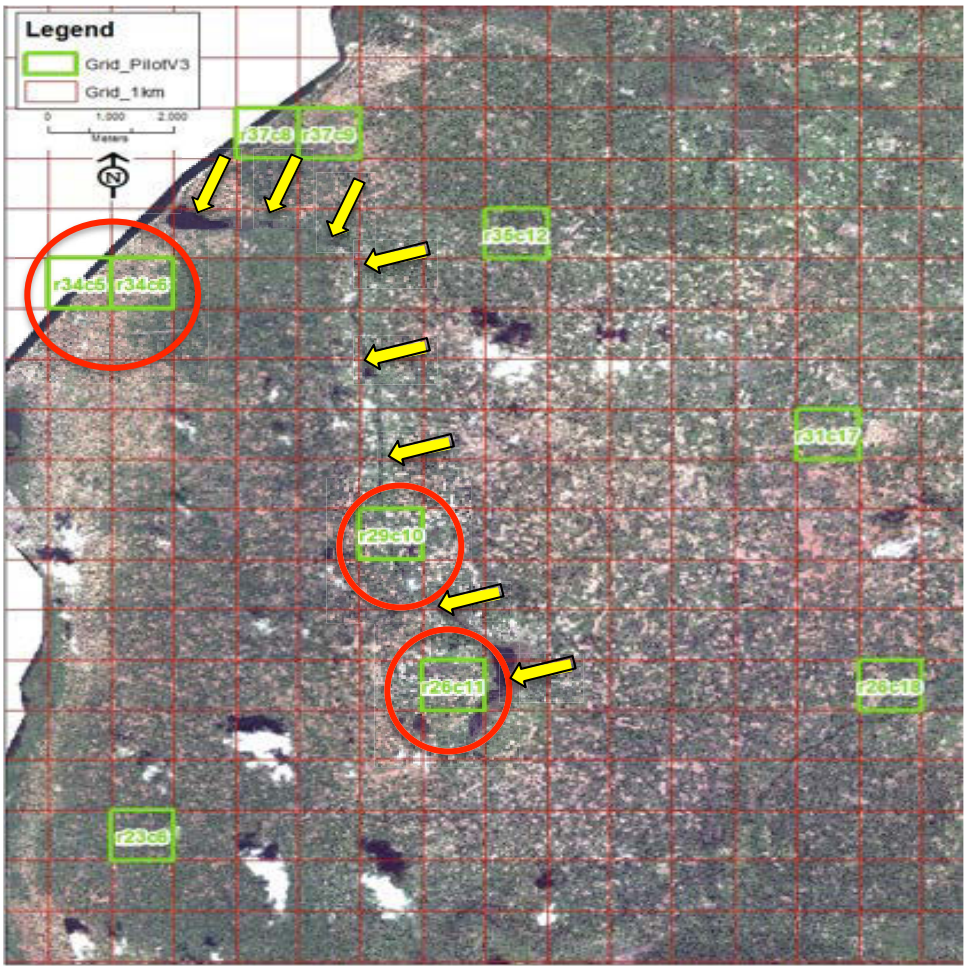

Figure 2.2. Satellite image of the study area in Nchelenge District. The initial 1-km^2 grids for ICEMR epidemiological and entomological surveys are highlighted in green. The white areas on the left side of the image represent Lake Mweru. The yellow arrows point to Kenani Stream that flows into Lake Mweru. The red circles denote the grids where mosquito collections were performed for thesis research: Katuna, Yenga, and Malulu villages are located in grids r34c5 and r34c6, Kapande B village is located in grid r29c10, and Kapande B village is located in grid r26c11.

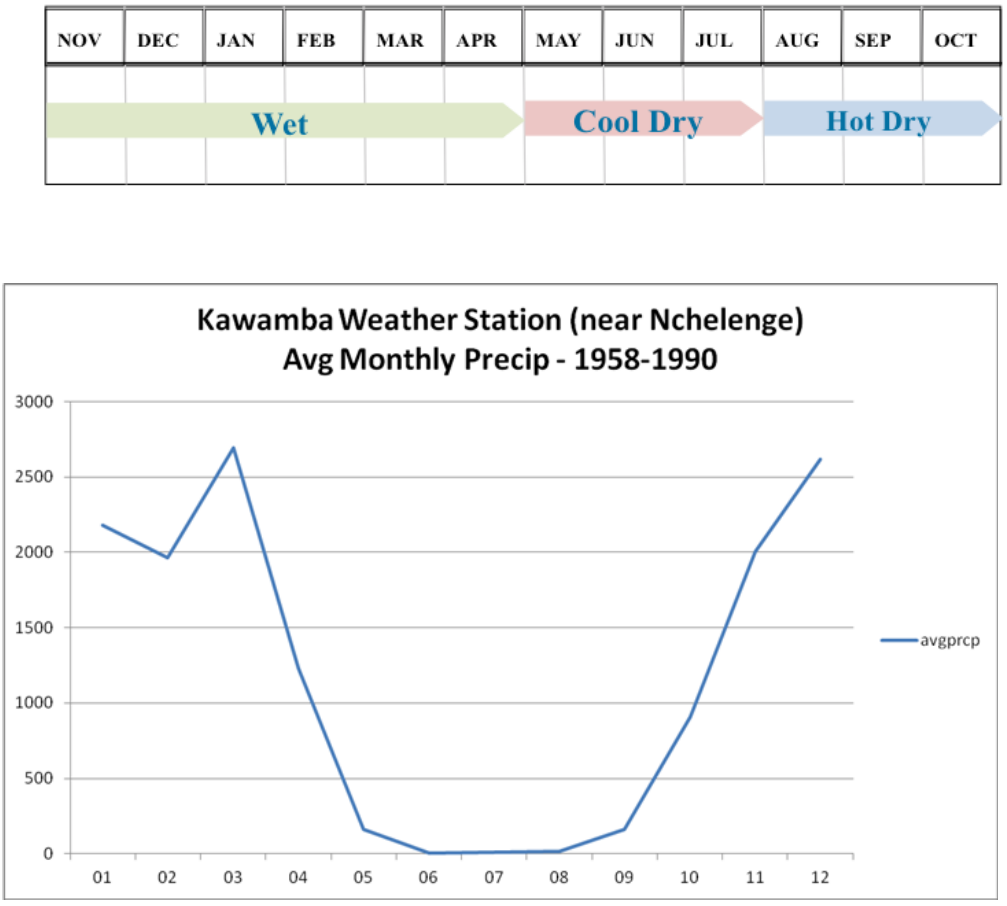

Figure 2.3. Seasons and Average Monthly Rainfall in Nchelenge District. The wet season is from November to May, the cool dry season is from May to August, and the hot dry season is from August to November. The rainfall also follows a seasonal pattern in Nchelenge.

Collection dates	4/2012 to 12/2013
Number of health centers	11
Confirmed malaria cases	94,905

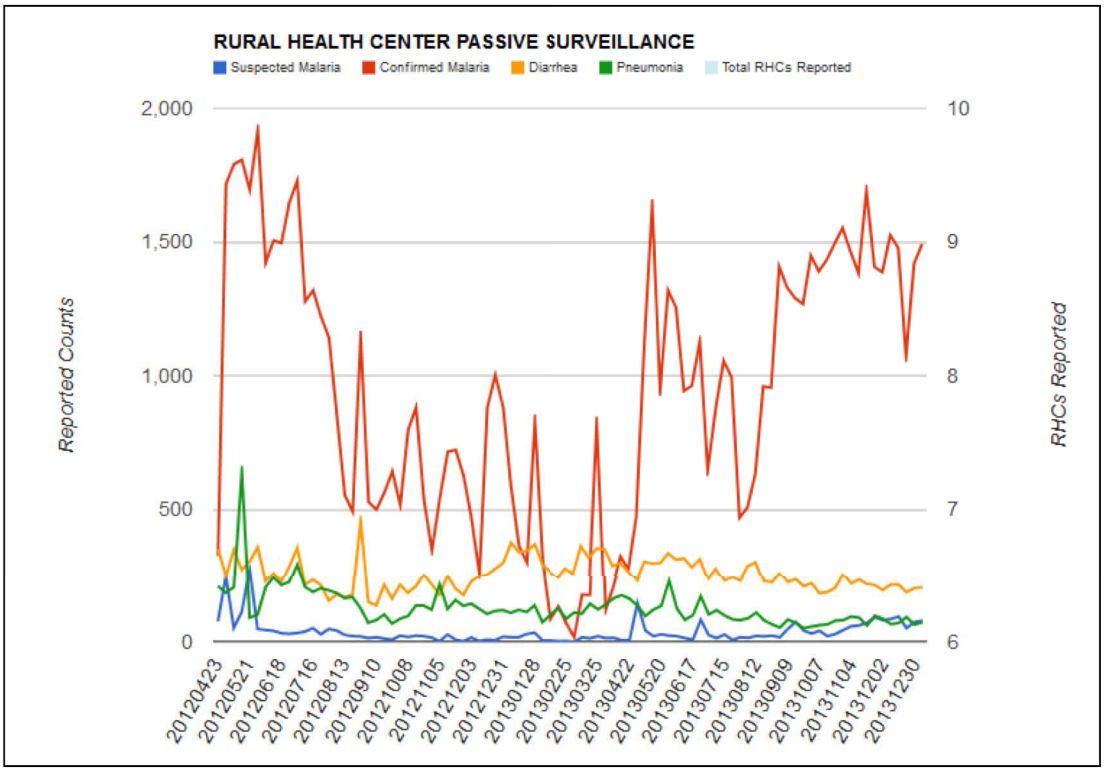

Figure 2.4. Reported counts of confirmed malaria by 11 health centers from April 2012 to December 2013. The number of malaria cases remains high throughout both the wet and dry seasons (southern Africa ICEMR REDCap data).

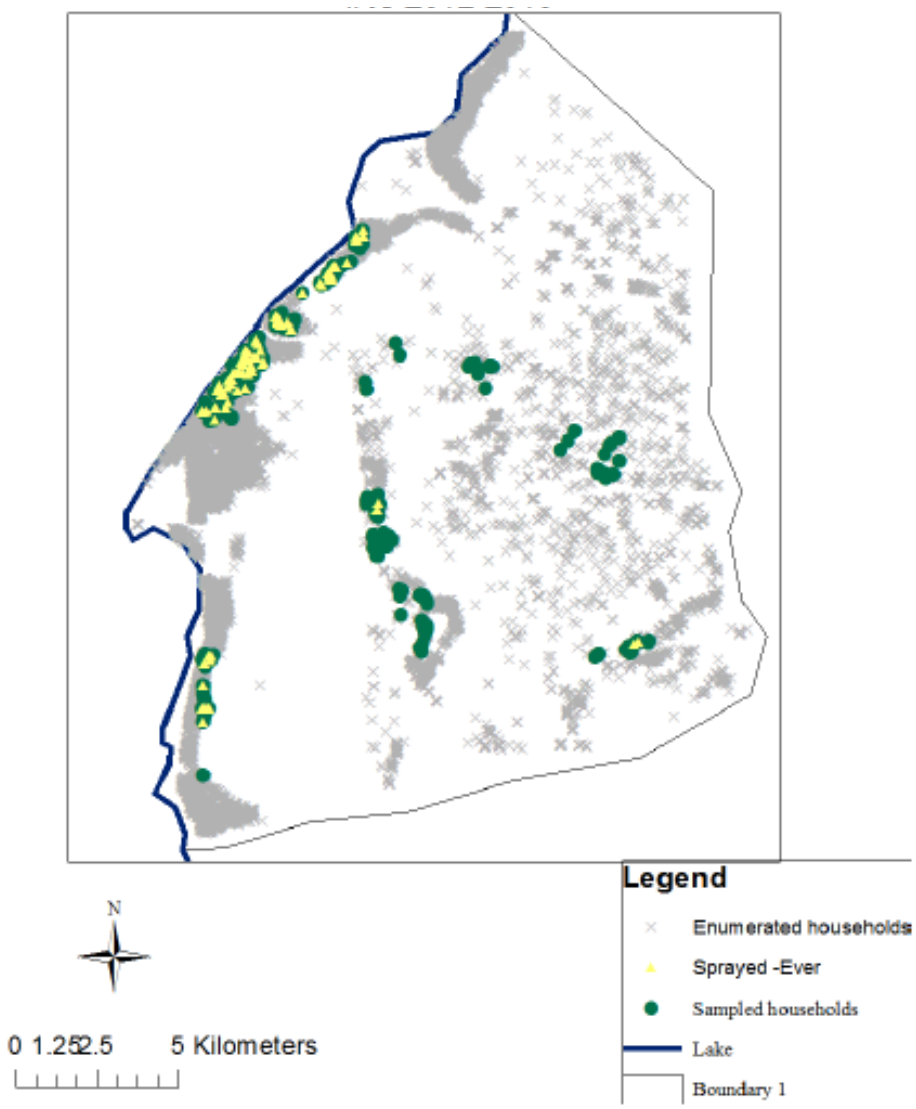

Figure 2.5. ICEMR surveyed households from 2012-2013 that reported ever receiving IRS. The majority of households are found along the main road that runs parallel to Lake Mweru. In contrast, fewer households in inland villages received IRS (Courtesy of Dr. Jessie Pinchoff, JHSPH).

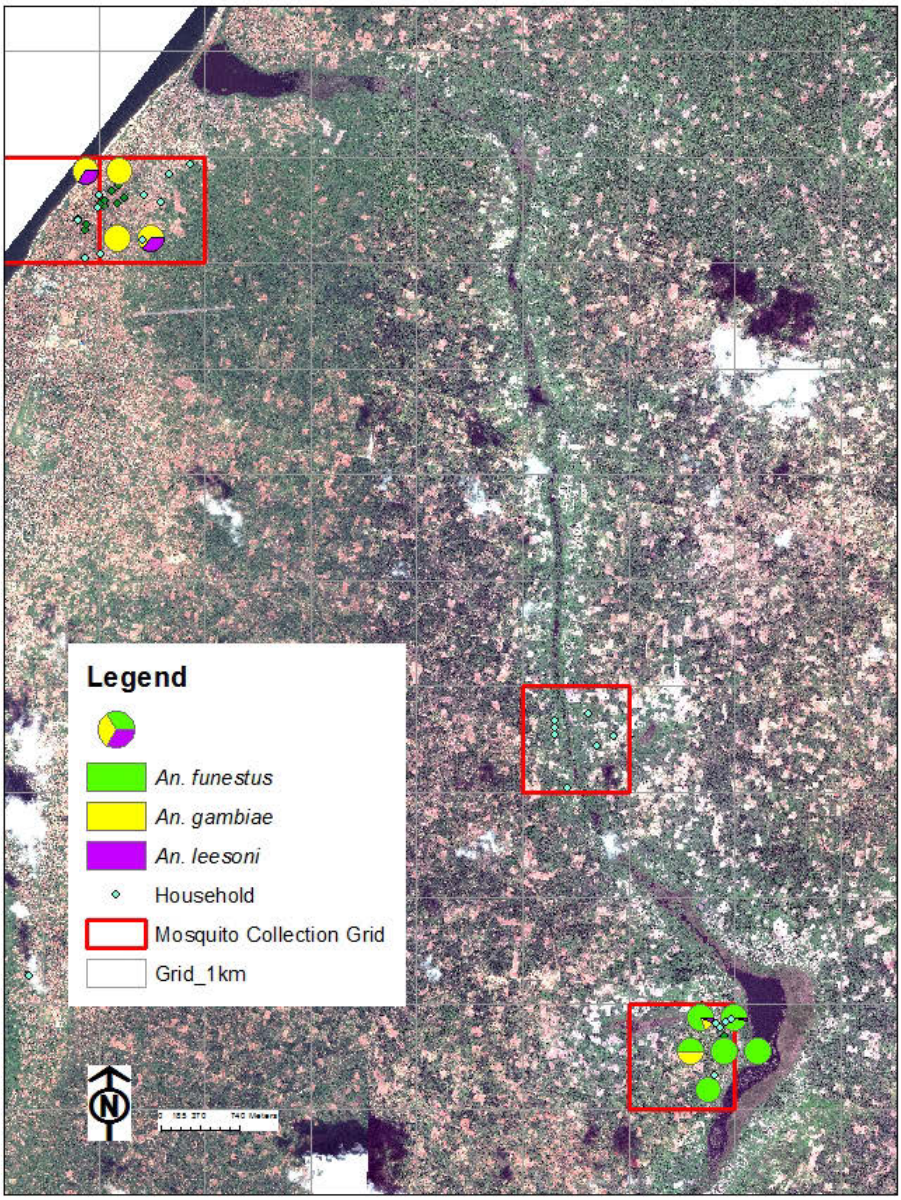

Figure 2.6. The proportion of *Anopheles* species caught per household that were sampled during the March 24-April 10, 2012 (wet) collection. Households were also sampled outside of study 1-km^2 grids (red), but were not included in this study. Species are denoted by color: *An. funestus* s.s. (green), *An. gambiae* s.s. (yellow), and *An. leesoni* (purple).

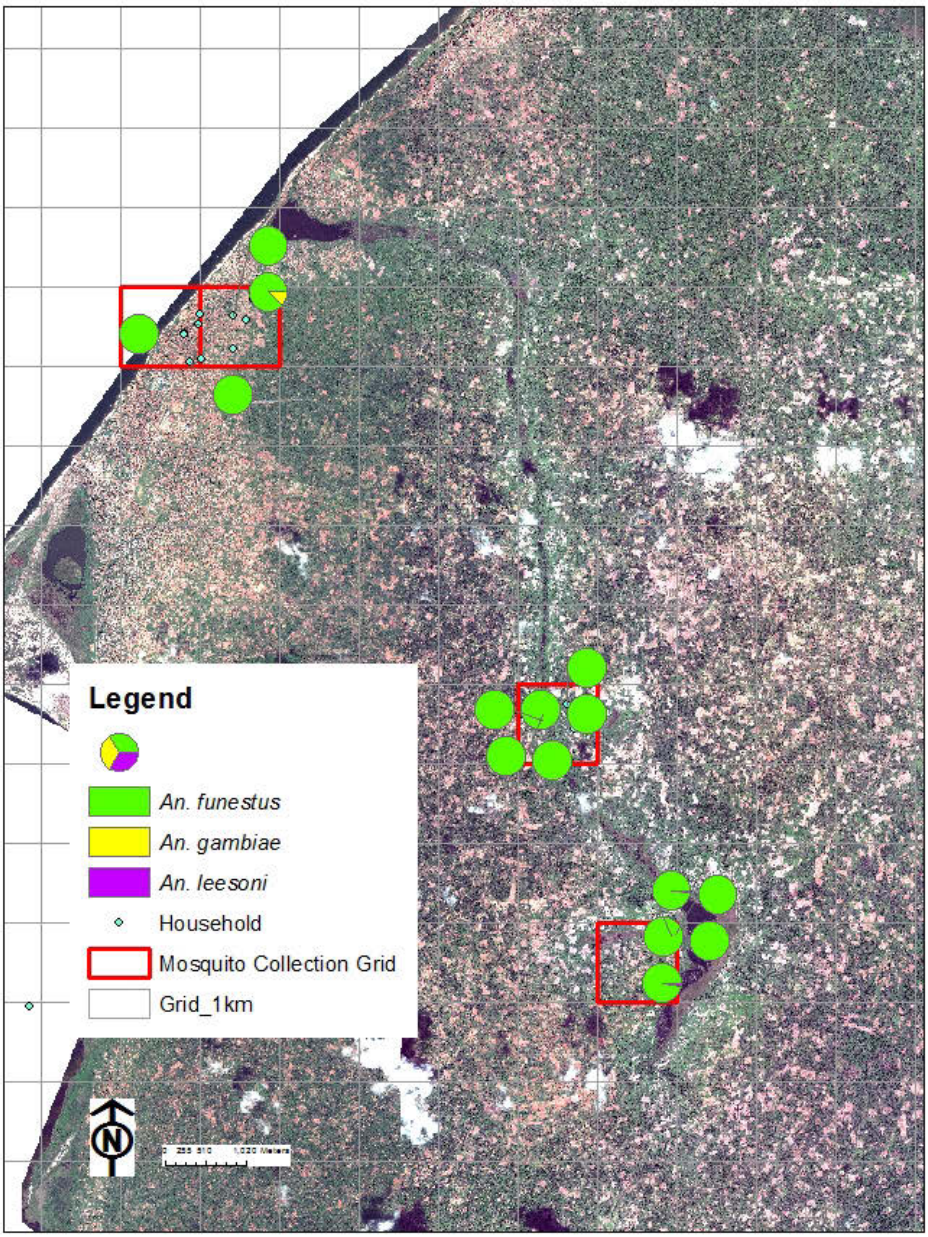

Figure 2.7. The proportion of *Anopheles* species caught per household that were sampled during the August 27-September 9, 2012 (dry) collection. Species are denoted by color: *An. funestus* s.s. (green), *An. gambiae* s.s. (yellow), and *An. leesoni* (purple).

