"Silent" malaria: Defining the infectious reservoirs of *Plasmodium falciparum* in communities of Southern Malawi

By

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Epidemiologic Sciences)
in the University of Michigan
2015

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List of Abbreviations

ACT	Artemisinin combination therapy
BT	Blantyre (District)
CA	California
CART	Classification and Regression Tree
cDNA	Complementary DNA
CI	Confidence interval
CK	Chikhwawa (District)
Ct	Cycle threshold
dCt	Delogged cycle threshold
df	Degrees of freedom
DNA	Deoxyribonucleic acid
EA	Enumeration area
EIR	Entomologic inoculation rate
G6PD	Glucose-6-phosphate dehydrogenase
gDNA	Genomic DNA
GDP	Gross domestic product
GPS	Global positioning system
Hb	Hemoglobin
HIV	Human immunodeficiency virus
HR	Hazard ratio
ICEMR	International Center of Excellence for Malaria Research
ID	Identification
IPTp	Intermittent preventive treatment in pregnancy
IRB	Institutional Review Board
IRS	Indoor Residual Spraying
IST	Intermittent Screening and Treatment
ITN	Insecticide-treated net
JICA	Japan International Cooperation Agency
KAP	Knowledge, Attitudes, and Practices
Km	Kilometers
LA	Lumefantrine-artemether
LDH	Lactate dehydrogenase
LOD	Limit of detection
MDA	Mass drug administration
mo	Months old
MO	Missouri
MoH	Ministry of Health
MOI	Multiplicity of infection

mRNA	Messenger RNA
MSAT	Mass screening and treatment
MSU	Michigan State University
N/A	Not applicable
NC	North Carolina
NIH	National Institutes of Health
NMCP	National Malaria Control Programme
NR	Not reported
OR	Odds ratio
PCA	Principal component analysis
PCR	Polymerase chain reaction
PMI	President's Malaria Initiative
POR	Prevalence odds ratio
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
QT-NASBA	Quantitative nucleic acid sequence-based amplification
RBC	Red blood cell
RDT	Rapid diagnostic test
REDCap	Research Electronic Data Capture
SAS	Statistical Analysis System
SD	Standard deviation
SES	Socioeconomic status
SP	Sulfadoxine-pyrimethamine
SSA	Sub-Saharan Africa
STA	Sub-traditional authority
TA	Traditional authority
TBI	Transmission blocking immunity
TO	Thyolo (District)
US$	United States Dollars
WBC	White blood cell
WHO	World Health Organization
yo	Years old

Abstract

Malaria is a vector-borne disease causing at least 200 million cases and 580,000 deaths annually. Frequent exposure to the *Plasmodium* parasites that cause malaria induces immunity that prevents symptoms, but not infection. In endemic areas, asymptomatic, or 'silent,' human infections are common, potentially representing persistent sources of transmission. To characterize such infectious reservoirs, cross-sectional surveys were undertaken seasonally across three diverse Districts in southern Malawi. Humans can infect mosquitoes that feed on their blood when it contains the *Plasmodium* gametocyte stage, but gametocytes occur at low densities, and are difficult to detect using traditional microscopy. Blood samples from a subset of the study population were tested using polymerase chain reaction (PCR) for *P. falciparum* genomic DNA and a novel quantitative reverse transcription-PCR (qRT-PCR) assay for stage-specific messenger RNA (mRNA) transcripts. The qRT-PCR assay was found to be valid for use in this community-based study population, and vastly improved sensitivity for detecting gametocytes over microscopy. These results were then used to analyze the population-level predictors of gametocytemia. Asymptomatically infected people were as likely to be gametocytemic as those reporting symptoms. School-aged children (6-15 years) were the most likely to be gametocytemic, though under-5-year-olds suffer the greatest malaria disease burden. Since untreated infections can persist for months, treatment-seeking behaviors were assessed for their potential to shorten duration of infectiousness. Treatment-seeking was common (>85%) among participants reporting recent fever, but few afebrile subjects sought treatment. Only 25% of *P. falciparum*-infected people had sought treatment in the previous two weeks. Appropriate testing and antimalarial treatment was most likely to occur at government/private facilities, but school-aged children and adults were more likely to use shops or other sources. These studies demonstrated that gametocyte carriage is common among asymptomatic

infections, and that these infections are unlikely to receive antimalarial treatment. School-aged children were key reservoirs of the infectious stage of the *Plasmodium* parasite. Malaria elimination interventions will need to address these human reservoirs in order to interrupt transmission, but control efforts will benefit from expanding coverage to school-aged populations.

CHAPTER 1 Introduction

1.1   Malaria epidemiology and parasite life cycle

Human malaria is a mosquito-borne disease caused by any of five species of *Plasmodium* protozoan parasites: *P. falciparum, P. vivax, P. malariae, P. ovale,* and *P. knowlesi.*[1] The early symptoms of malaria are non-specific, and may include periodic fever, rigors, myalgia, arthralgia, headache, cough, diarrhea, vomiting, and anemia. Those infections caused by *P. falciparum* can lead to more severe complications, including cerebral malaria with coma, convulsions, respiratory distress, severe anemia, and renal failure.[1,2] Infections caused by other *Plasmodium* species are not typically life-threatening, though *P. vivax* can rarely lead to splenic rupture, and may contribute more to mortality than previously thought.[1] In the latest World Malaria Report, the World Health Organization (WHO) estimated that there were 198 million cases and 584,000 deaths globally in 2013, with the majority of cases and 90% of deaths concentrated in sub-Saharan Africa (SSA) (Figure 1.1).[3] Although this represents significant progress toward malaria control in the past decade, there are still an estimated 3.2 billion people living at risk of infection, and 1.2 billion at high risk of disease (>1 in 1000 chance of getting malaria in the next year).[3]

Figure 1.1. Global incidence of malaria, 2013[3]
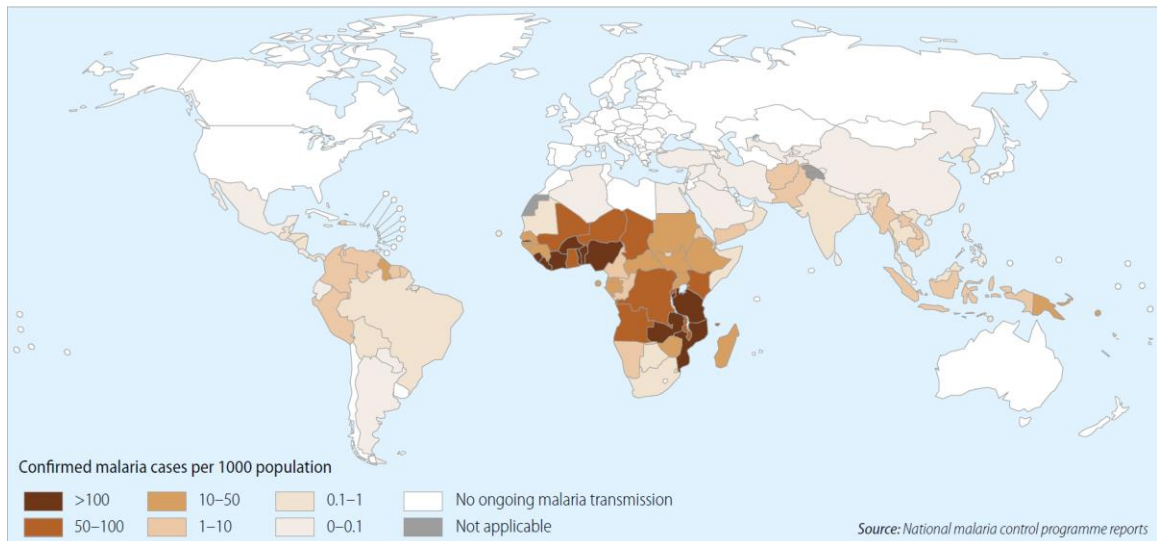
Nearly all transmission of *Plasmodium* is vector-borne, with an obligate phase of sexual reproduction taking place in mosquitoes of the genus *Anopheles* (Figure 1.2).[4] *Plasmodium* infections in mosquitoes are transmitted to humans by the bite of infected *Anopheles* mosquitoes, which primarily bite at night.[2] When an infected mosquito takes a blood meal, sporozoite forms of the parasite are injected with saliva from the mosquito salivary glands into the human bloodstream. In the initial, exo-erythrocytic stage of human infection, these sporozoites then invade hepatocytes in the human liver and develop into liver schizonts through asexual reproduction.[1,2,4] After 6 to 14 days, these liver schizonts rupture, releasing thousands of merozoites into the bloodstream to initiate the second asexual phase of reproduction, the erythrocytic stage of the life cycle.[1,2] In this erythrocytic cycle, merozoites invade red blood cells (RBCs), develop into trophozoites, and undergo asexual reproduction to produce blood schizonts, which also rupture and release merozoites into the bloodstream, continuing the cycle.[4] The periodic release of asexual merozoites into the bloodstream is responsible for the clinical manifestations of malaria disease;[1] however, these asexual stages of the parasite are not involved in transmission to other blood-feeding mosquitoes.

Figure 1.2. Life cycle of the Plasmodium parasite[4]
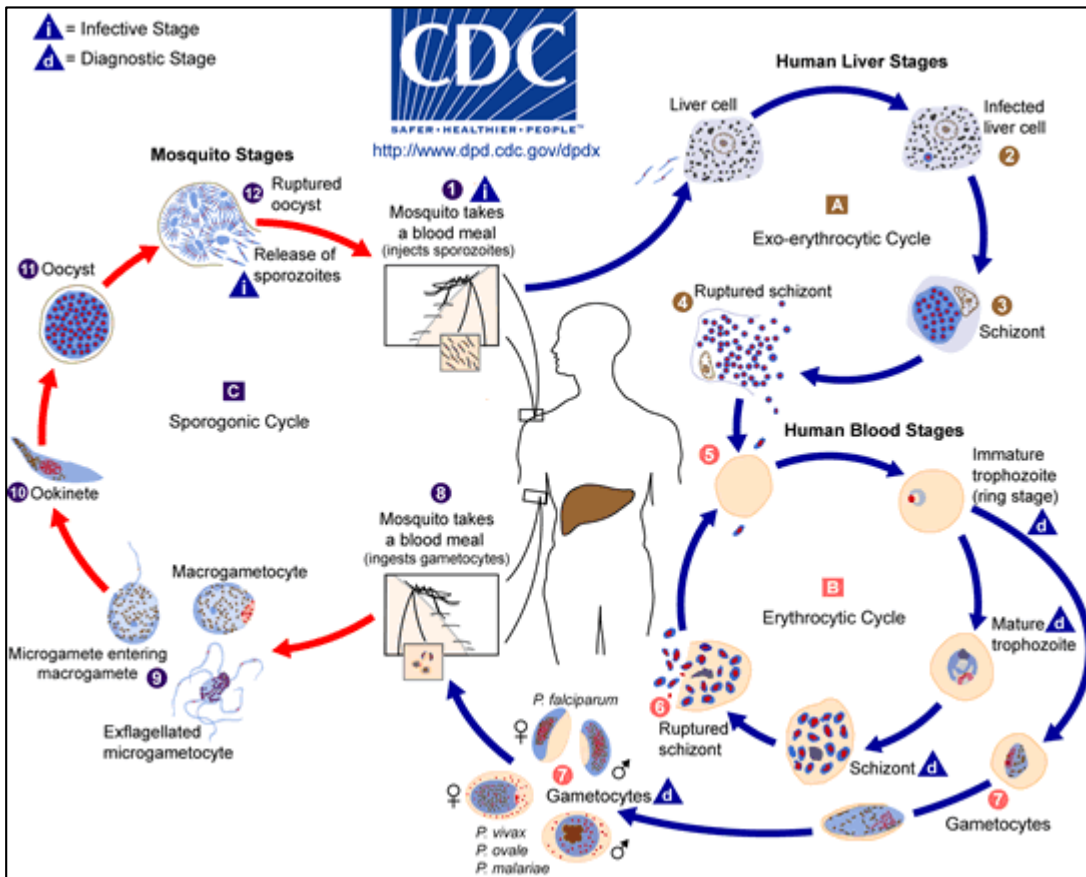
A small proportion of merozoites invade RBCs and, instead of continuing asexual reproduction, develop into the sexual stage gametocytes that are infectious to mosquitoes (Figure 1.3).[5] Usually gametocytes make up <5% of detectable circulating parasites, and the triggers that lead certain merozoites to commit to gametocytogenesis are poorly understood.[5] After invasion of a RBC, these sexually-committed merozoites go through five stages to develop into mature gametocytes over the course of one to two weeks.[5,6] Early gametocyte stages (I-III) are morphologically indistinguishable from asexual parasite stages by microscopy, and tend to sequester in the spleen and bone marrow.[5–7] By stage V, the mature gametocytes circulate in the bloodstream, and male (microgametocyte) and female (macrogametocyte) *P. falciparum* gametocytes are morphologically distinguishable.[5] Circulating gametocytes do not play a role in clinical manifestations of malaria disease,[2,5] but, because they infect mosquitoes, are critical for ongoing transmission.

Figure 1.3. Stages of P. falciparum gametocyte maturation[5]

Formation and maturation of *P. falciparum* gametocytes

I   IIa   IIb   III   IV   V
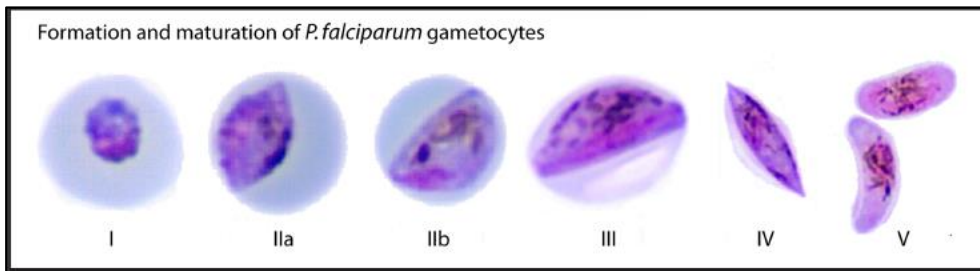
For a human to transmit a *Plasmodium* infection to an anopheline mosquito, the mosquito must ingest at least one male and one female stage V gametocyte during feeding (Figure 1.2).[4,5] Once inside the mosquito midgut, the sexual, sporogonic phase of the lifecycle takes place. The male microgametocyte exflagellates and merges with the female macrogametocyte to form an ookinete, which then penetrates the wall of the mosquito's midgut and becomes an oocyst in the hemocele.[1,2,4] Asexual division occurs within the oocyst over a period of days to weeks (the extrinsic incubation period) until the oocyst bursts, and the released sporozoites travel to the salivary glands, where they can infect a human during subsequent blood meals.[1,2,4]

1.2   Reservoirs of infection

Frequent human exposure to *Plasmodium* parasites confers some immune protection from symptomatic malaria disease, but does not lead to full protection against future infection.[8,9] Instead, there is thought to be a gradual development of a non-sterilizing immune response over time, and 'silent,' asymptomatic infections are common in endemic areas.[9–12] Given that asymptomatic infections are unlikely to be treated, this may lead to chronic carriage of the parasite that can last for months or even years,[13,14] rarely, if ever, prompting acute symptoms of malaria disease.[8,15]

Traditionally, light microscopy of stained blood smears on glass slides has been used to determine *Plasmodium* presence and diagnose malaria in clinical cases. The lower limit of detection (LOD) of microscopy is estimated to be between 10 and 40 parasites/µL, or more, depending on field conditions.[8,9,16,17] This is sufficiently sensitive for clinical diagnostic purposes, as the "pyrogenic threshold" for an infection to induce

fever varies with age and season, but has been estimated to be more than 200 parasites/µL.[15,18,19] This LOD, however, does not permit the detection of all human infections, some of which can be <<10 parasites/µL. Although some asymptomatic infections have sufficient parasite densities to be microscopically detectable, more sensitive molecular detection methods like polymerase chain reaction (PCR) reveal that infections with submicroscopic parasite densities are also highly prevalent in endemic populations.[9,20] Indeed, PCR-based methods typically detect twice as many cases as microscopy in endemic populations.[9,20]

As the Gates Foundation and the WHO have resurrected the goal of malaria eradication, the importance of asymptomatic human reservoirs of infection has recently drawn considerable attention as a potential impediment to such efforts.[8] As 'silent' carriers of the parasite, these people are difficult to identify and treat using existing methods in most contemporary health system infrastructures. The difficulty is only amplified by the large number of submicroscopic infections in some populations. Molecular techniques are needed to detect such human infections, but most facilities only have the capacity to perform more traditional diagnostics like microscopy, or even less sensitive rapid diagnostic tests (RDTs). Many facilities lack even these diagnostic methods, and distribute treatment presumptively.[21] Because such asymptomatically infected people pose a significant challenge to current intervention strategies, it is important to understand their contribution to ongoing transmission in different epidemiologic contexts. Characterizing the human infectious reservoir demands an understanding of the epidemiology of gametocytemia in both symptomatic and asymptomatic infections.

1.3   Detection and clearance of gametocytemia

Given their importance to transmission, surprisingly little is known about the population prevalence and individual-level dynamics of gametocytemia. This is due in part to their relative unimportance for clinical disease compared to asexual *Plasmodium* stages, and also due to the limited sensitivity of microscopy.[5,22] As gametocytes typically comprise a small proportion (<5%) of the total parasite burden, they frequently occur

beneath the LOD of microscopy, even in high-density clinical infections.[5,23] Higher blood density of gametocytes is associated with a higher probability of transmission to a mosquito in a blood meal, but even densities as low as one gametocyte/μL are capable of causing infection.[5,24–26] Molecular detection techniques that identify gametocyte-specific messenger RNA (mRNA) transcripts, such as quantitative nucleic acid sequence-based amplification (QT-NASBA) and quantitative reverse transcription – polymerase chain reaction (qRT-PCR), have emerged that enable detection of a greater proportion of transmissibly relevant gametocyte infections than microscopy. These molecular techniques can detect gametocytes at densities of less than one gametocyte/μL, and identify four- to seven-fold more gametocytemic infections than microscopy.[13,14,27–32]

Although such molecular detection techniques have existed for the past 15 years, they have rarely been used in community-based studies to characterize population prevalences of gametocytemia that include both asymptomatic and symptomatic infections.[13,14,27,28] QT-NASBA and qRT-PCR require sophisticated and reliable laboratory facilities and can be expensive, making it cost-prohibitive to apply them to study populations in which a large proportion of study participants are expected to be uninfected. Nonetheless, population-level gametocyte prevalence data are critical to understanding the role of the asymptomatic reservoir to ongoing parasite transmission.

For an intervention to effectively interrupt transmission from the human infectious reservoir, gametocytes should be completely cleared from the bloodstream. By eliminating the asexual stages that are the precursors for gametocytogenesis, successful antimalarial treatments, which eliminate the asexual stages that cause disease, can prevent the development of new gametocytes. Unfortunately, most antimalarials, including artemisinin combination therapies (ACTs), have limited effects on existing mature and immature gametocytes, typically allowing them to persist for up to a month after treatment.[5,33] Primaquine is a gametocytocidal drug that can potentially clear gametocytes in a week, but it is contraindicated for use in people with glucose-6-phosphate dehydrogenase (G6PD) deficiency, in whom it can cause hemolytic anemia.[5,34] The WHO has recommended incorporating a single low dose of primaquine with ACT to reduce duration of infectiousness in addition to treating clinical symptoms, but this has rarely been attempted in practice.[34] Antimalarial treatment with ACTs alone is common,

and has at least some potential for limiting the duration of infectiousness in gametocytemic people compared to natural clearance of infection. Population treatment patterns affect the duration of infectiousness, and are important to understanding the relative roles of symptomatic and asymptomatic cases in propagating *Plasmodium* transmission.

1.4   Research setting: Malawi

The research for this dissertation took place in Malawi, a small, land-locked nation in SSA (Figure 1.4). It is one of the poorest nations in the world, with a per capita gross domestic product (GDP) of US$667, and a human development index ranking of 164 out of 177 countries.[35] The most recent census in 2008 indicated a population estimate of about 13 million people, growing at about 2.8% annually since 1998.[36] The average life expectancy is 47 years for males and 46 years for females, with 46% of the population being <15 years of age.[35] The health sector of the country, like others in SSA, faces numerous challenges. The prevalence of human immunodeficiency virus (HIV) is estimated to be 12% among adults 15-49 years, with 28,000 incident cases of tuberculosis each year.[35] Although many Malawi Ministry of Health (MoH) services are provided for free, only 46% of the population lives within 5 km of a health facility, and nationally there are only two physicians and 59 nurses per 100,000 population.[35] Malaria persistently occurs throughout the country, with a seasonal pattern accompanying the annual rains that begin in November-December and continue through March-April.[21] An estimated 5 million cases occur each year, and about half of all inpatient hospitalizations are attributed to malaria.[21,37] *Plasmodium falciparum* is responsible for the vast majority of infections, but *P. ovale* and *P. malariae* infections have also been reported.[38] Data on the national morbidity and mortality of malaria are limited by poor public health infrastructure and inconsistent surveillance.[21]

Figure 1.4. Location of Malawi on the African continent

With support from the Global Fund and the President's Malaria Initiative (PMI), Malawi's National Malaria Control Programme (NMCP) has attempted to control malaria by achieving 85% coverage in high-risk groups with four key interventions: long lasting insecticide-treated nets (LLINs), intermittent preventive treatment during pregnancy (IPTp), indoor residual spraying (IRS) with insecticides, and prompt treatment with artemisinin-combination therapies (ACTs).[21,37] Distribution of LLINs targeted at pregnant women and children <5 yrs and the use of IPTp have had reasonably high

coverage, reaching >50% of the population in recent years.[21] IRS had been limited to certain pilot districts as of 2010, with nationwide coverage at <2%, though the MoH intended to scale up coverage in more recent years.[21] Since 2007, the government has recommended lumefantrine-artemether (LA) as the first-line ACT treatment for malaria.[21] Although diagnostic testing for fever is recommended, the MoH supports presumptive treatment for febrile children <5 yrs when diagnostic capacity is limited.[21,39] Treatment with LA has been provided for free at government health facilities since 2007, but the proportion of febrile people who rapidly receive antimalarials is estimated to remain low.[21] While there is evidence that all-cause, under-five mortality has decreased in the past decade, suggesting some success in aggressive implementation of interventions by the NMCP, malaria transmission remains a persistent threat. Passive surveillance systems have even reported an increase in diagnosed cases between 2005 and 2009, though this may be attributable in part to increased population size and improved case detection.[21,37] International funding and national commitment offer hope, but research is needed to guide more effective application of interventions to reduce the burden of malaria in Malawi.

To this end, one of the ten National Institutes of Health (NIH)-funded International Centers of Excellence for Malaria Research (ICEMR) was established in Malawi, and based in Blantyre, the second largest city in the country.[21,37] The Malawi ICEMR has built an epidemiologic research infrastructure in Blantyre and two of its more rural neighboring Districts: Thyolo and Chikhwawa.[21] Both Blantyre and Thyolo Districts are at relatively high elevations, but Chikhwawa District lies in the Shire Valley, a lowland area with more intense transmission of *P. falciparum*.[21] These three eco-geographically diverse zones have been the sites of cross-sectional surveillance studies at the end of each rainy and dry season since 2012. This infrastructure and active sampling serves as the basis for data that were analyzed in my dissertation

1.5   Dissertation aims

The purpose of the analysis reported herein was to characterize the community of potentially infectious human reservoirs of *Plasmodium* in southern Malawi. Data from a

repeated sample cross-sectional survey carried out at the end of each rainy and dry season in 2012 and 2013 by the Malawi ICEMR were used to address three specific aims. The first aim (reported in Chapter 2) was to validate the use of a novel qRT-PCR assay in conjunction with PCR testing to identify the prevalence of specific stages of *P. falciparum*, especially gametocytes, in a community-based study population. This recently developed assay distinguishes asexual ring stages, asexual trophozoites, immature gametocytes, and mature gametocytes based on expression of specific mRNA transcripts, but had not been applied to community samples to assess stage composition of infections in the general population. The results of this assay were then used to address aim two, that of characterizing the prevalence and predictors of gametocytemia across different seasons and eco-geographic settings (presented in Chapter 3). Reliable gametocyte prevalence data using sensitive molecular detection techniques had not been previously reported for this population, and contribute to the relatively small body of literature on the gametocyte status of asymptomatic human reservoirs of infection. These results help to evaluate the potential role of asymptomatic and submicroscopic infections in propagating *P. falciparum* transmission, and in maintaining a persistent malaria burden in endemic populations. Finally, for aim three, predictors of treatment-seeking and the use of antimalarial treatments were analyzed to identify gaps in treatment patterns (presented in Chapter 4). These results have implications for the duration of infection and potential infectiousness in different subsets of the population, and suggest the new tools and intervention changes that are necessary to interrupt transmission in the region. Overall, the goal of this dissertation is to contribute to the ongoing debate about the role of asymptomatic human reservoirs of *Plasmodium* infection, and to help identify research and development goals that will support further progress toward malaria control and elimination.

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CHAPTER 2 Assessment of a combined PCR and qRT-PCR testing strategy for the detection of *Plasmodium falciparum* gametocytes in a community-based study in southern Malawi

2.1   Abstract

Plasmodium falciparum gametocytes, the stages that are transmitted from infected humans to *Anopheles* mosquitoes, occur at low densities in people compared to asexual parasite stages, and are frequently beneath the threshold of detection of traditional light microscopy. More sensitive tools are needed to adequately characterize gametocyte carriage. This study evaluates the potential of using polymerase chain reaction (PCR) and a stage-specific quantitative reverse transcription-PCR (qRT-PCR) assay in series to detect parasite stage composition, especially gametocytemia, in a community-based study population.

Methods: Blood smears on slides for microscopy, dried blood spots on filter paper for PCR, and whole blood preserved in RNAprotect® (Qiagen, Valencia, CA) for qRT-PCR were collected from 1,511 people of all ages in cross-sectional surveys during the dry season 2012 and subsequent rainy season 2013 in southern Malawi. Blood spots were tested by PCR for *P. falciparum* genomic DNA (gDNA); messenger RNA (mRNA) from parasitemic individuals was then tested by qRT-PCR to detect five stage-specific transcripts. These results were compared to those from microscopy for *Plasmodium* parasitemia and gametocyte presence.

Results: Stage-specific mRNA transcripts were tested by qRT-PCR from 50 PCR-positive and 5 PCR-negative samples from the dry season 2012 and 162 PCR-positive and 9 PCR-negative samples from the rainy season 2013. The qRT-PCR testing corresponded well with PCR results: no parasite mRNA was detected in the PCR-negative samples, and mRNA for at least one stage of *P. falciparum* was detected in 98% of PCR-positive samples from the dry season and 86% from the rainy season. Quantification of the ring stage by qRT-PCR was strongly correlated with parasite

density estimates from microscopy. qRT-PCR detected more than three- to five-times as many gametocyte-positive infections as traditional microscopy. If qRT-PCR results are assumed to be the gold standard, the sensitivity of microscopy for detecting gametocytes was only 21%. This assay also enabled detection and stage determination of immature gametocytes from morphologically similar asexual ring stages. Immature gametocytes were frequently detected in the peripheral blood samples, often concurrent with mature gametocytes.

Conclusion: PCR for parasite gDNA followed by qRT-PCR for stage composition is a sensitive strategy for molecular detection of *P. falciparum* gametocytes in large, community-based study populations. The stage-specific qRT-PCR assay vastly improves the sensitivity of gametocyte detection over microscopy, and enables simultaneous distinction of both mature and immature gametocytes from asexual stage *P. falciparum* parasites. The implications for epidemiological studies of population-level *Plasmodium* transmission are considerable.

2.2   Introduction

Despite recent efforts to prevent and treat malaria, the disease continues to be a major source of global morbidity and mortality.[1,2] The recent Malaria Eradication Research Agenda evaluation argued that, although malaria incidence has decreased in some locations following scaling up of control efforts, eradication is not feasible with current knowledge and interventions.[3] Continued progress toward local elimination, particularly in holoendemic settings, requires effective control of transmission, including transmission to mosquito vectors. While asexual *Plasmodium* stages are responsible for the clinical presentation of malaria (i.e. fever),[4,5] transmission of *Plasmodium* to a susceptible mosquito vector only occurs when the sexual stage of the parasite, gametocytes, are taken up in a blood meal.[6] In part due to the limited clinical relevance, and in part due to the difficulties of gametocyte detection by traditional techniques, little is known about the human population-level prevalence of gametocytemia, or the dynamics of gametocytemia during the course of symptomatic and asymptomatic infections.[6,7]

Although traditional microscopy of Giemsa or Fields stained peripheral blood smears is generally considered adequately sensitive for detection of the asexual ring stages for clinical diagnosis of malaria disease, gametocytes frequently occur at "submicroscopic" blood concentrations.[6,8–10] The lowest limit of detection (LOD) by microscopy is approximately 10 parasites per microliter (µL), but may require as many as 40 or more parasites per μL depending on the film preparation, the skill of the reader, and the number of fields viewed.[11] In typical *P. falciparum* infections, the percentage of gametocytes in the total parasite burden is low compared to asexual stages, thought to average ≤5% in endemic areas;[12] however, submicroscopic gametocyte densities have been shown to be infectious to mosquitoes.[6,13,14] Therefore, microscopy is too insensitive to fully characterize the gametocytemic reservoir and assess its contributions to population-level transmission dynamics. Recently developed molecular techniques are required to provide more sensitive, accurate data for meaningful insights about population-level gametocytemia and potential human reservoirs for transmission to mosquitoes.

Gametocytes are not highly active metabolically, but researchers have identified a few genes, such as *Pfs25*[15] and *Pfg377*,[16] that are expressed exclusively in gametocytes. These gametocyte-specific molecular markers have been used to distinguish gametocytes from asexual stages in molecular testing. Such molecular methods, including quantitative nucleic acid sequence-based amplification (QT-NASBA) and quantitative reverse transcription polymerase chain reactions (qRT-PCR), can indicate the presence of gametocytes at densities of <1 gametocyte/µL, as demonstrated in recent studies.[4,8,15–20] These mRNA transcripts, however, are primarily expressed in mature (stage V) gametocytes,[11,16,18] thus failing to detect infections that had recently initiated gametocytogenesis and carried immature, developing gametocytes. Joice *et al.* recently developed a novel, stage-specific qRT-PCR assay that uses five key transcriptional primers that can detect and distinguish immature (stages I-III) and mature (stages IV-V) *P. falciparum* gametocytes from asexual ring and trophozoite stages, and infer overall stage composition in parasitemic blood samples.[21] The assay was previously evaluated for gametocyte detection compared to microscopy in samples from children with clinically severe malaria.[21] Building on this work, the present study sought to provide

additional evidence about the parasite stage composition in a community-based study population, with both uncomplicated malaria cases and asymptomatic infections.

As the qRT-PCR assay has high per sample costs (>US$50) and requires several days of processing, it may be infeasible for use in populations with low prevalence of *P. falciparum.* Therefore we applied a two-step molecular testing strategy in our community-based study population, screening first for individuals with *P. falciparum* gDNA using PCR and subsequently testing these for parasite stage distribution using the qRT-PCR assay. We evaluated the validity of using this two-step molecular testing strategy for the detection of population-based gametocyte prevalence by comparing the results of PCR vs. qRT-PCR for any parasite genomic material, and the combined molecular strategy vs. microscopy for the detection of gametocytemia. We hypothesized that the PCR and qRT-PCR results would correspond well, and that the strategy would provide an improvement in sensitivity for gametocyte detection compared to traditional microscopy. This testing strategy represents a new method for the detection of submicroscopic levels of both immature and mature *P. falciparum* gametocytes in other endemic communities.

2.3   Methods

The study was carried out under the auspices of the Malawi International Center of Excellence for Malaria Research (ICEMR), and all methods were approved by the independent Institutional Review Boards (IRB) of the University of Malawi College of Medicine, the University of Maryland, Baltimore, and Michigan State University (MSU). The University of Michigan IRB determined the expansion of gametocyte testing to be non-regulated based on existing approvals.

2.3.1   *Study design*

Data and samples were collected in three Districts of southern Malawi during biannual cross-sectional surveys at the conclusion of each rainy season (April-May) and dry season (September-October) from 2012 to 2014. This report analyzed samples collected during the dry season 2012 and subsequent rainy season 2013. The three Districts were expected to represent different transmission patterns. Blantyre is an urban District in the highlands with low transmission, Thyolo is a semi-rural District with

moderate transmission, and Chikhwawa is a rural District at low elevation with high transmission. A preliminary census was performed in each District to delineate enumeration areas (EAs) containing approximately 30 households each. Ten EAs were randomly selected from each District (excluding EAs on the Thyolo-Chikhwawa boundary) to be visited during each of the surveys. Thus, approximately 900 households were visited during each survey. As the average household size in Southern Malawi is 4.2 persons,[22] more than 3,500 individuals were expected to participate each round. The local survey team communicated closely with local health representatives and local chiefs to encourage high levels of participation.

At each visit, the field team interviewed household members in the local language (Chichewa) to obtain information about household-level variables and individual-level variables for all members of the household. The questionnaires sought data on demographics (i.e. age, sex, household size), house construction, use of malaria interventions (including details on insecticide treated net (ITN) quality and use, indoor residual spraying (IRS), intermittent preventive therapy in pregnancy (IPTp) and other treatment-seeking behaviors), and self-reported recent malaria symptoms (fever in the previous two weeks and a more extensive list of symptoms from the past 48 hours).

2.3.2 *Sample collection and preservation*

Malawian nurses on each field team measured the current axillary temperature and took peripheral blood samples from all subjects ≥6 months of age who were present and consented (or were assented) to participate in the study. During all surveys, the nurses collected ~0.5 mL of blood onto slides as thick smears for microscopy, onto filter paper for polymerase chain reaction (PCR), and into a Hemocue® device (Hb 201+ System, Hemocue, Inc) for immediate testing of hemoglobin levels for children 6 mos – 5 years old (yo). During the dry season 2012 survey, whole blood samples of approximately 50 µL were collected into 250 µL of RNAprotect® (Qiagen, Valencia, CA) for a subset of subjects in eight of the 30 EAs. RNA collection was expanded to include all subjects for the subsequent surveys. For the present study, we included all subjects in the same eight EAs from whom RNA samples were collected during the dry season 2012 and the subsequent rainy season 2013.

2.3.3 *Microscopy procedure*

Thick smears were air dried, methanol fixed, and Giemsa stained upon delivery to the laboratory at the end of each day of sample collection. Thick smears were read at 100X objective magnification by two trained microscopists, who recorded the number of parasites seen per 200 white blood cells (WBCs). The two reads were considered discrepant if one reader recorded presence of any *Plasmodium* parasites and the other did not, if one reader counted more than 10 times as many as the other when the lowest reader was less than 20 parasites seen, or if one reader counted more than twice as many as the other when both reads were 20 parasites or greater. Discrepant slides were sent to a third reader. All readers were blinded to the smear results recorded by the other readers. The final smear value was calculated as 0 if two readers reported no parasites. If at least two readers reported parasite counts, the final smear value per microliter was estimated using an assumed WBC count of 8000/µL, and calculating the geometric mean of the two positive reads multiplied by 40, using the two closest reads if all three were positive for parasites.

No additional efforts were integrated into the microscopy protocol in order to increase sensitivity for detection and quantification of gametocytes; however, all readers were asked to record dichotomously whether or not they observed at least one gametocyte while counting parasites per 200 white blood cells. Since the sensitivity of gametocyte detection by microscopy is known to be low[6,23,24], samples were considered to be microscopy positive for gametocytes when any one of the readers positively reported gametocytes. It was therefore possible for a sample to positive for gametocytes but have a final smear parasitemia estimate of 0 parasites per µL.

2.3.4 *Molecular testing*

PCR for *P. falciparum* gDNA: Dried blood spots on filter paper were stored individually in plastic bags with silica packets and kept in dry storage cabinets at the ICEMR-Malawi laboratory in the University of Malawi College of Medicine for up to 8 months before genomic DNA (gDNA) extraction and testing. Blood spots were cut from the filter paper into a 1.5 mL Eppendorf tube using scissors that were sterilized in methanol and DNA OUT™ (G-Biosciences, St. Louis, MO) between each sample. gDNA was fixed to the filter paper by submersion in 50 µL of molecular grade methanol for 15

minutes, after which the majority of the methanol was discarded and the filter paper was left out to dry completely for at least two hours. Once completely dry, 50 µL of DNase-free sterile water were added to extract the parasite gDNA from the papers by heating the tubes for 30 minutes at 99°C, vortexing at 15 minutes. The resulting gDNA samples were stored overnight at -20°C or used immediately for PCR.

Following extraction, these gDNA samples were used in PCR reactions targeting the *P. falciparum* lactate dehydrogenase (LDH) gene, as described by Rantala *et al.*[25] Each 25 µL reaction consisted of 12.5 µL Taqman® (Life Technologies™, Carlsbad, CA), 0.25 µL each of forward and reverse primers at 250 nM, 0.30 µL of probe at 300 nM, 10.7 µL nuclease-free water, and 1 µL of gDNA sample. Each plate was run with positive control wells of lab-cultured *P. falciparum* clone 3D7 samples at a known density of 2.7 parasite/µL and negative control wells of nuclease-free water. The plate was then placed in a 7300 Real Time PCR System (Applied Biosystems) machine for the following program: 1) 1 repetition of 2 minutes at 50.0°C, 2) 1 repetition of 10 minutes at 95.0°C, and 3) 50 repetitions of 15 seconds at 95.0°C followed by 1 minute at 60.0°C. All samples were run in duplicate and considered positive for *P. falciparum* if LDH had a clear amplification curve in at least one of the two wells.

qRT-PCR assay for stage-specific RNA expression: qRT-PCR was used to detect parasite stage-specific mRNA expression based on a novel assay that distinguishes ring stage parasites, trophozoites, immature (stage I-III) gametocytes, and mature (stage IV-V) gametocytes, as described by Joice *et al.*[21] The qRT-PCR assay was only performed to identify *P. falciparum* stage composition for subjects that had tested positive for any *P. falciparum* gDNA during PCR testing of filter papers. A few samples were initially recorded as PCR-positive through human errors in data entry; as these had been tested using the qRT-PCR assay but later proved to be PCR-negative during quality control review, they were evaluated as negative controls of the qRT-PCR assay.

The preserved RNA samples (approximately 50 µL whole blood in 250 µL RNAprotect®) were transferred to cold storage in a -80°C freezer within at most 48 hours, but typically less than 24 hours, of sample collection, and maintained there until thawed for RNA extraction and testing. After thawing, the samples were spun at full speed in a microfuge for 15 minutes at room temperature to obtain a visible pellet; the

spin was repeated under the same conditions if no pellet was initially visible. The supernatant was then extracted with a pipette and stored until the extraction proved successful. RNA was extracted from the selected samples using RNeasy Plus Mini-Kits® (Qiagen, Valencia, CA) and treated with RNase-free DNase Sets® (Qiagen, Valencia, CA) to eliminate all traces of parasite gDNA.

Reverse transcription was performed using Invitrogen Superscript complementary DNA (cDNA) Synthesis Kits® (Life Technologies™, Carlsbad, CA) in a double reaction compared to the Invitrogen protocol. First, 2 µL of 50 ng/µL random hexamers as the primer, 2 µL of 10 mM dNTP mix, and 16 µL of extracted RNA samples were combined to create 20 µL reactions. After briefly centrifuging the PCR strip, a Techne Thermocycler was used to incubate the samples for 5 minutes at 65°C, reducing to 4°C for 1 minute. Each RNA/primer sample was then combined with 20 µL of a cDNA synthesis mix that consisted of 4 µL of 10X RT buffer, 8 µL of 25 mM MgCl2, 4 µL of 0.1 M DTT, 2 µL of RNaseOut and 2 µL of Super Script III RT (200 U/µL). The samples were then returned to the Techne Thermocycler for 10 minutes at 25°C, 50 minutes at 50°C, and 5 minutes at 85°C. After centrifuging again to collect the sample, 2 µL of RNase H were added to each sample before returning it to the Thermocycler for 20 minutes at 37°C to complete the reverse transcription reaction. If qRT-PCR could not be performed immediately, then the resulting cDNA samples were temporarily stored overnight at -20°C.

The qRT-PCR assay involved the following stage-specific markers: 1) *PFE0065w* for the early asexual stage (ring stage), 2) *PF10_0020* for the late asexual stage (trophozoite), 3) *PF14_0748* for young gametocytes, 4) *PF14_0367* for mature gametocytes, and 5) *PF11_0209* as a constitutive protein. The assay was performed in triplicate for each stage-specific marker using 4 µL of sample per well, which required a dilution of the cDNA samples with 24 µL of nuclease-free water to obtain a volume of 66 µL for each cDNA sample. Primer/probe mixes were created by combining 18 µL of forward primers and 18 µL of reverse primers at a concentration of 900 nM, 5 µL of probe at a concentration of 250 nM, and 59 µL of nuclease-free water. These mixes were stored at -20°C in a dark box with minimal light exposure. Each well of the qRT-PCR reaction consisted of 10 µL of ABI TaqMan Gene Expression Master Mix® (Life

Technologies™, Carlsbad, CA), 1 µL of the primer/probe mix, 5 µL of nuclease-free water, and 4 µL of cDNA sample. Each plate included two types of negative controls: a set for each stage-specific marker using nuclease-free water instead of a cDNA sample, and a set of controls using reserved RNA from each sample that did not undergo reverse-transcription (RT-negative controls) that were tested only using the constitutive protein marker. The qRT-PCR program for each plate consisted of one repetition of 2 minutes at 50.0°C, one repetition of 10 minutes at 95.0°C, and 50 repetitions of 15 seconds at 95.0°C followed by 1 minute at 60.0°C.

The results of the qRT-PCR were recorded both dichotomously and quantitatively. Each sample was considered to be positive for a particular marker only if at least two of the three wells had a clear amplification curve. Quantification of the presence of each stage was estimated relative to the expression of the constitutive protein marker, *PF11_0209*. If at least two of the three wells for any stage-specific marker had an amplification curve, a delogged threshold cycle (dCt) value was calculated as 2^(-mean Cycle threshold [Ct]). The dCt value for each of the four stage markers (ring stage, trophozoite, immature gametocyte, and mature gametocyte) was then normalized against the dCt value of the constitutive protein marker. These four normalized dCt values could then be plotted for each sample as a rough estimate of the relative density of each stage of the *P. falciparum* parasite in the peripheral blood.

2.3.5   *Data management and analysis*

All study data were stored in the Research Electronic Data Capture (REDCap, Vanderbilt University) system hosted at the University of Malawi College of Medicine.[26] These data were downloaded and all statistical analyses were performed in Statistical Analysis System (SAS) versions 9.3 and 9.4 (SAS Institute, Cary, NC).

2.4   Results

2.4.1   *Samples tested*

Of 1,077 individuals from 249 study households during the dry season 2012 survey, there were 836 (77.6%) older than 6 months of age who were present and consented to give blood samples. Blood samples to test for *P. falciparum* RNA were collected from a subset of 628 (75.1%) of these subjects from 215 households during this

initial phase of RNA sampling. Of 1,017 individuals in 248 households from the same eight EAs that participated in the rainy season 2013 survey, RNA samples were obtained from all 905 (89.0%) individuals older than 6 months of age from 247 households who were present and consented to give samples. Consent documentation was lost for 22 individuals (11 from each of the two surveys); these 22 subjects were excluded from all analyses. Thus, there were 617 samples available to test for parasite RNA from the dry season 2012 and 894 from the rainy season 2013.

As discussed above, filter paper samples were tested by PCR for LDH to identify subjects with any *P. falciparum* parasitemia to be tested with qRT-PCR. There were three subjects for whom filter papers were unavailable, one from the dry season 2012 and two from the rainy season 2013. There were 50 subjects from the dry season 2012 and 162 from the rainy season 2013 that were positive for *P. falciparum* gDNA and subsequently had their RNA-preserved samples tested using the qRT-PCR assay for stage-specific *P. falciparum* mRNA expression. We tested five additional samples from the dry season 2012 and nine from the rainy season 2013 that were negative by PCR and were used to evaluate the specificity of the RNA assay. Figure 2.1 displays the sample testing scheme.