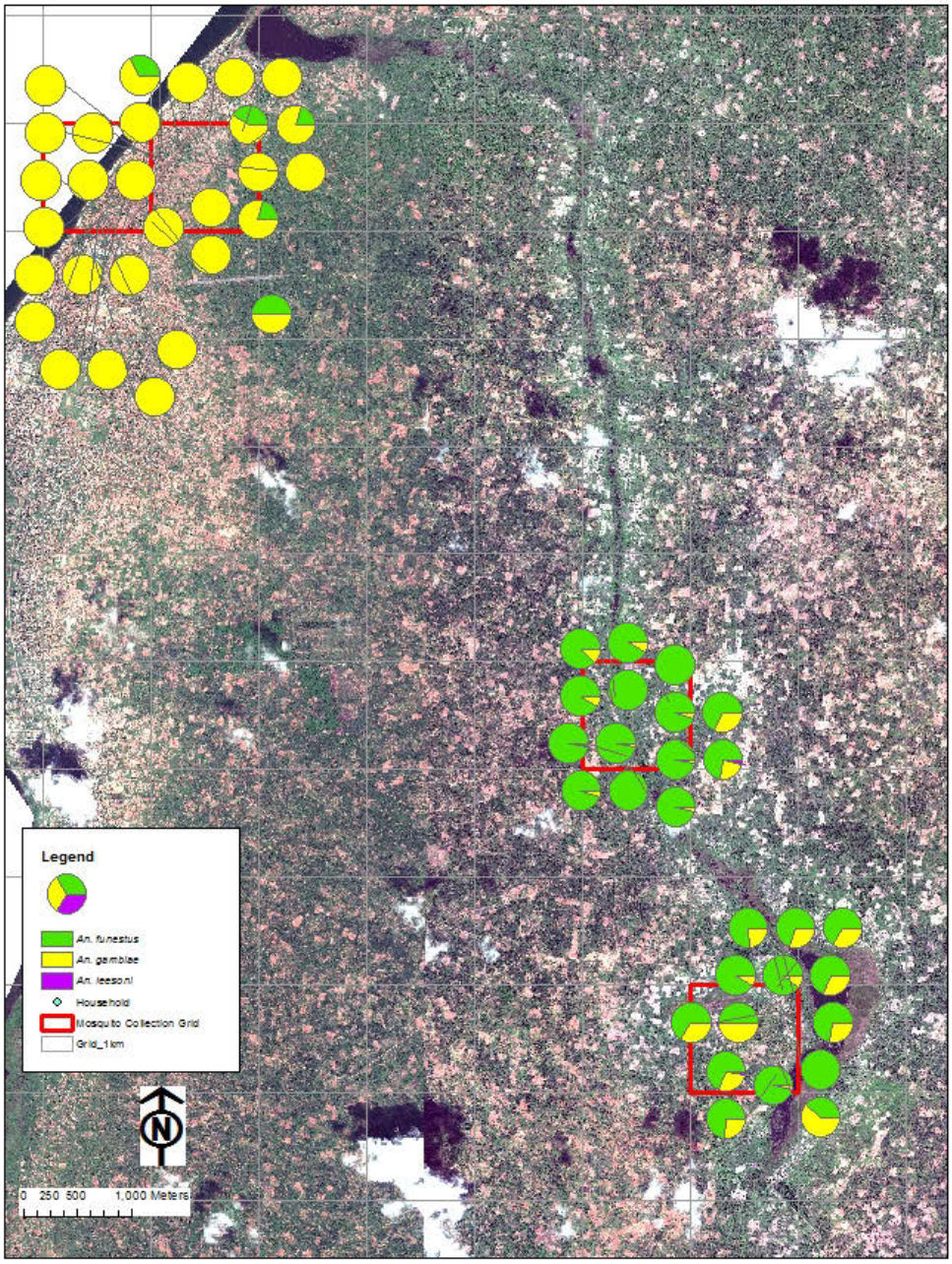

Figure 2.8. The proportion of *Anopheles* species caught per household that were sampled during the March 5-April 25, 2013 (wet) collection. Species are denoted by color: *An. funestus* s.s. (green), *An. gambiae* s.s. (yellow), and *An. leesoni* (purple).

Nchelenge March 24-April 10, 2012 (Wet): EIR Summary

***CDC LT (Standard)**

Species	Total Samples	SIR (%)	EIR (ib/p/6mo)
Overall	158		
An. funestus s.s.	134	1.8	3.7
An. gambiae s.s.	24	0.0	0.0

***CDC LT + PSC**

Species	Total Samples	SIR (%)	EIR (ib/p/6mo)
Overall	358		
An. funestus s.s.	324	1.3	7.3
An. gambiae s.s.	34	0.0	0.0

*** PSC only**

Species	Total Samples	SIR (%)	EIR (ib/p/6mo)
Overall	200		
An. funestus s.s.	190	0.8	10.6
An. gambiae s.s.	10	0.0	0.0

Table 2.1. Total abundance, sporozoite infection rate (SIR), and entomological inoculation rate (EIR) for *An. funestus* s.s. and *An. gambiae* s.s. based on collection method (CDC LT only, CDC LT + PSC, PSC only) from March 24-April 10, 2012 (wet).

Nchelenge August 27-September 9, 2012 (Dry): EIR Summary

***CDC LT only (Standard)**

Species	Total Samples	SIR (%)	EIR (ib/p/6mo)
Overall	787		
An. funestus s.s.	781	2.4	41.5
An. gambiae s.s.	6	0.0	0.0

***CDC LT and PSC**

Species	Total Samples	SIR (%)	EIR (ib/p/6mo)
Overall	1296		
An. funestus s.s.	1288	2.4	41.3
An. gambiae s.s.	8	0.0	0.0

***PSC only**

Species	Total Samples	SIR (%)	EIR (ib/p/6mo)
Overall	509		
An. funestus s.s.	507	2.4	41.1
An. gambiae s.s.	2	0.0	0.0

Table 2.2. Total abundance, sporozoite infection rate (SIR), and entomological inoculation rate (EIR) for *An. funestus* s.s. and *An. gambiae* s.s. based on collection method (CDC LT only, CDC LT + PSC, PSC only) from August 27-September 9, 2012 (dry).

Nchelenge March 5-April 25, 2013 (Wet): EIR Summary

***CDC LT only (Standard)**

Species	Total Samples	SIR (%)	EIR (ib/p/6mo)
Overall	856		
An. funestus s.s.	480	3.0	39.6
An. gambiae s.s.	376	2.5	5.9

***CDC LT and PSC**

Species	Total Samples	SIR (%)	EIR (ib/p/6mo)
Overall	1216		
An. funestus s.s.	695	3.2	45.8
An. gambiae s.s.	521	2.5	6.7

***PSC only**

Species	Total Samples	SIR (%)	EIR (ib/p/6mo)
Overall	360		
An. funestus s.s.	215	3.7	58.5
An. gambiae s.s.	145	2.6	8.5

Table 2.3. Total abundance, sporozoite infection rate (SIR), and entomological inoculation rate (EIR) for *An. funestus* s.s. and *An. gambiae* s.s. in a subsample based on collection method (CDC LT only, CDC LT + PSC, PSC only) from March 5-April 25, 2013 (wet).

Nchelenge March 5-April 25, 2013 (Wet): LAKESIDE EIR

***CDC LT only (Standard)**

Species	Total Samples	SIR (%)	EIR (ib/p/6mo)
Overall	117		
An. funestus s.s.	19	0.0	0.0
An. gambiae s.s.	98	1.5	0.60

***CDC LT and PSC**

Species	Total Samples	SIR (%)	EIR (ib/p/6mo)
Overall	133		
An. funestus s.s.	19	0.0	0.0
An. gambiae s.s.	114	1.2	0.60

***PSC only**

Species	Total Samples	SIR (%)	EIR (ib/p/6mo)
Overall	16		
An. funestus s.s.	0	0	0
An. gambiae s.s.	16	0	0

Table 2.4. Total abundance, sporozoite infection rate (SIR), and entomological inoculation rate (EIR) for *An. funestus* s.s. and *An. gambiae* s.s. in the lakeside villages (r34c5, r34c6; Katuna, Yenga, Malulu) based on collection method (CDC LT only, CDC LT + PSC, PSC only) from March 5-April 25, 2013 (wet). A subsample was performed for the entomological measurements.

Nchelenge March 5-April 25, 2013 (Wet): STREAMSIDE EIR

***CDC LT only (Standard)**

Species	Total Samples	SIR (%)	EIR (ib/p/6mo)
Overall	739		
An. funestus s.s.	461	3.9	51.5
An. gambiae s.s.	278	3.4	10.1

***CDC LT and PSC**

Species	Total Samples	SIR (%)	EIR (ib/p/6mo)
Overall	1083		
An. funestus s.s.	676	3.8	54.2
An. gambiae s.s.	407	3.5	10.8

***PSC only**

Species	Total Samples	SIR (%)	EIR (ib/p/6mo)
Overall	344		
An. funestus s.s.	215	4.4	58.5
An. gambiae s.s.	129	3.6	11.9

Table 2.5. Total abundance, sporozoite infection rate (SIR), and entomological inoculation rate (EIR) for *An. funestus* s.s. and *An. gambiae* s.s. in the streamside villages (r29c10, r26c11; Kapande B and Mutepuka) based on collection method (CDC LT only, CDC LT + PSC, PSC only) from March 5-April 25, 2013 (wet). A subsample was performed for the entomological measurements.

Chapter III

Underestimation of Foraging Behavior by Standard Field Methods in Malaria Vector Mosquitoes in Southern Africa

A**bstract.** Defining the anopheline mosquito vectors and their foraging behavior in malaria endemic areas is crucial for disease control and surveillance. The standard protocol for molecular identification of host blood meals in mosquitoes is to morphologically identify fed mosquitoes and then perform polymerase chain reaction (PCR), precipitin tests, or ELISA assays. The purpose of our study was to determine the extent to which the feeding rate and human blood indices (HBIs) of malaria vectors were underestimated when molecular confirmation by PCR was performed on both visually fed and unfed mosquitoes.

In association with the Southern Africa International Centers of Excellence for Malaria Research (ICEMR), mosquito collections were performed at three sites: Choma District in southern Zambia, Nchelenge District in northern Zambia, and Mutasa District in eastern Zimbabwe. All anophelines were classified visually as fed or unfed, and tested for blood meal species using PCR methods. The HBIs of visually fed mosquitoes were compared to the HBIs of overall PCR confirmed fed mosquitoes by Pearson's chi-square test of independence.

The mosquito collections consisted of *Anopheles arabiensis* from Choma, *An. funestus* sensu stricto, *An. gambiae* s.s., and *An. leesoni* from Nchelenge, and *An. funestus* s.s. and *An. leesoni* from Mutasa. The malaria vectors at all three sites had large human blood indices (HBI) suggesting high anthropophily. When only visually fed

mosquitoes tested by PCR for blood meal species were compared to testing those classified as both visually fed and unfed mosquitoes, it was found that the proportion fed was underestimated by up to 18.7%. For most *Anopheles* species at each site, there was a statistically significant relationship ($P < 0.05$) between the HBIs of visually fed mosquitoes and that of the overall PCR confirmed fed mosquitoes.

The impact on HBI of analyzing both visually fed and unfed mosquitoes varied from site to site. This discrepancy may be due to partial (interrupted) blood feeding behavior by mosquitoes, digestion of blood meals, sample condition, and/or expertise of entomology field staff. It is important to perform molecular testing on all mosquitoes to accurately characterize vector feeding behavior and develop interventions in malaria endemic areas.

Introduction

Malaria is a significant public health problem in Africa, killing hundreds of thousands of children annually [145]. In sub-Saharan Africa, *Plasmodium falciparum* malaria is the most common malaria parasite and is transmitted by mosquito species belonging to the *Anopheles* genus. The extent of vector-host association is one of the most important factors in predicting vectorial capacity [146, 147] and forms the basis for the Ross-MacDonald model and other contemporary models that estimate malaria transmission intensity [148-151]. The human blood index (HBI), or the proportion of blood meals taken on humans by mosquitoes, varies dramatically even within a single taxon, across localities and between seasons [149], and reflects differences in intrinsic host preferences, host availability, and accessibility [48, 152-155]. The HBI of malaria vectors is used to determine anthropophily, changes in feeding behavior, and even multiple blood feeding frequency [15, 153, 156-158]. Host preference studies have also been used to monitor the effectiveness of vector control programs by observing a reduction in blood feeding behavior, and have even served as evidence of control failure [159-162]. Additionally, the counts of fed mosquitoes from pyrethrum spray catches (PSCs) have been used as a correlate of biting rate in the estimation of the entomological inoculation rate (EIR), or the number of infectious bites per person per time period. Measurement of EIRs gives an estimation of transmission intensity in an area [163] and can be used to determine the contribution of each vector species to malaria transmission in a particular locale [99, 164]. Variation in EIRs over time and space is therefore often used to assess effectiveness of control and identify malaria foci [165].

In the field, one of the first steps in ascertaining the blood meal host is to visually identify and separate collected mosquitoes based on species morphology and feeding status. The mosquitoes that appear to be blooded are labeled as "fed" and it is these samples that are usually separated for blood meal analysis for identification of the host or simply counted if exclusive host association is assumed or capacity for host determination is unavailable. However, the possibility remains that some collected mosquitoes may have taken a small or partial blood meal or may have partially digested the blood and are indeed fed, but morphologically appear "unfed". Most importantly, these mosquitoes represent vectors that have bitten a host and therefore could have potentially vectored pathogens, but have evaded the "fed" count during field investigations. By not evaluating these mosquitoes for blood meal host, the blood feeding frequency and EIR may be significantly underestimated and HBI miscalculated leading to inaccurate interpretations of vector foraging behavior, parasite transmission, and malaria control. In this study, we performed mosquito collections in three distinct epidemiological areas in southern Africa: (1) Choma District, Southern Province, Zambia (controlled malaria transmission), (2) Nchelenge District, Luapula Province, Zambia (uncontrolled malaria transmission), (3) Mutasa District, Manicaland Province, Zimbabwe (resurgent malaria transmission). The specific aim was to estimate the disparity in morphological and molecular assessments of anopheline feeding status at all sites.

Materials and Methods

Study area.

These studies were carried out in association with the Johns Hopkins Southern Africa International Centers of Excellence for Malaria Research (ICEMR) project at three field sites: Choma District, southern Zambia (16.39292°S, 26.79061°E), Nchelenge District, northern Zambia (9° 19.115'S, 28° 45.070'E), and Mutasa District, eastern Zimbabwe (18° 23.161'S, 32° 59.946'E) (Figure 3.1) [166].

Choma District

In Choma District, collections were done within the catchment area of the Macha Mission Hospital, approximately 65 kilometers northeast from Choma town, Southern Province at a mean altitude of 1100 meters above sea level. Extensive malaria entomological and epidemiological studies have been conducted in this area since 2003 [166]. This area consists of mainly scrub bushland interspersed with seasonal streams (Miombo woodland) and the population consists of mainly cattle herders and subsistence farmers. There is a single rainy season each year (November to May), followed by a cool dry season (May to August) and a hot dry season (August to November). Vector control in the area relies on the use of long lasting insecticide-treated nets (LLINs). Household ownership of LLINs is estimated to be more than 90% and usage greater than 75% for across all age groups (unpublished data). Malaria transmission at this site is restricted to the rainy season. Households were randomly selected from a grid overlaid on satellite imagery and were either assigned to a longitudinal cohort of houses followed every other month or for cross-sectional studies samples in the interim months.

Nchelenge District

The field site in Nchelenge District, Luapula Province borders the Democratic Republic of Congo and lies along Lake Mweru. The area is located at a mean elevation of 807 meters above sea level in a marsh ecotype. The majority of the population in this area participates in subsistence farming and fishing. The seasons closely follow that of Choma District, although malaria transmission occurs year-round with a seasonal peak during the rains. Current vector control in this area includes LLIN distribution and indoor residual spraying (IRS) with an organophosphate. Net ownership and usage amongst study households is lower than that of Macha, with approximately 70% of households owning LLINs and usage across all age groups of approximately 50% (unpublished data). Longitudinal and cross-sectional households that were already enrolled in the ICEMR program and were also located within two defined 1-km^2 grids along both Lake Mweru and Kenani Stream were chosen for mosquito sampling.

Mutasa District

The study site in Mutasa District, Manicaland Province, Zimbabwe bordering Mozambique is an area marked by broad elevation changes, with a range of approximately 600 to 1300 meters above sea level. The majority of the population lives in Honde Valley. Subsistence farming occurs along streams and rivers, but there are several large tea estates within the district. Malaria transmission is seasonal, occurring most intensively during the wet season between November and April. Cool dry and hot dry seasons occur similarly to the study sites in Zambia. This area is targeted annually for IRS, and LLIN ownership is estimated at 88% and usage across all age groups at 70%

for the study households (unpublished data). Mosquito collections took place in ICEMR longitudinal and cross-sectional households that were randomly selected from 1-km^2 grids similar to the other sites.

Mosquito collection and handling.

Field collections took place from January 2012-December 2013 in Macha, March-April 2012 in Nchelenge, and December 2012-February 2013 in Mutasa. Mosquitoes were collected from consenting households using Center for Disease Control miniature light traps (CDC LTs; John W. Hock Ltd, Gainesville, FL, USA) at all sites, and additionally by PSCs in Nchelenge and Mutasa. Collection methods were approved by the Johns Hopkins Bloomberg School of Public Health IRB (#00003467) and in Zambia (TDRC/ERC/2010/14/11) and Zimbabwe (BRTI AP102/11). CDC LTs were hung indoors next to sleeping persons under LLINs, approximately 1.5 m above the floor, and would typically run from 6:00pm to 6:00am. PSCs were performed in the morning (6:00am-10:00am) in selected households, where white sheets were placed over floors and furniture, and an aerosol insecticide (100% synthetic pyrethroid) was applied towards the ceiling, eaves, and walls. After approximately 15 minutes, the sheets were taken out of each household and knocked down mosquitoes were collected.

Visual classification of bloodfed status.

All collected mosquitoes were killed by freezing. Using a dissecting microscope, female anopheline mosquitoes were morphologically identified to species (both vectors and non-vectors) using standard keys [156] and visually classified to feeding (abdominal) status ("fed" or "unfed"). Each mosquito was placed individually into a labeled 0.6 mL

microcentrifuge tube containing silica gel desiccant and cotton wool, and stored either at room temperature or frozen at -20°C until laboratory processing, which took place at both the Johns Hopkins University Bloomberg School of Public Health in Baltimore, Maryland and the Macha Research Trust in Macha, Zambia.

Classification of blood fed status by DNA techniques.

The head and thoraces of all anopheline mosquitoes were separated from the abdomen of each mosquito and DNA extraction of the abdomens was performed using a modified salt extraction [119]. Morphological identification of anopheline mosquitoes was confirmed using a PCR specific for members of the *An. gambiae* complex or *An. funestus* complex [121, 122]. All specimens collected in Nchelenge and Mutasa were tested for blood meal species by PCR whilst in Macha only those determined to be the vector *An. arabiensis* were analyzed due to the large number of specimens collected over the 2-year period. Specimens were tested using the Kent et al. multiplex PCR, which differentiates potential mammal host bloods through amplification of the cytochrome b gene of the mitochondrial genome producing a range of species specific bands from 132 to 680 base pairs [119]. Samples that did not amplify a band(s) for blood meal host were then tested with a more sensitive Fornadel et al. PCR and restriction fragment length polymorphism (RFLP) assay [167]. In brief, the Fornadel et al. PCR is used to amplify a 98 base pair region from the cytochrome b gene of the mitochondrial genome of the mammalian host, followed by a restriction enzyme digest that is specific to that animal host [167].

Statistical analysis.

Pearson chi-square test of independence using STATA version 11 was performed to determine if the proportion of fed mosquitoes is the same or different between methods (visual classification or the overall PCR confirmation). A *P* value less than 0.05 was considered statistically significant.

Results

Composition of Anopheles species.

Choma District

From January 2012 to December 2013, 643 female *An. arabiensis* were collected from 113 traps across 69 different households in Choma District. All collected anophelines had their morphological identities confirmed by molecular methods, of which *An. arabiensis* comprised 67%, and the other 33% was made up of other non-vector anophelines.

Nchelenge District

From March-April 2012 in Nchelenge District, 411 *Anopheles* were collected from 18 CDC light traps and 15 PSCs from 31 households and morphological identity was confirmed by PCR analysis. *Anopheles funestus* s.s. accounted for 83.4% of the total collection followed by *An gambiae* s.s. (8.8%) and *An. leesoni* (7.8%).

Mutasa District

From December 2012-February 2013, 84 *Anopheles* were collected in Mutasa District from 43 CDC light traps and 14 PSCs from 13 households. Morphological

identifications in the field were confirmed by molecular methods. The collection was composed of 97.6% *An. funestus* s.s. and 2.4% *An. leesoni*.

Determination of blood feeding frequency, blood meal source, and HBI for visually fed anophelines.

Choma District

In the collection, 11.7% (75/643) of *An. arabiensis* were classified visually as fed and of those 75, 48 (64%) were confirmed by both the Kent et al. PCR and Fornadel et al. PCR methods, giving a feeding rate of 7.5% (Table 3.2). Of the 48 blood fed confirmed *An. arabiensis*, 46 had fed on humans or mixed human/animal blood meal to give an HBI of 0.96. One of these specimens was found to have a mixed blood meal of human and goat.

Nchelenge District

Of the collected *Anopheles* species, 32.4% (111/343) of *An. funestus*, 25% (9/36) of *An. gambiae,* and 18.8% (6/32) of *An. leesoni* were scored as visually fed and all were molecularly confirmed by both the Kent et al. and Fornadel et al. PCR methods (Table 3.2). Of 126 blood fed *Anopheles*, 111 *An. funestus*, 9 *An. gambiae*, and 5 *An. leesoni* had fed on humans. One specimen of *An. leesoni* had also taken a goat blood meal. The HBIs for both *An. funestus* and *An. gambiae* were 1.00. *An. leesoni* had a lower human blood index of 0.75.

Mutasa District

Of the collected *Anopheles* species, 30.5% (25/82) of *An. funestus* were scored as visually fed and all were molecularly confirmed by both the Kent et al. and Fornadel et al. PCR methods (Table 3.2). None of the collected *An. leesoni* were visually fed. Of the 25 blood fed *An. funestus*, 24 had fed on human blood and 1 had fed on goat. The resulting HBI was 0.96 for *An. funestus*. None of the *An. leesoni* caught were classified as fed by any method.

Determination of blood feeding frequency and blood meal source for visually unfed anophelines.

Choma District

The Kent et al. PCR method revealed 3.9% (22/568) of *An. arabiensis* previously scored visually as unfed had actually taken blood meals (Table 3.2). It was also found that one *An. arabiensis* had fed on cow and two *An. arabiensis* had fed on goat. There was also one mixed human and dog blood meal detected. Of those classified as unfed by both morphology and the Kent et al. PCR, the more sensitive Fornadel et al. PCR method revealed that a further 11.5% (63/546) of *An. arabiensis* had actually taken human or other non-human blood meals.

Nchelenge District

Of those *Anopheles* that appeared unfed in the field which were subsequently tested for blood meal source by the Kent et al. PCR method, 0.9% (2/232), 3.7% (1/27), and 7.7% (2/26) of unfed *An. funestus* s.s., *An. gambiae* s.s., and *An. leesoni* respectively

were positive for human and goat blood meals in Nchelenge (Table 3.2). No other animal host was detected. The Fornadel et al. PCR method revealed that further 8.3% (19/230), 15.4% (4/26), and 16.7% (4/24) of *An. funestus*, *An. gambiae*, and *An. leesoni* respectively, previously classified as unfed by morphology and the Kent et al. PCR had actually taken human and/or goat blood meals.

Mutasa District

Unlike Choma District and Nchelenge District, molecular testing of visually unfed *Anopheles* by the Kent et al. PCR did not reveal any additional fed mosquitoes. However, the Fornadel et al. PCR revealed that 5.3% (3/57) of the visually unfed *An. funestus* had taken human (2/3) and goat blood meals (1/3) (Table 3.2). No additional blood meals were detected by the Kent et al. or the Fornadel et al. PCR methods in the visually unfed *An. leesoni*.

Overall blood feeding frequency and HBI of Anopheles.

Choma District

Combining the outcomes of the PCRs carried out on anophelines visually scored as fed and unfed revealed that the actual proportions of fed *An. arabiensis* was 22.1% (Table 3.2). Therefore, visual scoring alone may result in blood feeding rates being underestimated as much as 10.4% compared to PCR detection of blood meals. If determination of host by Kent et al. PCR was limited to those mosquitoes determined visually as fed, HBI was calculated as 0.96, but if all mosquitoes were analyzed using both PCR methodologies, 124/142 *An. arabiensis* had fed on humans, some with mixed animal/human blood meals. This resulted in a reduction in the estimated HBI for *An.*

arabiensis to 0.87. Chi-square test results for *An. arabiensis* detected a significant relationship between the visually fed status and the overall PCR confirmed fed status (df = 1; $X^2 = 144.4$; $P < 0.05$).

Nchelenge District

Of those *Anopheles* specimens classified visually both as fed and unfed, combining the results of the Kent et al. and Fornadel et al. PCR methods, revealed that the actual proportions of fed *An. funestus* s.s., *An. gambiae* s.s., and *An. leesoni* were 38.5%, 38.9%, and 37.5% respectively in Nchelenge (Table 3.2). Using just visual assessment of blood feeding status could, therefore, underestimate blood feeding frequency by as much as 18%. After accounting for these blood meals detected in visually unfed *Anopheles*, the HBIs for *An. funestus* and *An. gambiae* remained at 1.00, whereas *An. leesoni* was higher at 0.80. Chi-square test results indicate a significant relationship between the visually fed status and the overall PCR confirmed fed status for all malaria vectors in this area (*An. funestus* s.s.: df = 1, $X^2 = 267.7$; $P < 0.05$; *An. gambiae* s.s.: df =1, $X^2 = 21.2$; $P < 0.05$; *An. leesoni*: df = 1, $X^2 = 16.2$; $P < 0.05$).

Mutasa District

The PCR results for both visually fed and unfed *Anopheles* reveals that the overall proportion of fed mosquitoes was 35.4%, suggesting that visual assessment alone underestimated blood feeding rates by up to 4.9% (Table 3.2). After detection of goat blood meals in visually unfed *An. funestus*, the HBI for *An. funestus* was reduced to 0.96. Chi-square test revealed a significant relationship between the visually fed status and the

overall PCR confirmed fed status for *An. funestus* in this area (df = 1; $X^2 = 168.3$; $P < 0.05$).

Discussion

Through entomological investigations in Choma, *An. arabiensis* has been identified as the primary malaria vector of *P. falciparum* transmission and analysis of blood feeding was restricted to samples identified as this vector [168]. Although this vector is known for its zoophilic behavior in many parts of Africa, it has been found to be highly anthropophilic in Choma. After molecular testing, the human blood index of *An. arabiensis* decreased due to the identification of blood meals from other animal hosts such as goats and cows in the mosquitoes that were visually unfed. This indicates that *An. arabiensis* takes occasional blood meals on non-human hosts, although many of these may be small meals where the mosquito does not feed to repletion or is partially digested. Although still highly anthropophilic, these previously undetected blood meals dilute the reported rates of anthropophily for this species [167].

In Nchelenge, *An. funestus* s.s. is the most abundant species followed by *An. gambiae* s.s. and *An. leesoni*. Preliminary field collections in the area have confirmed *An. funestus* s.s. and *An. gambiae* s.s. to be the primary and secondary vectors of *P. falciparum* transmission (See Chapter II). The malaria parasite has not been detected in *An. leesoni* in Nchelenge. However, the role of *An. leesoni* as a malaria vector in other parts of Africa suggests its potential as a secondary vector in this region and further investigation is warranted [169]. The human blood indices of both *An. funestus* s.s. and *An. gambiae* s.s. remained the same after molecular testing on all mosquitoes regardless

of abdominal status, indicating that they are highly anthropophilic vectors. However, after testing all *An. leesoni* for blood meal host, the updated HBI increased suggesting greater anthropophily than would have been estimated if only visually classified specimens had been analyzed.

In Mutasa District, the primary malaria vector of *P. falciparum* is *An. funestus* s.s. (unpublished data). The human blood index of *An. funestus* was reduced slightly after molecular testing of both visually fed and unfed mosquitoes due to detection of additional goat blood meals, but confirms the high anthropophily of this species in eastern Zimbabwe. None of the collected *An. leesoni* were visually fed or molecularly confirmed as fed. As a result, we were not able to determine the blood meal source and resulting HBI of this potential vector species.

For basic malaria vector studies, identifying the host of mosquito blood meals is a crucial step in estimating vector transmission potential and intensity of malaria transmission. When mosquito collections take place in the field, it is common practice to have trained personnel identify each mosquito and classify the abdominal status by morphology. Once in the laboratory setting, normally only those mosquitoes labeled as "fed" are tested for blood meal host, and even then only if the infrastructure and financial support exists to conduct these assays. However, in this study, we demonstrated that a significant proportion of visually classified "unfed" mosquitoes have detectable blood meals by PCR methods. The Kent et al. and Fornadel et al. PCR protocols used in this study amplify different portions of the cytochrome B gene, but the Fornadel et al. PCR is more sensitive by being able to detect small and/or degraded blood meals up to 60 hours post-feeding in laboratory experiments [119, 167]. A large proportion of visually

"unfed" mosquitoes were found to be blooded by the Kent et al. PCR and a further number were found to be fed by the Fornadel et al. PCR assay. By only testing the visually "fed" mosquitoes for blood meal host identification, the true proportion of fed mosquitoes in a collection may be underestimated by as much as 18%. We have demonstrated this trend in three epidemiological distinct sites in southern Africa. Conversely, it was also observed in the Choma site that a small proportion of the visually fed mosquitoes did not contain a blood meal as determined by the Kent et al. and Fornadel et al. PCR protocols. This may occur because of incorrect classification of the specimen, desiccation of specimens resulting in dark pigmentation that can be mistaken for blood in mosquitoes, specimens with enlarged abdomens may actually be gravid or half gravid, or contain a sugar meal. It may also be due to the inherent limitation of the PCR assays used [119, 167].

The molecular confirmation of "unfed" mosquitoes actually being fed may be due to several reasons. Firstly, it may indicate partial or interrupted feeding behavior, resulting in a blood meal size that is undetectable by the human eye. In the field, host defensive behaviors can interrupt a mosquito's ability to reach repletion [170]. Previous field studies using unrestrained hosts in stable traps found that a large proportion of *Culex tarsalis* mosquitoes were attracted to the bait, but took partial or no blood meals [171, 172]. Similarly, laboratory-reared mosquitoes also experienced decreased feeding success due to defensive host behaviors [170, 173, 174]. Another factor that may result in partial or reduced blood feeding is vector control; at all three sites of our study, vector control such as LLINs and/or IRS have been implemented in response to which mosquitoes may limit their duration of contact with a host to avoid insecticides [169].

Consequently, mosquitoes may be unable to reach repletion during feeding and must take multiple blood meals during a gonotrophic cycle. This has important implications for estimating vector potential and malaria transmission risk in endemic areas [15, 170, 175]. However, this study was not designed to assess feeding behavior pre- and post-intervention. Clearly, further research needs to be done to ascertain the extent of anopheline partial blood feeding behavior in Africa. In addition to partial feeding, mosquitoes may have undergone partial digestion of the blood meal such that the volume remaining is not easily detectable by eye. Visual assessment of blood feeding status may also be hindered by sample condition such as desiccation or damage. Additionally, personnel must be trained to correctly assess the abdominal status.

Overall, if all collected mosquitoes are not tested for blood meal host, then the proportion fed, HBI, and even EIR may be miscalculated and the accuracy of vector studies may be diminished. The proportion fed in a collection can be an important component for testing and evaluating vector control interventions such as LLINs, IRS, or spatial repellants. Efficacy may be determined by observing a reduction in feeding behavior by vectors as well as changes in other parameters such as deterrency, entry/exit behavior and mortality rates [176-180]. The HBI, a component of vectorial capacity, provides crucial information about mosquito feeding patterns and vector-host association [181]. Furthermore, an incorrect estimate of the number of fed mosquitoes can lead to a miscalculation of biting rates and therefore EIR. The relationship between EIR and malaria prevalence is not direct, but EIR can range from 0 to 1500 infective bites per person per year in endemic parts of Africa [182, 183]. Thus, it can be a useful measurement in relating malaria endemicity and transmission intensity [182, 184].

Accuracy in the calculations for HBI and EIR are essential for defining malaria transmission and dynamics in affected locales [185], and for guiding appropriate control strategies and assessing their effectiveness. Based on this study, we predict that in areas with highly anthropophilic vectors such as Nchelenge and Mutasa Districts, the HBI will show little or no change, but a potentially large effect on the EIR, when testing for blood meal source in all mosquitoes. However, in areas with both anthropophilic and zoophilic vectors such as Choma, testing the blood meal source in all mosquitoes could affect both the HBI and EIR.

The present study illustrates the importance of testing morphologically unfed and fed mosquitoes for identification of host blood meal. By not testing all mosquitoes in a collection, inaccurate measurement of the HBI and even the EIR may result. We showed that misestimation of the HBI occurred when restricting testing to only those visually fed, even at sites with very different vector compositions and epidemiology. Both the HBI and EIR contribute to our understanding of malaria transmission intensity by *Anopheles* mosquitoes; these parameters not only help direct control efforts, but also provide tools for surveillance by assessing potential changes in foraging behavior in response to vector control or other ecological changes. The visually unfed mosquitoes that have detectable blood meals by molecular methods may suggest partial feeding behavior, a response to vector control measures, partial blood meal digestion that is undetectable by eye, or errors in interpreting unfed or fed abdomens by personnel. Although performing molecular techniques to identify host blood meals in both morphologically fed and unfed mosquitoes is ideal for increased accuracy in measurements of anopheline foraging behavior and estimation of EIR, it may pose a challenge for resource limited countries to

be able to perform such extensive testing. As a result, it is suggested that sub-sampling and extrapolation can be used for morphological and molecular determination of host blood meal in order to more accurately characterize mosquito feeding behavior in malaria endemic areas.

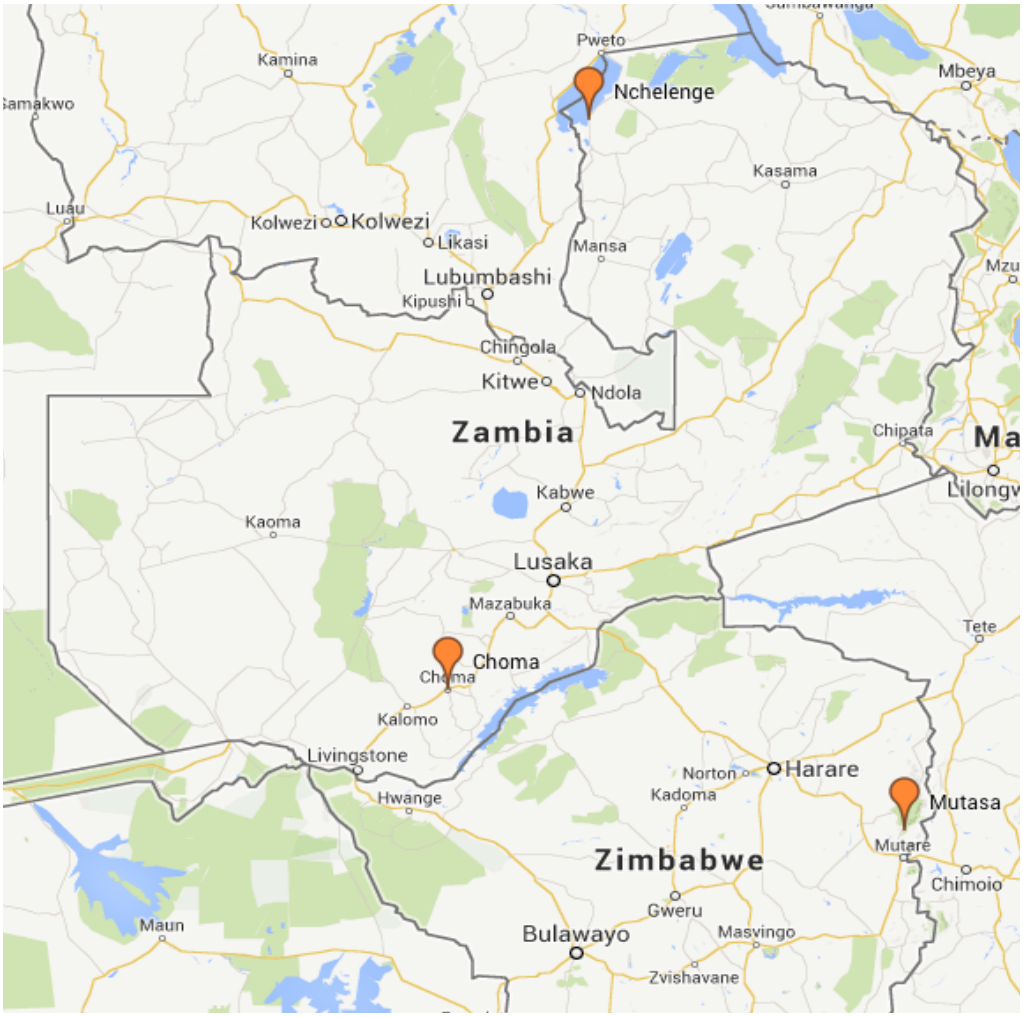

Figure 3.1. The southern Africa ICEMR field sites. Nchelenge District is in northern Zambia and represents unsuccessful malaria control, Choma District is in southern Zambia and represents successful malaria control, and Mutasa District is in eastern Zimbabwe and represents resurgent malaria.

Collection	An. Vector Species	Fed visually (%)	Fed visually and confirmed molecularly[#] (%)	HBI[*]	Unfed Visually but Fed by Kent PCR(%)	Unfed Visually and by Kent PCR but Fed by Fornadel PCR (%)	Combined PCR confirmed Fed[a] (%)	Underestimation of blood feeding frequency[b] (%)	Updated HBI[c]
Macha (n=643†)	arabiensis (n=643)	11.7	7.5	0.96	3.9	11.5	22.1	10.4	0.87
Nchelenge Mar-Apr 2012 (n=411)	funestus s.s. (n=343)	32.4	32.4	1.00	0.86	8.26	38.5	6.1	1.00
	gambiae s.s. (n=36)	25.0	25.0	1.00	3.7	15.4	38.9	13.9	1.00
	leesoni (n=32)	18.8	18.8	0.75	7.7	16.7	37.5	18.7	0.80
Mutasa Dec 2012 (n= 84)	funestus s.s. (n=82)	30.5	30.5	0.96	0.0	5.26	35.4	4.9	0.93
	leesoni (n=2)	0.0	0.0	---	0.0	0.0	0.0	---	---

† Restricted to *An. arabiensis*

Confirmation by both Kent et al. [119] and/or Fornadel et al. [167] PCRs

* HBI based on Kent et al. PCR of visually fed mosquitoes

a Combined results of Kent et al. and Fornadel et al. PCRs run on visually fed and unfed mosquitoes

b Difference in blood feeding frequency of only visually fed mosquitoes and the combined PCR confirmed fed mosquitoes

c HBI based on molecularly determined fed mosquitoes (e.g. Analyzing all mosquitoes caught, whether visually fed or unfed)

Table 3.1. Abdominal status and human blood indices (HBI) determined by molecular assays of visually fed and unfed anophelines at three field sites in southern Africa

Chapter IV

Multiple Blood Feeding Behavior and *Plasmodium falciparum* Infection Complexity in *Anopheles* Mosquitoes in Northern Zambia

Abstract. The interactions among *Anopheles* mosquitoes, the human host, and the malaria parasite were investigated by characterizing multiple blood feeding behavior and the genetic diversity of *Plasmodium falciparum* in infected mosquitoes in Nchelenge District, Zambia. Located in the northern part of Zambia in Luapula Province and borders the Democratic Republic of Congo (DRC), Nchelenge experiences holoendemic transmission despite the use of vector control methods. Mosquito collections were performed from March-April 2013 (wet season) using Center for Disease Control light traps (CDC LTs) and pyrethroid spray catch (PSC) collections. The dominant malaria vectors of *P. falciparum* are *Anopheles funestus* sensu stricto and *An. gambiae* s.s. during the wet season. In addition, the entomological inoculation rate (EIR) was 39.6 infectious bites/person/6 months (ib/p/6mo) for *An. funestus* and 5.9 ib/p/6mo for *An. gambiae*. However, the EIR, a measure of transmission intensity by malaria mosquitoes, makes the assumption that a mosquito takes only one bite per gonotrophic cycle. In Nchelenge District, it was demonstrated that in the March-April 2013 wet collection, both *An. funestus* and *An. gambiae* take multiple human blood meals at similar rates, 23.2% and 25.7% respectively, and that both vectors have a tendency to bite human males. A crucial component in the calculation of EIR is the sporozoite infection rate (SIR), or the proportion of mosquitoes that are infected with the malaria parasite. Interestingly, the parasite within the mosquito can be genetically diverse. In the March-April 2013 collection, the overall *P. falciparum* complexity of infection (COI) in infected

mosquitoes was 6.4 with a range of 1-14 clones. Together, multiple blood feeding behavior and *P. falciparum* COI may be used for surveillance of vector behavior, especially in response to control, and also emerging parasite clones that may successfully be vectored to the human population.

Introduction

Affecting an estimated 198 million people worldwide, malaria is a major public health problem and the burden is disproportionately higher in sub-Saharan Africa, where *Anopheles funestus* sensu stricto and *An. gambiae* s.s. are the most important vectors of *Plasmodium falciparum* [1]. Between 2000 and 2013, there was a 58% decrease in malaria cases in children under the age of 5 years in Africa and despite a 43% increase in population over the last ten years, the infection rate of both symptomatic and asymptomatic decreased from 173 million to 128 million, a 26% reduction [1]. Many of these improvements are attributed to increased coverage of vector control interventions (Long lasting insecticide-treated nets and indoor residual spray), accessibility to rapid diagnostic tests (RDTs), and artemisinin-based combination therapy (ACT) [1]. Especially as implementation of vector control continues, it will be imperative to continue surveillance activities of vector foraging behavior and malaria parasite transmission to assess the effectiveness of control measures.

The proportion of human blood meals in a mosquito collection, the human blood index (HBI), has been used to monitor the success of control programs, changes in foraging behavior, and even the multiple blood feeding rate [15, 108, 118, 158]. Moreover, the entomological inoculation rate (EIR), the number of infectious bites per person per time period, is an essential measurement of transmission intensity by anopheline vectors and is calculated as the product of the human biting rate and the *P. falciparum* sporozoite infection rate (SIR) [163, 186]. A basic assumption of the EIR is that a mosquito bites once and takes a single blood meal per gonotrophic cycle [15, 187]. However, if mosquitoes exhibit multiple blood feeding behavior, then the human biting

rate increases and subsequently the EIR are underestimated, and most importantly the increased risk of malaria transmission within the human population goes unrecognized [15, 187]. Furthermore, an increased biting rate decreases the vector population size needed to sustain malaria parasite transmission, and smaller populations tend to be more difficult to control and eliminate [188]. Accordingly, vector control programs that aim to reduce human infection by decreasing the *Anopheles* population may not be effective because a single mosquito can contribute to multiple human infections. Basic ecological modeling of arthropod disease vectors has demonstrated that an underestimation of the proportion of people bitten may lead to a 2-4 fold increase in the basic reproductive number (Ro), the number of infected individuals resulting from a single infectious person [106, 107]. When the EIR is less than 100 ib/p/yr in an endemic area, and immunity and a finite population are included in a malaria transmission model, the basic Ro in a population may actually decrease. This suggests that more bites are focused on individuals who are already infected [105]. If the annual EIR is higher, then the Ro will increase with little to no heterogeneity in biting behavior [105]. Thus, it is crucial to take into account multiple blood feeding behavior when calculating the EIR to better define the Ro of malaria transmission and the extent of heterogeneity, or inequality of risk.

Multiple blood feeding can occur due to natural disturbances in the environment, as well as host defensive behaviors that affect the ability of mosquitoes to take a single full blood meal for oogenesis [57, 172]. Vector control measures such as indoor residual spraying (IRS) and long-lasting insecticide treated nets (LLINs) may also deter mosquitoes from reaching repletion in order to avoid contact with insecticides [169]. Interestingly, laboratory reared insecticide resistant *An. arabiensis* were found to

maintain metabolic resistance with age when taking multiple blood meals versus those that did not feed or took only a single blood meal, suggesting that taking more than one blood meal is beneficial for the longevity of female anopheline mosquitoes [189]. Heterogeneity in malaria transmission risk can also result from inherent host attractiveness to mosquitoes, proximity to breeding sites, host age and body size, and bed net use and quality [15]. The rate at which mosquitoes take multiple blood meals differs among species and may be further driven by ecology. In places where *Aedes aegypti* reside and dengue virus is the major public health concern, multiple blood feeding behavior has been routinely observed and associated with heterogeneities in biting behavior, where a certain proportion of individuals or gender are bitten more often [58]. For example, *Ae. aegypti* in Puerto Rico and south central Thailand were found to have multiple blood feeding rates of 18% and 45% respectively [58, 59]. In Puerto Rico, it was found that there was a feeding bias towards young adults and males, whereas in south central Thailand, it was found that non-residents visiting collection households were more likely to be bitten, highlighting the importance of human movement among and within communities in dengue transmission [58, 59]. Similarly, multiple blood feeding and heterogeneity in foraging behavior has also been detected in *Anopheles* vectors of malaria in Africa. In western Kenya, DNA profiling of human blood samples and mosquito blood meals within a household was performed, and it was found that the multiple blood feeding frequency of *An. funestus* and *An. gambiae* was 14% and 11% respectively [57]. The same study also revealed that only 20% of profiled human hosts were contributing to more than 50% of all blood meals, and young adults were more likely to be bitten than older adults and children [57]. If adults were sleeping under an

untreated bed net, while children remained unprotected, it appeared that the bites were redirected to children [57]. There were no epidemiological or ecological differences in human-vector contact between highland and lowland areas [57]. Similarly, multiple blood feeding rates and inequality in biting behavior were investigated in southern Zambia pre- and post- insecticide treated net (ITN) distribution. The dominant vector in Macha, Southern Province, Zambia is *An. arabiensis* and is anthropophilic despite high coverage of ITNs. The multiple blood feeding rate decreased from 18.9% to 9.1% pre- and post- ITN distribution, and it was demonstrated that the difference was due to heterogeneity in biting behavior, where mosquitoes fed on people who were not protected [187]. Before ITN distribution in 2007, 20% of individuals contributed to 40% of mosquito blood meals, whereas post ITN distribution, 25% of individuals contributed to 78.1% of mosquito blood meals [187]. Although there were no differences observed in the human gender preference by the population of *An. arabiensis*, there was significant spatial clustering of households with large densities of *An. arabiensis*, which may make those households appropriate targets for future malaria control activities [187]. By characterizing *Anopheles* multiple blood feeding behavior, the EIR and heterogeneity in risk are better defined to focus interventions on high-risk individuals and/or households.