Figure 2.1. Sample sizes for qRT-PCR testing, ICEMR-Malawi cross-sectional survey
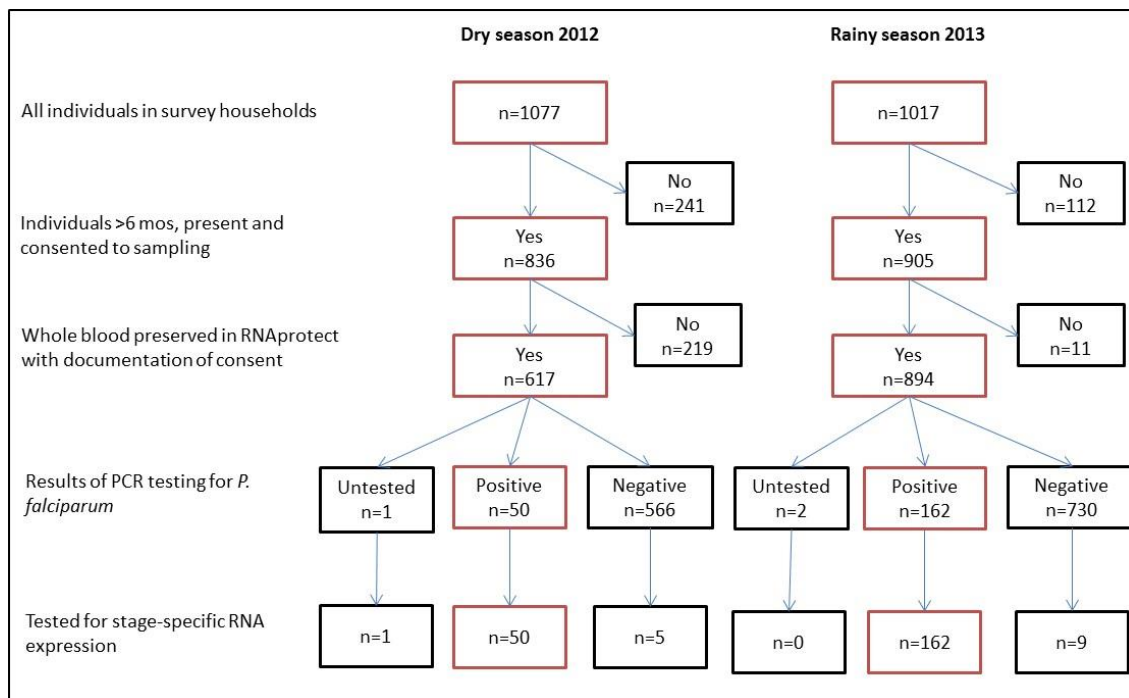
2.4.2 *Sensitivity and specificity of stage-specific RNA assay*

Only two (2) and 14 samples during the dry and rainy seasons, respectively, were recorded as positive by microscopy and negative for *P. falciparum* gDNA by PCR; none of these were tested by the qRT-PCR assay for *P. falciparum* mRNA expression. Among the samples that were PCR-positive for *P. falciparum* gDNA, 35 of 50 from the dry season 2012 (70.0%) and 66 of 162 from the rainy season 2013 (40.7%) were recorded as negative for any parasite stage by microscopy, suggesting that *P. falciparum* infections with submicroscopic peripheral parasitemia were common in this study population. The results of the qRT-PCR assay corresponded well with the results from PCR for *P. falciparum* gDNA. As expected, no parasite RNA was detected in any of the 14 samples that were negative for parasite gDNA by PCR. Conversely, *P. falciparum* RNA from at least one stage of the parasite (or the constitutive marker) was detected in 49 of 50 samples (98%) from dry season 2012 and 140 of 162 samples (86.4%) from rainy season 2013.

Precise quantification of the density of each stage of the marker was not attempted; however, the dCt for the ring stage marker and the microscopy-based parasite density estimates were highly correlated. Both variables were normalized by log-transformation after adding one to eliminate zero values, and entered into a simple linear regression model to assess the predictive value of the qRT-PCR ring marker results for overall parasite density per microliter (Figure 2.2). The qRT-PCR output and parasite density by microscopy were highly correlated (F=196.7, p<0.0001). Despite a strong correlation overall, the samples that were PCR-positive but submicroscopic did not show consistently lower dCt values by the qRT-PCR assay than the microscopy positive samples.

Figure 2.2. Log-transformed parasite density from light microscopy vs. log-transformed dCt of the ring stage from the qRT-PCR assay for all PCR positive samples in which the ring stage was detected, n=183
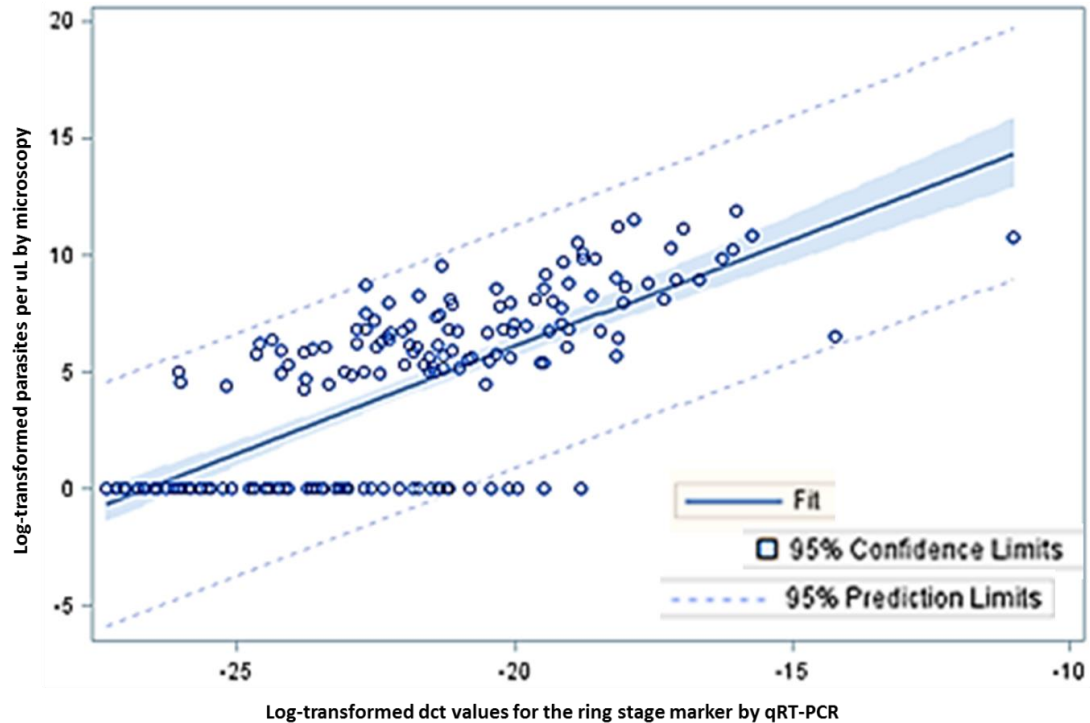
2.4.3 *Stage-specific marker detection*

The results of the qRT-PCR assay for each marker in the PCR-positive samples from the dry season and the estimated prevalence of the stage among the PCR-positive samples and in the entire study population are reported in Table 2.1. The ring stage marker was found in all but two samples: one was negative for all mRNA markers, and the other tested positive only for the constitutive protein marker, *PF11_0209*. The constitutive protein marker was not detected in 11 samples in which at least one other stage of the parasite tested positive. Immature gametocytes were detected in 22 samples: 3.6% of the total population, and 44.0% of the samples in which *P. falciparum* gDNA was detected by PCR. Mature gametocytes were detected in 21 samples: 3.4% of the total population, and 42.0% of the samples in which *P. falciparum* was detected by PCR. Trophozoites were the least likely stage to be detected in these peripheral blood samples, and found in only 12 samples.

Table 2.1. Summary of qRT-PCR results from dry season 2012

Stage	RNA positive (n)	Prevalence in PCR+ samples (n = 50)	Prevalence in total population (n = 617)
Ring stage	48	96.0%	7.8%
Trophozoites	12	24.0%	1.9%
Immature gametocytes	22	44.0%	3.6%
Mature gametocytes	21	42.0%	3.4%

There were 16 samples that tested positive for both the immature and mature gametocyte markers, with six (6) that tested positive for only the immature gametocyte marker and five (5) that tested positive only for the mature gametocyte marker (Figure 2.3). Thus, there were a total of 27 samples in which either immature gametocytes, mature gametocytes, or both were detected, representing 4.3% of the total population, and 54.0% of the samples in which *P. falciparum* was detected by PCR.

Figure 2.3. Co-occurrence of trophozoites, immature gametocytes, and mature gametocytes in samples from the dry season 2012*
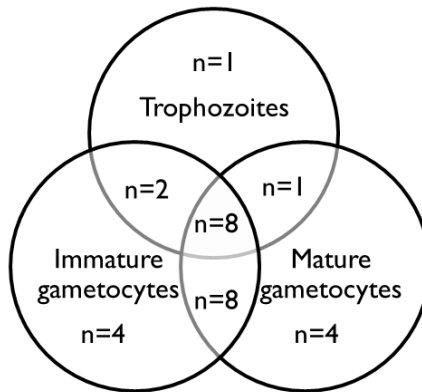
*Ring stages were present in all 28 of these samples, and expressed in 20 additional samples where no other stages were present

The corresponding results for the rainy season are presented in Table 2.2. None of the five mRNA markers were detected in 22 of 162 (13.6%) samples tested. The ring stage marker was detected in all but two of the remaining 140 samples: one of these tested positive for both immature and mature gametocytes (as well as the constitutive

protein marker), and the other tested positive only for mature gametocytes. The constitutive protein marker was not detected in 19 samples in which at least one other stage of the parasite tested positive.

Table 2.2. Summary of qRT-PCR results from rainy season 2013

Stage	RNA positive (n)	Prevalence in PCR+ samples (n = 158)	Prevalence in total population (n = 894)
Ring stage	138	87.3%	15.4%
Trophozoites	46	29.1%	5.1%
Immature gametocytes	70	44.3%	7.8%
Mature gametocytes	78	49.4%	8.7%

During the rainy season, there were 65 samples (41.1%) that tested positive for both the immature and mature gametocyte markers, with five (5) and 13 samples positive for only the immature or mature marker, respectively (Figure 2.4). Thus, there were a total of 83 samples in which either immature gametocytes, mature gametocytes, or both were detected, representing 9.3% of the total population, and 52.5% of the samples in which *P. falciparum* was detected by PCR.

Figure 2.4. Co-occurrence of trophozoites, immature gametocytes, and mature gametocytes in samples from the rainy season 2013*
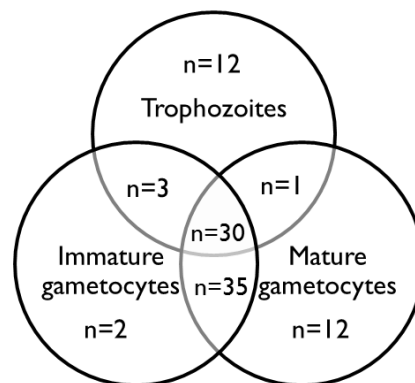
*Ring stages were present in all but two (2) of these 95 samples, one that had both mature and immature gametocytes, and one that had only mature gametocytes. The ring stage marker was expressed in 43 additional samples where none of these other stages were detected.

The sensitivity and specificity of microscopy for the detection of gametocytes in thick smears were calculated compared to the PCR/qRT-PCR testing strategy (Tables 2.3-2.4). There were six samples for which a microscopist recorded gametocytemia that were negative for *P. falciparum* gDNA by PCR, and therefore were not tested by the qRT-PCR assay or included in Figures 2.5-2.7 above. The qRT-PCR assay results for the early and/or late stage gametocyte marker corresponded well with the microscopy results for gametocyte presence in all other PCR positive samples that were tested. qRT-PCR detected either the early or late stage gametocyte marker in 23 of 29 samples that were recorded as positive for gametocytes by at least one microscopist and tested by the qRT-PCR assay: all four (4) samples from the dry season 2012 and 19 of 25 samples from rainy season 2013. Alternatively, the qRT-PCR assay demonstrated a marked increase in sensitivity compared to microscopy, identifying an additional 23 and 64 early or late stage gametocyte-positive samples from the dry and rainy seasons, respectively. If the molecular testing results are assumed to be the gold standard, microscopy had a high specificity, but very low sensitivity for the detection of gametocytes. During the dry seasons, the sensitivity was 14.8% and specificity was 99.8%; during the rainy seasons, the sensitivity was 22.9% and specificity was 98.6%. Overall, this represented a sensitivity of 20.9% and a specificity of 99.1%. The molecular testing strategy detected more than five times as many gametocytemic infections in the population during the dry season and more than three times as many gametocytemic infections in the population during the rainy season.

Table 2.3. Microscopy vs. molecular testing strategy (PCR/qRT-PCR) for detection of any stage of gametocytes, dry season 2012

| | | **Microscopy results** | | |
		Gametocyte +	Gametocyte -	Total
Molecular	Gametocyte +	4	23	27
results	Gametocyte -	1	589	590
	Total	5	612	617

Table 2.4. Microscopy vs. molecular testing strategy (PCR/qRT-PCR) for detection of any stage of gametocytes, rainy season 2013

		Microscopy results		
		Gametocyte +	Gametocyte -	Total
Molecular	Gametocyte +	19	64	83
results	Gametocyte -	11	800	811
	Total	30	864	894

Quantitative estimates of the relative presence of each stage of the parasite are shown in comparison to the parasite densities estimated by microscopy for dry season samples in Figure 2.5 and rainy season samples in Figures 2.6-2.7. The results are only presented for the 38 dry season samples and 121 rainy season samples in which the constitutive protein was detected by qRT-PCR (enabling the calculation of normalized dCt values for each other stage). The presence of gametocytes was indicated dichotomously by microscopists while counting parasites per 200 WBCs, and is also presented in panel A of Figures 2.5-2.7.

Figure 2.5. Comparison of microscopy and stage-specific qRT-PCR assay results for 38 subjects* from dry season 2012
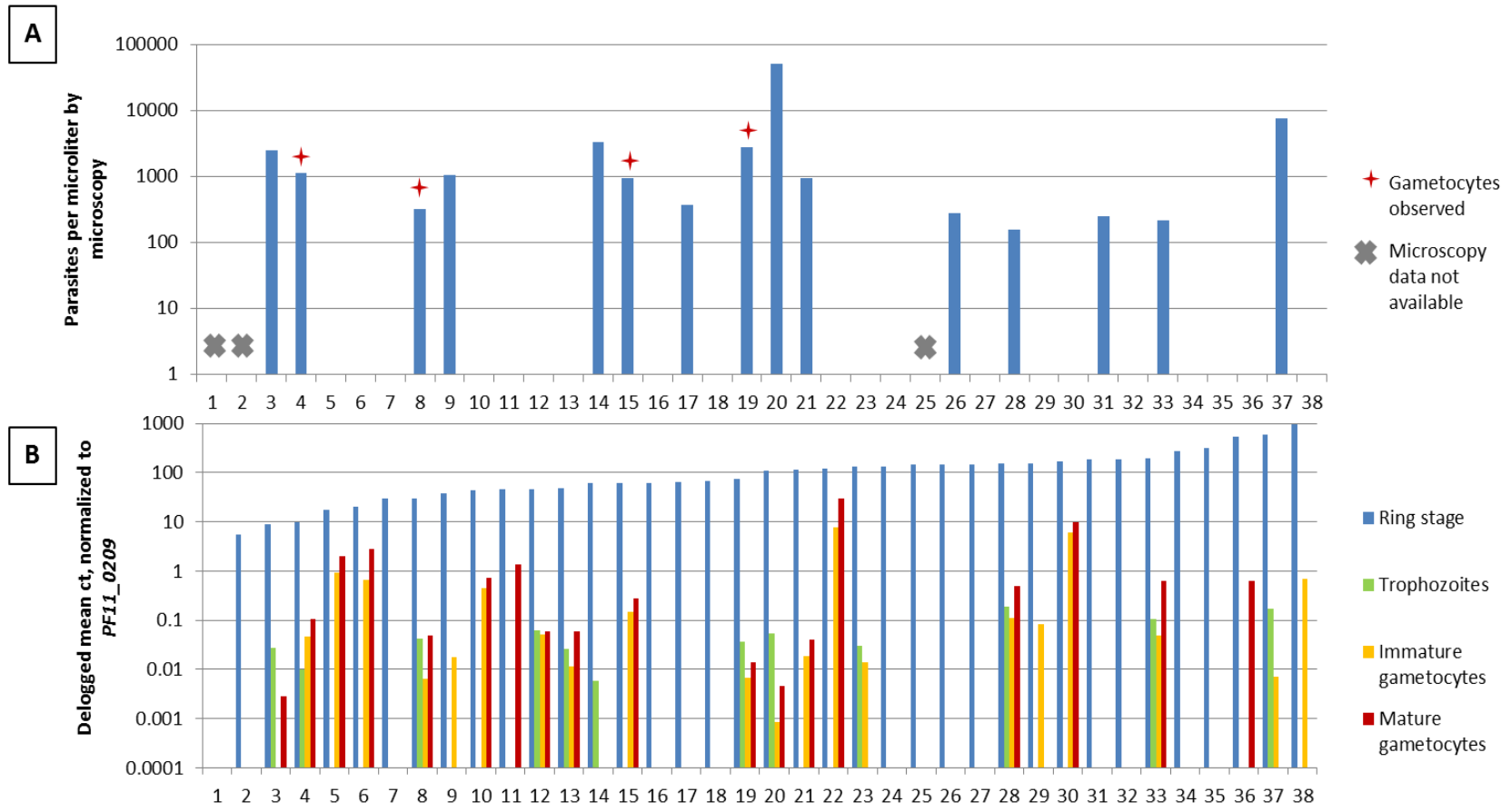
A. Total parasite density and gametocyte presence as detected by microscopy

B. Delogged mean CT values of four stage-specific markers by qRT-PCR (normalized to *PF11_0209*)

PF11_0209 was not detected in 12/50 subjects, so relative threshold calculations could only be performed for 38.

Figure 2.6. Comparison of microscopy and stage-specific qRT-PCR assay results for 60 subjects with lowest delogged mean Ct values for the ring stage marker normalized to *PF11_0209** from rainy season 2013
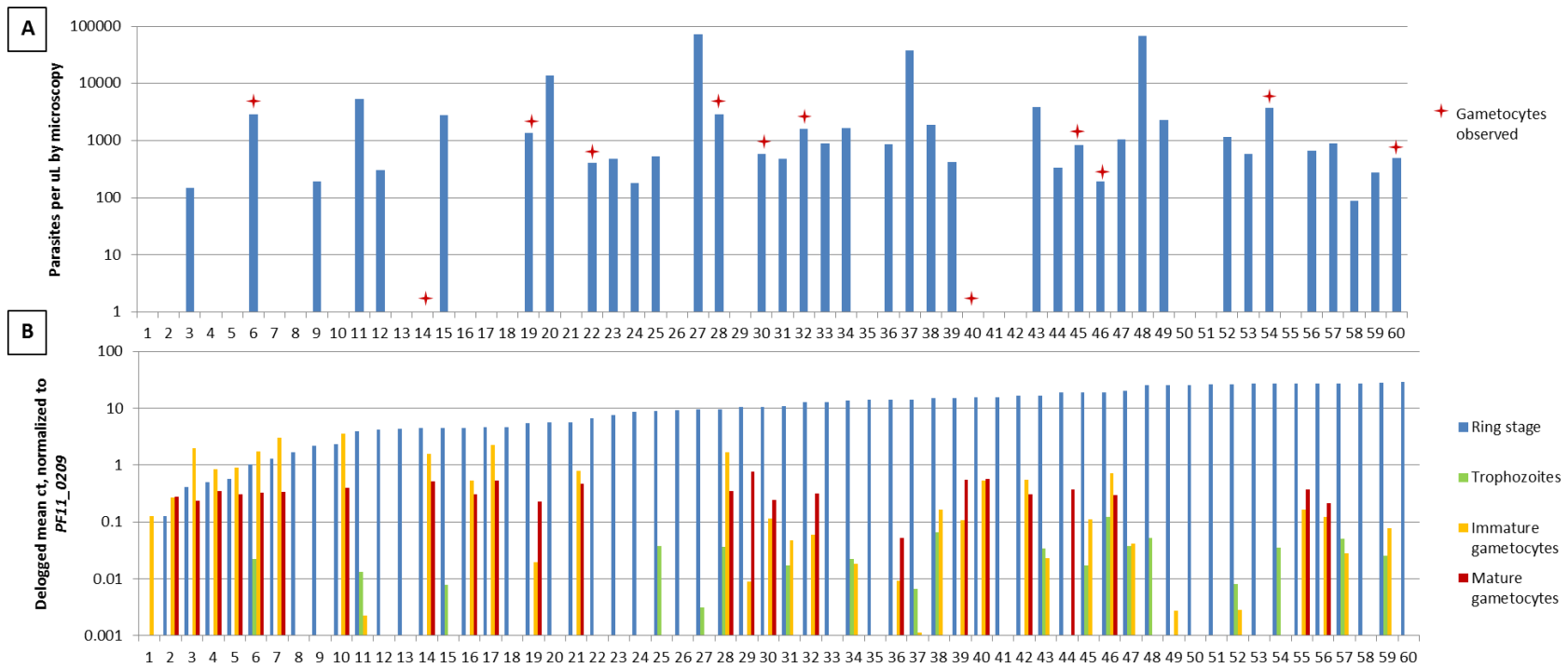
A. Total parasite density and gametocyte presence as detected by microscopy
B. Delogged mean CT values of four stage-specific markers by qRT-PCR (normalized to *PF11_0209*)
*PF11_0209 was not detected in 37/158 subjects, so relative threshold calculations could only be performed for 121.

Figure 2.7. Comparison of microscopy and stage-specific qRT-PCR assay results for 61 subjects with highest delogged mean Ct values for the ring stage marker normalized to *PF11_0209** from rainy season 2013
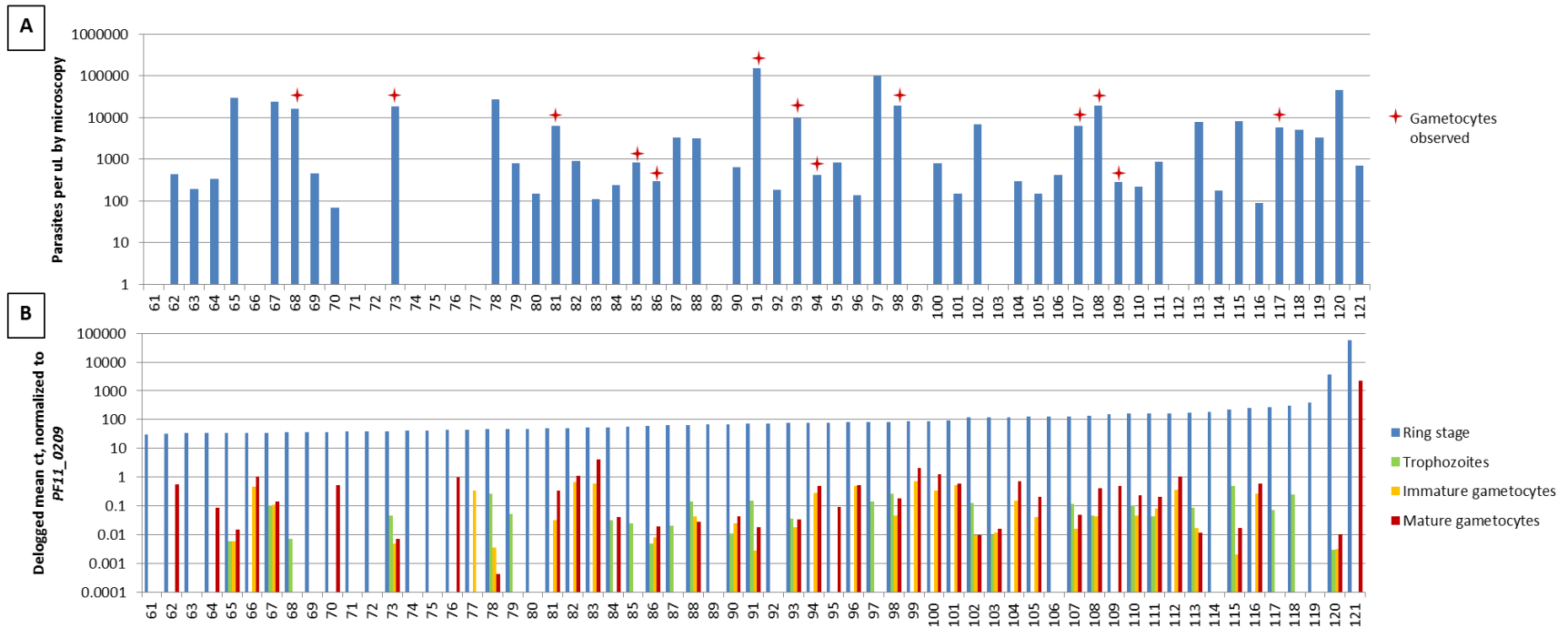
A. Total parasite density and gametocyte presence as detected by microscopy
B. Delogged mean CT values of four stage-specific markers by qRT-PCR (normalized to *PF11_0209*)
   *PF11_0209 was not detected in 37/158 subjects, so relative threshold calculations could only be performed for 121.