Though the effect of the malaria parasite on mosquito feeding behavior has not been well studied in the field [15, 190], research has revealed that the foraging behavior of *Leishmania*-infected sandflies, *Yersinia pestis*-infected rat fleas, and trypanosome-infected tsetse flies is impaired, leading to an inability to obtain full blood meals and increased probing. Laboratory-based studies of altered feeding behavior by parasites in the malaria system have shown that sporozoites can lower apyrase activity, as well as

increase duration of probing, number of probes, and the likelihood of probing occurring [60, 190, 191]. However, the aforementioned studies were conducted using unnatural vector-host systems and anesthetized hosts, and the number of probes does not increase the likelihood of an infection event [60]. A field study in Tanzania demonstrated that *P. falciparum* infected *An. gambiae* sensu lato were more likely to feed on more than one host than uninfected *An. gambiae* s.l., 22% versus 10% respectively [60]. Likewise, both naturally infected *An. gambiae* s.l. and *An. funestus* were likely to probe more often and for a longer duration than their uninfected counterparts in western Kenya [190]. Accordingly, *P. falciparum*'s ability to manipulate *Anopheles* foraging behavior may be a key mechanism of ensuring endemicity within a human population.

The *P. falciparum* sporozoites found in infected *Anopheles* mosquitoes can be further characterized in the context of multiple blood feeding behavior. The *P. falciparum* complexity of infection (COI) is defined as the genetic diversity of a single malaria parasite species [83, 192]. The production of multiple parasite clones occurs exclusively in the mosquito midgut and has been correlated with the frequency of cross-mating and subsequent meiotic recombination [76, 82]. Studies in Cameroon have suggested that in comparison to monoclonal infections, multiclonal infections in mosquitoes are found at lower parasitemias, more likely to evade the mosquito immune defenses, and perhaps be more efficiently vectored to human hosts [74]. This may also lead to competition among clones that have the potential to influence parasite structure and result in the transmission of parasites that confer drug resistance or changes in virulence [74, 76, 193, 194]. Circulation through the vector-host-parasite system relies on the proportion of clones that successfully survive the liver and blood stages to produce

gametocytes [195]. Both the mosquito biting rate and liver stage immunity vary with age and environment such that spatial and temporal dynamics are important to capture in COI studies [195, 196]. In the holoendemic village of Dielmo in Senegal, it was found that infection complexity in asymptomatic individuals was related to the number of parasite clones that were able to successfully begin the blood stage cycle [196]. In a malaria transmission model where multiclonal infection occurs due to super infection, or multiple mosquitoes with different clones contribute to human infection, a lower number of infective mosquito bites reflects a lower number of clones detected in peripheral circulation [196]. Recently, however, it has been observed that the parasite clones in infected individuals are actually related haplotypes, suggesting that an infectious mosquito can successfully transmit multiple clones in a single bite [197]. A major drawback to these studies is that they do not account for multiple blood feeding behavior by mosquitoes, which increases the human biting rate, and thus a potential increase in the number of different clones received or frequency of clones transmitted by a mosquito. As a result, the anopheline multiple blood feeding frequency and *P. falciparum* COI together in infected mosquitoes can provide crucial information about foraging behavior and our understanding of parasite transmission.

Located along Lake Mweru in northern Zambia and bordering the Democratic Republic of Congo (DRC), Nchelenge District experiences intense malaria transmission year-round. Both LLIN distributions and IRS campaigns have been implemented in Nchelenge since 2006, but the area still remains at high risk for malaria [7]. Collections in 2012 and 2013 have shown that the major vectors of *P. falciparum* transmission in this area are *An. funestus* and *An. gambiae*, both of which are highly anthropophilic and

display both spatial and temporal dynamics (See Chapter II). Temporally, *An. funestus* is the dominant vector and has a higher entomological inoculation rate than *An. gambiae* in both the wet and dry seasons. In contrast, *An. gambiae* population increases in abundance during the wet season, but then decreases dramatically to almost zero and has no contribution to transmission in the dry season. Spatially, during the wet season, *An. funestus* is the dominant vector in villages near Kenani Stream, a large stream that flows into Lake Mweru, and *An. gambiae* is the dominant vector in villages along Lake Mweru (See Chapter II) (Figure 4.5). Despite LLIN and IRS programs, *An. funestus* and *An. gambiae* together maintain high year-round transmission.

The specific aims of this study were to investigate the overall multiple blood feeding frequency of *An. funestus* and *An. gambiae* and its impact on EIR, host gender preference by both vector species, and the COI in mosquitoes harboring the malaria parasite in Nchelenge District, Zambia. Additionally, we investigated if *P. falciparum* infection in mosquitoes influences multiple blood feeding behavior. We sought to identify any multiple blood meals in morphologically unfed but PCR confirmed fed mosquitoes, and performed comparisons of vector multiple blood feeding rates based on collection methods (CDC LT only, CDC LT and PSC, PSC only). This study provides evidence of one of the highest rates of multiple blood feeding behavior by major anopheline vectors in sub-Saharan Africa, as well as a description of multiple *P. falciparum* clones present in mosquitoes and presumably transmitted with each bite in Nchelenge District. This information will be especially important as future vector control programs are implemented in Nchelenge. Our findings may be valuable for surveillance

of *Anopheles* foraging behavior and infection complexity of the malaria parasite circulating in other parts of Africa.

Materials and Methods

Study Area.

This study was conducted in collaboration with the Johns Hopkins Southern Africa International Centers for Excellence in Malaria Research (ICEMR) project which is run at three field sites: Choma District, southern Zambia, Nchelenge District, northern Zambia, and Mutasa District, eastern Zimbabwe (See Figure 4.1). The focus of the research reported here is in Nchelenge District, Luapula Province, in Zambia (9° 19.115'S, 28° 45.070'E) at an elevation of approximately 807 meters above sea level and marsh ecotype (Figure 4.2). Nchelenge District lies along the eastern perimeter of Lake Mweru, which serves as a border between the southeastern part of the Democratic Republic of Congo (DRC) and the northern part of Zambia. Kenani Stream, a large stream that flows from south to north through the study area into Lake Mweru. The region experiences three seasons: a single rainy season from November to May, a cool dry season from May to August, and a hot dry season from August to November (Figure 4.3). Annual rainfall follows a seasonal pattern, with a peak of 2700 mm during the rain months and approximately 0 mm during the dry months (Figure 4.3). Although passive surveillance data from 11 health centers in the district suggest some seasonality in confirmed malaria cases, the overall rates throughout the year are high and characterize the region as holoendemic (Figure 4.4). Mosquito sampling was performed at longitudinal and cross-sectional households enrolled in the ICEMR program located

within two defined 1-km^2 grids along both Lake Mweru and another two 1-km^2 grids inland near Kenani Stream. Five villages were involved in this study: three villages in grids r34c5 and r34c6 (Katuna, Yenga, and Malulu) near the lake and two villages in grids r29c10 and r26c11 (Kapande B and Mutepuka) inland along the stream (Figure 4.2). These villages are representative of the local demography and landscape, and frequent movement of local people from fishing to farming (seasonally), as well as high malaria incidence. LLIN and IRS programs were initiated in 2006 and 2007 respectively [6]. In 2012, another LLIN distribution campaign took place and resulted in an average coverage of 1.24 nets per person [7]. IRS with a carbamate insecticide was conducted in 2013, but was applied mainly to households found along the major road that runs along Lake Mweru (personal communication with Dr. Mike Chaponda, TDRC, 2014). Recently, an IRS campaign with the organophosphate pirimiphos methyl (Actellic 300 CS, Syngenta Limited) began in September 2014 and was chosen due to high resistance of *Anopheles* vectors in the area to most insecticide classes (Personal communication with Dr. Douglas Norris, JHBSPH, and Mbanga Muleba, TDRC, 2014).

Mosquito Collection and Handling.

From March 5-April 25, 2013 (wet), mosquitoes were collected by Center for Disease Control light traps (CDC LTs) and pyrethroid spray catches (PSCs) in three villages along Lake Mweru (Yenga, Katuna, and Malulu) and two inland villages along Kenani Stream (Kapande B and Mutepuka). Collection methods were approved by the Johns Hopkins Bloomberg School of Public Health IRB (#00003467) and by a Zambian IRB (TDRC/ERC/2010/14/11). In the March-April 2013 collection, lakeside and streamside villages were sampled intensely on alternate days each week. CDC LTs were

performed on Monday, Wednesday, and Friday, and PSCs on Tuesday, Thursday, and Saturday during each collection week. Briefly, CDC LTs were hung indoors next to sleeping persons under LLINs, approximately 1.5 m above the floor and would typically run from 6:00pm to 6:00am. PSCs were performed in the morning, between 6:00am and 10:00am, in selected households where white sheets were placed over all surfaces and a 100% synthetic aerosol pyrethroid was applied towards the ceiling, eaves, and walls,after which the home was closed. After approximately 15 minutes, the sheets were removed from the homes and knocked down mosquitoes were collected. All field-caught mosquitoes were killed immediately by freezing. The female anopheline mosquitoes were separated and morphologically identified to species using a dissecting microscope and dichotomous key [97]. Up to three mosquitoes were placed in each labeled 0.6 mL microcentrifuge tube containing silica gel desiccant and cotton wool, and stored either at room temperature or frozen at -20°C until laboratory processing, which took place at Johns Hopkins University Bloomberg School of Public Health in Baltimore, Maryland.

DNA Preparation and PCR.

The head and thorax of each collected anopheline were separated from the abdomen and a modified salt extraction method was used to extract DNA from the abdomen. The morphological identification of anopheline mosquitoes was confirmed using a polymerase chain reaction (PCR) specific for members of the *An. gambiae* complex or *An. funestus* complex [121, 122]. If neither PCR was able to identify the mosquito species, then a PCR targeting the ITS2 gene was used [198]. The ITS2 PCR amplifies a portion of the intergenic spacer region 2 of the ribosomal DNA, and has a

range of base pair sizes that are specific to *Anopheles* species outside of the *An. gambiae* and *An. funestus* complexes [121].

All specimens, both morphologically fed and unfed, were tested for blood meal host using the Kent al. 2005 multiplex PCR, which targets the cytochrome b gene of the mitochondrial genome producing a range of mammalian host specific bands from 132-680 base pairs [119]. A modification to the Kent PCR enhanced detection of the human host; the original Kent et al human forward primer was replaced with a pair of forward and reverse primers that amplify a unique 193 base pair region of the human cytochrome b gene. The forward primer is FOR16068: 5'- GAC TCA CCC ATC AAC AAC CG -3' and the reverse primer is REV16260: 5'- GGC TTT GGA GTT GCA GTT GA -3'.

A more sensitive PCR was performed on samples that did not amplify a band(s) for blood meal host by Kent et al PCR [11]. The Fornadel et al. 2010 PCR amplifies a 98 base pair region of the cytochrome b gene of the mitochondrial genome of the mammalian host, followed by a restriction fragment length polymorphism (RFLP) assay specific to an animal host of interest [11].

DNA from mosquito abdomens was also used to test for the presence of the *P. falciparum* parasite by PCR. The Norris et al. PCR, which was developed in the laboratory, amplifies a small portion of the cytochrome b gene of *P. falciparum*, is more sensitive and reliable than the commonly used Snounou et al. PCR, and has an expected fragment size of 183 base pairs [34, 125].

Enzyme-linked Immunosorbent Assay (CSP-ELISA) for Plasmodium falciparum Detection

The CSP-ELISA method as previously described by the Malaria Research and Reference Reagent Resource Center (MR4) was used to detect *P. falciparum* circumsporozoite protein (CSP) in the mosquito head and thorax. Briefly, a 96-well U-bottom plate is incubated overnight with *P. falciparum* CSP capture antibody. Then, each mosquito head and thorax is homogenized using a pestle, and added to the plate (38 mosquitoes per plate) with CSP capture monoclonal antibody. Following a two-hour incubation, the plate is washed 7 times, and then the CSP monoclonal antibody with a conjugated peroxidase tag is added to the plate and incubated for one hour. The plate is then washed 7 times, and ABTS solution is added to visualize the presence of CSP in the mosquito head and thorax for one hour. If CSP protein is present in the mosquito head and thorax, then the well containing an individual mosquito will turn green and suggests that the mosquito was infected with sporozoites and therefore infectious. The plate is then placed in a spectrophotometer set at 400-nanometer wavelength for analysis. The values associated with each mosquito that are two times the average of the negative controls on the plate are considered to be CSP or *P. falciparum* positive. Due to the large number of anophelines caught in the March-April 2013 collection, a representative sub-sample of mosquitoes was chosen for CSP-ELISA. The sporozoite infection rate (SIR) was calculated as the proportion of infectious mosquitoes confirmed by ELISA and the total mosquitoes tested in each collection.

Microsatellite analysis.

Multiple blood feeding behavior was determined by amplifying and sequencing human microsatellites in human fed anophelines. A subsample of human blooded *Anopheles* mosquitoes was used for microsatellite analysis. Four loci were used to determine alleles, as well as the gender of the host. Primers fluorescently labeled with HEX and FAM were used to amplify the CTT (CSF1PO, THO1), Penta D STR (Penta D), and Silver-STR (D13S317) loci [15, 57-59, 199]. Minor modifications of the primers by Jiang et al. 2012 [199] were made to all primers compared to those used in previous studies [15, 57-59, 199] (Table 4.6). The 20 µl PCR reaction for each microsatellite contained 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 µM dNTPs, 2.0 units Taq polymerase, 20 pmol each forward and reverse primers, and 2 µl template DNA.

To determine the gender of human blood meals, the Amelogenin locus was amplified using FAM-labeled primers as previously described [200] (Table 4.6). The 50 µl PCR reaction for Amelogenin contained 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 400 µM dNTPs, 2.0 units Taq polymerase, 25 pmol each forward and reverse primers, and 2 µl template DNA. One microliter from the CSF1PO, THO1, Penta D, and D13S317 PCR reactions, and 2 µl of the Amelogenin PCR reaction were multiplexed together with 15 µl formamide and 0.5 µl GeneScan-500 Rox size standard (Applied Biosystems Inc., Foster City, CA), and incubated at 95°C for 5 minutes. The samples were then prepared for shipment and subsequent fragment analysis to the DNA Analysis Facility on Science Hill at Yale University, New Haven, Connecticut.

Sequencing results were sent back to and analyzed at Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland.

Plasmodium falciparum complexity of infection (COI)

Using mosquito specimens that were positive for *P. falciparum* by PCR and/or ELISA, the COI was determined by genotyping the polymorphic loci of merozoite surface proteins 1 and 2 (*msp1* and *msp2*) and the glutamate receptor protein (*glurp*) of the parasite [194]. The repetitive regions block 2 of *msp1* and block 3 of *msp2* were amplified by nested PCR, and the RII repeat region of *glurp* by semi-nested PCR [194]. In the first nested reaction, the primer pairs correspond to conserved regions of the polymorphic regions of each gene [194, 201]. The first reaction product was then used in the second nested reaction as the template for six subsequent and separate reactions, each of which uses a specific primer pair to detect allelic variants from the K1, MAD20, and RO33 families of *msp1* block 2, the FC27 and 3D7/IC families of *msp2* block 3, and the RII block of *glurp* [194]. Following gel electrophoresis, the genotypes were characterized by the base pair sizes using FluorChem Image Analyzer (Protein Simple). For each isolate, the number and size of genotypes, as well as the *msp1* and *msp2* allelic families were described.

An infection was considered monoclonal if a single PCR fragment was detected for at least one locus [194]. If more than one PCR fragment was detected for any of the loci: *msp1*, *msp2*, or *glurp*, then the infection was considered polyclonal [194]. The number of bands for *msp1* and *msp2* were determined by adding the bands observed for the K1, MAD20, RO33 families, and the FC27 and 3D7/IC families respectively [74, 75,

194, 202]. The largest number of bands at whatever locus was considered the overall COI of that infection [74, 75, 194, 202]. The alleles for each family were placed into bins with a 40 bp width to determine the number of distinct alleles [203-207]. The mean COI was calculated by dividing the sum of each sample's overall COI by the number of positive samples.

Statistical Analysis.

Pearson's chi-square test of independence was used to compare the multiple blood feeding rates between *An. funestus* and *An. gambiae* in Nchelenge District during the March-April 2013 collection. Differences in gender preference by each vector species were determined by exact binomial test where P=0.50. Due to small sample sizes, Fisher's exact test was used to determine any overall relationships in multiple blood feeding rates of *P. falciparum* infected mosquitoes, "unfed" but fed mosquitoes, and among the COI of each loci. Negative binomial regression was used to further investigate the overall COI among antigenic markers, as well as to compare the COI of each locus and the overall COI measurements between *An. funestus* and *An. gambiae*. Detection of parasite clones at each locus was compared by logistic regression. Statistical significance was defined as a p-value less than or equal to 0.05. All statistical analyses were performed using STATA version 11.

Results

Species Identification.

In the March 5-April 25, 2013 collection (wet), 2989 *Anopheles* mosquitoes were caught from 77 households, a majority of which were *An. funestus* (80.9%, n=2417),

followed by *An. gambiae* (18.9%, n=564), and *An. leesoni* (0.2%, n=9). Overall, *An. funestus* was the most abundant anopheline species in the 2013 wet season collection, followed by *An. gambiae* and *An. leesoni* (See Chapter II).

Spatial differences in *Anopheles* abundances were observed between the villages along Lake Mweru and inland villages near Kenani Stream in the March-April 2013 collection (See Chapter II). A total of 133 anopheline were collected in the lakeside villages (Katuna, Yenga, and Malulu) and composed of mainly *An. gambiae* (85.7%, n=114), with the remainder *An. funestus* (14.3%, n=19). In contrast, in roughly the same number of collections, 2,856 anophelines were collected at the streamside villages (Kapande B and Mutepuka), which were composed of 2,397 *An. funestus* (83.9%), 450 *An. gambiae* (15.8%), and 9 *An. leesoni* (0.32%). In the lakeside villages during the wet season, the most abundant anopheline species was *An. gambiae*, but was the second most abundant species in the streamside villages. *An. funestus*, however, was the most abundant species in the streamside villages and the second most abundant species in the lakeside villages. *An. leesoni* was only found in the streamside collections.

Blood feeding behavior.

Regardless of being morphologically classified as fed, all *Anopheles* mosquitoes were tested for mammalian host blood meal using the Kent et al. 2005 and the Fornadel et al. 2010 PCRs. This was performed due to the possibility that there may be mosquitoes that appear "unfed", but are actually fed as determined by the molecular assays.

In the March-April 2013 collection, 18.4% (444/2417) *An. funestus* and 17.7% (100/564) *An. gambiae* were visually fed and positive for human and/or goat blood

meals. Of the anophelines that were classified as "unfed", 6.7% (164/2437) fed on human and/or goat hosts: 153 *An. funestus* and 11 *An. gambiae*. Human blood meals were detected in 95.4% (146/153) and 72.7% (8/11) of *An. funestus* and *An. gambiae* respectively. Mixed human and goat blood meals were detected in 7 *An. funestus* blood meals and 3 *An. gambiae*. No fed *An. leesoni* were identified. The HBIs for *An. funestus* and *An. gambiae* were 0.95 and 0.93 respectively.

Plasmodium falciparum detection.

Both PCR and ELISA methods were used to detect *Plasmodium falciparum* in the abdomen and the head and thorax of collected anophelines respectively. In the March-April 2013 collection, *P. falciparum* was detected in 60 mosquitoes composed of 39 *An. funestus* and 21 *An. gambiae*: 27 ELISA positive, 25 PCR positive, and 8 ELISA and PCR positive.

Multiple blood feeding.

A subsample of human blooded *An. funestus* and *An. gambiae* were used for human microsatellite genotyping in all collections. *An. leesoni* was not included because of small sample size. In the March-April 2013 collection, 491 human fed *Anopheles* mosquitoes were analyzed for human microsatellites, of which 376 (76.6%) were successfully genotyped at more than one locus. This sample set was composed of 263 *An. funestus* and 113 *An. gambiae* from both CDC LTs and PSC traps. Fed mosquitoes that were detected by the Fornadel et al. PCR made up 71.3% of the failed samples (82/115). Of the four loci, CSF1PO had the lowest failure rate, 35.8% (176/491), and PentaD had the highest failure rate, 58% (285/491). The overall multiple blood frequencies for *An.*

funestus and *An. gambiae* were 23.2% (n=61/263) and 25.7% (n=29/113), and were not statistically significant (P>0.05). The multiple blood feeding frequencies of *An. funestus* and *An. gambiae* collected by CDC LT only or PSC only were also determined. In CDC LT only collections, 26.9% (18/67) of *An. funestus* and 28.9% (11/38) of *An. gambiae* took more than one blood meal (P>0.05). In PSC only collections, 21.9% (43/196) of *An. funestus* and 24.0% (18/75) of *An. gambiae* took more than one blood meal (P>0.05). When collection methods were compared for *An. funestus* and *An. gambiae*, there was no statistical significance in the multiple blood feeding rates (P>0.05).

When the multiple blood feeding frequencies for each species were compared between the lakeside and streamside villages in the March-April 2013 collection, the rates were similar between both areas, though it must be noted that the collection sizes of each vector for the lakeside was small: 22.2% (2/9) lakeside and 23.2% (59/254) streamside for *An. funestus* respectively and 25% (7/28) lakeside and 26.8% (22/85) streamside for *An. gambiae* respectively. There was no statistical significance in multiple blood feeding rates between the lakeside and streamside for *An. funestus* (P>0.05). *An. gambiae* near the stream were 0.15 times less likely to have taken multiple blood meals than near the lake (95% CI: 0.05-0.46, P<0.05). The multiple blood feeding behavior was then measured for *P. falciparum* positive and human fed mosquitoes to determine if the parasite modulates feeding behavior; the rates were 42.9% (3/7) and 20% (1/5) for *An. funestus* and *An. gambiae* respectively, but were not significant between species (P>0.05). Like the lake versus stream comparison, the sample size for malaria parasite positive mosquitoes was small for each anopheline species. Surprisingly, multiple blood meals were found in visually "unfed" mosquitoes, 3.8% (2/52) *An. funestus* and 26.8%

(11/41) *An. gambiae*, but the proportion of multiple blood meals detected was not statistically significant within species (P>0.05).

Human Gender Preference.

The gender of human blood meals detected in *Anopheles* mosquitoes collected in March-April 2013 collection was determined by genotyping the Amelogenin locus, a single copy gene located on the human X and Y chromosomes and can be used to differentiate human sex. Of the 491 samples tested, 399 (81.3%) successfully amplified the Amelogenin locus. The proportion of human blood meals identified as male was higher than that of females: 53.5% (154/283) and 45.6% (129/116) for *An. funestus* and 56.9% (66/116) and 43.1% (50/116) for *An. gambiae* respectively, but were not significant (P>0.05). When the proportion of male blood meals taken by *An. funestus* and *An. gambiae* were compared, they were not different (P>0.05). The same trend of predominately male blood meals was observed for human fed *An. funestus* and *An. gambiae* in the CDC LT only and PSC only collections, lakeside and streamside villages, *P. falciparum* infected mosquitoes, and visually "unfed" but fed mosquitoes, but were not found to be statistically different (P>0.05 within and among species).

P. falciparum complexity of infection (COI) in Anopheles mosquitoes.

Both PCR and ELISA *P. falciparum* confirmed anophelines were used to investigate the complexity of infection (COI). In the March-April 2013 collection, the rate of successful amplification of *msp1*, *msp2*, and *glurp* loci in 60 *P. falciparum* infected mosquitoes was 71.7% (n=43), 78.3% (n=47), and 40% (n=24) respectively, and only 16.9% (n=11) samples failed when all four loci were combined. The genotyped

mosquitoes comprised of 21 *An. gambiae* and 39 *An. funestus*. In the successfully amplified samples, multiple clones and single clones were detected in 46 (93.9%) and 3 (6.1%) infected mosquitoes respectively. The overall range of parasite clones for *An. funestus* and *An. gambiae* were similar: 1-12 and 1-14 parasite clones respectively. The mean COIs of *An. funestus* and *An. gambiae* were 6.1 and 6.8 respectively ($P>0.05$), and the overall mean COI was 6.4.

The mean COIs of the *msp1*, *msp2*, and *glurp* loci were investigated for relationships and differences. The mean COI was 5.3, 5.4, and 1.6 for *msp1*, *msp2*, and *glurp* respectively. The *msp1* and *msp2* COIs were found to be independent of each other ($P>0.05$), whereas there was a statistical association between the *msp1* and *glurp* COIs, and the *msp2* and *glurp* COIs ($P<0.05$). Differences in COIs among antigen markers revealed that the *msp1* and *msp2* COIs were 5 times and 5.3 times that of the overall glurp COI ($P<0.05$), but there was no statistical significance between the *msp1* and *msp2* COIs ($P>0.05$).

The COIs of the three loci were compared for each vector species, as well as between species. In *An. funestus*, the COIs of *msp1* and *msp2* were 6.3 and 6.2 times higher than that of *glurp* ($P<0.05$), but there was no statistical significance between *msp1* and *msp2* COIs ($P>0.05$). Similarly, in infected *An. gambiae*, the *msp1* and *msp2* COIs were 5.5 and 7.6 times that of *glurp* ($P<0.05$), but no statistical significance was observed between *msp1* and *msp2* ($P>0.05$). When the *msp1*, *msp2*, and *glurp* loci were compared between *An. funestus* and *An. gambiae*, no statistical significance in COI was observed ($P>0.05$).

Detection of clones among the *msp1*, *msp2*, and *glurp* markers, as well as allelic families within a locus was compared. For each allelic family, the numbers of successfully genotyped samples were 40 (66.7%) for K1, 24 (40%) for MAD20, 26 (43.3%) for RO33, 45 (75%) for FC27, and 38 (63.3%) for IC/3D7. Parasite clones were more likely to be detected at the *msp1* and *msp2* loci than *glurp* (OR=4.5, 95% CI: 2.06-9.81, P<0.05 for *msp1*; OR=5.4, 95% CI: 2.43-12.10, P<0.05 for *msp2*). There was no significant significance in the ability to detect parasite between the *msp1* and *msp2* loci (P>0.05). When the presence of parasite in the *msp1* locus was further defined, there was a lower chance of detecting clones in the MAD20 and RO33 loci compared to K1 (OR=0.33, 95% CI: 0.16-0.70, P>0.05 for MAD20; and OR=0.38, 95% CI: 0.18-0.80, P<0.05 for RO33). The FC27 and 3D7 families of *msp2* did not differ in the detection of the malaria parasite in infected mosquitoes (P>0.05).

Spatial differences in the lakeside and streamside COIs were also explored. In the lakeside villages, only 3 infected *Anopheles* mosquitoes were collected, all of which were *An. gambiae*. In the streamside villages, 57 infected anophelines were collected and composed of 39 *An. funestus* and 18 *An. gambiae*. The average COI between the lake and stream for both vectors species was 12.3 and 5.1 respectively, and was close to significance (P=0.055). There was no statistical significance in infected *An. gambiae* collected at lakeside and streamside villages (P>0.05)

The number of distinct genotypes detected at each antigen marker was observed. Twenty-nine *msp1* genotypes were detected: 11 (37.9%) K1, 8 (27.6%) MAD20, and 10 RO33 (34.5%) (Table 4.7). Additionally, there were 36 distinct *msp2* genotypes: 20 (55.6%) FC27 and 16 (44.4%) 3D7 (Table 4.7). For *glurp*, 12 unique genotypes were

observed (Table 4.7). The K1 and FC27 families showed greater parasite diversity, suggesting that these two families were predominant during the March-April 2013 collection.

Discussion

The foraging behavior of *Anopheles* vectors in Nchelenge District was investigated, especially in the context of malaria parasite transmission intensity. The March-April 2013 collection revealed *An. funestus* s.s. and *An. gambiae* s.s. as the primary and secondary malaria vectors respectively, both of which were highly anthropophilic. It was found that *An. funestus* and *An. gambiae* exhibit multiple blood feeding behavior at rates of 23.2% and 25.7% respectively when CDC LT and PSC collections were combined. Statistical differences in the multiple blood feeding frequencies between vectors was not observed, indicating that *An. funestus* and *An. gambiae* feed on more than one person at relatively the same rates. These rates are much higher than the 11-14% multiple feeding rates for *An. gambiae* s.s. and *An. funestus* s.s. reported from western Kenya [57], 10% rate for *An. gambiae* s.s. in Nigeria [208], as well as the 9% rate for *An. funestus* s.l. in Tanzania [209]. Spatially, it was predicted that there would be differences in multiple blood feeding behavior for both vectors because of the relative abundances of each vector in the lakeside and streamside villages (See Chapter II). Our results demonstrate that there was no difference in the multiple blood feeding frequencies of *An. funestus* between the lakeside and streamside sites, whereas *An. gambiae* had a higher rate in the lakeside villages, though the small sample size should be noted. Multiple blood feeding rates of both vectors were also compared among collection methods: CDC LT only, CDC LT and PSC, and PSC only, and no differences were

observed. This suggests that the multiple blood feeding rates of both *Anopheles* mosquitoes are consistent across these common collection methods, a finding that has not been explored elsewhere. The presence of multiple blood meals in CDC LT collections, which theoretically target foraging unfed mosquitoes, is unexpected and should be studied further. An increase in the frequency of blood meals taken by a mosquito can be due to several factors including host defensive behavior, response to vector control, and/or *P. falciparum* infection, all of which reduce interactions between the host and vector such that the vector must bite multiple times in order to get a full blood meal.

Host defensive behaviors can influence the number of blood meals taken by a single mosquito. Previous field studies with *Culex tarsalis* mosquitoes showed attraction of unrestrained host baits in stable traps, but only partial or no blood meals were detected [210]. Laboratory studies have also illustrated the effect of host defensive behavior on feeding failure of *Anopheles* mosquitoes [170, 172, 210, 211]. It is also important to acknowledge differences in human behavior, sleeping arrangements, body size within a population.

Furthermore, vector control measures, which limit human-vector contact, can be detrimental to *An. funestus* and *An. gambiae* [209]. In Tanzania, multiple blood meals in An. *funestus* s.l. and An. *gambiae* s.l. were detected in households without insecticide treated nets (ITNs) compared to households with ITNs, suggesting that ITNs provided substantial personal protection from mosquito bites [209]. Additionally, in western Kenya, *An. funestus* and *An. gambiae* also bit more than one person in areas where people do not sleep under bed nets or have any other means of disrupting mosquito contact [57]. There have been instances where blood fed anophelines were collected regardless of

control measures such as ITNs and/or indoor residual spraying (IRS), but multiple blood feeding behavior was not investigated in those studies [15, 41, 180, 187, 209]. Control efforts can alter the feeding behaviors of *Anopheles* mosquitoes, demonstrating their remarkable ability to adapt to various conditions. In Senegal, it was observed that after an ITN distribution campaign, pyrethroid resistant *An. gambiae* shifted from being endophagic in the late night to exophagic in early evening. Likewise, *An. funestus* and *An. gambiae* had greatly reduced nocturnal feeding activity inside households and increased biting rate outdoors after ITN distribution in Tanzania [212]. *An. faurauti* in the Soloman Islands has become a predominately outdoor feeder after the introduction of ITNs and IRS [176]. In contrast, *An. funestus* and *An. gambiae* in Kenya continued to bite indoors and late night despite the use of ITNs [168]. Unfortunately, the multiple blood feeding frequencies of anophelines in these studies were not investigated pre-ITN and pre-IRS. The collection performed in this study took place in households with long lasting insecticide treated nets (LLINs) and IRS. Insecticide resistance testing in Nchelenge has revealed high resistance to pyrethroid and carbamate by *An. funestus* and *An. gambiae*, as well as DDT by *An. gambiae* [8]. The dramatically reduced effect of the insecticides may not only be a factor in continued indoor feeding, but may have also contributed to the high multiple blood feeding rates of both vectors due to partial or interrupted feeding such that they must bite more than once in order to reach repletion. There has also been some evidence that the malaria parasite in *An. gambiae* may increase multiple blood feeding behavior [60, 190], but this was not observed in a recent study of *An. arabiensis* in southern Zambia [15]. In Nchelenge, although the sample size was small, there was no apparent effect of *P. falciparum* infection and multiple blood feeding

behavior compared to uninfected *An. funestus* and *An. gambiae*. Surprisingly, multiple blood meals were detected in morphologically unfed mosquitoes that were PCR confirmed as fed. This may indicate the sensitivity of the assay to detect multiple meals in a digested blood meal, which may suggest an increased frequency of disruptions in a vector's ability to fully imbibe its human host due to a variety of aforementioned influences (ie. Host defensive behavior, vector control, and parasite modulation of vector feeding behavior).

The method used to observe multiple hosts in a single blood meal was based on the three-allele method previously described by Norris et al. 2010 [15]. Previous multiple blood feeding studies in Kenya and Tanzania used 6-locus and 8-locus fingerprints respectively because several loci increase the detection of numerous alleles that can be matched to fingerprints of human blood samples [207, 209]. By using the three-allele method, two biases are present that underestimate the true proportion of blood meals from multiple humans; first, inbreeding in a population decreases the number of distinct alleles present in a blood meal [15] and second, PCRs that fail lead to decreased detection of unique alleles and thus exaggerate the bias [15]. The simulation model ultimately showed that with a missed detection rate of 30-32%, the bias is only 3-5% percent as long as three or more loci are included [15]. In the method used for this study, the missed detection rate was smaller, 23%, suggesting that the bias will be lower than that predicted by the Norris et al. 2010 simulation model. It should be noted that a large proportion of failed samples were anophelines that were visually unfed but PCR confirmed fed; genotyping failure was likely due to low human DNA concentration in these mosquito abdomens.

The epidemiologic impact of multiple blood feeding behavior by accounting for an increase in the human biting rate results in a significant increase in the reproductive number R_o, the number of new infections that arise from a single infected individual. For example, when a 20% daily multiple blood feeding rate is accounted for in the vectorial capacity equation, the predicted result is a 44% increase in the number of new infectious bites [92, 213]. Accordingly, the human biting rate (HBR) is a relatively sensitive component of the vectorial capacity equation. A simpler, more practical method that directly corresponds to malaria risk is the entomological inoculate rate (EIR). The EIR is defined as the number of infective bites per individual per time period, and is used to define the intensity of malaria transmission by a vector species. The EIR is calculated by multiplying the HBR measured from CDC LT collections and the *P. falciparum* sporozoite infection rate in mosquitoes. If CDC LT raw counts are used for the HBR, the EIR will be underestimated if mosquitoes bite more than once in a single gonotrophic cycle. The high multiple blood meal rates observed in *An. funestus* and *An. gambiae* has a potentially large effect on the EIR. In Nchelenge District from March-April 2013, the EIRs for *An. funestus* and *An. gambiae* were 39.6 ib/p/6mo and 5.9 ib/p/6mo respectively. If multiple blood feeding is taken into account, 23.2% for *An. funestus* and 25.7% for *An. gambiae*, the human biting rate and therefore EIR increases. The resulting EIRs would increase to 48.8 ib/p/6mo for *An. funestus* and 7.4 ib/p/6mo for *An. gambiae*. The risk of malaria transmission by *Anopheles* vectors in an endemic area is severely underestimated if multiple blood meals are excluded from EIR measurements.

The detection of *P. falciparum* sporozoites in *Anopheles* mosquitoes not only provides the sporozoite infection rate for EIR calculations, but it can also reveal the

diversity of the parasite transmitted to the human host. In the context of multiple blood feeding behavior, malaria parasite infection complexity highlights the important role that mosquitoes play in the production of unique clones in the midgut and successful survival of those clones through effective transmission to multiple humans due to an increased human biting rate. The March-April 2013 collection revealed multiple clones in over 90% of infected anophelines. The number of clones detected ranged from 1-12 and 1-14 clones in *An. funestus* and *An. gambiae* respectively. The overall COI in infected vectors was 6.4, signifying a very high transmission setting, and no spatial differences in COI between the lakeside and streamside villages was observed [75, 192, 214]. The parasite COI reported in this study is higher than the COIs reported in infected human blood samples in other parts of Africa, 3.7 in Tanzania, 3.4 in Cote d'Ivoire, 3.2 in Mauritania, 3.0 in Uganda, 2.0 in western Kenya, 1.9 in eastern Sudan, 1.5 in Nigeria and the Gambia [75, 76, 194, 202, 204, 207, 215, 216]. Both infected *An. funestus* and *An. gambiae* in Nchelenge have similar COIs 6.1 and 6.8 respectively, which is consistent with vector distributions and their implicit interactions with human hosts. Additionally, 77 unique *P. falciparum* genotypes were identified; a large number of different genotypes were detected at the *msp1* and *msp2* loci. There have been recent reports of *P. falciparum* and *P. ovale* co-infections (Personal communication with Dr. Douglas Norris), and detection of mixed infections and its influence on *P. falciparum* COI would certainly add to the body of knowledge regarding the complex interaction of multiple infections, development of clones in the mosquito, and possible clinical outcomes.

Infection multiplicity in mosquitoes can be used to test the effectiveness of vector control efforts on chronic infection as it reflects the potential number of clones

circulating throughout a population, as well as novel clones that may contribute to clinical outbreaks. For example, in Tanzania, there was a 17% decrease in malaria infection prevalence in ITN users, but there was no difference in *P. falciparum* COI between users and non-users [217]. A similar finding was also observed in western Kenya [81]. Both studies suggest that ITNs may not have any effect on the protective immunity established in high transmission settings. The effectiveness of ITNs against a vector population is not only threatened by insecticide resistance, but also undermined by the ability of mosquitoes to take single or multiple blood meals and thus allowing continued transmission of parasite clones. Identification of genetically distinct malaria parasite clones in mosquitoes may also be used to predict the potential inoculation of novel clones that may exhibit resistance to drugs, increased virulence, or other variations that may produce clinical outbreaks [74, 76, 193, 194]. Several studies have suggested that multiclonal infections can predict the risk of clinical malaria, which is established by the rate of new infections, the number of clones inoculated at each mosquito bite, and the duration of infections [195, 218-221]. Multiclonal infections have been associated with chronic asymptomatic human infection, which has been the predominant outcome observed in infected individuals residing in Nchelenge (personal communication with Dr. Mike Chaponda, TDRC) [75, 192, 214]. Because COI is known to decrease in adults compared to children due to age and acquired immunity of *P. falciparum*, it will be interesting to investigate this difference in Nchelenge and the influence of mosquito foraging behavior. In Senegal, the infection complexity in asymptomatic individuals was related to the parasite clones that were able to reach the human blood stage; this suggested that a lower number of infective bites would lead to a lower number of clones

being detected in human peripheral blood [196]. However, multiple blood feeding behavior by a vector may actually lead to numerous clones being received from infectious individuals; conversely, a single infectious vector biting multiple humans may lead to a large number of clones detected in human blood circulation, inoculation of novel clones with varying potential to influence clinical outcome, and may actually contribute to stable circulation of numerous clones. It will also be important to assess the importation of novel parasite clones due to migration patterns of individuals living in Nchelenge, where movement between the lake and stream areas is common.

Because the genotyping experiments were performed on the abdominal DNA of PCR and/or ELISA confirmed anophelines, it is possible that the observed COI is an overestimation due to meiotic recombination and simply provides the maximum number of clones that could be transmitted to the human population. However, a study in the Gambia observed multiple parasite clones in infected mosquitoes that were not detected in infected human blood samples and determined that the rate of distinct parasite alleles in mosquitoes was much higher than expected by *P. falciparum* meiotic recombination patterns [76]. As a result, they concluded that there are parasite clones that exist below the limit of detection, but thrive inside the mosquito and contribute to the production of new genetically diverse clones [76]. It will therefore be crucial in future studies to compare the parasite diversity between infected mosquitoes and infected human blood samples. The number of distinct genotypes may have been underestimated by choosing a conservative bin width of 40 bp, which was used to discern bands on multiple gels with varying electrophoretic migration of DNA fragments [206].

The identification of human male or female blood in fed *Anopheles* vectors can reveal heterogeneities in risk and potentially target control strategies. In Nchelenge District, *An. funestus* and *An. gambiae* took a slightly larger proportion of blood meals from males than females, but it was not significant, suggesting that both males and females are bitten approximately equally. There were also no spatial differences of male and female blood meals between the lake and stream. Similar research in southern Zambia also found no significant difference in the biting preference of *An. arabiensis* [187], whereas other studies of *An. funestus* and *An. gambiae* in Kenya and Tanzania observed a feeding bias towards young children and males respectively [207, 209]. DNA profiling of household inhabitants would have provided further details about the human age groups of human blood meals. Our finding is not concordant with a Nchelenge human malaria risk model that suggests that adolescent males are at greater risk of being infected than other age groups, likely due to decreased ITN use (unpublished, Dr. Jessie Pinchoff). Further studies will need to be conducted to better understand gender preference of mosquitoes, household members, ITN use, and human malaria epidemiology.

The characterization of multiple blood feeding behavior and *P. falciparum* infection complexity in *An. funestus* and *An. gambiae* in Nchelenge District from March-April 2013 revealed substantially high rates and *P. falciparum* complexity respectively compared to other parts of Africa. It was also shown that both human males and females receive approximately the same number of bites from both vectors. In combination with multiple blood feeding behavior, the COI results suggest that a tremendous number of clones are undergoing recombination events in the mosquito and being transmitted to

multiple hosts per gonotrophic cycle. Considering a recent IRS campaign and LLIN distribution, it will be crucial to investigate any spatial and temporal changes in the multiple blood feeding frequencies of both anopheline vectors, human gender preference, and *P. falciparum* genetic diversity post-control to identify heterogeneities in malaria risk and changes in parasite COI that may contribute to our understanding and development of effective malaria control.

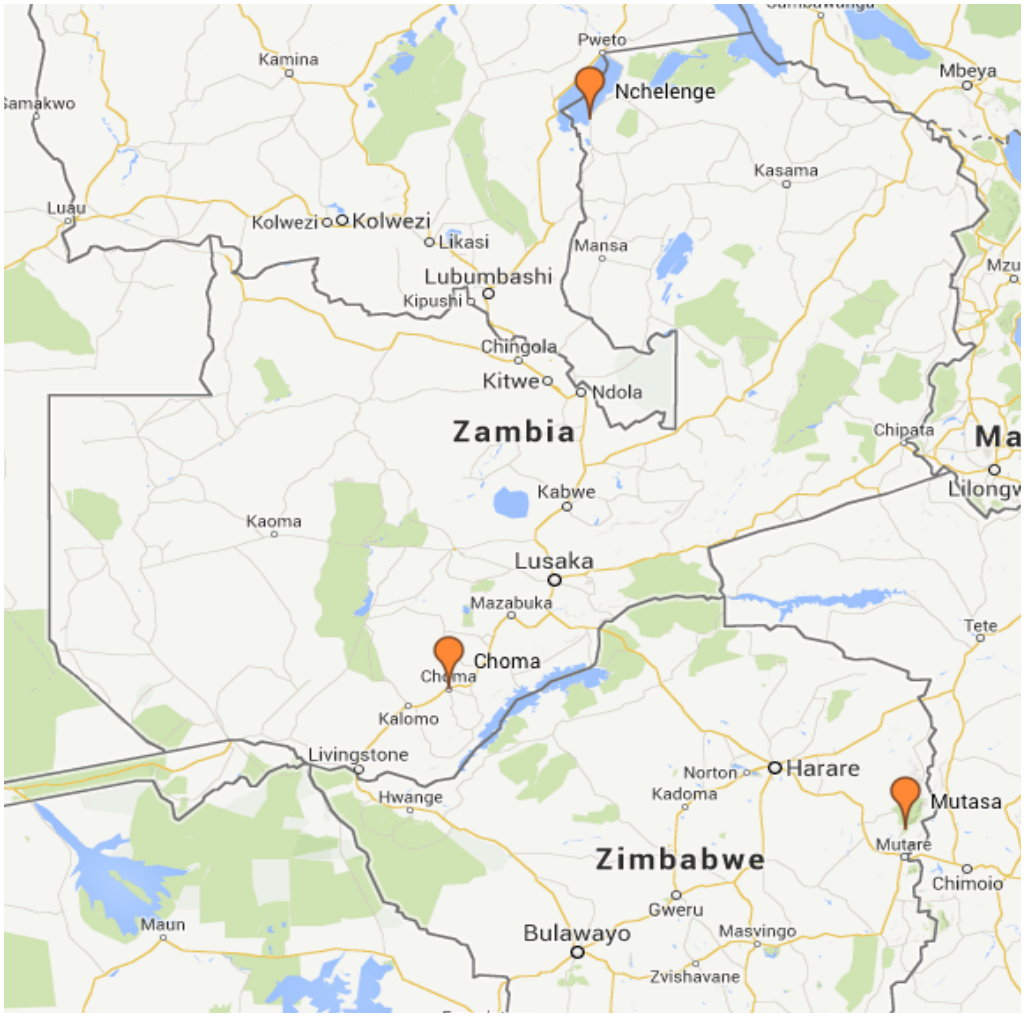

Figure 4.1. The southern Africa ICEMR field sites. Nchelenge District is in northern Zambia and represents a site with unsuccessful malaria control, Choma District in southern Zambia represents successful malaria control, and Mutasa District in eastern Zimbabwe represents resurgent malaria.