Previously published results from the use of this qRT-PCR assay (with a slightly expanded mRNA marker panel) showed the stage-specific parasite composition in 58 children with severe malaria from the research ward at the Queen Elizabeth Central Hospital in 2011.[21] These results are reproduced in Figure 2.8. In that severe malaria cohort, the parasite stage composition differed from those presented in the community-based infections shown in Figures 2.5-2.7, which included both symptomatic and asymptomatic infections. The ring stage predominated in both study populations, being detected in all severe cases and most community-based infections, as described above. However, trophozoites were far more common in the severe malaria cohort (47 of 58 samples, 81%) than the community-based infections from this study (48 of 208 samples 23%), and gametocytes of any stage were much more common in the community-based infections (110 of 208 samples, 52.9%) than the severe malaria cohort (6 of 58 samples, 10.3%).

Figure 2.8. Parasite stage composition in pediatric severe malaria patients from Blantyre in 2009 (reproduced from Joice _et al._Figure 4A)[21]
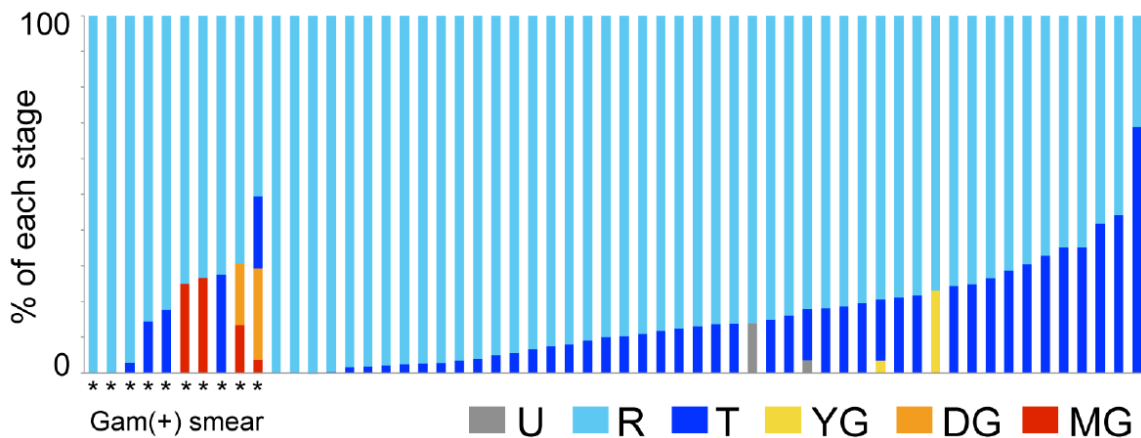
U	Undefined stage
R	Ring stages
T	Trophozoites
YG	Young gametocytes (stage I)
DG	Developing gametocytes (stages II-IV)
MG	Mature gametocytes (stage V)

2.5   Discussion

Malaria's resistance to the recent expansion of intervention efforts suggests a need for new approaches to understand and interrupt transmission. Past research on transmission has been limited by traditional microscopy's lack of sensitivity for the detection of the *P. falciparum* parasite, particularly the transmissible gametocyte stage. Molecular techniques have emerged that begin to address these limitations, but a broader spectrum of options is needed to supplement our ability to answer different questions about the drivers of transmission. The new qRT-PCR assay developed by Joice *et al.* provides another tool in the arsenal of transmission research, with sensitive and specific detection of a broad spectrum of the *P. falciparum* life cycle stages in human infections, including both immature and mature gametocytes. Previous research established that the assay had a high degree of sensitivity and specificity in clinical samples. The results of the current study indicate that, when combined with screening for parasitemic samples using PCR testing for *P. falciparum* gDNA, the assay can also be used effectively to detect gametocytemia in community-based study populations. The two-step PCR and qRT-PCR testing strategy enabled the detection of three- to five-times as many gametocytemic infections in the population as traditional microscopy, and, compared to previous molecular gametocyte detection efforts, further provided the ability to distinguish between immature and mature gametocyte presence and potentially quantify their relative expression.

2.5.1   *Comparison of PCR and qRT-PCR results*

Applications of the qRT-PCR assay in other study populations has indicated that its mRNA detection may be more sensitive than the PCR assay for detection of parasite gDNA (unpublished data from communications with Dr. Matthias Marti)); however, in this study, the sensitivity for stage-specific parasite detection was limited by the two-step testing design using PCR for initial sample screening. In this nonclinical study population, the majority of samples were expected to be aparasitemic, making it inefficient and cost-prohibitive to test every sample with the stage-specific qRT-PCR assay, for which the per sample cost is >US$50. Testing in series with the qRT-PCR assay only after a positive result for parasite gDNA by PCR limited the sensitivity for stage-specific parasite detection to that of the PCR for LDH, which has a LOD of

approximately 2.7 parasites per µL in the ICEMR-Malawi laboratory. For instance, as they were PCR-negative, none of the 16 samples discussed above that were PCR-negative and microscopy-positive were tested with the qRT-PCR assay, which may have enabled identification of samples that were false negative by PCR. Though the use of the qRT-PCR assay in all samples could theoretically have higher sensitivity for gametocyte detection, the two-step testing strategy lowered the study costs enough to be feasible. Moreover, the LOD of 2.7 parasites per µL by the molecular testing strategy still constitutes a vast improvement over that of microscopy of thick smears, which has an LOD of about 10-40 parasites per µL under ideal conditions.[11]

Regardless of this limitation, the results of the qRT-PCR assay appeared to correspond well to the PCR results. While specificity could not be fully evaluated for all PCR-negative samples, the qRT-PCR assay was negative for all stage-specific markers in the 14 PCR-negative samples that were tested. Regarding sensitivity, the qRT-PCR assay positively identified mRNA expression for at least one stage of *P. falciparum* in 189 of 208 (90.7%) of the samples that tested positive for *P. falciparum* gDNA by PCR. The 23 samples which were identified as positive for *P. falciparum* gDNA but negative for all stage-specific mRNA were investigated further.

Using the microscopy results on parasite density for comparison, all 23 PCR-positive and qRT-PCR-negative individuals appeared to have very low densities of parasitemia, with 22 being submicroscopic and only one recorded as parasite positive by microscopy. Thus, the levels of parasite genetic material may have been near the LOD of the molecular assays, where there is a degree of stochastic error in detection. Furthermore, some mRNA expression was, in fact, detected in four of these samples, but since only one of three wells in the plate tested positive, the sample was considered negative for analysis purposes. This supports the hypothesis that these samples may have had low densities of parasitemia that approached the LOD. These results may also reflect a true difference in the persistence of parasite gDNA vs. mRNA in recently cleared infections, though the cross-sectional sampling method limited our ability to assess such a difference through the course of individual infectsions. We investigated whether any of these subjects had recently received an antimalarial that might have contributed to clearance of the infection, but none reported use of any antimalarial (including

lumefantrine artemether, chloroquine, quinine, or sulfadoxine pyrimethamine) in the two weeks prior to sample collection. Though we were unable to conclusively identify the reason(s) for the discrepancies among the 23 PCR-positive and qRT-PCR negative individuals, they comprised a small number of the tested subjects, and overall the sensitivity for *P. falciparum* mRNA detection by qRT-PCR compared to gDNA detection by PCR was high.

2.5.2 *The PCR/qRT-PCR testing strategy for stage-specific parasite detection*

True validation of the two-step, PCR and qRT-PCR testing strategy for the detection of each specific stage of the parasite was limited by the lack of an adequate 'gold standard' value for each sample in the population, since microscopy is known to be less sensitive than molecular testing strategies. For instance, when the PCR assay is treated as the gold standard for any stage *P. falciparum* detection, the sensitivity of microscopy was only 30.0% during the dry season and 59.3% during the rainy season in this subset of eight EAs. Submicroscopic infection is explored in greater detail in another publication that considers all 30 EAs from the corresponding surveys.[27] Furthermore, the microscopists recorded the presence of gametocytes if they were observed, but did not quantify the gametocytemia, and no microscopy data were collected regarding the presence of the trophozoite stage or the distinction between immature and mature stages of gametocytes. While further improvements in sensitivity may be possible, the comparison of the PCR/qRT-PCR results with microscopy do confirm the expected increase in sensitivity for gametocyte detection, with microscopy detecting only 20.9% of the molecularly-identified gametocytemic samples.

Assessment of the validity of the two molecular tests compared to microscopy results was somewhat complicated by the presence of multiple species of *Plasmodium* in the area. Though *P. falciparum* is predominant, *P. ovale* and *P. malariae* infections have also been recorded in the region.[28] Microscopists counted parasites seen without identifying the species of *Plasmodium*, but could conceivably detect any species. On the other hand, both PCR and qRT-PCR were specific to the detection of *P. falciparum*. There were 16 samples in the total study population of 1,511 individuals that were counted as positive by microscopy but negative by PCR; these may be samples that were false negatives for *P. falciparum* by molecular testing, samples that were infected with

other species of the *Plasmodium* parasite, or samples that were false positive by microscopy. Based on the available information, we were unable to delineate the ultimate source of these discrepancies, but even if all were truly parasitemic, the number of false negatives by PCR was still very small (≤1% in the study population).

Surprisingly, there were 12 samples in which gametocytes were reported by microscopy that were not identified as gametocytemic by the PCR/qRT-PCR testing strategy: six that were PCR-negative and did not get tested for *P. falciparum* mRNA by the assay, and six that were tested by the qRT-PCR and determined to be negative for both the immature and mature gametocyte markers. Of the six untested PCR-negative samples, four were ultimately determined to be aparasitemic in the final smear calculation after resolving discrepancies among the readers. The other two subjects had three or fewer total parasites counted by each reader, and may represent either false positives or non-*falciparum* infections; however, it is possible that the sample testing strategy led us to falsely identify these samples as negative for the presence of gametocytes. Of the six PCR-positive samples, there were four in which the marker for either immature or mature gametocytes was detected in only one of three wells by qRT-PCR, suggesting that they may have been true gametocyte positive infections at very low densities. Defining as positive any samples with an amplification curve in at least one well by the qRT-PCR assay may indeed increase the sensitivity for identifying very low density gametocyte infections, but could also reduce specificity. Given the small number of apparent false negatives and large number of gametocyte positives that were not identified by microscopy, using two of three wells to identify positive samples by the qRT-PCR assay appears to be sufficient to improve upon the sensitivity of microscopy in this study population without a loss in specificity.

2.5.3 *Quantification of* P. falciparum *stage-specific densities*

There were several limitations for using the stage-specific qRT-PCR assay to estimate the quantitative blood density of each stage. Ideally, each plate would have been run with a positive control in which each stage was present at a known concentration in order to estimate the relative concentration of the test samples by comparing the test sample dCt values to those of the positive control. No such positive controls were available. Furthermore, while we attempted to consistently preserve 50 µL of blood from

each individual participant, it was not possible to guarantee the precision of this quantity in the field collection procedures, so there may have been some variation in the amount of starting material for each sample tested. Such variation would lead to a difference in the amount of parasite genetic material tested for each sample (and the resulting dCt values) that is not reflective of true differences in parasitemia. Nevertheless, the dCt values still offer a rough estimate of the relative densities of parasitemia for each sample, as demonstrated by the high correlation between the dCt value for the ring stage and the estimated parasite density by microscopy ($p < 0.0001$).

One surprising feature of the relationship between the dCt value of the ring stage marker by qRT-PCR and the parasite density recorded by the microscopists was that submicroscopic infections (those that were negative by microscopy) did not have consistently lower dCt values than microscopically-detected infections. This may reflect a limitation of microscopic slide readings from a non-clinical population with a large proportion of infections having low peripheral densities, and the lack of granularity inherent in estimating smear density per microliter as 40 times the number of parasites observed. Despite the limitations of the microscopy data, the statistical model did confirm that the qRT-PCR results for the ring stage marker were strongly correlated with the observed parasite density in peripheral blood, which suggests that the dCt values of other stage-specific mRNA markers are also correlated with the quantitative blood density of their respective stages. While our collaborators continue to refine a linear model to predict the relative stage composition of a given individual's infection based on the output from the qRT-PCR assay, the ring stage marker results support the possibility of comparing the dCt values of the other stage-specific markers to estimate the relative density of a particular parasite stage across different individual subjects based on the currently available data alone.

Comparison of the results of the assay in this community-based study population differ considerably from those that were previously reported for a cohort of severe malaria cases in a pediatric population in Blantyre from 2009.[21] Gametocytes were far more common in our community infections than the severe malaria cases, while trophozoites expression was much more common in the severe cases than the community-based infections. The severe malaria cases may represent the stage

distribution of relatively new infections, whereas the community-based samples considered here were collected cross-sectionally, and will represent prevalent infections of any duration, up to multiple months. While a *P. falciparum* infection could potentially develop gametocytes at any point in time, if gametocytemia persists once it develops, then older infections would be more likely to be gametocytemic. This would have profound implications for the relative importance of asymptomatic infections to the human infectious reservoir, since they tend to be untreated and of long duration. Further research is needed to understand the reasons for these distinct presentations of parasite stages, especially research involving longitudinal sampling over the course of individual infections. In particular, such data would be useful to understand the relationship between the age of an infection and the development of gametocytemia/potential contribution to the population-level infectious reservoir, or any association between parasite stage composition and symptom severity.

2.5.4 *Conclusions*

Ultimately, despite a number of limitations for validation, the results presented here indicates that this molecular testing strategy involving PCR for gDNA and a novel, stage-specific qRT-PCR assay is effective for the detection of four stages of *P. falciparum* infections in community-based study populations. Like other molecular testing strategies, such as the detection of *Pfs25* mRNA transcripts from mature gametocytes using QT-NASBA or RT-PCR, this assay shows a marked improvement in sensitivity compared to traditional light microscopy. The use of markers that span exon-exon junctions ensures stage specificity, and use of a panel of such markers makes this test uniquely suited to answering questions about complex parasite stage dynamics within human hosts, including the ability to distinguish sexual from asexual stage presence, and mature from immature gametocyte presence. Future publications could use this assay for research into parasite life cycle presence in the course of both symptomatic and asymptomatic infections to investigate their relationship to disease severity and duration. In Chapter 3, the results of this assay will be analyzed in regression models to determine the predictors of gametocytemia across the different study settings and seasons, in an effort to better understand population-level transmission dynamics and potential reservoirs for *P. falciparum* infection in southern Malawi.

2.6   References

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CHAPTER 3 Moving beyond microscopy: Epidemiology and predictors of the human gametocyte reservoir based on molecular detection methods in communities of southern Malawi

3.1   Abstract

Asymptomatic *Plasmodium falciparum* infections in humans are common in areas where malaria is endemic. The extent to which asymptomatic infections contribute to ongoing transmission remains unclear. The presence of mature *P. falciparum* gametocytes is a prerequisite for transmission, but population-level prevalence of human gametocytemia is poorly characterized due to the difficulty in measuring gametocyte carriage, which frequently occurs at submicroscopic densities. This study applied more sensitive molecular detection techniques to characterize the epidemiologic patterns of the human gametocytemic reservoir in communities of southern Malawi.

Methods: Blood samples were collected from ~30 households in each of eight geographically diverse enumeration areas (EAs) during two cross-sectional surveys: one at the end of the dry season 2012 and one at the end of the following rainy season 2013. About 616 people in 2012 and 892 in 2013 were bled for molecular parasite testing. Presence of mature gametocytes was determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) for all people who were polymerase chain reaction (PCR)-positive for *P. falciparum* genomic DNA (gDNA). Multilevel logistic regression models were used to identify the predictors of gametocytemia in the total population, and among people with prevalent *P. falciparum* infections, based on data from household- and individual-level surveys.

Results: The overall prevalence of mature gametocyte carriage in the study population was 3.4% during the dry season and 8.7% during the rainy season, with substantial EA-level heterogeneity. Nearly half of PCR-positive *P. falciparum* infections carried detectable mature gametocytes by qRT-PCR, regardless of recent symptom status.

People who lived in unfinished houses, owned bednets, or were of lower socioeconomic status tended to have higher prevalence of gametocytemia in the total population, and among *P. falciparum*-infected people, though only a few associations were statistically significant after adjustment. School-aged children, 6-15 years old (yo), had significantly higher odds than adults (≥16 yo) of being gametocytemic when infected, and were significantly more likely to be gametocytemic than either younger children (<5 yo) or adults in the total population.

Conclusions: School-aged children are an important gametocyte reservoir, and may contribute significantly to transmission, despite suffering less of the burden of malaria disease than younger children. Factors that increase the risk of being infected also tended to increase the odds of being gametocytemic given infection, though the individual associations were not statistically significant in the study population. Asymptomatic cases are frequently gametocytemic and therefore a potential source for perpetuating transmission to mosquitoes. Malaria elimination strategies will need to consider these human reservoirs, but there may be opportunities for targeting interventions through schools to treat this school-aged group with the highest gametocyte prevalence.

3.2   Introduction

As the global malaria burden has proven somewhat intractable to our best efforts to prevent infection and treat the disease, attention has been increasingly drawn to the existence of asymptomatic and submicroscopic *Plasmodium* infections.[1–6] Such infections are widespread in areas where malaria is endemic, but the extent of their infectiousness and contribution to transmission dynamics is not fully understood.[7] Transmission from an infected human to a mosquito requires only that at least one male and one female gametocyte, the sexual stages of the parasite, are ingested in a blood meal.[8] As the average size of a blood meal for Anopheline mosquitoes is approximately two to three microliters (µL),[9] human blood density of one gametocyte per µL would be a theoretical lower limit for parasite transmission. Though the probability that a given mosquito will develop infectious oocysts after taking a blood meal decreases with diminishing gametocyte density in human hosts, even gametocyte densities <1/µL have

been found to be potentially infectious to mosquitoes.[8,10] Humans with asymptomatic or submicroscopic infections may have low-density gametocytemia and contribute significantly to ongoing transmission, particularly if they are disproportionately likely to be bitten by mosquitoes, or maintain gametocytemia for long durations compared to symptomatic cases. Indeed, previous research found that individuals could remain gametocytemic through months of a dry season with negligible transmission.[11,12] New data on the population-level dynamics of gametocytemia will improve our understanding of the potential infectiousness of human reservoirs for *Plasmodium* infection in endemic communities, including those people with submicroscopic and asymptomatic infections.

Few studies have reported on the prevalence of gametocytemia in communities where people are apparently healthy, and most of these studies have relied on microscopy to detect gametocytes. The lower limit of detection of microscopy ranges between 10 and 40 parasites/µL, depending on slide preparation, the skill of the reader, and the number of fields viewed.[13] Conclusions from previous research regarding the gametocytemic human reservoir are limited by the low sensitivity of microscopy, which cannot detect all gametocyte infections of potentially infectious densities. Modern molecular testing techniques that detect gametocyte-specific mRNA transcripts, such as quantitative reverse transcription polymerase chain reaction (qRT-PCR) and quantitative nucleic acid sequence based amplification (QT-NASBA), offer a vastly improved sensitivity over that of microscopy.[8,14,15] Some studies attempted to improve sensitivity by increasing the number of fields viewed or white blood cells (WBCs) counted by a microscopist, but none has approached the sensitivity achieved by molecular methods, which can potentially detect <1 parasite/µL.[15] Studies based in clinical populations alone are also insufficient for characterizing the human infectious reservoir, because asexual stage parasites, and not gametocytes, are associated with the occurrence of symptoms. There is a need for improved estimates of the gametocyte carriers using molecular detection in community-based samples that include asymptomatic cases across a variety of transmission settings. These data will aid public health workers in identifying who are the important infectious human reservoirs and intervening effectively to interrupt transmission.

Of the relatively small number of prior studies that reported gametocyte epidemiology for community-based samples, most reported a monotonic decrease in gametocyte prevalence by age.[16–27] The majority of these used microscopy alone for gametocyte detection, and all involved samples collected before 2006. The current study is the first to report the epidemiology of gametocytemia among people living in southern Malawi using a sensitive, stage-specific qRT-PCR assay.

While direct measures of infectiousness from mosquito blood-feeding were not available, the current studysought to identify the potentially infectious (a.k.a. gametocytemic) human reservoirs, and assess the predictors of gametocytemia in a community-based population across two seasons and a variety of geographic zones with varying transmission intensities. We hypothesized that people with asymptomatic infections are as likely to be gametocytemic as people who reported no recent malaria symptoms. We also hypothesized that the odds of being gametocytemic when infected would decrease with age, but that the association would be weaker in sites with lower transmission intensity, where less frequent exposures might delay the development of immune responses to gametocytes.

3.3   Methods

The study was carried out under the auspices of the Malawi International Center of Excellence for Malaria Research (ICEMR), and all methods were approved by the independent Institutional Review Boards (IRB) of the University of Malawi College of Medicine, the University of Maryland, Baltimore, and Michigan State University (MSU). The University of Michigan IRB determined this gametocyte study expansion to be non-regulated based on existing approvals.

3.3.1   *Study design*

Data were collected in three Districts of southern Malawi during biannual cross-sectional surveys at the conclusion of each rainy season (April-May) and dry season (September-October) from 2012 to 2014. These three Districts represent different transmission patterns. Blantyre is a more urbanized District in the highlands with expected low transmission, Thyolo a semi-rural District with expected moderate transmission, and Chikhwawa a rural District at low elevation with expected high

transmission. Compact segments of approximate 30 households were chosen for each of 10 enumeration areas (EAs) per District; EAs and compact segments were randomly selected using two-stage cluster sampling, excluding any EAs on the District border between Thyolo and Chikhwawa, and Chikhwawa EAs at >500 m elevation or Thyolo EAs at <500 m elevation. Thus, approximately 900 households were selected for study and visited during each survey. As the average household size in southern Malawi is 4.2 persons,[28] more than 3,500 individuals were expected to participate each round of surveillance. The survey team returned to the same compact segments for each round, having communicated closely with local health representatives and EA chiefs to encourage high levels of participation.

At each visit, the field team interviewed household members in the local language (Chichewa) to obtain information about household-level variables and individual-level variables for all members of the household. A standardized questionnaire sought data on household demographics (i.e. age, sex, relationship of all members), house construction, use of malaria interventions [including details on insecticide treated net (ITN) quality and use, indoor residual spraying (IRS), intermittent preventive therapy in pregnancy (IPTp) and other treatment-seeking behaviors], and self-reported recent symptoms of disease.

3.3.2   *Sample collection and preservation*

Malawian nurses on each field team measured the axillary temperature and took peripheral blood samples from all subjects ≥6 months old (mo) who were present and consented (or were assented) to participate in the study. During all surveys, the nurses collected ~0.5 mL of blood onto slides as thick smears for microscopy, onto filter paper for DNA isolation, and into a Hemocue® device (Hb 201+ System, Hemocue, Inc) for immediate testing of hemoglobin levels for children 6 mos - 5 yrs old (yo). Initial RNA sample collection was undertaken during the dry season 2012 survey for a convenience-based subset of eight of the 30 EAs: two from Blantyre and three each from Thyolo and Chikhwawa. RNA collection was expanded to include all subjects for the subsequent surveys. To preserve RNA, whole blood samples of approximately 50 μL were collected into 250 μL of RNAprotect® (Qiagen Inc., Valencia, CA). For qRT-PCR testing for the present study, we included all subjects in the eight EAs from whom RNA samples were

collected during the dry season 2012, and the same eight EAs during the subsequent rainy season 2013 (Figure 3.1).

The selected EAs differed from the excluded EAs for their respective Districts on a number of variables, including age distribution, parasite prevalence, and socioeconomic status (SES), and were not considered to be representative of each District as a whole. Thus, District was not included as a potential predictor in statistical analyses. Instead, we approximated the relative *P. falciparum* transmission intensity for each specific EA using PCR data from the first cross-sectional study survey during the 2012 rainy season. The 30 study EAs were classified into tertiles of baseline *P. falciparum* prevalence. The lowest tertile included EAs with 0 to <8%, the middle tertile included those with 8 to <15%, and the highest tertile included those with ≥15% PCR-based prevalence estimates in the rainy season 2012. RNA was sampled from two of the EAs in the lowest tertile: Ngabu EA117 from Chikhwawa and Chimaliro EA052 from Thyolo; four of the EAs in the middle tertile: Makhwira EA051 from Chikhwawa, Nchilamwela EA058 from Thyolo, and Misesa EA014 and Soche West Ward EA020 from Blantyre; and two of the EAs from the highest tertile: Lundu EA019 from Chikhwawa and Kapichi EA056 from Thyolo. The baseline prevalence tertile value was assigned to all individuals residing in households within a given EA in the two subsequent surveys included in this qRT-PCR analysis, the dry season 2012 and rainy season 2013 surveys.

Figure 3.1. Enumeration Areas (EAs) included in each survey of the ICEMR-Malawi Cross-Sectional Study

3.3.3 *Microscopy procedure*

Thick smears were air dried, methanol fixed, and Giemsa stained upon delivery to the lab at the end of each day of sample collection. Thick smears were read at 100X objective magnification by two trained microscopists, who recorded the number of parasites seen per 200 white blood cells (WBCs). The two reads were considered discrepant if one reader recorded presence of any *Plasmodium* parasites and the other did not, if one reader counted more than 10 times as many as the other when the lowest reader was less than 20 parasites seen, or if one reader counted more than twice as many

as the other when both reads were 20 parasites or greater. Discrepant slides were sent to a third reader. All readers were blinded to the smear results recorded by the other readers. The final smear value was calculated as zero if two readers reported no parasites. Using an assumed WBC count of 8000/µL, the final smear value per microliter was estimated as the geometric mean of the two positive reads multiplied by 40, using the two closest reads if all three were positive for parasites.

Readers were asked to record dichotomously whether or not they observed at least one gametocyte while counting parasites per 200 WBCs. Samples were considered to be microscopy-positive for gametocytes when any one of the readers reported seeing any gametocyte(s). It was therefore possible for a sample to be positive for gametocytes but have a final smear value of zero parasites per µL when the various reads were discrepant.

3.3.4   *Molecular testing*

To identify gametocytemic individuals, samples were tested in series: all individuals for whom blood on a filter paper sample tested positive by PCR for gDNA from the *P. falciparum* lactate dehydrogenase (LDH) gene were classified as parasitemic, and their whole blood sample with preserved RNA was tested by qRT-PCR for the expression of *P. falciparum* stage-specific mRNA. The molecular testing methods were described in detail in Chapter 2; briefly, for PCR of the filter papers, blood spots were fixed by submersion in methanol for 15 minutes, then left to dry for at least two hours. Parasite gDNA was then extracted by adding 50 µL of DNase-free water and heating at 99°C for 30 minutes, vortexing at 15 minutes. These gDNA samples were used in PCR reactions targeting the *P. falciparum* LDH gene, as described by Rantala *et al*.[29] Each 25 µL reaction consisted of 12.5 µL Taqman® (Life Technologies™, Carlsbad, CA), 0.25 µL each of forward and reverse primers at 250 nM, 0.30 µL of probe at 300 nM, 10.7 µL nuclease-free water, and 1 µL of gDNA sample. All samples were run in duplicate and considered dichotomously positive for *P. falciparum* if LDH had a clear amplification curve in at least one of the two wells.

For individuals with filter papers that were PCR-positive, qRT-PCR was used to detect parasite stage-specific mRNA expression based on a novel assay that distinguishes ring stage parasites, trophozoites, immature (stage I-III) gametocytes, and mature (stage IV-V) gametocytes, as described by Joice *et al*.[30] The preserved RNA samples were kept

in a -80°C freezer until thawed for RNA extraction and testing. After thawing, samples were spun in a microfuge at room temperature to obtain a pellet. RNA was extracted from the selected samples using RNeasy Plus Mini-Kits® (Qiagen Inc., Valencia, CA) and treated with RNase-free DNase Sets® (Qiagen Inc., Valencia, CA) to eliminate all traces of parasite gDNA. Reverse transcription was performed using Superscript complementary DNA (cDNA) Synthesis Kits® (Life Technologies™, Carlsbad, CA) in a double reaction compared to the standard protocol, using 50 ng/µL random hexamers as the primer. If qRT-PCR could not be performed immediately, the resulting cDNA samples were temporarily stored overnight at -20°C.

The qRT-PCR assay involved the following stage-specific markers: 1) *PFE0065w* for the early asexual stage (ring stage), 2) *PF10_0020* for the late asexual stage (trophozoite), 3) *PF14_0748* for immature gametocytes, 4) *PF14_0367* for mature gametocytes, and 5) *PF11_0209* as a constitutive protein. The assay was performed in triplicate for each stage-specific marker using 4 µL of cDNA sample per well. Primer/probe mixes were created by combining 18 µL of forward primers and 18 µL of reverse primers at a concentration of 900 nM, 5 µL of probe at a concentration of 250 nM, and 59 µL of nuclease-free water. These mixes were stored at -20°C in a dark box with minimal light exposure. Each well of the qRT-PCR reaction consisted of 10 µL of ABI TaqMan Gene Expression Master Mix® (Life Technologies™, Carlsbad, CA), 1 µL of the primer/probe mix, 5 µL of nuclease-free water, and 4 µL of cDNA sample. The results of the qRT-PCR were recorded as positive for a particular marker only if at least two of the three wells had a clear amplification curve.

3.3.5 *Data management and analysis*

All study data were stored in the Research Electronic Data Capture (REDCap) system (Vanderbilt University) hosted at the University of Malawi College of Medicine.[31] Some new variable creation and all statistical analyses were performed in Statistical Analysis System (SAS) versions 9.3 and 9.4 (SAS Institute, Cary, NC).

Socioeconomic status (SES) variables were collected at the household level. Ten variables pertaining to SES were combined into a single SES indicator variable based on the method suggested by Filmer and Pritchett.[32] The ten variables were each input into a Principal Component Analysis (PCA) based on the following scoring system: Ownership

of each of six items (radio, bike, car, house, phone, and television) was indicated by a value of 1 if the household owned at least one of the item and 0 if it did not. If the home was powered by electricity, it received a score of 1 for that variable, and 0 if it was not. If the head of household earned any income, that was indicated with a value of 1. If the household reported a shortage of food in the previous month, it received a score of 0; the lack of a food shortage in the previous month received a score of 1. Finally, the highest level of education achieved by either the head of household or spouse was ranked as a 0 for 'No schooling,' 1 for any years completed of Standard 1-8, or 2 for completing anything beyond Standard 8. Higher scores on each component therefore represented greater wealth or SES. PCA was performed in SAS using *proc factor* to obtain weights for each item of the ten individual indicators to evaluate their relative importance in contributing to the total household score. These weights were obtained from Factor 1, which had an eigenvalue of 2.55. The PCA was calculated using 3,599 households in the entire dataset from four surveys. These weights were then applied to each of the ten components to calculate an overall weighted SES index score for each household during each survey. The PCA-weighted SES index score was applied to all individuals within a household. The individuals from all 30 EAs from a given survey were grouped into quartiles by the SES index scores, including 3,861 participants from dry season 2012, and 3,754 participants from rainy season 2013. Quartiles of SES were determined for each survey independently to minimize the impact of any potential changes in population SES over time. The subset of eight EAs where RNA was sampled was extracted from the quartiles assigned to the entire dataset for a given survey, leading to uneven sample sizes for each quartile within this analysis.

Household construction was assessed separately from the overall SES index, as it may be directly associated with *Plasmodium* infection if the household materials increase exposure to *Anopheles* vectors. The roof, floor, and walls of the house were each classified as natural, rudimentary, or finished. Natural roofs were those made of thatch/leaf or sticks/mud or missing entirely; rudimentary roofs were made of rustic mat/plastic sheet, reed/bamboo, or wood planks; finished roofs were those made of corrugated iron/metal sheets, wood, cement fiber, cement/concrete, or roofing shingles. Natural floors were those made of earth/sand or dung; rudimentary floors were those

made of wood plants or palm/bamboo; finished floors were those made of parquet/polished wood, vinyl or asphalt strips, ceramic tiles, cement, or wall-to-wall carpet. Natural walls were those that were made of cane, sticks, bamboo, or reeds, or missing entirely; rudimentary walls were those that were made of bamboo/wood with mud, carton/plastic sheets/sacks, mud, plywood, or stone with mud; finished walls were those made of bricks, cement, cement blocks, covered adobe, plaster, stone with lime/cement, or wood planks/shingles. A household overall was categorized as 'finished' if at least two of the roof, walls, and floor were classified as finished rather than natural or rudimentary, and 'unfinished' if none or only one of the three was finished. The status of the eaves was included as a separate variable, categorized as 'open' or 'closed.'

Age groups were defined as young children (6 mos – 5 yrs), school-aged children (6 – 15 yrs), or adults ($\geq$16 yrs). We identified whether or not each household owned at least one bednet, and which members of the household had slept under the bednet on the previous night.

Symptoms of malaria were defined as reported history of any fever in the previous two weeks, any fever, rigors/chills, or headache in the previous 48 hours, or an axillary temperature $\geq$37°C at the time of the survey. Data also were collected on the use of any treatments in the previous two weeks for all subjects, including the antimalarials lumefantrine-artemether (LA), chloroquine, quinine, and sulfadoxine-pyrimethamine (SP)/fansidar.

Prevalent gametocyte carriers were identified as those that tested positive for any *P. falciparum* gDNA by PCR and subsequently tested positive for the immature gametocyte marker, the mature gametocyte marker, or both. Regression analyses focused on carriage of mature gametocytes as the outcome, as those individuals represented the potentially infectious reservoir at the time of the survey, and there was no certainty that subjects with immature gametocytes alone would necessarily develop a mature gametocytemic infection. Univariate analyses of the potential predictors of mature gametocytemia were performed using chi-squared tests of association when possible, or Fisher's exact tests for the variables with expected values of five or fewer. Classification and regression tree (CART) techniques were attempted, but failed to produce a tree with nodes due to the low prevalence of gametocytemia in the total population. Logistic

regression models were used to evaluate the potential predictors of gametocytemia in the total study population, and among the subpopulation that was parasitemic by PCR, in the context of multivariable adjustments. Multilevel models were explored to account for clustering at the household and EA levels and prevent overestimation of statistical significance, using the *proc glimmix* command in SAS.

3.4 Results

3.4.1 *Samples included in RNA testing and analysis*

RNA samples were tested and analyzed for eight EAs included in the ICEMR-Malawi Cross-Sectional Study, two from Blantyre and three each from Thyolo and Chikhwawa (see Figure 3.1). The schematic of samples collected and included in the current molecular analysis of gametocyte prevalence is presented in Figure 3.2. Of 1,077 individuals from 249 study households in those eight EAs during the dry season 2012 survey, there were 836 (77.6%) ≥6 months of age who were present and consented to give blood samples, though only 825 had samples available for analysis. Samples of gDNA on filter paper and mRNA in RNAprotect for molecular testing were only collected from a subset of 616 (74.7%) of these subjects from 215 households during this initial more limited phase of RNA sampling. Of 1,017 individuals in 248 households from the same eight EAs that participated in the rainy season 2013 survey, RNA samples were obtained from all 905 (89.0%) individuals ≥6 months of age from 247 households who were present and consented to give samples, though only 894 were available for analysis. An additional two subjects from rainy season 2013 were excluded as they lacked filter paper samples to enable PCR testing for parasite gDNA. Thus, 616 individuals from 214 households from the dry season 2012 and 892 individuals from 247 households from the rainy season 2013 formed the study population for molecular analysis. Of these, there were 50 samples that tested positive for *P. falciparum* parasite gDNA by PCR from the dry season 2012 and 162 from the rainy season 2013 that were subsequently tested by qRT-PCR for stage-specific *P. falciparum* RNA expression.

Figure 3.2. Sample sizes included in the molecular testing of gametocytemia from two seasons of the ICEMR-Malawi Cross-Sectional Study
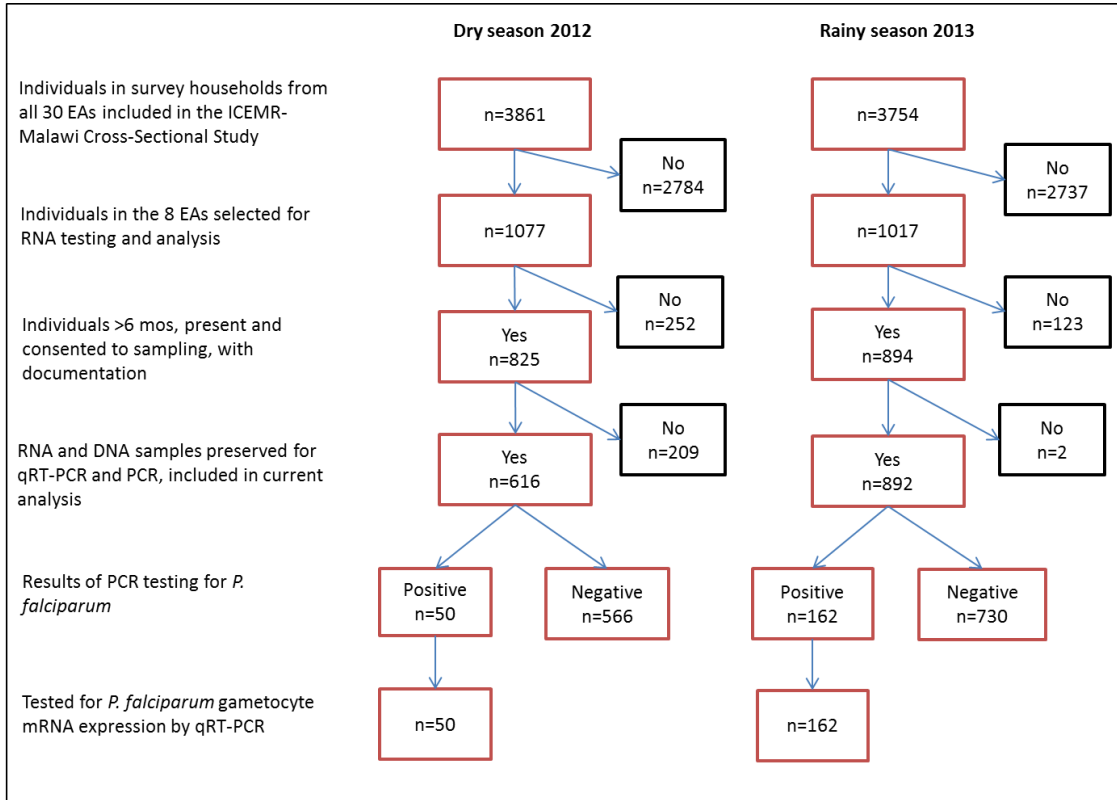
Individuals within each of the eight included EAs who provided samples included in this molecular testing were compared to the subjects from the same eight EAs that were not included. Both gDNA for PCR and RNA for potential qRT-PCR were available for only 58.3% of individuals in the dry season 2012, but this expanded to 89.0% of individuals in the rainy season 2013, largely due to the intentional addition of RNA sampling to the study protocol. During the dry season 2012, RNA sampling proportions were low among school-aged children 6 – 15 yrs of age (57.1%) and adults ≥16 yrs (57.7%) relative to those among young children 6 mos – 5 yrs of age (70.0%) (X^2=10.69, p=0.005) and among men (48.2%) relative to women (66.8%) (X^2=38.08, p<0.0001). These relationships were generally consistent within each of the eight EAs. The sampling proportions were not significantly different for individuals from households made of finished vs. unfinished materials (X^2=1.60, p=0.21) or for different wealth quartiles (X^2=3.13, p=0.37), though the proportion of individuals sampled from the highest wealth quartile tended to be slightly lower than the lower wealth quartiles. A greater proportion

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of individuals who had a temperature $\geq 37.5°C$ or reported a fever within the previous two weeks were sampled for RNA (64.8%) than people with no measured/reported fever (56.6%) ($X^2=4.43$, p=0.04). While the RNA sampling coverage was much higher overall during the rainy season 2013, the sampling proportions followed similar patterns. Children 6 mos – 5 yrs had the highest coverage (99.0%), followed by school-aged children 6 – 15 yrs (93.9%), and adults ≥ 16 yrs (83.2%) ($X^2=48.23$, p<0.0001). The sampling proportion continued to be higher among women (96.3%) than men (79.5%) ($X^2=72.31$, p<0.0001) and among individuals with a measured temperature $\geq 37.5°C$ or reported fever within the previous two weeks (94.2%) compared to those without one (87.9%) ($X^2=5.60$, p=0.02). There was again no significant difference in the sampling proportion for individuals from households with finished materials compared to those with unfinished materials ($X^2=1.45$, p=0.22). The highest two wealth quartiles had slightly lower sampling coverage than the lowest two wealth quartiles, but the difference was small and not statistically significant ($X^2=5.01$, p=0.17).

3.4.2 *Prevalence of parasitemia and gametocytemia*

The results for immature, mature, or any stage gametocyte prevalence for each of the sampled EAs differed by season (Table 3.1), as did the prevalence of mature gametocytes as a proportion of all PCR-positive parasitemic samples by EA and season (Figure 3.3). The prevalence of gametocytemia, like the prevalence of any parasitemia, was generally higher during the rainy season 2013 than the dry season 2012 in a given EA, though Figure 3.3 seems to indicate that a greater *proportion* of parasitemic infections carried mature gametocytes during the dry season than the rainy season in most EAs. Many participants who had mature gametocytes also carried immature gametocytes and vice versa, particularly during the rainy season 2013. A majority of the 23 participants with immature gametocytes also had mature gametocytes during the dry season, but a greater proportion of them had immature gametocytes alone (7 of 23) than during the rainy season (5 of 70).

Table 3.1. Prevalence of gametocytemia by EA and season, as determined by qRT-PCR testing of blood samples during two surveys in 2012 and 2013 in southern Malawi.