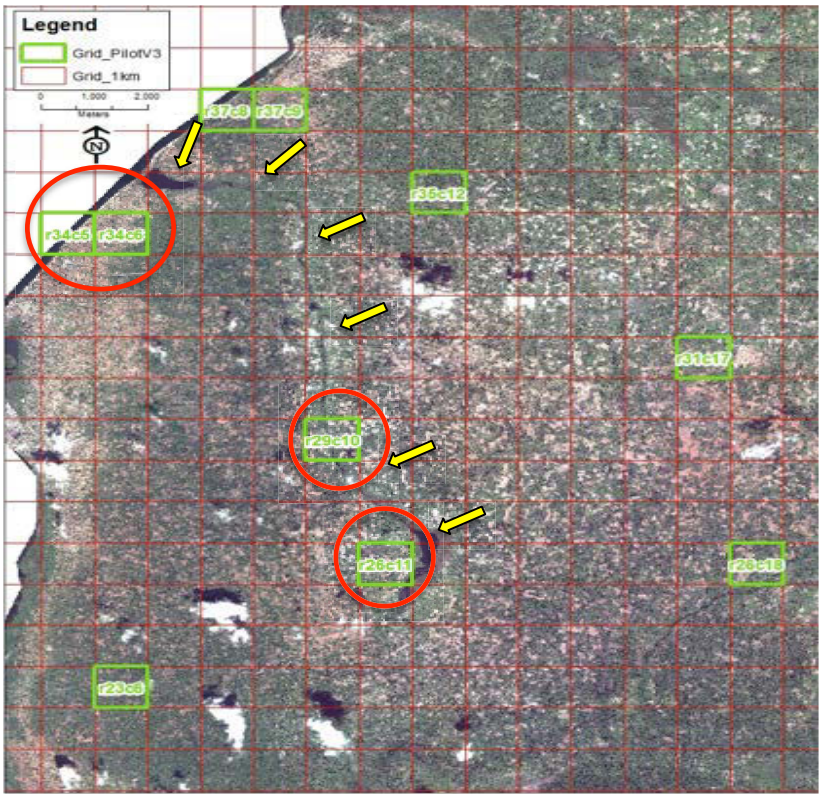

Figure 4.2. Satellite image of the study area in Nchelenge District. The initial 1-km^2 grids for ICEMR epidemiological and entomological surveys are highlighted in green. The white areas on the left side of the image represent Lake Mweru. The yellow arrows point to Kenani Stream that flows into Lake Mweru. The red circles denote the grids where mosquito collections were performed for thesis research: Katuna, Yenga, and Malulu villages are located in grids r34c5 and r34c6, Kapande B village is located in grid r29c10, and Kapande B village is located in grid r26c11.

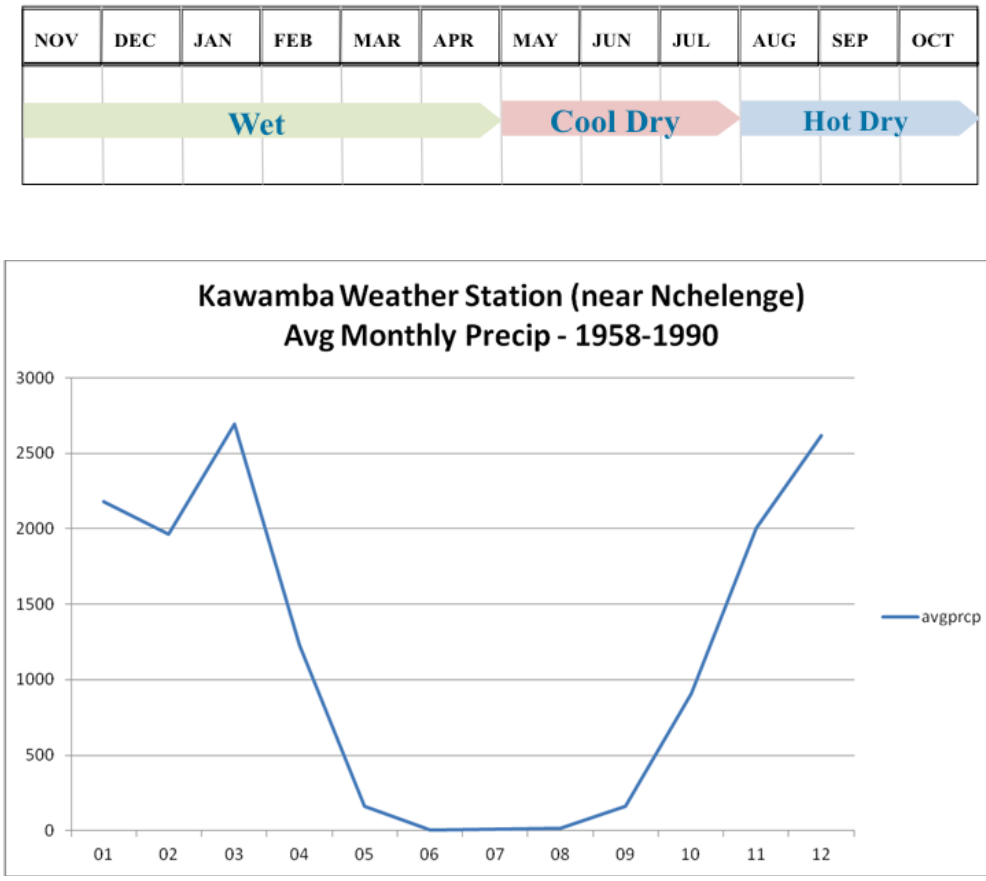

Figure 4.3. Seasons and Average Monthly Rainfall in Nchelenge District. The wet season is from November to May, the cool dry season is from May to August, and the hot dry season is from August to November. The rainfall also follows a seasonal pattern in Nchelenge.

Collection dates	4/2012 to 12/2013
Number of health centers	11
Confirmed malaria cases	94,905

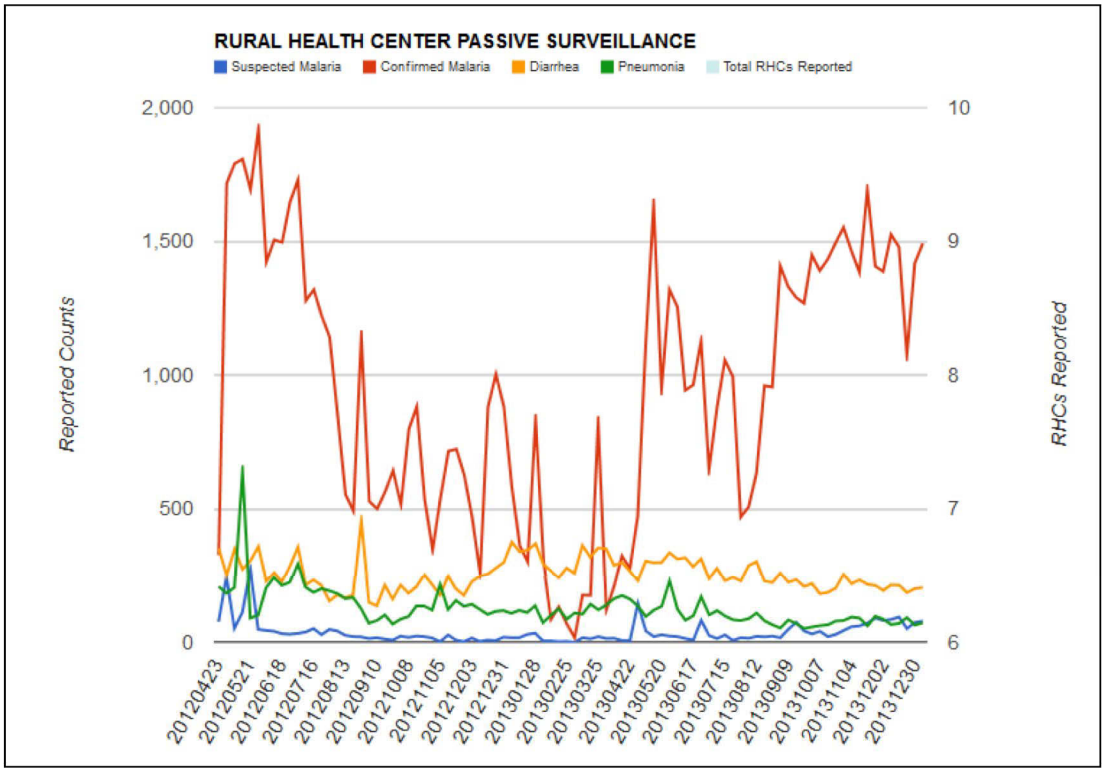

Figure 4.4. Reported counts of confirmed malaria by 11 health centers from April 2012 to December 2013. The number of malaria cases remains high throughout both the wet and dry seasons (southern Africa ICEMR REDCap data).

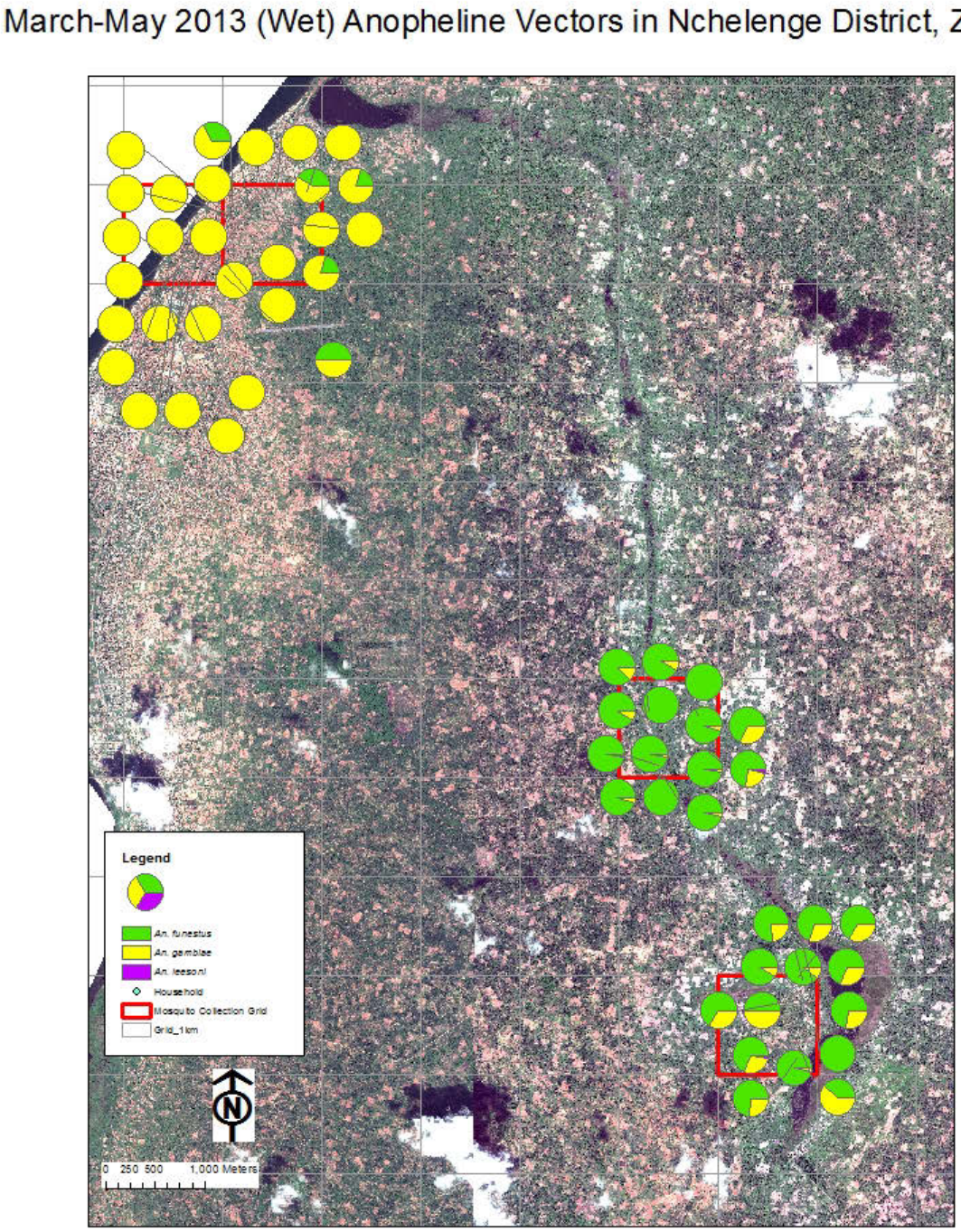

Figure 4.5. The proportion of *Anopheles* species caught per household that were sampled during the March 5-April 25, 2013 (wet) collection. Species are denoted by color: *An. funestus* s.s. (green), *An. gambiae* s.s. (yellow), and *An. leesoni* (purple).

Primer	Primer Sequence
CSF1PO A	5’- /5HEX/ACT CCA GGG CAG TGT TCC A -3’
CSF1PO B	5’- AGC CCA TTC TCC AGC CTC C -3’
D13S317 A	5’- /5HEX/CAT GGT ATC ACA GAA GTC T -3’
D13S317 B	5’- CCA AAA AGA CAG ACA GAA AGA TAG -3’
PentaD A	5’-/5HEX/AAG TAG GAT CAC TTG AGC CTG -3’
PentaD B	5’-CAA GTC CTT TTT TAG ATA TGT GA -3’
THO1 A	5’- /56-FAM/ATT CAA AGG GTA TCT GGG CTC TG -3’
THO1 B	5’- TGG GCT GAA AAG CTC CCG ATT AT -3’
Amelogenin A	5’- /56FAM/CCC TGG GCT CTG TAA AGA ATA GT -3’
Amelogenin B	5’- ATC AGA GCT TAA ACT GGG AAG CTG -3’

Table 4.1. Microsatellite loci and their corresponding primers for multiple blood feeding assay and gender preference from human engorged *Anopheles* mosquitoes.

Nchelenge District: Mar-May 2013 (wet)				
Genes	**# Positive (%)**	**COI**	**# Fragments**	**# Distinct genotypes**
msp1		5.3		**29**
K1	40 (66.7)		65	11
MAD20	24 (40)		47	8
RO33	43.3 (26)		44	10
msp2		5.4		**36**
FC27	45 (75)		108	20
IC/3D7	38 (63.3)		62	16
glurp	24 (40)	1.6	38	**12**
Overall COI		**6.4**		**77**

Table 4.2. Overview of *P. falciparum* complexity of infection (COI) in *Anopheles* mosquito vectors in Nchelenge District.

Chapter V

Conclusions

This dissertation is the first intense evaluation of mosquito vectors and their roles in a high malaria transmission setting in northern Zambia. Mosquito collections were performed in Nchelenge District, Luapula Province, to determine the major anopheline species contributing to transmission, their foraging behaviors, and the intensity of transmission of each species in the wet and dry seasons. Temporal and spatial dynamics of the malaria vectors were also explored to understand their contributions to year-round transmission. The traditional field method for the classification of engorged *Anopheles* mosquitoes and subsequent PCR determination of the mammalian host were examined, and its implications for the entomological inoculation rate (EIR) and human blood index (HBI) were acknowledged. Additionally, the foraging behavior was further defined to expose heterogeneities in malaria transmission, as well as to characterize *P. falciparum* infection complexity in anophelines to better understand their roles in the continued circulation of parasite clones within a human population.

There is a scarcity of research on the vectors and their contribution to holoendemic malaria in Nchelenge District. During the 20th century, there were few publications regarding the flight ranges of unidentified mosquitoes, the identification of *An. funestus* sensu lato and *An. gambiae* s.s. breeding sites, and the potential panmictic population made up of *An. gambiae* complex members A, B, and C, which are recognized as *An. gambiae, An. arabiensis* and *An. quadriannulatus*. More extensive research over the last decade has been performed in primarily in southern Zambia and also at sentinel

sites for detection of vector insecticide resistance. In Nchelenge District, long lasting insecticidal net (LLIN) and indoor residual spray (IRS) campaigns have been implemented since 2007, but the area still experiences some of the highest disease prevalence rates in the country. As a result, it has been imperative to identify and characterize the malaria vectors in Nchelenge to guide more effective control measures in this highly endemic area.

Preliminary collections revealed that the potential vectors could be *An. funestus* s.s. and *An. gambiae* s.s., but more extensive collections were needed to confirm the two species and examine their behaviors in context of human malaria transmission. The work in this dissertation supports the hypothesis that the two main mosquito vectors of *P. falciparum* malaria in Nchelenge District are *An. funestus* and *An. gambiae*, both species are highly anthropophilic, and transmit malaria intensely year-round. *An. leesoni* was also collected, but in lesser numbers than *An. funestus* and *An. gambiae*, and none were infected, suggesting that it is a non-vector or at most an infrequent vector.

Additionally, there are temporal and spatial heterogeneities of malaria vector composition. During the wet season, *An. funestus* is the predominant vector with secondary contribution from *An. gambiae*. The EIR for *An. funestus* was also significantly higher than that of *An. gambiae*. Whereas, during the dry season, *An. funestus* remains the dominant vector, while the *An. gambiae* population severely declines. This is likely due to differences in breeding site preferences of both *Anopheles* mosquitoes and changes that occur at each site in the dry season, which will require extensive oviposition surveys and hydrology studies. In general, *An. funestus* tends to breed in vegetative, semi-permanent or permanent water bodies such as swamps and

ponds; such an area in Nchelenge is Kenani Stream that flows into Lake Mweru. In contrast, *An. gambiae* prefers temporary sunlit breeding habitats such as puddles, animal footprints, and ground depressions formed by heavy rains. During the dry season, these temporary breeding sites disappear, affecting the growth of the *An. gambiae* population. As a result, *An. funestus* continues to transmit *P. falciparum* into and through the wet season. Within Nchelenge District, there are two ecologically distinct areas that affect vector species composition and transmission impact: Areas along Lake Mweru and areas located inland and near Kenani Stream. The hypothesis was further supported by the observation of *An. gambiae* predominately near Lake Mweru, whereas *An. funestus* and *An. gambiae* are the main vectors along Kenani Stream. The difference in abundances between the two geographically distinct locations was significant during the wet season, where the abundances of both *An. gambiae* and *An. funestus* found in lakeside villages was lower than those of the streamside villages. Together with the EIR, streamside villages where collections took place are considered areas to have more intense parasite transmission by anophelines than the villages along the lake. The same pattern was observed during the dry season, except there were few *An. gambiae* caught in households, suggesting that *An. funestus* was transmitting effectively at areas near both the lake and stream.

One of the most crucial entomological measurements is the HBI, human biting rate (HBR), and subsequent EIR. Collectively, these parameters help elucidate vector foraging behavior and transmission potential by identification of the host blood meal, the average number of bites taken by a single mosquito that is used for defining the intensity of transmission, EIR. We sought to determine if traditional field methods of

morphological classification of blood fed mosquitoes and separation of those samples for PCR confirmation underestimate the true proportion of engorged mosquitoes. This premise was substantiated by the finding that visually unfed mosquitoes contained blood meals that were detected by PCR at all three ICEMR sites in southern Africa, including Nchelenge, and that there was a significant relationship between visually fed mosquitoes and overall PCR confirmed mosquitoes. As a result, it was deduced that depending on the transmission setting and the presence of both anthropophilic and zoophilic *Anopheles* species, the HBI could be underestimated. Additionally, if pyrethroid spray catch (PSC) collections are used to determine the human biting rate, not testing the morphologically unfed vectors will lead to an underestimation of the number of mosquitoes taking human blood meals. Since the HBR is an essential component of the EIR, the result may be inaccurate measurements of mosquito transmission intensity. Moreover, the proportion of human fed mosquitoes can be an important component of vector control testing and evaluation, whereby efficacy is determined by a reduction in feeding behavior as well as other changes such as mortality rates, entry/exit behavior, and deterrency.

Previous work in southern Zambia demonstrated changes in multiple blood feeding behavior and human gender preference of *An. arabiensis* pre- and post-ITN distribution; There was a reduction in multiple blood feeding that reflected heterogeneity of risk in individuals protected and not protected by nets, as well as no significant bias for human gender. Our study demonstrated that both *An. funestus* and *An. gambiae* take multiple blood meals per gonotrophic cycle, and the frequencies, 23.2% and 25.7% respectively, are among the highest recorded in sub-Saharan Africa. There was no significant difference in multiple blood feeding between both species. The implication of

this finding is that by biting multiple individuals, the human biting rate increases and results in a dramatic underestimation of the EIR. The reproductive ratio R_o, the number of individuals that become infected from a single infected individual, is also underestimated as an infectious mosquito that takes multiple blood meals transmits the malaria parasite to more than one person. Furthermore, studies in Kenya, Tanzania, and southern Zambia have investigated the possibility of human gender preference by *Anopheles* mosquitoes. In Nchelenge, it was revealed that both *An. funestus* and A*n. gambiae* bite both males and females at approximately the same rate. However, the lack of human DNA fingerprints from collection households makes it difficult to conclude that all males and females are at risk because it may differ depending on other factors such as age group and inherent human behaviors that place people at risk.

Another crucial component of the EIR is the sporozoite infection rate (SIR), or the proportion of infected mosquitoes in a collection. A more detailed look at the transmission of *P. falciparum* in infected mosquitoes revealed the presence of multiple clones. In *An. funestus* and *An. gambiae*, the overall complexity of infection (COI), 6.1 and 6.8 respectively, of the malaria parasite did not differ statistically and ranged from 1-12 and 1-14 clones respectively. The overall COI was 6.4, an exceptionally high number of potential clones circulating within Nchelenge District. It should be noted that there could be potential overestimation of the overall COI in infected mosquitoes because of the inability to distinguish haploid and diploid parasites by PCR. However, the implication of the COI is the transmission of multiclonal parasites with each mosquito bite. In the context of multiple blood feeding behavior, each bite has the potential to transmit an average of 6 parasite clones in Nchelenge, resulting in the accelerated spread,

development and stability of various *P. falciparum* clones. Additionally, when an *Anopheles* mosquito takes a blood meal from an infectious individual, the various clones undergo meiotic recombination events in the mosquito midgut and are also in competition with each other, resulting in a potential change in parasite structure and production of novel clones that confer drug resistance and/or virulence. The frequency of these new clones is largely dependent on the number of infective bites received by an individual and the number of clones transmitted with each bite. Accordingly, an infectious vector that bites multiple humans could influence the rate of development of novel clones with undefined clinical outcomes. The detection of *P. falciparum* clones in the mosquito could be a possible method for surveillance of emergent medically relevant clones and as a measure of endemicity temporally and spatially, especially in response to vector control.

The anopheline foraging behavior studies presented in this dissertation have opened new avenues for future malaria research in Nchelenge District and for malaria control efforts throughout the country. The spatial differences in species abundance between the lake and stream for both *An. funestus* and *An. gambiae* introduces the questions of where oviposition sites are located, ecological differences between locations, and if there is heterogeneity in risk for households located closest to those oviposition areas. Furthermore, the identification of mosquito breeding sites may lead to recommendations for or against larvaciding as an appropriate intervention to reduce the burden of risk in some areas. Also, CDC LT and PSC traps in Zambia captured a large proportion of human fed anophelines that also took multiple blood meals despite the use of LLINs by household inhabitants. As a result, there is a need to better understand the use and effectiveness of LLINs in households, and to also identify which individuals are

bitten more often than others. Genetic profiling of blood from household members would provide important information to identify individuals who are bitten, therefore harboring and donating *P. falciparum* gametocytes, and direct interventions to limit human exposure. The considerable COI detected in *Anopheles* mosquitoes should be compared to the COI in infected human blood samples for agreement, spatial and temporal changes, and also to determine if meiotic recombination overestimated the COI or if there is equal transmissibility of parasite clones. Furthermore, cytogenetics of *P. falciparum* clones in *Anopheles* mosquitoes would reveal patterns of gene exchange among clones within the mosquito that vary by geographical location and/or season, as well as provide evidence of previous bottlenecks. It would also be interesting to verify if infected individuals who are bitten more often than others by DNA fingerprinting of mosquito blood meals have different infection complexities than others.

The recent IRS campaign implemented after this dissertation's studies requires an assessment of *Anopheles* in sprayed households, their foraging behavior, and contribution to transmission intensity, as well as spatial and temporal changes. The IRS campaign has focused on households located along Lake Mweru, but the results of this dissertation indicate that areas more inland and along the stream would benefit the greatest from vector control due to the greater abundance of and higher intensity of transmission by *Anopheles funestus* and *An. gambiae*. There have been reports in other African countries, where vector control lead to shifts in mosquito feeding behavior, such that people remained at risk. As such, our research findings have set the baseline vector dynamics and bionomics for comparisons with future vector control campaigns. The collections performed for this dissertation were conducted indoors. Accordingly, it will be crucial to

also perform outdoor HLCs, exit/entry and resting traps post-IRS. Moreover, changes in the multiple blood feeding frequency post-IRS may indicate heterogeneity of risk for certain individuals, which may also affect the parasite complexity of infection. In addition to vector measures, COI in infected mosquitoes may also indicate the effect of vector control on the malaria parasite, by either reducing or maintaining existing clones. Any changes observed will be important for surveillance and strategy for future malaria control efforts.

The studies performed as part of this dissertation have provided the framework for future vector research in Nchelenge District by characterizing the foraging behavior of *An. funestus* and *An. gambiae* in detail to determine their roles in intense malaria transmission in this area, any inequalities of risk both temporally and spatially, and identification of parasite clones inoculated with each infectious bite. The knowledge gained from these studies has also established a reference point for evaluation of the long-term impact of IRS or other control efforts. Overall, the findings of this dissertation have resulted in the establishment of baseline research for future entomological studies, and more importantly, recommendations for future vector control in Nchelenge.

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Appendix A

Differentiation of the *Anopheles gambiae* complex by PCR

This PCR uses 4 primers that in combination produce three differentially-sized amplicons of the ribosomal DNA spacer region of *An. gambiae* complex mosquiotoes. The expected product sizes are as follows: *An. gambiae* s.s. (~390 bp), *An. arabiensis* (~315 bp), and *An. quadriannulaus* (~150 bp).

Primers:

UN: 5'- GTG TGC CCC TTC CTC GAT GT -3'
GA: 5'- CTG GTT TGG TCG GCA CGT TT -3'
AR: 5'- AAG TGT CCT TCT CCA TCC TA -3'
QD: 5'- CAG ACC AAG ATG GTT AGT AT -3'

PCR Program: (SCOTT)

1. 94ºC 2 min
2. 94ºC 30 sec
3. 50ºC 30 sec
4. 72ºC 30 sec
5. Go to 2, 29x
6. 72ºC 7 min
7. 4ºC forever

Reaction Mixture:	**25 µL**	**20 µL**	**12 µL**
10X	2.5 µL	2.0 µL	1.25 µL
dNTPs 2.5 mM	2.0 µL	1.6 µL	1.0 µL (final conc 200 µM each)
AR	3.0 µL	2.4 µL	1.5 µL (150 pmol)
QD	3.0 µL	2.4 µL	1.5 µL (150 pmol)
GA	0.5 µL	0.4 µL	0.25 µL (25 pmol)
UN	1.0 µL	0.8 µL	0.5 µL (50 pmol)
Taq	0.75 µL (1.5 U)	0.6 µL	0.38 µL
dH_20	fill to total reaction mix volume		

Use between 0.5 and 1 µL of template DNA.

Reference:

Scott, J.A., W.G. Brogdon and F.H. Collins. 1993. Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction. Am. J. Trop. Med. Hyg. 49(4): 520-529.

Appendix B

Differentiation of the *Anopheles funestus* complex by PCR

This PCR differentiates species of the *An. funestus* complex based on variation in the ITS2 region of nuclear rDNA. There is a universal forward primer and seven species-specific primers. The expected product sizes are as follows: *An. funestus* (505 bp), *An. leesoni* (146 bp), *An. vaneedeni* (587 bp), *An. parensis* (252 bp), *An. rivulorum* (411 bp), *An. rivulorum*-like (313 bp), and *An.funestus*-like (390 bp). Because the expected amplicons from *An. rivulorum* and *An. funestus*-like are too close in size to be effectively visualized on an agarose gel, only one of these primers should be used at a time in the reaction mixture.

Primers:

UV: 5'- TGT GAA CTG CAG GAC ACA T -3'
FUN: 5'- GCA TCG ATG GGT TAA TCA TG -3'
VAN: 5'- TGT CGA CTT GGT AGC CGA AC -3'
RIV: 5'- CAA GCC GTT CGA CCC TGA TT -3'
PAR: 5'- TGC GGT CCC AAG CTA GGT TC -3'
LEES: 5'- TAC ACG GGC GCC ATG TAG TT -3'
RIVLIKE: 5'- CCG CCT CCC GTG GAG TGG GGG -3'
FUNLIKE (MalaFB) 5′- GTT TTC AAT TGA ATT CAC CAT T -3′

PCR Program: (FUNESTUS)

1.	94ºC	2 min
2.	94ºC	30 sec
3.	45ºC	30 sec
4.	72ºC	40 sec
5.	Go to 2	29x
6.	72ºC	5 min
7.	4ºC	forever

Reaction Mixture:	**25 µL**
10X	2.5 µL
dNTPs 2.5 mM	2.0 µL (final conc 200 µM each)
UV	0.3 µL (33 pmol each primer)
FUN	0.3 µL
VAN	0.3 µL
RIV (or FUNLIKE)	0.3 µL
PAR	0.3 µL
LEES	0.3 µL

RIVLIKE	0.3 µL
Taq	0.8 µL (1.6 U)
dH_20	16.9 µL

Use 1 µL of template DNA

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Appendix C

ITS2 rDNA PCR

This PCR is very robust and therefore can be used to check the quality of DNA extractions. It targets the ITS2 region of nuclear rDNA and produces amplicons of varying sizes depending on mosquito species. It can be used in tandem with the Funestus PCR to identify ambiguous samples. Because ITSA binds to the conserved 5.8S rDNA and ITS2B binds to the 28S rDNA, this PCR can be used to sequence samples from almost any anopheline mosquito for species identification. ITS2B1, a novel, alternate primer, binds slightly downstream from ITS2B and produces a slightly larger amplicon that can be used to sequence through the entire ITS2.

Expected product sizes for different mosquito species:
Funestus group:

An. leesoni ~520 bp
An. parensis ~ 620 bp
An. vaneedeni ~ 830 bp
An. rivulorum and *rivulorum*-like ~520 bp
An. longipalpis ~620 bp and ~900 bp
An. funestus and *funestus*-like ~850 bp

Other species:

An. rufipes, maculipalpis, and *pretoriensis* ~500 bp
An. theileri ~ 520 bp
An. coustani ~620 bp
An. gambiae complex ~600 bp
An. squamosus – doesn't react

Primers:

ITS2A: 5'- TGT GAA CTG CAG GAC ACA T -3'
ITS2B: 5'- TAT GCT TAA ATT CAG GGG GT -3'
ITS2B1: 5'- GTC CCT ACG TGC TGA GCT TC -3'

Note: Only the ITS2B and ITS2B1 primers work well for sequencing.

PCR Program: (ITS2)

1.	94ºC	2 min
2.	94ºC	30 sec
3.	50ºC	30 sec
4.	72ºC	40 sec
5.	Go to step 2	39x
6.	72ºC	10 min
7.	4ºC	forever

Reaction Mixture:	**25 µL**
10X	2.5 µL
dNTPs 2.5 mM	2.0 µL (final conc. 200 µM each)
ITS2A	0.3 µL (30 pmol)
ITS2B	0.3 µL (30 pmol)
Taq	2.0 U
dH_20	fill to 25 µL

Use 1 µL of template DNA.

Reference:

Koekemoer, L.L., L. Kamau, R.H. Hunt, M. Coetzee. 2002. A cocktail polymerase chain reaction assay to identify members of the *Anopheles funestus* (Diptera: Culicidae) group. Am. J. Trop. Med. Hyg. 6(6): 804-811.

Appendix D

M/S Form Differentiation of *Anopheles gambiae* s.s. by PCR

This PCR diagnostic differentiates the M-form and S-form of An. gambiae s.s. by amplifying the a portion at the 5' end of the rDNA intergenic spacer region. The S-form will have a band at 475 bp and the M-form will have a band at 727 bp. Hybrid M/S form will have two bands at 475 bp and 727 bp.

Primers

R5:	5'- CGA ATT CTA GGG AGC TCC AG - 3'
R3:	5' - GCC AAT CCG AGC TGA TAG CGC - 3'
Mopint:	5' - GCC CCT TCC TCG ATG GCA T - 3'
B/Sint:	5' - ACC AAG ATG GTT CGT TGC - 3'

PCR Program (MSDIFF)

1.	94°C	10 min
2.	94°C	30 s
3.	63°C	30 s
4.	72°C	30 s
5.	Go to Step 2	x24
6.	72°C	7 min
7.	4°C	forever

Reaction Mixture	**25 µL**
10X	2.5 µL
dNTPs 2.5 mM	1.0 µL
R5	0.5 µL
R3	0.5 µL
Mopint	0.4 µL
B/Sint	0.25 µL
Taq	2 U
dH_2O	17.35 µL

Use 2 µL DNA (from abdomen extraction eluted in 50 µL dH_2O).

Reference

Favia, G et al., 2001. Molecular characterization of ribosomal DNA polymorphisms discriminating among chromosomal forms of *Anopheles gambiae* s.s. *Insect Molecular Biology* 10(1): 19-23.

Appendix E

Mammalian host blood meal species identification by PCR

This multiplexed PCR diagnostic differentiates between potential mammal host bloods in engorged mosquitoes. Species-specific products are amplified from the cytochrome b gene of the mitochondrial genome. Expected product sizes are as follows: Human (334 bp), Cow (561 bp), Dog (680 bp), Goat (132 bp), Pig (453 bp). Host source can be detected out to 30 hours post feeding.

Primers:

PIG573F:	5'- CCT CGC AGC CGT ACA TCT C -3'
HUMAN741F:	5'- GGC TTA CTT CTC TTC ATT CTC TCC T -3'
GOAT894F:	5'- CCT AAT CTT AGT ACT TGT ACC CTT CCT C -3'
DOG368F:	5'- GGA ATT GTA CTA TTA TTC GCA ACC AT -3'
COW121F:	5'- CAT CGG CAC AAA TTT AGT CG -3'
UNREV1025:	5'- GGT TGT CCT CCA ATT CAT GTT A -3'

PCR Program: (BLOOD)

1.	95ºC	5 min
2.	95ºC	1 min
3.	56ºC	1 min
4.	72ºC	1 min
5.	Go to step 2	39x
6.	72ºC	7 min
7.	4ºC	forever

Reaction Mixture:	**25 µL**
10X	2.5 µL
dNTPs 2.5 mM	1.0 µL (final conc. 100 µM each)
UNREV1025	0.5 µL (50 pmol of each primer)
PIG573F	0.5 µL
HUMAN741F	0.5 µL
GOAT894F	0.5 µL
DOG368F	0.5 µL
COW121F	0.5 µL
Taq	2.0 U
dH_20	fill to 25 µL

Use up to 3 μL of template DNA (from abdomen extraction eluted in 50 μL dH_20).

Reference:

Kent RJ, Norris DE, 2005. Identification of mammalian blood meals in mosquitoes by a multiplexed polymerase chain reaction targeting cytochrome b. *Am J Trop Med Hyg 73:* 336-342.

Appendix F

Mammalian small blood meal species PCR and RFLP

This PCR targets the cytochrome b gene and produces a 98 bp amplicon that can be differentially digested or sequenced to identify host species. The enzyme digest allow identification of mammalian host sources from partially digested mosquito blood meals (out to 60 hours post feeding), as well as partially degraded DNA extractions.

Primers:

UNIFORA: 5'- TCC AAA CAA CRM AGC ATA ATA TT -3'
UNREV1025: 5'- GGT TGT CCT CCA ATT CAT GTT A -3'

Note: both primers work for sequencing

PCR Program: (BLOOD55)

1.	95ºC	5 min
2.	95ºC	1 min
3.	55ºC	1 min
4.	72ºC	1 min
5.	Go to step 2	39x
6.	72ºC	7 min

Reaction Mixture:	**25 µL**
10X	2.5 µL
dNTPs 2.5 mM	1.0 µL (final conc. 100 µM each)
UNIFORA	0.5 µL (50 pmol)
UNREV1025	0.5 µL (50 pmol)
Taq	2.0 U
dH_20	fill to 25 µl

Use 2 µL of DNA template (from abdomen extraction eluted in 50 µL dH_20)

Enzyme Digests:

Save 6 µL of the PCR products to run as undigested controls on a 3% agarose gel alongside the digested amplicons.

Fnu4HI digest – human

Buffer 4	2.5 µL
dH_20	7.5 µL
1 U enzyme	0.2 µL
PCR product	15 µL

BanII digest – cow

Buffer 4	2.5 µL
dH_20	7.5 µL
1 U enzyme	0.1 µL
PCR product	15 µL

MspI digest – dog

Buffer 2	2.5 µL	dH_20	7.5 µL
2 U enzyme	0.1 µL	PCR product	15 µL

NsiI digest – goat

Buffer 3	2.5 µL
dH_20	7.5 µL
1 U enzyme	0.1 µL
PCR product	15 µL

SpeI digest – pig

Buffer 2	2.5 µL
dH_20	7.5 µL
BSA (100x)	0.025 µL
1 U enzyme	0.1 µL
PCR product	15 µL

All digests are carried out at 37°C for at least 3 hrs but can be left overnight.

Notes:

1. For field-collected mosquitoes digests can be done sequentially in order of the most likely host sources.

2. Master mix can be tripled for a total reaction volume of 78 μL. This will allow one to perform up to 4 digests of the amplified product with enough undigested sample leftover as a control.
3. Some cows have a point mutation in the BanII target site, so that the PCR product will not be digested. These can be identified by sequencing.

Reference:

Fornadel, C.M., D.E. Norris. 2008. Increased endophily by the malaria vector *Anopheles arabiensis* in southern Zambia and identification of digested blood meals. Am. J. Trop. Med. Hyg. 79(6): 876-80.

Appendix G

Universal vertebrate cytochrome b PCR

This PCR has an expected product size of 358 base pairs and can be used to amplify any vertebrate cytochrome b gene for ID by sequencing. This PCR can be used to amplify blood meal DNA from host species not recognized by the avian or mammalian blood PCRs.

Primers:
L14816: 5′-CCATCCAACATCTCAGCATGATGAAA-3′
H15173: 5′- CCCCTCAGAATGATATTTGTCCTCA-3′

PCR Program: (UNIVCYTB)

1.	95ºC	11 min
2.	94ºC	30 sec
3.	50ºC	45 sec
4.	72ºC	45 sec
5.	Go to step 2	29x
6.	72ºC	5 min
7.	4ºC	forever

Reaction Mixture:	**25 µL**
10X	2.5 µL
dNTPs 2.5 mM	2.5 µL (final conc. 250 µM each)
L14816	0.3 µL (30 pmol)
H15173	0.3 µL (30 pmol)
Taq	2.0 U
dH_20	fill to 25 µl

Use between 2 µL of template DNA.

Reference:

W. Parson, K. Pegoraro, H. Niederstätter, M. Föger and M. Steinlechner. 2002. Species identification by means of the cytochrome b gene. *International Journal of Legal Medicine*. 114: 23-28.

Appendix H

Small *Plasmodium* product PCR diagnostic

This PCR specifically reacts with a portion of the cytochrome b gene of *Plasmodium falciparum* and can be used to test for infected mosquitoes. The expected product size is 183 bp. *Plasmodium vivax* cross-reacts with these primers and will produce a similarly-sized amplicon.

Primers:
PFcytbLongF: 5'- ATACATGCACGCAACAGGTGCTTCTC -3'
PFcytbLongR: 5'- CAATAACTCATTTGACCCCATGGTAAGAC -3'

PCR Program: (PFNEW1) – 3 hrs 17 min

1.	95ºC	5 min
2.	95ºC	30 sec
3.	58ºC	50 sec
4.	72ºC	40 sec
5.	Go to step 2	59x
6.	72ºC	5 min
7.	4ºC	forever

Reaction Mixture:	**25** µL
10X	2.5 µL
dNTPs 2.5 mM	1.5 µL (final conc. 150 µM each)
cytbLongF	0.5 µL (50 pmol)
cytbLongR	0.5 µL (50 pmol)
Taq	2.0 U
dH_20	fill to 25 µl

Use 1-2 µL DNA template (from abdomen extraction eluted in 50 µL dH_20).

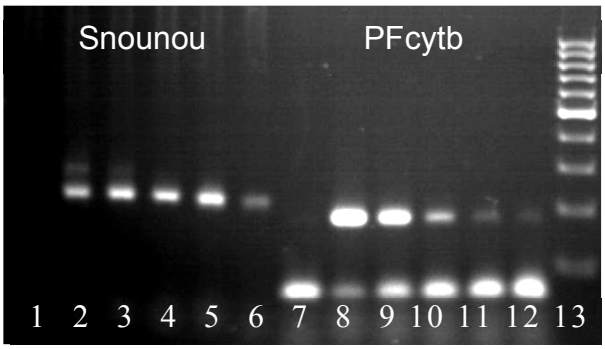


Figure O.1. Comparison of Snounou et al. PCR and PFcytb PCR. Snounou et al. products run on a 2% agarose gel. Lanes 1 and 7, HPLC H_20; Lanes 2 and 8, 10^4 parasites/μL; Lanes 3 and 9, 10^3 parasites/μL; Lanes 4 and 10, 10^2 parasites/μL; Lanes 5 and 11, 10 parasites/μL; Lanes 6 and 12, 1 parasites/μL; Lane 13, 100 bp DNA ladder

Appendix I

Multiplicity of Infection (MOI) of *Plasmodium falciparum* in Infected Mosquitoes

Distinct strains of *P. falciparum* can be distinguished by genotyping of the polymorphic regions *msp1*, *msp2*, *glurp*, and *csp* loci. A nested PCR strategy is used for all loci, except for CSP which is a single PCR reaction. The primary round amplifies parasite DNA using *Plasmodium*-specific oligonucleotide primers. Product from the primary round is subsequently used as template for secondary amplification using *P. falciparum* family-specific primers, yielding size variants within distinct allelic families of MSP1 (K1, MAD20, RO33), MSP2 (FC27 and IC/3D7) and GLURP. These size polymorphs can be resolved by simple agarose electrophoresis and visualized under UV transillumination. The purpose of this protocol is to determine multiplicity of infection of *P. falciparum* in infected anopheline mosquitoes (confirmed by ELISA and/or PCR).

Primary Reaction Primers

MSP1

M1-OF: 5- CTAGAAGCTTTAGAAGATGCAGTATTG -3
M1-OR: 5- CTTAAATAGTATTCTAATTCAAGTGGATCA -3

MSP2

M2-OF: 5- ATGAAGGTAATTAAAACATTGTCTATTATA -3
M2-OR: 5- CTTTGTTACCATCGGTACATTCTT -3

GLURP

G-OF: 5- TGAATTTGAAGATGTTCACACTGAAC -3
G-OR: 5- GTGGAATTGCTTTTTCTTCAACACTAA -3

Secondary Reaction Primers

MSP1

K1-family specific

M1-KF: 5- AAATGAAGAAGAAATTACTACAAAAGGTGC -3
M1-KR: 5- GCTTGCATCAGCTGGAGGGCTTGCACCAGA -3

MAD20-family-specific

M1-MF: 5- AAATGAAGGAACAAGTGGAACAGCTGTTAC -3
M1-MR: 5- ATCTGAAGGATTTGTACGTCTTGAATTACC -3

RO33-family-specific

M1-RF: 5- TAAAGGATGGAGCAAATACTCAAGTTGTTG -3
M1-RR: 5- CATCTGAAGGATTTGCAGCACCTGGAGATC -3

MSP2

FC27-family-specific

M2-FCF: 5- AATACTAAGAGTGTAGGTGCARATGCTCCA -3

M2-FCR: 5- TTTTATTTGGTGCATTGCCAGAACTTGAAC -3

3D7/IC-family-specific

M2-ICF: 5- AGAAGTATGGCAGAAAGTAAKCCTYCTACT -3

M2-ICR: 5- GATTGTAATTCGGGGGATTCAGTTTGTTCG -3

GLURP

G-NF: 5- TGTTCACACTGAACAATTAGATTTAGATCA -3

G-OR: 5- GTGGAATTGCTTTTTCTTCAACACTAA -3

PCR Program: (PfMOIM1-GL)

1. 94ºC	2 min
2. 94ºC	45 sec
3. 60.0ºC	45 sec
4. 65ºC	1 min
5. Go to Step 2	x39
6. 65ºC	3 min

Reaction Mixture:	**25ul**
10X	2.5 ul
dNTPs	2.0 ul
Forward Primer	0.3 ul
Reverse Primer	0.3 ul
Taq	1.0 U
dH20	Fill to 25 ul
DNA	3.0 ul

Reference:

Southern Africa ICEMR SOP Number S0001: *Plasmodium falciparum* MSP1, MSP2 and GLURP Genotyping.

Kiwuwa, MS, Ribacke, U, Moll, K, Byarugaba, J, Lundblom, K, Farnert, A, Fred, K, and Mats Wahlgren, 2013. Genetic diversity of *Plasmodium falciparum* infections in mild and severe malaria of children from Kampala, Uganda. *Parasitol Res* 112: 1691-1700.

Appendix J

Human Microsatellite PCR for Multiple Blood Feeding Analysis

This PCR can be used to amplify human microsatellite DNA from mosquito blood meals for molecular fingerprinting and detection of multiple blood meals and sex differentiation. CSF1PO, D13, THO1, and Penta D work best for differentiating individuals in the Nchelenge population. Each PCR is run separately, and then multiplexed together.