		Dry Season 2012				Rainy Season 2013		
	n	Immature gametocytes (%)	Mature gametocytes (%)	Any gametocytes (%)	n	Immature gametocytes (%)	Mature gametocytes (%)	Any gametocytes (%)
Total	616	23 (3.7)	21 (3.4)	28 (4.6)	892	70 (7.9)	78 (8.7)	83 (9.3)
Blantyre								
Soche West Ward EA020	84	3 (3.6)	4 (4.8)	4 (4.8)	100	6 (6.0)	6 (6.0)	6 (6.0)
Misesa EA014	90	2 (2.2)	3 (3.3)	4 (4.4)	95	3 (3.2)	3 (3.2)	3 (3.2)
Thyolo								
Kapichi EA056	50	2 (4.0)	2 (4.0)	3 (6.0)	116	11 (9.5)	13 (11.2)	13 (11.2)
Nchilamwela EA056	85	4 (4.7)	4 (4.7)	4 (4.7)	119	11 (9.2)	13 (10.9)	13 (10.9)
Chimaliro EA052	85	0 (0.0)	0 (0.0)	0 (0.0)	123	2 (1.6)	1 (0.8)	2 (1.6)
Chikhwawa								
Ngabu EA117	81	1 (1.2)	1 (1.2)	1 (1.2)	113	8 (7.1)	8 (7.1)	8 (7.1)
Lundu EA019	56	8 (14.3)	4 (7.1)	8 (14.3)	113	22 (19.5)	27 (23.9)	30 (26.6)
Makhwira EA051	85	3 (3.5)	3 (3.5)	4 (4.7)	113	7 (6.2)	7 (6.2)	8 (7.1)

EA   Enumeration area

ICEMR   International Center of Excellence for Malaria Research

qRT-PCR   Quantitative reverse transcription polymerase chain reaction

Figure 3.3. Prevalence of *P. falciparum* parasitemia by PCR for each EA by season, with gametocyte presence indicated by qRT-PCR results among those that were parasitemic
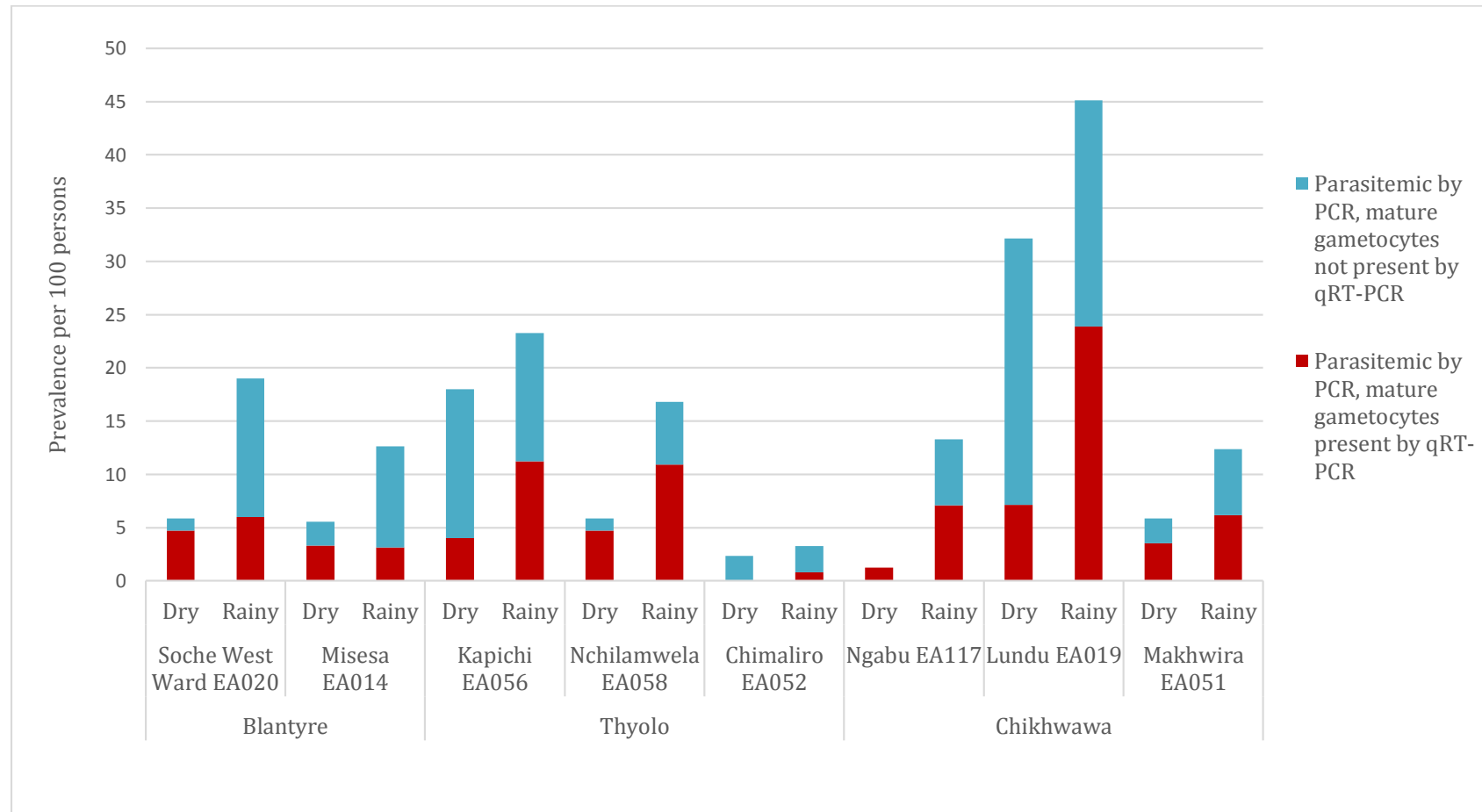
EA	Enumeration area
PCR	Polymerase chain reaction
qRT-PCR	Quantitative reverse transcription polymerase chain reaction

In the total study population of 1,508 subjects, 99 (6.56%) tested positive for mature gametocytes by qRT-PCR: 21 of 616 (3.41%) from the dry season 2012 and 78 of 892 (8.74%) from the rainy season 2013. By microscopy, gametocyte prevalences of only 0.84% during the dry season 2012 (5 of 597 subjects), and 3.36% during the rainy season 2013 (30 of 892 subjects) were identified. Of the 1,508 subjects, 127 were judged positive for any parasitemia by microscopy. More than half of the microscopically parasitemic subjects had mature gametocytes by qRT-PCR (66 of 127, 52.0%), but these represented only 66.7% of all gametocytemic individuals in the study population as detected by the molecular testing strategy, meaning that approximately one-third of the gametocytemic reservoir carried submicroscopic *P. falciparum* infections.

3.4.3 *Predictors of gametocytemia in the total study population*

The distribution of mature gametocytemia by various characteristics of the study population is presented in Table 3.2, with the total prevalence of *P. falciparum* parasitemia by qPCR presented for comparison. Though molecular gametocyte data were not available for the first cross-sectional survey from the rainy season (April-May) 2012, EAs that had high prevalence of parasitemia in the rainy season 2012 continued to have relatively higher prevalence of parasitemia and had higher prevalences of mature gametocytemia in the subsequent dry season 2012 and rainy season 2013. The prevalence of gametocytemia was 2.5% in the individuals from EAs in the lowest tertile, 5.6% in individuals from the middle tertile, and 13.7% prevalence in individuals from the highest prevalence tertile at baseline. The prevalence of gametocytemia was higher in the males included in the study (7.6% vs. 5.9% in females), but the difference was not statistically significant (p=0.21). Household construction (p<0.001) and SES quartile (p=0.01) were both associated with differences in gametocyte prevalence. Individuals in households constructed with at least two of the three finished materials categories (walls, floors, and roof) had a lower gametocyte prevalence (4.2%) than individuals in households with fewer finished materials (8.7%). SES quartile was inversely related to gametocyte prevalence, with the highest prevalence of gametocytemia in individuals from the lowest quartile (8.5%) and the lowest prevalence of gametocytemia in individuals from the highest quartile (2.1%). The prevalence of parasitemia and gametocytemia was lower in individuals from households that did not own any bednets compared to individuals from

households that did own bednets, whether or not the individuals used the bednets on the previous night. Among individuals within the households that owned any bednets, the prevalence of parasitemia and gametocytemia was higher among the individuals that did not sleep under a net on the previous night. Bednet status was significantly associated with parasitemia (p<0.01), but there was a slightly higher probability that the observed association with gametocytemia may have been attributable to chance (p=0.08). None of the considered combinations of symptoms was associated with prevalence of gametocytemia, and few subjects had recently been treated with an antimalarial, limiting the sample size for further analysis.

Age category was strongly associated with the prevalence of both parasitemia and gametocytemia (p<0.0001 for each). The prevalence of mature gametocytemia in school-aged children (12.1%) was high compared to that of young children (4.4%) or adults (3.4%). There are approximately 2.8 million (21.5% of the national population) children ≤5 yo, 3.5 million (26.8%) school-aged children 6 – 15 yo, and 6.8 million (51.8%) adults ≥16 yo in Malawi, according to the most recent national census from 2008.[28] Applying the age-specific gametocyte prevalences found in this study to the age structure of the total population leads to an estimated 777,178 gametocytemic individuals nationwide, with 15.9% of these being young children ≤5 yo, 54.5% being school-aged children 6 – 15 yo, and 29.6% being adults ≥16 yo.

Table 3.2. Characteristics of the study population and prevalence of parasitemia and gametocytemia by molecular testing, ICEMR-Malawi Cross-Sectional Study

	n	*P. falciparum* parasitemia [α]		Mature gametocytemia	
	n	n (%)	p-value	n (%)	p-value
Total	1508	212 (14.1%)		99 (6.6%)	
Microscopy results					
Positive for any parasitemia	127	111 (87.4%)		66 (52.0%)	
Negative for any parasitemia	1362	97 (7.1%)	**<0.0001**	33 (2.4%)	**<0.0001**
Season					
Dry 2012	616	50 (8.1%)		21 (3.4%)	
Rainy 2013	892	162 (18.2%)	**<0.0001**	78 (8.7%)	**<0.0001**
EA transmission intensity [β]					
Low	402	22 (5.5%)		10 (2.5%)	

	n	*P. falciparum* parasitemia [α]		Mature gametocytemia	
		n (%)	p-value	n (%)	p-value
Medium	771	85 (11.0%)		43 (5.6%)	
High	335	105 (31.3%)	**<0.0001**	46 (13.7%)	**<0.0001**
Sex					
Male	580	89 (15.3%)		44 (7.6%)	
Female	928	123 (13.3%)	0.26	55 (5.9%)	0.21
Age (mean 17.3 yrs)					
Young children, 6 mos - 5 yrs	341	32 (9.4%)		15 (4.4%)	
School-aged children, 6 - 15 yrs	511	114 (22.3%)		62 (12.1%)	
Adults, ≥16 yrs	656	66 (10.1%)	**<0.0001**	22 (3.4%)	**<0.0001**
SES Quartile [γ]					
Lowest	434	63 (14.5%)		37 (8.5%)	
2nd	406	70 (17.2%)		28 (6.9%)	
3rd	425	62 (14.6%)		29 (6.8%)	
Highest	239	16 (6.7%)	**<0.01**	5 (2.1%)	**0.01**
Household characteristics [δ]					
Unfinished	809	142 (17.6%)		70 (8.7%)	
Finished	699	70 (10.0%)	**<0.0001**	29 (4.2%)	**<0.001**
Eaves					
Open	387	71 (18.4%)		34 (8.8%)	
Closed	1121	141 (12.6%)	**<0.01**	65 (5.8%)	**0.04**
Bednet use					
Slept under a net previous night	969	131 (13.5%)		65 (6.7%)	
Net available but not used	364	66 (18.1%)		29 (8.0%)	
No nets in household	175	15 (8.6%)	**<0.01**	5 (2.9%)	0.08
IRS in previous 12 months					
Yes	334	43 (12.9%)		23 (6.9%)	
No	1165	166 (14.3%)	0.20	76 (6.5%)	0.71
Fever in the previous 2 weeks					
Yes	305	39 (12.8%)		19 (6.2%)	
No	1202	173 (14.4%)	0.47	80 (6.7%)	0.79
Fever, rigor, or headache in the previous 48 hours					
Yes	193	28 (14.5%)		12 (6.2%)	
No	1315	184 (14.0%)	0.84	87 (6.6%)	0.83

	n	*P. falciparum* parasitemia [α]		Mature gametocytemia	
		n (%)	p-value	n (%)	p-value
Any treatment sought in the previous 2 weeks					
Yes	241	25 (10.4%)		13 (5.4%)	
No	1267	187 (14.8%)	0.07	86 (6.8%)	0.42
Antimalarial taken in the previous 2 weeks					
LA	56	6 (10.7%)		2 (3.6%)	
Other antimalarial [ε]	10	3 (30.0%)		1 (10.0%)	
None	1442	203 (14.1%)	0.23	96 (6.7%)	0.47

EA   Enumeration area
ICEMR   International Center of Excellence for Malaria Research
IRS   Indoor residual spraying
LA   Lumefantrine artemether
SES   Socioeconomic status

Bolded p-values are those <0.05.
α   Parasitemia as detected by PCR for *P. falciparum* lactate dehydrogenase (LDH)
β   Tertiles of parasite prevalence established for all 30 EAs from the first survey (rainy season 2012) data were used as a proxy estimate of transmission intensity in the EA. See Methods for details.
γ   SES indicator calculated using technique based on Filmer & Pritchett (2001).[22] See Methods section for details
δ   Roof, wall, and floor materials were each classified as finished or unfinished (rudimentary/natural); 'finished' households were those with 2 or 3 finished materials, 'unfinished' households were those with 0 or 1
ε   'Other' antimalarials included chloroquine, quinine, or sulfadoxine-pyrimethamine

As mentioned above, microscopists only identified five gametocytemic subjects during the dry season 2012 and 30 during the rainy season 2013. However, results based on microscopy still indicated a statistically significant difference in the prevalence of gametocytemia by age (X^2=6.99, 2 d.f., p=0.03), and a borderline significant association between prevalence of gametocytemia and household materials (X^2=3.12, 1 d.f., p=0.08). Nevertheless, microscopy failed to identify the same patterns of association with EA transmission intensity and found no association between gametocyte prevalence and SES quartile (data not shown).

Logistic regression models were used to explore the predictors of gametocytemia in the total population (Table 3.3). Season, EA transmission intensity, and age category all significantly improved the model fit and were included in adjusted models to test all other potential predictors. SES quartile and household characteristics significantly

improved the model fit when included independently, but after adjustment for household characteristics, the association of SES quartile with gametocytemia was attenuated and had a borderline significant p-value. Models were built that tested interaction of the other potential predictors with the EA transmission intensity (as approximated by the parasitemia prevalence tertile from the preceding rainy season 2012 survey), but small sample sizes led to unstable estimates, and the interactions could not be included in the final model. The best predictive model included season, EA transmission intensity, age category, and household characteristics.

Adjustment for the predictors in the final model generally led to attenuation of the prevalence odd ratios (PORs) compared to the estimates from unadjusted models, but most of the key predictors identified from univariate analysis remained statistically significant after adjustment. The odds of gametocytemia during the rainy season were 2.48 times those of the dry season (95% confidence interval (CI): 1.50 – 4.12). The odds of gametocytemia for individuals living in EAs that had a moderate intensity of parasite prevalence in the baseline survey were 2.58 times those of individuals living in the low prevalence EAs (95% CI: 1.27 – 5.26), and the odds of gametocytemia in individuals in the high prevalence EAs were 5.05 times those of the low prevalence EAs (95% CI: 2.47 – 10.35). The odds of gametocytemia among young children 6 mos – 5 yrs were slightly elevated compared to the odds in adults ≥16 yrs (POR=1.25, 95% CI: 0.63 – 2.47), though the difference was not statistically significant. The odds in school-aged children 6 – 15 yrs were 4.00 times those of adults ≥16 yrs (95% CI: 2.40 – 6.68). The odds of gametocytemia among individuals in households made of unfinished materials were 1.95 times those of individuals in households made of finished materials (95% CI: 1.20 – 3.18). The odds of gametocytemia for individuals from households that owned at least one net were higher than those of households that did not own any nets, though neither difference was statistically significant after adjustment for other key predictors. The odds of gametocytemia were slightly higher for the individuals that lived in households that owned a net but did not sleep under it the previous night than the individuals that did sleep under a net, but the difference in PORs relative to individuals who did not own a net was very small after adjustment (2.26 vs. 2.18, respectively). Relative to the highest SES quartile, all other quartiles had elevated odds of gametocytemia, but after adjustment

for season, age category, EA transmission intensity, and household characteristics, these were of only borderline statistical significance. Controlling for these variables changed the direction of the association between fever in the previous 2 wks and gametocytemia, with an unadjusted POR of 0.93 and an adjusted POR of 1.33; however, the model did not preclude the possibility that this observed association was due to chance (95% CI: 0.77 – 2.30).

The potential predictors were also tested in a multilevel model that included random intercepts at the household- and EA-level in order to more accurately estimate associations and conservative standard errors that accounted for potential clustering within the data. Unknown EA level factors accounted for a statistically significant portion of the unexplained variance in the model that included season, EA transmission intensity, age category, and household characteristics (p=0.01). Though the household level clustering did not account for a statistically significant part of the remaining variance (p=0.12), the random intercept at the household level was retained in the final model to achieve conservative estimates of the standard error and avoid overestimation of the significance of each predictor. All multilevel models were adjusted for season, EA transmission intensity, age category, and household characteristics, despite some loss of statistical significance. As expected, accounting for the household and EA level clustering led to lower precision for the fixed effect estimates compared to the model without random intercepts, though the direction and magnitude of the estimates was fairly consistent. The odds of gametocytemia during the rainy season remained significantly higher than those during the dry season in the multilevel model (POR=2.41, 95% CI: 1.43 – 4.09), as did the odds of gametocytemia among school-aged children relative to those among adults (POR=4.16, 95% CI: 2.47 – 7.00). The odds of gametocytemia among individuals from EAs with medium and high transmission intensity were higher than those among individuals from EAs with low transmission intensity, but both fixed effect estimates were imprecise in the multilevel model, and only the POR for living in an EA with high transmission intensity remained statistically significant (POR=5.52, 95% CI: 1.00 – 30.42). Living in a household made of unfinished materials was associated with higher odds than living in a finished household, but the POR was only of borderline statistical significance in the multilevel model (POR=1.71, 95% CI: 0.98 – 2.98).

Table 3.3. Logistic regression modeling of the predictors of mature gametocyte carriage in the ICEMR-Malawi Cross-Sectional Study from two surveys, n=1508

	Unadjusted POR (95% CI)	Adjusted POR (95% CI)*	Adjusted POR, multilevel model (95% CI)*
Season			
Dry, 2012	1.00 (ref)	1.00 (ref)	1.00 (ref)
Rainy, 2013	**2.72 (1.66 - 4.45)**	**2.48 (1.50 – 4.12)**	**2.41 (1.43 – 4.09)**
EA transmission intensity [α]			
Low	1.00 (ref)	1.00 (ref)	1.00 (ref)
Medium	**2.32 (1.15 – 4.66)**	**2.58 (1.27 – 5.26)**	2.81 (0.65 – 12.25)
High	**6.24 (3.10 – 12.57)**	**5.05 (2.47 – 10.35)**	**5.52 (1.00 – 30.42)**
Age			
Young children, 6 mos-5 yrs	1.33 (0.68 – 2.59)	1.25 (0.63 – 2.47)	1.18 (0.59 – 2.34)
School-aged children, 6-15 yrs	**3.98 (2.41 – 6.57)**	**4.00 (2.40 – 6.68)**	**4.16 (2.47 – 7.00)**
Adults, ≥16 yrs	1.00 (ref)	1.00 (ref)	1.00 (ref)
Household characteristics [β]			
Finished	1.00 (ref)	1.00 (ref)	1.00 (ref)
Unfinished	**2.19 (1.40 – 3.42)**	**1.95 (1.20 – 3.18)**	1.71 (0.98 – 2.98)
Bednet use			
Slept under net previous night	2.44 (0.97 – 6.12)	2.18 (0.84 – 5.63)	2.13 (0.79 – 5.77)
Net available but not used	**2.94 (1.12 – 7.74)**	2.26 (0.83 – 6.11)	2.45 (0.87 – 6.95)
No nets in household	1.00 (ref)	1.00 (ref)	1.00 (ref)
SES Quartile [γ]			
Lowest	**4.36 (1.69 – 11.25)**	2.60 (0.94 – 7.18)	2.65 (0.90 – 7.80)
2nd	**3.47 (1.32 – 9.10)**	2.20 (0.79 – 6.13)	2.11 (0.72 – 6.20)
3rd	**3.43 (1.31 – 8.98)**	2.71 (1.00 – 7.33)	**2.81 (1.00 – 7.90)**
Highest	1.00 (ref)	1.00 (ref)	1.00 (ref)
Eaves			
Closed	1.00 (ref)	1.00 (ref)	1.00 (ref)
Open	**1.57 (1.02 – 2.41)**	1.24 (0.76 – 2.02)	1.15 (0.67 – 1.97)
Fever in previous 2 wks			
Yes	0.93 (0.56 – 1.56)	1.33 (0.77 – 2.30)	1.29 (0.73 – 2.27)
No	1.00 (ref)	1.00 (ref)	1.00 (ref)
Any antimalarial taken in previous 2 wks[δ]			
Yes	0.67 (0.21 – 2.17)	0.85 (0.25 – 2.84)	0.79 (0.23 – 2.69)
No	1.00 (ref)	1.00 (ref)	1.00 (ref)

	Unadjusted POR (95% CI)	Adjusted POR (95% CI)*	Adjusted POR, multilevel model (95% CI)*
IRS in previous yr			
Yes	0.96 (0.83 – 1.12)	0.97 (0.86 – 1.10)	N/A[ε]
No	1.00 (ref)	1.00 (ref)	
Sex			
Male	1.30 (0.86 – 1.97)	1.10 (0.71 – 1.69)	1.12 (0.72 – 1.74)
Female	1.00 (ref)	1.00 (ref)	1.00 (ref)

CI   Confidence interval
EA   Enumeration area
IRS   Indoor residual spraying
N/A   Not applicable
POR   Prevalence odds ratio
SES   Socioeconomic status

Bolded values are those where the 95% CI does not contain 1.0.
*Adjusted for season, EA transmission intensity, age category, and household characteristics (finished vs. unfinished)
α   Tertiles of parasite prevalence established for all 30 EAs from the first survey (rainy season 2012) data were used as a proxy estimate of transmission intensity in the EA. See Methods for details.
β   Roof, wall, and floor materials were each classified as finished or unfinished (rudimentary/natural); 'finished' households were those with 2 or 3 finished materials, 'unfinished' households were those with 0 or 1
γ   SES indicator calculated using technique based on Filmer & Pritchett (2001).[22] See Methods section for details
δ   Antimalarials included were lumefantrine artemether, chloroquine, quinine, or sulfadoxine-pyrimethamine
ε   The model did not converge.

3.4.4 *Predictors of gametocytemia among PCR-positive parasitemic individuals*

Logistic regression models were also used to explore the predictors of gametocytemia among only the 212 subjects that were parasitemic by PCR (Table 3.4). As there was again a possibility of clustering at the household- and EA-levels, multilevel models that included random intercepts for these levels were tested. Neither random intercept explained a significant amount of the variance in gametocytemia among the parasitemic population. Therefore, there did not appear to be household- or EA-level clustering in the data that required the use of multilevel modeling techniques, and the final adjusted models did not include random intercepts at these levels.

The only statistically significant predictor in unadjusted models was age category. Similar to the prevalence of gametocytemia in the total population, the odds of gametocytemia among school-aged children with parasitemia were 2.38 times the odds of

gametocytemia among adults with parasitemia (95% CI: 1.27 – 4.48). The odds of gametocytemia among young children with parasitemia were also elevated compared to adults with parasitemia, but less so than those of school-aged children, and the difference was not statistically significant (POR=1.82, 95% CI: 0.76 – 4.39). Age category was therefore included in the adjusted models for all other potential predictors.

EA transmission intensity did not appear to have a strong association with gametocytemia among parasitemic individuals, with the odds among individuals in EAs of medium intensity being weakly higher and in EAs of high intensity being weakly lower than those of individuals in EAs of low intensity, and neither difference was statistically significant. For the other predictors in general, the directions of the associations with gametocytemia among parasitemic individuals mirrored the directions of the associations with gametocytemia among the population in general, though none of the individual predictors other than age category reached the level of statistical significance at $\alpha < 0.05$. SES quartile had some of the largest magnitude PORs, despite being of borderline statistical significance. Controlling for age category, the odds of gametocytemia among parasitemic individuals in the lowest SES quartile were 3.24 times higher than the odds among parasitemic individuals in the highest SES quartile (95% CI: 0.99 – 10.62). The odds of gametocytemia among parasitemic individuals in the second and third quartiles were also elevated compared to those in the highest wealth quartile, though the associations were weaker and were not statistically significant (Table 3.4).