Expected PCR product sizes:

HEX-labeled
CSF1PO: 280-308 bp
D13S317: 173-205 bp
Penta D: 373-446 bp

FAM-labeled
THO1: 159-198 bp
Amel: 104, 109

Primer sets:

CSF1PO A: 5'- /5HEX/ACT CCA GGG CAG TGT TCC A -3'
CSF1PO B: 5'- AGC CCA TTC TCC AGC CTC C -3'
D13S317 A: 5'- /5HEX/CAT GGT ATC ACA GAA GTC T -3'
D13S317 B: 5'- CCA AAA AGA CAG ACA GAA AGA TAG -3'
Penta D A: 5'-/5HEX/AAG TAG GAT CAC TTG AGC CTG -3'
Penta D B: 5'-CAA GTC CTT TTT TAG ATA TGT GA -3'

THO1 A: 5'- /56-FAM/ATT CAA AGG GTA TCT GGG CTC TG -3'
THO1 B: 5'- TGG GCT GAA AAG CTC CCG ATT AT -3'
AMEL A: 5'- /56FAM/CCC TGG GCT CTG TAA AGA ATA GT -3'
AMEL B: 5'- ATC AGA GCT TAA ACT GGG AAG CTG -3'

PCR Program: (HMNMICR)

1. 96ºC 4 min
2. 94ºC 1 min
3. 60ºC 1 min
4. 70ºC 1.5 min
5. Go to Step 2 39x
6. 70ºC 45 min

7. 4ºC forever

Reaction Mixture:	**20 ul**
10X	2.0 ul
dNTPs 2.5 mM	1.6 ul (final conc. 200 uM each)
forward primer	0.2 ul (20 pmol)
reverse primer	0.2 ul (20 pmol)
Taq	2.0 U
dH20	fill to 20 ul

Use 2.0 ul DNA template.

Multiplex 1.0 ul each PCR product (CSF1PO, D13S317, THO1, Penta D) and 1.5 ul Amelogenin product with 15 ul formamide and 0.5 ul ROX ladder. Incubate at 95ºC for 3 minutes. HEX-labeled samples will be green, FAM-labeled samples will be blue.

Package and send samples to Yale University DNA Analysis Facility on Science Hill by FedEx, UPS, or DHL (Attn: Carol Mariani, 170 Whitney Ave., ESC Room 150, New Haven, CT 06511; phone- 203-432-7394).

References:

Norris, LC, Fornadel, CM, Hung WC, Pineda, FJ, and DE Norris. 2010. Frequency of multiple blood meals taken in a single gonotrophic cycle by *Anopheles arabiensis* mosquitoes in Macha, Zambia. *Am J Trop Med Hyg.* 83(1):33.

Scott, TW, Githeko, AK, Fleisher, A, Harrington, LC, and G Yan. 2006. DNA profiling of human blood in Anophelines from lowland and highland sites in western Kenya. *Am J Trop Med Hyg.* 75: 231-237.

Jiang, X, He, J, Jia, F, Shen, H, Zhao, J, Chen, C, Bai, L, Liu, F, Hou, G, and F Guo. 2012. An integrated system of ABO typing and multiplex STR testing for forensic DNA analysis. *Forensic Science International: Genetics.* 6: 785-797.

Appendix K

Marriott DNA extraction procedure

1. If specimens are dried, rehydrate them in a 1.5 mL microfuge tube containing 20 µL HPLC H_20 for 10 minutes. If specimens are frozen begin the procedure from Step 2.
2. Add 100 µL of Bender buffer directly into the tube with the specimen and homogenize until there are no recognizable mosquito parts. Place used pestles in 1M NaOH.
3. Incubate homogenized samples at 65ºC for 1 hour.
4. Add 15 µL cold 8M potassium acetate to each sample. Mix gently and incubate on ice for 45 minutes. (Procedure may be stopped here overnight.)
5. Spin samples in a microcentrifuge (14,000 rpm) for 10 minutes, and then transfer the supernatant to a new 1.5 mL microfuge tube.
6. Add 300 µL 100% ethanol (2X volume) to each supernatant to precipitate DNA. Mix well by inverting the tube. Incubate samples at room temperature for 5 minutes.
7. Centrifuge samples (14,000 rpm) for 15 minutes. Following this spin there should be a small pellet of DNA at the bottom of the tube.
8. Carefully remove the supernatant and discard it, leaving the pellet behind in the tube. Let the pellets dry completely before resuspending – residual ethanol can interfere with PCR later.
9. Resuspend pellets in 50 µL HPLC H_20 for head/thorax or abdomen extractions (100 µL for whole mosquitoes). Ideally, store overnight at 4ºC before use. Store DNA permanently at -20ºC.

Pestle washing: To prevent DNA contamination in PCR-based analyses, pestles should be soaked in 1M NaOH after use. They should then be washed in soapy water, rinsed off in distilled water, and autoclaved before they are used again.

Required solutions:

1. Bender Buffer
0.1 M NaCl (5 mL from a 1M stock solution – need to make this stock solution)
0.2 M sucrose (3.42 grams)
0.1 M Tris-HCl (5 mL from a 1M stock)
0.05 M EDTA ph 9.1 (5 mL from a 0.5M stock)
0.5% SDS in DEPC water (0.25 mL from a 0.1M stock)

Making up 50 mL Bender buffer. First add 3.42 grams dry sucrose to a 50 mL conical tube. Then add the proper amounts of the other ingredients, listed above in parentheses. Fill to a final volume with DEPC water and filter with a 0.2 micron filter before using. Store at room temperature.

To make 1 M NaCl stock solution, add 2.9 grams dry NaCl into 50 mL HPLC H_20 and vortex. Stock solutions of the liquid reagents should come in the molar concentrations listed.

Appendix L

CSP (Circumsporozoite protein) ELISA

This assay detects *Plasmodium falciparum* CSP protein in mosquito samples. CSP is only expressed during the sporozoite stage of malaria development, so this assay detects only sporozoite-positive mosquitoes, which are capable of transmitting malaria. The monoclonal capture antibody nonspecifically binds to the ELISA plate, after which the addition of blocking buffer prevents nonspecific binding of other proteins. After the addition of mosquito homogenate, the capture antibody binds to CSP and holds it during subsequent wash steps. After the monoclonal antibody is added, it also binds CSP and remains after washing. This antibody is conjugated to a peroxidase which catalyzes ABTS indicator solution, turning the solution green, while negative samples remain uncolored.

Adapted from the Malaria Research and Reference Reagent Resource Center (MR4) Methods in *Anopheles* Research Manual, available at http://www.mr4.org/Publications/MethodsinAnophelesResearch/tabid/336/Default.aspx
Limited amounts of *Plasmodium falciparum* positive controls, capture antibodies, and conjugated antibodies are available free of cost through the MR4 website ((MR #890)).

Materials

PBS (phosphate buffered saline, available from MMI Dept.)
BSA (bovine serum albumin) (A7906)
Casein (Sigma C7078)
Phenol red (Sigma P4758)
IGEPAL CA-630 (Sigma I3021)
Tween (Fisher BP337)
P.f. capture MAb (MR #890)
P.f. conjugate MAb (MR #890)
P.f. CSP positive control (MR #890)
Glycerol (Sigma G6279)
ABTS solution (Kirkegaard Perry)
10% SDS (sodium dodecyl sulfate) (Gibco #15553-035)
96-well U bottom vinyl ELISA plates (Corning #2797)

Solutions

Blocking Buffer (BB): 250 mL
250 mL PBS
2.5 g BSA

1.25 g casein
50 µl 0.1 g/mL phenol red stock

Stir ~3 hours until dissolved. Store overnight at 4°C or freeze for future use. Store BSA at 4°C.

BB: IG-630 (mosquito grinding buffer): 5 mL
5 mL BB
25 µl IGEPAL CA-630 detergent

PBS: Tween (wash buffer): 500 mL
500 mL PBS
0.25 mL Tween

MAb (monoclonal antibody) stock
Dissolve lyophilized antibody in 1:1 dH_2O: glycerol, following instructions on the bottle. Store antibody at -20°C. Make the following antibody dilutions immediately prior to use:
Capture antibody: 40 µl stock in 5 mL PBS—this is enough for one 96-well plate
Conjugated antibody: 10 µl stock in 5 mL BB—this is enough for one 96-well plate

P.f. positive control stock
Resuspend *Plasmodium falciparum* CSP protein in 250 µl BB (vial I)
Take 10 µl from vial I, dissolve in 990 µl BB (vial II, 100x dilution)
Take 10 µl from vial II, dissolve in 990 µl BB for working stock (vial III, 10,000x dilution)

For the positive control serial dilution, add 100 µl from vial III to a plate well. Transfer 50 µl of this to the next well down, mix well with 50 µl BB. Using a new pipet tip, transfer 50 µl to the next well down, mix well with 50 µl BB, etc., resulting in 1X, 2X, 4X, 8X, 16X, 32X, 64X, and 128X positive control dilutions.

Mosquito homogenate
Grind each whole mosquito in 50 µl BB: IG-630. Rinse pestle twice with 100 µl BB, for a total of 250 µl mosquito homogenate. Mosquito homogenates can be prepared in advance and stored at -20°C.

Negative controls
Homogenize uninfected colony mosquitoes as above for negative controls.

ABTS solution
Immediately before use, mix 1:1 Solution A (ABTS) and Solution B (Hydrogen

Peroxide), 100 µl per well, 10 mL total per 96-well plate. Store at 4°C, throw away remaining solution after assay is finished.

Stop Solution
1% SDS (1 mL 10% SDS in 9 mL dH_2O for one 96-well plate)

Plate Setup

	1	2	3	4	5	6	7	8	9	10	11	12
A	neg	(+) 1x	(+) 1x	3	7	7	15	15	23	23	31	31
B	neg	(+) 2x	(+) 2x	3	8	8	16	16	24	24	32	32
C	neg	(+) 4x	(+) 4x	4	9	9	17	17	25	25	33	33
D	neg	(+) 8x	(+) 8x	4	10	10	18	18	26	26	34	34
E	1	(+) 16x	(+) 16x	5	11	11	19	19	27	27	35	35
F	1	(+) 32x	(+) 32x	5	12	12	20	20	28	28	36	36
G	2	(+) 64x	(+) 64x	6	13	13	21	21	29	29	37	37
H	2	(+) 128x	(+) 128x	6	14	14	22	22	30	30	38	38

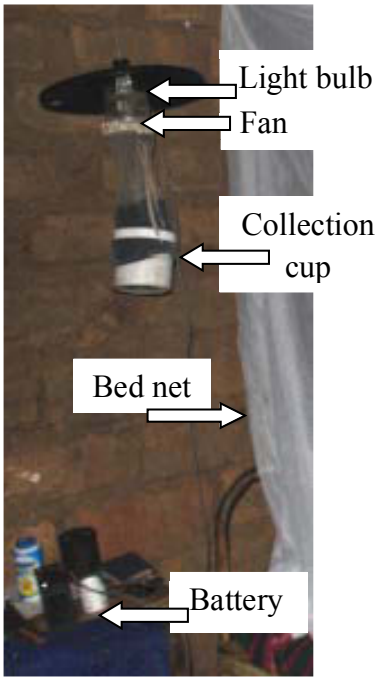
ELISA Protocol
Note: All incubations are carried out at room temperature.

1. Add 50 µl capture MAb solution to each well (40 µl MAb in 5 mL PBS). Cover and incubate overnight.
2. Remove solution by knocking plates upside-down. Fill wells with BB (~220-250 µl) and incubate for 1 hour.
3. Remove solution and add 50 µl mosquito homogenate, positive controls, and negative controls to their respective wells. Run all mosquito samples in duplicate. Add 50 µl BB to any empty wells. Incubate for 2 hours.
4. During the 2 hour incubation:
   - Prepare the ABTS solution (mix solutions A and B)
   - Dilute the conjugate MAb in BB as described above (10 µl MAb in 5 mL BB).
   - Confirm enzyme activity by mixing 5 µl conjugate MAb with 100 µl ABTS. A dark green color should begin developing within a few minutes.
5. Remove mosquito homogenate. Wash plate 7 times with PBS-Tween using a plate washer.
6. Add 50 µl conjugate MAb to each well, incubate for 1 hour.
7. Remove conjugate MAb, wash 7 times with PBS-Tween.
8. Add 100 µl ABTS solution to each well and incubate for 60 minutes.
9. Add 100 µl Stop Solution to each well and read plate absorbance at 405 nm.
10. The absorbance cut-off for positive samples in 2X the average absorbance of the negative controls

Appendix M

Centers for Disease Control light trap field protocol

Centers for Disease Control (CDC) light traps (John W. Hock Company, Gainesville, Florida) are employed to collect host-seeking mosquitoes. Mosquitoes are lured to the trap by a combination of light and attractive bait (e.g., a person under a bed net). When mosquitoes fly towards the light bulb, a small fan blows them down into a collection cup.



1. Charge all batteries that will be needed for a night's collections.
2. Hang CDC traps in selected sleeping houses approximately 1.5 meters off the floor next to a bed net. Place a piece of paper with the traps location (household and sleeping house number) inside the cup before hanging.
3. Instruct the owner of the house to connect the battery to the trap before they go to sleep around 19:00.
4. Make sure the owner understands that all people sleeping in the trapping room need to sleep under a bed net. If extra bed nets are needed they will be provided for the night.
5. The trap should be left in place until a JHMRI field team member returns the next morning by 07:00. First tie the bag shut to prevent mosquitoes from escaping. Next, unhook the battery and take down the trap.
6. Bring the traps back to the lab and kill the mosquitoes by placing the cups in a freezer.
7. Recharge the batteries.

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Shiff, C.J., J.N. Minijas, T. Hall, R.H. Hunt, and S. Lyimo. 1995. Malaria infection potential of anopheline mosquitoes sampled by light trapping indoors in coastal Tanzanian villages. *Medical and Veterinary Entomology* 9: 256-262.

Appendix N

Pyrethroid spray catch field protocol

Pyrethrum spray catches are used for estimating the relative population densities of mosquitoes that rest indoors after taking a blood meal. Engorged specimens may also be used to determine the human blood indices of different species. Collections need to be conducted during the morning hours before mosquitoes move outside to find other resting or breeding sites.

1. Collection teams of 3-4 people will perform spray catches from 06:00-10:00 in selected sleeping houses.
2. A white sheet will be spread inside the sleeping room to cover the entire floor and furniture, including beds and tables. Food, drinking water, and animals should be removed before spraying. In addition, windows and large gaps under the eves should be blocked to prevent mosquito escape.
3. Once the room is prepared a team member will spray the ceiling and walls with a pyrethroid spray (e.g., locally-purchased DOOM Super® (Adcock Ingram Ltd., Bryanston, South Africa)).
4. Close the door to the room or block the doorway with a curtain of material.
5. Let the room sit for 15 minutes undisturbed while the aerosol kills the insects. The collection team will be able to work on multiple houses at one time.
6. After the 15 minutes, enter the sleeping room with torches and carefully remove and take the white sheets outdoors. Using forceps, collect all mosquitoes that are on the sheet. All mosquitoes from each house should be placed in the same tube with a slip of paper indicating the household and house number of the collection.
7. Once back in the lab collection tubes are placed in the freezer to kill any mosquitoes that might have just been knocked down.

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PERSONAL DATA

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EDUCATION AND TRAINING

Degree/Year	*Institution and Field*
Study Abroad - 2007 (3 months)	Indiana University Summer Program, Graz, Austria
Study Abroad - 2009 (7 months)	University of Western Australia, Perth, Australia
BS (2006 - 2010)	Indiana University, Bloomington, Indiana Microbiology
BA (2006 - 2010) Studies	Indiana University, Bloomington, Indiana Germanic
PhD - Current (2010-2015)	Johns Hopkins Bloomberg School of Public Health, The W. Harry Feinstone Department of Molecular Microbiology and Immunology, Baltimore, Maryland Dissertation: "Foraging Behavior of Anopheles Mosquitoes in Nchelenge District, Zambia"
Specialized Training	
November 2010	Animal Care and Use Training, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland
February 2010	Blood-borne Pathogen Training, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland

August 2011	Human Subjects Research Training, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland

HONORS AND RESEARCH AWARDS

August 2006 - May 2010	Herman B Wells Merit Scholar, Indiana University, Bloomington, Indiana
August 2006 - May 2010	Honor Student, Indiana University, Bloomington, Indiana
August 2010 - 2011	Howard Hughes Medical Institute Integrated Freshman Learning Experience (IFLE), Indiana University, Bloomington, Indiana
August 2007 - 2009	Science, Technology, and Research Scholars (STARS), Indiana University, Bloomington, Indiana
May 2008 - August 2008	Microbiology Undergraduate Research Award, Indiana University, Bloomington, Indiana
August 2010 - August 2012	NIH T32 Training Grant (2T32AI007417-16), Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland
August 2010 - August 2012	Martin Frobisher Fellowship Fund, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland
September 2012 - September 2014	Johns Hopkins Malaria Research Institute (JHMRI) Pre-doctoral Fellowship, Johns Hopkins Bloomberg School of Public Health Baltimore, Maryland
August 2012 - September 2012	Global Health Established Field Placement Award, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland
September 22 - 27, 2013	Society of Vector Ecology (SOVE) Travel Award, La Quinta, California

RESEARCH EXPERIENCE

August 2006 - 2008	Undergraduate Research, Department of Biology, Indiana University, Bloomington, Indiana Project: MAP65 family and microtubule dynamics in *Arabidopsis thaliana.* Mentor: Dr. Sidney Shaw
July 2008 - May 2009	Co-Investigator, Children's Health Services Research, Indiana University School of Medicine, Indianapolis, Indiana Project: Provider characteristics and disparities in chlamydia testing. Supervisor: Dr. Sarah Wiehe
October 2008 - January 2009	Undergraduate Research, School of Public and Environmental Affairs (SPEA), Indiana University, Bloomington, Indiana Project: Women's reproductive health and policy. Supervisor: Dr. Nicole Quon
August - December 2009	Honors Thesis, School of Public and Environmental Affairs (SPEA), Indiana University, Bloomington, Indiana Dissertation: "Assessing STIs and HIV Screening, Prevention, and Treatment Services for Incarcerated Women in the United States". Mentor: Dr. Nicole Quon
September - November 2009	Undergraduate Research, Center for Sexual Health Promotion, Indiana University, Bloomington, Indiana Project: Epidemiology of sexual practices among women. Supervisors: Dr. Michael Reese and Kristin N. Jozkowski
March - August 2010	Research Assistant, Department of Biology, Indiana University, Bloomington, Indiana Project: Measuring beta lactamase activity in clinical bacteria strains. Supervisor: Dr. Karen Bush

FIELD EXPERIENCE

August 2011 (2 weeks)	Field Training Course, Valles Caldera National Preserve, Valles Caldera, New Mexico

March 2012 - May 2013	Thesis research, Nchelenge District, Luapula Province, Zambia
August 2012 (2 weeks)	Entomology laboratory training for local scientists, Macha, Southern Province, Zambia
May 2013 (1 week)	Entomology laboratory training for local scientists, Macha, Southern Province, Zambia

TEACHING EXPERIENCE

July - August 2009	Freshman Student Mentor, Howard Hughes Medical Institute Integrated Freshman Learning Experience (IFLE), Indiana University, Bloomington, Indiana
October - December 2011	Teaching Assistant, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland Course: Public Health Ecology
October - December 2012	Teaching Assistant, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland Course: Public Health Ecology
January - March 2013	Teaching Assistant, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland Course: Introduction to Vector Biology
August 2012 - Present	Laboratory Research Mentor for 4 Undergraduate and 3 Graduate Students, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland

PUBLICATIONS

Das, S., Henning, T.C., Simubali L., Mamini, E., Lukwa, N., Muleba, M., Norris, D.E., and Jennifer C. Stevenson. Underestimation of foraging behavior in field-caught anopheline mosquitoes in southern Africa. *Malaria Journal* 2015.

Das, S., Muleba, M., and Douglas E. Norris. Spatial and temporal dynamics of malaria vectors in Nchelenge District, Zambia. (in preparation)

Das, S., Muleba, M., and Douglas E. Norris. Multiple blood feeding behavior and *Plasmodium falciparum* complexity of infection in *Anopheles* mosquitoes in Nchelenge District, Zambia. (in preparation)

PRESENTATIONS

Oral

1. Das, S., Norris L.C., and D.E. Norris. Blood feeding behavior of *Culex quinquefasciatus* from CDC light traps in Macha, Zambia. Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, 2011.

2. Das, S., Norris L.C., and D.E. Norris. Identification and characterization of malaria vectors in Northern Zambia. Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, 2012.

3. Das, S. and D. E. Norris. Geographical heterogeneity of malaria vector dynamics and bionomics during the wet season in Nchelenge District, Zambia. Society of Vector Ecology International Congress 2013, La Quinta, California.

4. Das, S. and D. E. Norris. Heterogeneity of malaria transmission by *Anopheles gambiae* and *An. funestus* in Nchelenge, Zambia. Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, 2013.

Poster

1. Das, S., Norris L.C., and D.E. Norris. Multiple blood feeding behavior and population structure in Anopheles mosquitoes in Northern Zambia. American Society for Tropical Medicine and Hygiene Conference 2011, Philadelphia, Pennsylvania.

2. Das, S., Norris L.C., and D.E. Norris. Malaria vector bionomics and human malaria transmission dynamics in Nchelenge District, Zambia. American Society for Tropical Medicine and Hygiene Conference 2012, Atlanta, Georgia.

3. Das, S. and D.E. Norris. Seasonality of malaria vectors in Nchelenge District, Zambia. Johns Hopkins Bloomberg School of Public Health World Malaria Day 2013, Baltimore, Maryland.

4. Das, S. and D.E. Norris. Malaria vector bionomics during the dry season in Nchelenge, Zambia. Johns Hopkins Bloomberg School of Public Health Global Health Day 2013, Baltimore, Maryland.

5. Das, S. and D.E. Norris. Heterogeneity of malaria vector dynamics and bionomics in Nchelenge District, Zambia. American Society for Tropical Medicine and Hygiene Conference 2013, Washington DC.

6. Das, S., Muleba, M., Eng, S., Kessler, M., D.E. Norris. Seasonality of multiple blood feeding behavior in anopheline mosquitoes and implications for malaria transmission in Nchelenge District, Zambia. American Society for Tropical Medicine and Hygiene Conference 2014, New Orleans, Louisiana.

MEMBERSHIPS

Society

2011 - Present	American Society of Tropical Medicine and Hygiene
2011 - Present	Student Representative, Maryland Tropical Medicine Dinner Club
2012 - Present	American Society of Microbiology
2013 - Present	Society of Vector Ecology

Organizations, Clubs, and Activities

March 5, 2011	Volunteer, Health Fair at Elmer A. Henderson Elementary School, Baltimore, Maryland
March 2011 - Present	Volunteer, My Sister's Place Women's Center (MSPWC), Baltimore, Maryland
August 2012 - Present	Writer for Feinstones' Notes (Biannual Newsletter), The W. Harry Feinstone Department of Molecular Microbiology and Immunology Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland
October 2013 - September 2014	Faculty Liasion, The W. Harry Feinstone Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland
October 2013 - September 2014	Member at Large, Student Assembly, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland
August 2014 - Present	Secretary, Lab Sciences Student Group, Johns Hopkins