Table 3.4. Predictors of gametocytemia among individuals that tested positive for *P. falciparum* parasitemia by PCR, n=212

	Unadjusted POR (95% CI)	Adjusted POR (95% CI)*
Season		
Dry, 2012	1.00 (ref)	1.00 (ref)
Rainy, 2013	1.28 (0.68 – 2.43)	1.39 (0.72 – 2.69)
EA transmission intensity [α]		
Low	1.00 (ref)	1.00 (ref)
Medium	1.23 (0.48 – 3.15)	1.12 (0.43 – 2.97)
High	0.94 (0.37 – 2.36)	0.86 (0.33 – 2.23)
Age		
Young children, 6 mos - 5 yrs	1.77 (0.75 – 4.18)	1.82 (0.76 – 4.39)

	Unadjusted POR (95% CI)	Adjusted POR (95% CI)*
School-aged children, 6 - 15 yrs	**2.38 (1.27 – 4.48)**	**2.33 (1.22 – 4.43)**
Adults, ≥16 yrs	1.00 (ref)	1.00 (ref)
Household characteristics [β]		
Finished	1.00 (ref)	1.00 (ref)
Unfinished	1.38 (0.77 – 2.45)	1.45 (0.80 – 2.61)
Bednet use		
Slept under a net previous night	1.97 (0.64 – 6.08)	1.89 (0.60 – 5.99)
Net available but not used	1.57 (0.48 – 5.09)	1.29 (0.39 – 4.34)
No nets in household	1.00 (ref)	1.00 (ref)
SES Quartile [γ]		
Lowest	3.13 (0.97 – 10.09)	3.24 (0.99 – 10.62)
2nd	1.47 (0.46 – 4.68)	1.56 (0.48 – 5.04)
3rd	1.93 (0.60 – 6.22)	2.13 (0.65 – 6.97)
Highest	1.00 (ref)	1.00 (ref)
Eaves		
Closed	1.00 (ref)	1.00 (ref)
Open	1.07 (0.61 – 1.90)	1.24 (0.69 – 2.25)
Fever in previous 2 wks		
Yes	1.11 (0.55 – 2.21)	1.07 (0.52 – 2.19)
No	1.00 (ref)	1.00 (ref)
Any antimalarial taken in previous 2 wks[δ]		
Yes	0.67 (0.21 – 2.17)	0.48 (0.12 – 2.03)
No	1.00 (ref)	1.00 (ref)
IRS in previous yr		
Yes	0.96 (0.85 – 1.07)	0.96 (0.86 – 1.07)
No	1.00 (ref)	1.00 (ref)
Sex		
Male	1.21 (0.70 – 2.09)	1.12 (0.64 – 1.96)
Female	1.00 (ref)	1.00 (ref)

CI   Confidence interval
IRS   Indoor residual spraying
PCR   Polymerase chain reaction
POR   Prevalence odds ratio
SES   Socioeconomic status
Bolded values are those where the 95% CI does not contain 1.0.
*Age category was adjusted for SES quartile. All other values were adjusted for age category.
α   Tertiles of parasite prevalence established for all 30 EAs from the first survey (rainy season 2012) data were used as a proxy estimate of transmission intensity in the EA. See Methods for details.
β   Roof, wall, and floor materials were each classified as finished or unfinished

	Unadjusted POR (95% CI)	Adjusted POR (95% CI)*

(rudimentary/natural); 'finished' households were those with 2 or 3 finished materials, 'unfinished' households were those with 0 or 1

γ SES indicator calculated using technique based on Filmer & Pritchett (2001).[22] See Methods section for details

δ Antimalarials included were lumefantrine-artemether, chloroquine, quinine, or sulfadoxine-pyrimethamine

3.5   Discussion

As molecular techniques for the detection of gametocytes have only emerged in the last 10-15 years, relatively little epidemiologic data is available to characterize the population-level dynamics of gametocytemia with high sensitivity. The current study contributes to a growing knowledge base concerning gametocyte infection patterns using cross-sectional results from a community-based, all-ages population, and evaluates who are potential infectious reservoirs across multiple seasons and settings with different transmission intensity. The results confirm that a large proportion of the human parasitemic reservoir in these endemic areas carry mature gametocytes and have the potential to contribute to ongoing transmission, even in a community-based, nonclinical population with many asymptomatic individuals. In fact, recent malaria symptoms were not associated with the odds of gametocytemia, either in the total population or among those who were parasitemic. This study also identified high odds of gametocytemia among *P. falciparum*-infected school-aged children relative to young children or adults, and estimated that more than half of the gametocytemic reservoirs nationwide are school-aged children (6 – 15 yo). This finding was in contrast to many previous publications, which suggested that gametocytemia was most common in *P. falciparum* infections of younger children and decreased monotonically with age. Identification and treatment of the asymptomatic gametocytemic reservoir will be a critical step in any efforts aimed at malaria elimination and eventual eradication, but our findings suggest that targeting interventions at school-aged children may be an efficient strategy for reducing the number of human gametocytemic reservoirs.

Asymptomatic infections were as likely to be gametocytemic as symptomatic infections. No combination of malaria-related symptoms (including fever, headache, and rigors) was associated with gametocytemia, suggesting that asymptomatic infections can potentially contribute to ongoing transmission, and that it would be insufficient to focus

treatment on symptomatic people or clinical cases to eliminate infectious human reservoirs. More sensitive rapid diagnostic tests are needed for field detection of asymptomatic reservoirs of parasitemia in endemic communities. In the absence of new detection tools, better understanding of the predictors of gametocytemia can help target testing and intervention strategies.

The most important individual-level predictor of gametocytemia in the study population was age. School-aged children (6 – 15 yrs) had notably elevated odds of being both gametocytemic and parasitemic in comparison to young children and adults, even after adjustment for other key predictors and after accounting for household- and EA-level clustering in the data. Other individual-level variables collected in the study showed low value for prediction of gametocytemia in the total population. Age was also the strongest, and only statistically significant, predictor of gametocytemia among parasitemic individuals. The odds of gametocytemia for school-aged children 6 – 15 yrs with parasitemia were 2.33 times the odds of gametocytemia in adults ≥16 yrs, and the difference was statistically significant with a 95% CI of 1.22 – 4.34. The odds of a gametocytemia in parasitemic young children (≤5 yrs) were slightly higher than those of adults ≥16 yrs (POR=1.82, 95% CI: 0.76 – 4.39), but this difference was not statistically significant. While immune reaction to *P. falciparum* is thought to stimulate the parasite to "invest" in gametocytogenesis, the low odds of gametocytemia in *P. falciparum* infections among adults suggest that a lifetime of recurrent infection in an endemic area may ultimately lead to effective anti-gametocyte immune responses. However, this would lead us to expect the highest odds of gametocytemia in the youngest age groups, which was not observed.

Of the relatively small number of prior studies that reported gametocyte epidemiology for community-based samples, most did report a monotonic decrease in gametocyte prevalence by age.[16–27] The majority of these used microscopy alone for gametocyte detection, and all involved samples collected before 2006 across a wide variety of nations in both Africa and Asia. Only two of these studies reported an age-distribution similar to that found in the current study. In a study from holoendemic rural villages in Burkina Faso testing 410 individuals for gametocytemia using QT-NASBA for *Pfs25* mRNA, Ouédraogo *et al.* reported the highest odds of gametocyte prevalence

among children 5 – 9 yrs, followed by those 10 – 14 yrs.[33] The odds of gametocyte prevalence were also elevated among children <5 yrs compared to adults, but were slightly lower than those of the school-aged children. However, no statistical tests were reported comparing gametocyte prevalence across the different age groups of children; age was assessed as a linear predictor. In a study in Thailand, Coleman *et al.* reported microscopy-based gametocytes per 500 WBCs in 6,494 subjects from a low prevalence area with mixed species infections.[34] The prevalence of *P. falciparum* gametocytemia peaked in the 10 – 15 yo age group, but the authors did not report any statistical testing related to age. No previous studies have reported on the epidemiology of gametocytemia in Malawi. Differences between our results and previous findings may be attributable to the improved sensitivity of the molecular assays, geographic variations in parasite epidemiology, or possibly a true shift in the age-distribution of gametocytemia compared to the populations in earlier studies. Many recent interventions have focused on the populations that suffer the greatest burden of malaria disease, namely pregnant women and children <5 yo. It is possible that increasing coverage of bednets and other interventions in the past decade has minimized the frequency of infection in children <5 yo, resulting in a delay in the development of transmission-blocking immunity (TBI) to gametocytes, and correspondingly higher likelihood of gametocytemia in *P. falciparum*-infected school-aged children. Additional research with longitudinal sample collection in one geographic region, with consistent methodology and data on antibody responses, is necessary to disentangle the causal relationships; regardless, the results have implications for malaria elimination efforts, with school-aged children currently representing the majority of the gametocytemic reservoir in Malawi, suggesting a need for interventions to reduce the infection prevalence in these older children in order to interrupt transmission..

Populations with lower SES in our study tended to harbor a higher prevalence of gametocytes than populations with higher SES. Aspects of household construction that could increase exposure to mosquito vectors explained part of this association, as the magnitude of the estimated association between SES quartile and gametocytemia was attenuated after controlling for household construction characteristics. Although household characteristics did not account for the entirety of the association between SES quartile and prevalence of gametocytemia, the remaining association was not statistically

significant and did not demonstrate a clear monotonic trend as SES quartile decreased. Although the association had borderline statistical significance after using a multilevel model to account for household- and EA-level clustering, individuals in households that were made of all unfinished or only one finished material (of walls, floor, and roof) had higher odds of gametocytemia than individuals in households made of at least two finished materials. While general economic improvements would likely contribute to a reduction in the burden of *Plasmodium* infections and malaria disease, areas with relatively low SES and high proportions of rudimentary/unfinished households may represent another effective target for *Plasmodium* screening and intervention activities.

EAs that had higher *P. falciparum* prevalence during the baseline survey in rainy season 2012 tended to have higher prevalence of gametocytemia in the two subsequent surveys included here. After accounting for clustering at the EA and household levels, the estimated association between the variables representing EA transmission intensity and gametocytemia remained high, despite loss of precision. This provides some evidence in favor of consistently elevated prevalences at some sites over time, though further data collection would be required to explain the specific EA-level variables responsible for these consistently elevated prevalences (i.e. proximity to standing water sources, elevation, ITN coverage, etc). Identification of infectious 'hot-spots' through parasite surveillance could therefore guide future interventions to efficiently reduce the human infectious reservoir. For instance, sites identified as harboring a large prevalence of gametocytemic/parasitemic individuals can theoretically be targeted for interventions to reduce the prevalence of gametocytemia during the dry season, in order to interrupt the transmission cycle before mosquito populations increase with the arrival of the seasonal rains. To be effective, good surveillance data would be needed to identify the target, high prevalence areas where transmission intensities are high.

Our results suggest a high degree of across- and within-District heterogeneity in both the prevalence of *P. falciparum* parasitemia overall, and gametocytemia specifically. Molecular testing for gametocytes was performed in a limited selection of only eight EAs of ~30 households each across a large geographic area (see Figure 3.1). The 10 EAs per District that were included in the ICEMR-Malawi Cross-Sectional Study were randomly selected, and more likely to be representative of their respective Districts. The eight EAs

chosen for qRT-PCR testing were chosen based on logistical considerations, not random selection, and individuals in the qRT-PCR tested EAs differed from those in other study EAs on a number of key variables. This suggests that any simple descriptive epidemiology results from this study cannot be extrapolated to estimate the local prevalence of gametocytemia in each District, and that *Plasmodium* surveillance activities will need to have a high degree of granularity in order to identify all potential 'hot spots' of transmission. While the high degree of heterogeneity may limit our ability to assign relevant average prevalences across the larger geographic areas sampled in the study, nonetheless, analyses about the associations of various predictors with gametocytemia within this study should still be internally valid, and may be generalizable to other populations, particularly to those with similar degrees of *P. falciparum* endemicity.

There is some potential for selection bias within each sampled EA, as not all individuals were present and some were not included in the gametocyte RNA sampling. Subjects that had recently been ill with a reported fever were more likely to participate than those who were not. Young children <5 yrs were also more likely to be sampled than individuals in older age categories, and women were more likely to be sampled than men. This could have led to selection bias in estimating the associations of age and sex with the odds of gametocytemia if the older children/adults and men who were present at home during the day tended to be those who were sick, and had higher prevalences of infection than their unsampled counterparts. This would lead to overestimation of the odds of gametocytemia in men and school-aged children/adults relative to women and young children. However, in this community-based sample, recent symptom status had no association with the odds of either gametocytemia or any *P. falciparum* parasitemia, so there is little evidence to support a relationship between the sampling proportions by age or sex and the measured study outcome of gametocytemia. Thus, selection bias is unlikely to significantly impact the analysis of the predictors of gametocytemia.

The cross-sectional nature of the data introduced a number of limitations. By identifying only prevalent infections and not incident infections, the associations reported above may be related to differences in risk for developing a gametocytemic *P. falciparum* infection, differences in the duration of gametocyte carriage, or both. This distinction

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may not matter in terms of identifying which infectious reservoir groups to target for interventions, but could influence the types of interventions required to effectively reduce the size of that reservoir population. If the predictor is related more to the risk of developing a new gametocytemic infection, it will be more successful to emphasize interventions targeted at preventing exposure to mosquitoes, through the use of ITNs, IRS, or elimination of vector breeding sites. If the increase prevalence associated with a predictor is related more to longer durations of gametocytemia, the priority should be to target high risk groups with screening and treatment interventions to shorten these durations. In reality, the prevalence differences are likely due to combinations of duration and risk, requiring a multipronged intervention approach, but greater emphasis on certain strategies in different high prevalence groups could improve the efficiency for transmission reduction.

These cross-sectional measurements also limit assessment of trends in infectiousness over time, in particular the extent to which there is active *P. falciparum* transmission during the dry season vs. whether gametocytes simply persist in certain people until transmission resumes with the next rainy season. Previous research has indicated that certain individuals might harbor long-term gametocytemic infections in areas of highly seasonal transmission.[11,12] Longitudinal data would also enable clonal identification that could help determine whether specific individuals or groups of individuals contribute disproportionately to seasonal transmission cycles. If this does occur, the ability to identify these chronically gametocytemic infections might further improve targeted treatment to more efficiently reduce transmission.

Assessment of the impacts of anti-malaria interventions was also limited by the cross-section nature of the data and the nonrandom distribution of interventions in the study population. IRS was performed in 2012 by the government only in Chikhwawa District, creating issues of collinearity with EA-level variables and likely contributing to the failure of convergence of the multilevel model that included IRS as a coefficient. The prevalence of gametocytemia was higher in individuals from households that owned at least one bednet than for households that did not, suggesting not that bednets increased the risk of gametocytemia, but likely via a reverse causal relationship, where households with high malaria risks were more likely to obtain and/or receive bednets. This perhaps

indicates successful logistics for distribution of bednets in the area, but the temporal ambiguity of the cross-sectional data limits the opportunity to evaluate the potential protective impact that bednet distribution could have for reduction of the gametocytemic reservoir in other study populations. In households that owned nets, the odds of gametocytemia were higher for those people who did not sleep under a bednet the previous night than for those who did (POR, multilevel model=1.15, 95% CI: 0.70 – 1.91), but the difference was small and not statistically significant (p=0.58). Bednet coverage was high in this population, with 88.4% of study participants living in a household that owned at least one net, suggesting that net distribution alone is insufficient to eliminate the infectious reservoir.

Beyond the lack of longitudinal data, one limitation of the current study was the sample size. At 1,508, the overall study population was large, but only 99 individuals were classified as having mature gametocytes. Despite interest in potential interaction of the predictors of gametocytemia with transmission intensity, the sample size was too small to enable adequate testing of interaction terms in multiple regression, leading to very unstable estimates. As settings with low transmission intensity will inherently have small sample sizes, this limitation may be difficult to overcome without attempts to oversample areas with low transmission.

Another important limitation of the present study was that the identification of individuals with mature gametocytes in their peripheral blood only confirms *potential* infectiousness. Density of gametocytes in the peripheral blood is known to be associated with infectiousness to mosquito vectors,[8,10] so submicroscopically infected gametocyte carriers may not necessarily contribute to the infectious reservoir to the same degree as their microscopically parasitemic counterparts, and their role in the population transmission dynamics remains uncertain.[7] The current study did not directly demonstrate the infectiousness of gametocyte-positive samples through the use of membrane feeding assays, though other research has indicated that submicroscopic infections with gametocyte densities of <1 gametocyte/µL are capable of transmitting *P. falciparum* to naïve vectors.[8,10,35] The relationship between gametocyte density and proportion of mosquitoes infected is nonlinear, with surprisingly frequent transmission of these very low density infections.[8] Future research using dynamic mathematical models could

perform sensitivity analyses to estimate the number of new infections caused by low density asymptomatic infections using a combination of findings from our population epidemiologic study with membrane-feeding data from laboratory settings.

In addition to the density of gametocytemia, the contribution of a gametocytemic individual to the infectious reservoir will also be driven by the duration of gametocyte carriage and the frequency of biting encounters with uninfected *Anopheles* mosquitoes, among other possible factors. Even if a low density, asymptomatic infection infects a smaller percentage of the mosquitoes that take a blood meal than a higher density symptomatic infection, the absolute number of infected mosquitoes could be higher for the asymptomatic infection if a greater number of mosquitoes take a blood meal from that person over the full course of their infection. If mosquito vectors tend to bite gametocytemic individuals at a higher rate than uninfected or infected but not gametocytemic individuals, it would increase the relative importance of these individuals to ongoing transmission, even when their gametocyte density is low. Ongoing research with the ICEMR-Malawi Cross-Sectional Study involves expanded vector collection within study households to assess potential variations in the household-level distributions of vectors, and blood meal testing to assess potential variations in biting patterns in relation to the gametocyte status of individuals within the households and surroundings. Months of persistent gametocyte carriage could also increase the total number of mosquitoes exposed to the asymptomatic infection. Further research with longitudinal data is needed to better characterize the duration of gametocytemia in infections of all parasite densities, but, again, future studies with dynamic models can investigate the relative amount of new mosquito infections stemming from asymptomatic vs. symptomatic human infections under different realistic assumptions and conditions.

Because an individual must be parasitemic in order to be gametocytemic, the predictors of gametocytemia in the population as a whole are influenced by the predictors of any *P. falciparum* infection. As mentioned above, we also evaluated the predictors of gametocytemia *among* parasitemic individuals to determine which individuals are more likely to carry gametocytes when infected, and found that only age category was a statistically significant predictor. Previous research has led to the hypothesis that certain external 'stressors,' such as co-infection with multiple clones (multiplicity of infection,

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[MOI]), antibody response to the parasite, and the use of non-curative treatment, may increase the parasite's investment in gametocytes.[8] Results of the current study offer mixed interpretations regarding the factors that may stimulate the parasite to develop gametocytes. EA-level variables did not necessarily support the hypothesis that MOI was associated with increased odds of gametocytemia. Neither the rainy season nor increased EA transmission intensity, both of which would likely increase clonal competition within individuals, were associated with the odds of gametocytemia among parasitemic subjects. Household- and EA-level variation did not contribute to the overall variance in gametocyte status when random intercepts were included in multilevel models. While these cross-sectional data are limited in developing causal inference regarding the stimulation and timing of gametocytogenesis within individuals, our study provides no evidence that such relationships play a significant role in the population-level dynamics of the gametocytemic reservoir.

On the other hand, interpretation of the individual- and household-level predictors of gametocytemia is more consistent with the 'stress' hypothesis for gametocytogenesis. Individual- and household-level variables that increased the odds of being parasitemic also tended to increase the odds of being gametocytemic when infected, though the magnitudes of the associations were weak and none were individually statistically significant. These results are consistent with the hypothesis in so far as increased risk of infection may indicate greater MOI and higher frequency of previous infections, thereby stimulating an immune response to *P. falciparum* infection. However, in a small subset of 30 gametocytemic and 30 non-gametocytemic samples that were PCR-positive for *P. falciparum* and matched on age, season, microscopically evaluated parasite density, and anemia status (for children <5), MOI was not associated with increased odds of gametocytemia.[36] While the current study results may be consistent with the 'stressor' hypothesis for gametocytogenesis, they cannot be interpreted as such with confidence due to the lack of statistical significance and temporal ambiguity regarding frequency of infection in specific individuals.

Despite the limitations, this analysis contributed to the small body of literature that used sensitive molecular detection methods to characterize the epidemiology of gametocytemia in an all-age, community-based study population. Further research is

needed to confirm whether the observed relationships are consistent in different geographic areas, but these results can complement the growing attempts to define the role of the asymptomatic reservoirs in perpetuating *Plasmodium* transmission, and ultimately aid in the design of efficient interventions to eliminate malaria disease.

Even in an area of Malawi with high coverage of bednets, our results show that interventions targeting primarily children <5 yrs will have limited success at interrupting transmission, since school-aged children in our populations had both higher odds of being parasitemic and higher odds of being gametocytemic given parasitemia. Longitudinal research is also needed to evaluate whether the higher prevalence of gametocytemia among school-aged children reflects higher risk (incidence) of developing gametocytemia or longer duration of gametocytemia. Chapter 4 of this dissertation will explore treatment-seeking behaviors to estimate potential differences in duration of infection, but this strategy is limited compared to longitudinal study designs for understanding duration of asymptomatic infections. The identification of older children as a key infectious reservoir lends itself to an opportunity for expanding school-based distribution of intervention materials or increased education on avoiding/treating infection. Whether a school-based intervention would reach the most at-risk students requires study, but such an option may be promising in areas with relatively high rates of school attendance through the later teenage years.

The confirmation that asymptomatic infections are frequently gametocytemic highlights them as a key challenge for efforts aimed at malaria elimination. There is an urgent need for new tools to help rapidly and sensitively detect all *Plasmodium* infections in endemic communities, and for new antimalarial drugs with strong gametocytocidal effects to enable large scale treatment without encouraging resistance against the first line clinical treatments. Without such new tools, malaria elimination may be impossible to achieve in most endemic regions. Intervention efforts should expand to cover older children and young adults, who are important reservoirs of infection, despite experiencing relatively lower incidence of clinical disease. Even using existing intervention strategies, such targeting could potentially help reduce the persistence of *Plasmodium* transmission and help achieve malaria control.

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CHAPTER 4 Treatment-seeking behavior and use of antimalarials in three Districts of southern Malawi: Relevance for potential *Plasmodium* transmission

4.1   Abstract

Untreated *Plasmodium* infections can persist for months, with potentially long infectious periods. Antimalarial treatment is key for clearance of infections, thus limiting the duration of their contribution to transmission. Treatment-seeking behaviors play an important role in determining prompt and proper antimalarial interventions. Thus, we studied treatment-seeking in three eco-geographically diverse Districts of southern Malawi to assess potential intervention needs that would reduce the human infectious reservoir and make progress toward malaria elimination.

Methods: Approximately 3,000 individuals from 900 households in three Districts of southern Malawi were included in each of four cross-sectional surveys. Participants were questioned about fever and treatment-seeking in the two weeks prior to each survey. Multilevel logistic regression models were used to analyze the predictors that a febrile person would seek any treatment, that a person seeking treatment would attend a government or private clinic as opposed to another source, and that a person seeking treatment would receive an antimalarial. Cox regression was used to assess differences in the time from fever onset until treatment was sought.

Results: Most people who reported a recent fever had sought treatment (>85%), but few afebrile people had (5.5%). Among those who did seek treatment, few afebrile people (<5%) and few febrile people who went to shops or other local treatment sources (<8%) received any antimalarial drug. Antimalarial use was most common among febrile people who had visited a government or private clinic (>40%). Among febrile people, greater distance to health facilities was associated with less frequent treatment seeking in the urban Blantyre District and semi-rural Thyolo District, but not in the rural Chikhwawa District. Overall, greater distances to health facilities were associated with

lower odds of seeking treatment at a government or private clinic instead of a shop or other local source. The proportion of febrile people who sought treatment decreased and the time from fever onset to treatment-seeking increased with age category in all three Districts, but school-aged children (6 – 15 years old (yo)) and adults (≥16 yo) were more likely to attend a shop or other local source than young children (≤5 yo), who were significantly more likely to be taken to a government or private facility. Of individuals who sought treatment but did not receive an antimalarial, 6.9% that had gone to a government or private facility, and 16.8% that had gone to a shop or other local source, tested positive for *P. falciparum* by PCR when surveyed. Diagnostic blood testing was not performed on >45% of individuals at a government or private facility and >90% of individuals at a shop or other local source. All children ≤15 yo were more likely to receive an antimalarial than adults at either source of treatment and whether or not they were diagnostically tested. Only a quarter of participants that tested positive for *P. falciparum* by PCR at the time of survey reported seeking any treatment in the previous two weeks.

Conclusions: Afebrile people in Malawi rarely seek treatment, so asymptomatic reservoirs of *Plasmodium* infection are likely to have long durations of infection. Among those that do seek treatment, diagnostic testing and antimalarial treatment are not always provided, particularly among those who seek treatment only at shops and other local sources rather than government or private clinics. Diagnostic testing capacity and antimalarial drug availability should be increased at local sources of care to reach a greater proportion of the infectious reservoir, but it is unlikely that malaria elimination can be achieved in such highly endemic areas until more sensitive diagnostic techniques and safer gametocytocidal drugs are readily available.

4.2   Introduction

As international efforts have begun to shift towards a goal of malaria elimination and eradication, attention has increasingly focused on interrupting *Plasmodium* transmission by eliminating reservoirs of infection in addition to treating cases.[1–5] Mass screening and treatment (MSAT) interventions (also known as Intermittent Screening and Treatment [IST]) have been attempted in some settings with limited success, possibly due

to the presence of undetected, low-density infections that perpetuate transmission, and the use of antimalarials with limited gametocytocidal properties.[6–9] Mass drug administration (MDA) has also been recommended as a strategy to overcome limitations in the sensitivity of parasite detection methods.[10] These strategies prompt concerns about promotion of drug resistance and the potential for adverse reactions, particularly among people who are glucose-6-phophate dehydrogenase (G6PD) deficient for whom the primary gametocytocidal drug, primaquine, is counterindicated.[11] There is a need for improved methods of both screening and treatment in order to approach elimination. Meanwhile, a better understanding of transmission dynamics based on which human groups are important reservoirs of infection could help target future interventions for more effective interruption of transmission.

Broadly, the contributions of infected humans to ongoing transmission depends on whether or not they carry infectious gametocytes, how many Anopheline mosquitoes bite these gametocytemic people, how likely those mosquitoes are to develop infectious sporozoites given that they took a blood meal, and how likely these now infectious mosquitoes are to subsequently bite a new human. Each of these broad components is influenced by a number of additional factors that contribute to a person's relative importance in maintaining transmission.

Recent results from the Malawi International Center of Excellence for Malaria Research (ICEMR) cross-sectional study have shown that both submicroscopic parasitemia and asymptomatic parasitemia are common in communities of southern Malawi.[12] Furthermore, Chapter 3 of this dissertation demonstrated that gametocyte carriage, especially submicroscopic gametocytemia, is common among these prevalent infections. The importance of these potentially infectious reservoirs to ongoing transmission, however, is difficult to assess. Other researchers have found that individuals with very low-density gametocytemia can transmit the infection to *Anopheles* vectors, but that the probability of infecting a given mosquito increases with greater gametocyte density in the human peripheral blood.[11,13] However, low density gametocyte infections may still be important if these people are bitten by a large number of mosquitoes, whether due to disproportionate exposure to mosquito bites in a given time frame, or a long duration of gametocyte carriage.

Duration of infectiousness is key to understanding human contributions to infection transmission, but this remains poorly characterized for *Plasmodium*, particularly in natural settings. Clinical trials are the best recent sources of data on infection durations, and show that mature and immature gametocytes are resistant to sulfadoxine-pyrimethamine (SP), chloroquine, amodiaquine, and quinine, allowing gametocytemia to persist for up to a month after treatment, even when clearance of asexual stage parasites prevents additional gametocytogenesis.[11,14] Artemisinin combination therapies (ACTs) appear to be more effective against immature gametocytes, leading to lower prevalence of gametocytemia in the weeks post-treatment than for non-ACT regimens, but still have limited effect on mature gametocytes and may also allow gametocyte persistence for several weeks.[11,14] It is difficult to obtain data on the duration of gametocytemia in untreated infections, as even an asymptomatic infection should be treated when detected, to prevent both potential development into a symptomatic case and potential transmission to other vulnerable individuals. Data from the 1950s indicated that untreated or inadequately treated *P. falciparum* parasitemia persisted for an average of 222 days, and could last for more than a year.[15] More recent evidence from population-level molecular testing for gametocytemia in an area with highly seasonal transmission found that submicroscopic gametocyte carriage could persist for months through the long dry season in untreated infections.[16,17] Thus, treatment with an effective antimalarial is critical for limiting the duration of infectiousness and thereby reducing an individual's potential contribution to ongoing transmission.

In Malawi, Ministry of Health (MoH)-provided health services are largely free, and the recommended first-line treatment for *Plasmodium* infections is a course of lumefantrine-artemether (LA), an ACT.[18,19] The MoH recommends that diagnosis be made by microscopy wherever possible, with rapid diagnostic testing (RDT) as a potential alternative, in patients with fever or recent history of fever or any illness that can be a complication of malaria (coma, convulsions, anemia, jaundice, etc). Presumptive treatment is not recommended for adults unless both microscopy and RDTs are unavailable. Presumptive treatment is considered acceptable for children under 5 years old (yo), though laboratory confirmation is still suggested. In addition to MoH sources,

antimalarial drugs may also be available through non-governmental, private sources, including health care facilities and local shops.

A repeated sample cross-sectional study in the communities of three Districts of southern Malawi collected data on the actual treatment-seeking practices of individuals in the two weeks prior to the surveys at the end of each rainy and dry season in 2012 and 2013. The current analysis aims to: (1) assess the general predictors for seeking any form of treatment across the four surveys, (2) analyze the predictors of seeking treatment at a government or private facility, as opposed to a traditional healer, community health worker, or shop, (3) determine which individuals are likely to receive antimalarials given that they sought care. Although longitudinal blood samples from participants were not available, treatment-seeking behaviors and use of antimalarials have implications for the potential duration of infectiousness in the community-based human reservoirs for *Plasmodium* infection. The results will help identify gaps in the treatment of human reservoirs that are unchecked by current standard practices in southern Malawi, and suggest directions for future interventions to interrupt transmission.

Previous analyses of this study population found that school-aged children were an important reservoir of parasitemia overall,[12] as well as gametocytemia specifically (Chapter 3). We hypothesized that these elevated prevalences compared to younger children may be related to lower odds of receiving prompt and adequate antimalarial treatment, leading to longer duration of disease in school-aged children than younger children. We also hypothesized that recently afebrile individuals, including those with *P. falciparum* infections, were unlikely to seek any type of treatments, or to receive antimalarial treatments when they did seek care.

4.3   Methods

The study was carried out under the auspices of the Malawi ICEMR, and all methods were approved by the independent Institutional Review Boards (IRB) of the University of Malawi College of Medicine, the University of Maryland, Baltimore, and Michigan State University (MSU).

4.3.1 *Study design*

Data were collected in three Districts of southern Malawi during biannual cross-sectional surveys at the conclusion of each rainy season (April-May) and dry season (September-October) in 2012 and 2013. These three Districts represent different transmission patterns. Blantyre is a more urbanized District in the highlands with expected low transmission, Thyolo a semi-rural District with expected moderate transmission, and Chikhwawa a rural District at low elevation with expected high transmission. Compact segments of approximately 30 households were chosen within each of 10 enumeration areas (EAs) per District; EAs and compact segments were randomly selected using two-stage cluster sampling, excluding any EAs on the District border between Thyolo and Chikhwawa, Chikhwawa EAs at >500 m elevation, or Thyolo EAs at <500 m elevation. Thus, approximately 900 households were selected for study and visited during each survey. As the average household size in southern Malawi is 4.2 persons,[20] more than 3,500 individuals were expected to participate in each round of surveillance. The survey team returned to the same compact segments and roughly the same households for each round, having communicated closely with local health representatives and EA chiefs to encourage high levels of participation.

At each visit, the field team interviewed household members in the local language (Chichewa) to obtain information about household-level variables and individual-level variables for all members of the household, both present and absent. A standardized questionnaire sought data on household demographics (i.e. age, sex, relationship of all members), house construction, use of malaria interventions [including details on insecticide treated net (ITN) quality and use, indoor residual spraying (IRS), intermittent preventive therapy in pregnancy (IPTp) and other treatment-seeking behaviors], and self-reported recent symptoms of disease. Participants were asked about whether any member of the household had experienced a fever in the previous two weeks, whether they had sought treatment or medical advice related to any illness or health concern in the previous two weeks, where such treatment was obtained, whether a finger or heel stick was performed there to diagnostically test for *Plasmodium* infection, and to identify any medications that were taken in the previous two weeks.

4.3.2 *Sample collection and testing*

Malawian nurses who were members of each field team measured the axillary temperature and took finger-pricked peripheral blood samples from all subjects ≥6 months old (mo) who were present and consented (or were assented) to participate in the study. During all surveys, the nurses collected ~0.5 mL of blood onto slides as thick smears for microscopy, onto filter paper for DNA isolation, and into a Hemocue® device (Hb 201+ System, Hemocue, Inc) for immediate testing of hemoglobin levels for children 6 mo – 5 yo. RNA-protected sample collection was undertaken during the dry season 2012 survey for a convenience-based subset of eight of the 30 EAs. This "RNA collection" was expanded to include all subjects for the subsequent surveys. To preserve parasite RNA, whole blood samples of approximately 50 µL were collected into 250 µL of RNAprotect® (Qiagen Inc., Valencia, CA).

Thick smears were air dried, methanol fixed, and Giemsa stained upon delivery to the lab at the end of each day of sample collection. Thick smears were read at 100X objective magnification by two trained microscopists, who recorded the number of parasites seen per 200 white blood cells (WBC). The two reads were considered discrepant if one reader recorded presence of any *Plasmodium* parasites and the other did not, if one reader counted more than 10 times as many as the other when the lowest reader counted less than 20 parasites, or if one reader counted more than twice as many as the other when both reads counted 20 parasites or greater. Discrepant slides were sent to a third reader. All readers were blinded to the smear results recorded by the other readers and any information about the subject. The final smear value was calculated as zero if two readers reported no parasites. Using an assumed WBC count of 8000/µL, the final smear value per microliter was estimated as the geometric mean of the two positive reads multiplied by 40, using the two closest reads if all three were positive for parasites.

Molecular tests for the presence of *P. falciparum* genomic DNA (gDNA) were performed using polymerase chain reaction (PCR). The methods were described in detail in Chapter 2; briefly, for PCR of the filter papers, blood spots were fixed by submersion in methanol for 15 minutes, then left to dry for at least 2 hours. Parasite gDNA was then extracted by adding 50 µL of DNase-free water and heating at 99°C for 30 minutes, vortexing at 15 minutes. These gDNA samples were used in PCR reactions targeting the

P. falciparum lactate dehydrogenase (LDH) gene, as described by Rantala *et al*.[21] Each 25 μL reaction consisted of 12.5 μL Taqman® (Life Technologies™, Carlsbad, CA), 0.25 μL each of forward and reverse primers at 250 nM, 0.30 μL of probe at 300 nM, 10.7 μL nuclease-free water, and 1 μL of gDNA sample. All samples were run in duplicate and considered dichotomously positive for *P. falciparum* if LDH had a clear amplification curve in at least one of the two wells.

4.3.3   *Data management and analysis*

All study data were stored and managed in the Research Electronic Data Capture (REDCap) system (Vanderbilt University) hosted at the University of Malawi College of Medicine.[22] Questionnaire responses during the first survey from the rainy season 2012 were collected on paper in the field and later entered into REDCap at the ICEMR-Malawi offices by two independent study personnel. REDCap functions were used to compare the double-entered data, and the original paper records were used to resolve any discrepancies in data entry. All data for the subsequent three surveys were collected electronically in the field on android-based tablets using OpenDataKit (http://opendatakit.org) and uploaded to REDCap at the end of each day of field data collection. Geographic locations of study households were captured using the global positioning system (GPS) embedded in the tablets during the fourth survey in the dry season of 2013. Roughly the same households had been included in all prior surveys, though it is possible that there was some variation in specific household participation during the preceding surveys. Geographic data were managed and all maps created using ArcMap version 10.2.1 (Esri, Redlands, CA).

Several variables were created for evaluation as potential predictors of treatment-seeking outcomes. Distance to health care facilities was calculated to estimate access to health care. The 'mean center' method in ArcMap was used to estimate the centroid of the compact segment of ~30 households sampled for each EA. Locations of health care facilities were collected by the Japanese International Cooperation Agency (JICA), and the types of facilities were separated into two categories. Dispensaries, maternity wards, or dispensary/maternity wards were grouped into one category; 'other' health facilities included central hospitals, district hospitals, hospitals, primary health centers, urban health centers, and district health offices. Other key characteristics of these facilities,

such as antimalarial drug availability and quality of care were unknown, but the locations nonetheless allow a rough estimation of access to health care. The proximity analysis tool in ArcMap was used to calculate the Euclidean distance in kilometers (km) from the centroid of the study households in each EA to the nearest dispensary/maternity facility and 'other' facility.

Plasmodium transmission intensity was estimated for each EA using prevalence from the first survey as a proxy estimate. PCR data from the original cross-sectional study survey during the 2012 rainy season were used to classify the 30 study EAs into tertiles of baseline *P. falciparum* prevalence. The lowest tertile included EAs with 0% to <8%, the middle tertile included those with 8% to <15%, and the highest tertile included those with ≥15% PCR-based prevalence estimates in the rainy season 2012. The baseline prevalence tertile value was assigned to all individuals residing in households within a given EA.

Socioeconomic status (SES) variables were collected at the household level. Ten variables pertaining to SES were combined into a single indicator variable based on the method suggested by Filmer and Pritchett.[23] The ten variables were each input into a Principal Component Analysis (PCA) based on the following scoring system: Ownership of each of six items (radio, bike, car, house, phone, and television) was indicated by a value of 1 if the household owned at least one of the item and 0 if it did not. If the home was powered by electricity, it received a score of 1 for that variable, and 0 if it was not. If the head of household earned any income, that was indicated with a value of 1. If the household reported a shortage of food in the previous month, it received a score of 0; the lack of a food shortage in the previous month received a score of 1. Finally, the highest level of education achieved by either the head of household or spouse was ranked as a 0 for 'No schooling,' 1 for any years completed of Standard 1-8, or 2 for completing anything beyond Standard 8. Higher scores on each component therefore represented greater wealth or SES. PCA was performed in SAS using *proc factor* to obtain weights for each of the ten individual indicators to indicate their relative importance in contributing to the total household SES index score. These weights were obtained from Factor 1, which had an eigenvalue of 2.55. The PCA was calculated using 3,599 households in the entire dataset from four surveys. These weights were then applied to

each of the ten components to calculate an overall weighted SES index score for each household. The PCA-weighted SES index score was applied to all individuals within a household. The individuals were then grouped into quartiles of SES index scores for each survey separately, to minimize the impact of any potential changes in overall SES over time. All analyses were based on the individual's SES quartile within the study population of each survey.

Household construction was assessed separately from the overall SES index, as it may be directly associated with *Plasmodium* infection if the household materials increase exposure to *Anopheles* vectors. The roof, floor, and walls of the house were each classified as natural, rudimentary, or finished. Natural roofs were those made of thatch/leaf or sticks/mud or missing entirely; rudimentary roofs were made of rustic mat/plastic sheet, reed/bamboo, or wood planks; finished roofs were those made of corrugated iron/metal sheets, wood, cement fiber, cement/concrete, or roofing shingles. Natural floors were those made of earth/sand or dung; rudimentary floors were those made of wood plants or palm/bamboo; finished floors were those made of parquet/polished wood, vinyl or asphalt strips, ceramic tiles, cement, or wall-to-wall carpet. Natural walls were those that were made of cane, sticks, bamboo, or reeds, or missing entirely; rudimentary walls were those that were made of bamboo/wood with mud, carton/plastic sheets/sacks, mud, plywood, or stone with mud; finished walls were those made of bricks, cement, cement blocks, covered adobe, plaster, stone with lime/cement, or wood planks/shingles. A household overall was categorized as 'finished' if at least two of the roof, walls, and floor were classified as finished rather than natural or rudimentary, and 'unfinished' if none or only one of the three was finished.

We also created a number of individual-level variables. Not all subjects could be given an exact age in months or years, but most subjects in this analysis could be categorized as either: young children (≤5 yo), school-aged children (6 – 15 yo), or adults (≥16 yo). The ownership of bednets was assessed at the household level; in households that owned at least one bednet, we identified which individuals had slept under a net on the previous night.

This analysis considered several outcomes related to treatment-seeking. Interviewers asked whether each subject had sought treatment or medical advice for any

type of illness or condition in the two weeks previous to the survey, and, if so, what source(s) of treatment. Participants who had visited a government hospital, government health center, government health post, government mobile clinic, private hospital/clinic, private pharmacy, private doctor, or private mobile clinic were classified as having visited any government or private facility for this analysis. Participants who had not visited a government or private facility but who had gone to a community health worker, shop, traditional practitioner, or 'other' (primarily described as taking medications that they already owned or taking them from neighbors and family) were classified as having visited 'other' sources of care. Participants were also asked to report any medications taken in the previous two weeks, including the antimalarials LA, chloroquine, quinine, and SP/Fansidar. Subjects were classified as having sought treatment in the previous two weeks if they had visited any type of facility, reported taking any medication, or reportedly sought treatment or took medication even though the interviewee did not know the source of treatment or type of medication taken. Interviewees were asked to report any fever in the previous two weeks or any fever, rigors/chills, headache, diarrhea, nausea/vomiting, cough, abdominal pain, rash, or other symptoms in the previous 48 hours. The field nurses measured the temperature of any subjects who were present at the time of survey and consented to sample collection. Subjects were classified as febrile for this analysis only if they reportedly had a fever in the previous two weeks, as this was the relevant time period for the data on treatment-seeking. Subjects that reported seeking treatment for fever were also asked about the number of days that had passed since the onset of fever before they sought care.

All data analysis was performed using Statistical Analysis Systems (SAS) version 9.3 and 9.4 (SAS Institute, Cary, NC). Analyzed outcomes were either binomial (sought treatment or not, sought treatment at a government or private facility versus other source, and received an antimalarial or not) or time to event (time from onset of fever to seeking treatment). All predictors were categorical except the distances to health facilities, which were estimated continuously in kilometers, and non-normally distributed. For univariate analyses of binomial outcomes, Wilcoxon-Mann-Whitney and Chi-squared tests of association were used for the continuous predictors and categorical predictors, respectively. Fisher's exact test was used to obtain p-values when any cells had expected

values of less than five. Logistic regression was used to analyze the predictors that a person sought treatment given that they reported a fever in the previous two weeks. Multilevel logistic regression models with random intercepts were used to account for clustering of treatment-seeking behaviors at the household and EA levels. Among those who reported being febrile and seeking treatment in the previous two weeks, multilevel logistic regression was also used to assess the predictors of seeking treatment at a government or private clinic as opposed to with a community health worker, shop, traditional practitioner, or other source, again including random intercepts for the household and EA levels. For those individuals who reported having a fever in the previous two weeks, the Kaplan-Meier method was used to plot the time in days from the onset of fever to seeking treatment, stratified by various potential predictors. The Log-Rank test was used to test for homogeneity of the stratified survival curves, and Cox proportional hazards regression was used to calculate unadjusted and adjusted hazard ratios (HRs) for the key predictors. The results of the Cox proportional hazards analysis were very similar to those of the multilevel logistic regression analysis of the overall odds of seeking treatment, and therefore were not included here. We also analyzed the predictors that an individual would receive an antimalarial among the subpopulation that had sought treatment in the previous two weeks, using multilevel logistic regression with random intercepts at the EA- and household-levels. Interactions of interest, particularly the interaction of District with other key predictors, were explored in each regression, and retained in the final adjusted models when statistically significant or important for interpretation.

4.4 Results

4.4.1 *Description of study population*

The current analysis includes data collected during four surveys. Questionnaires were completed for 3,459 individuals from 879 households from rainy season 2012, 3,861 individuals from 909 households from dry season 2012, 3,754 individuals from 921 households from rainy season 2013, and 3,670 individuals from 924 households from dry season 2013. Consent documentation was lost or not obtained for 794 individuals from rainy season 2012, 383 from dry season 2012, 433 from rainy season 2013, and 482 from

dry season 2013; after these subjects were excluded, 2,665 individuals from 820 households, 3,478 individuals from 908 households, 3,321 individuals from 917 households, and 3,188 individuals from 922 households, respectively, were included in the final analysis. Basic characteristics of the study population from each survey are presented in Table 4.1. As household respondents were asked to provide details about all members, including those not present at the time of survey, some aspects of treatment-seeking were unknown for a large proportion of the study population, and PCR and microscopy results were not available for absent subjects. There was a notable increase in bed net ownership between the first and second surveys, reflecting a MoH bed net distribution campaign that occurred between the ends of the rainy and dry seasons of 2012.

Table 4.1. Characteristics of the study population from four surveys of the ICEMR-Malawi Cross-Sectional Study, 2012 - 2013

	Rainy season 2012 n (%)	Dry season 2012 n (%)	Rainy season 2013 n (%)	Dry season 2013 n (%)
Total	2,665	3,478	3,321	3,188
District				
Blantyre	838 (31.4%)	1,110 (31.9%)	1,050 (31.6%)	999 (31.3%)
Thyolo	879 (33.0%)	1,135 (32.6%)	1,103 (33.2%)	1,035 (32.5%)
Chikhwawa	948 (35.6%)	1,233 (35.5%)	1,168 (35.2%)	1,154 (36.2%)
Sex				
Male	1,053 (39.5%)	1,539 (44.3%)	1,356 (40.8%)	1,285 (40.3%)
Female	1,611 (60.5%)	1,939 (55.8%)	1,965 (59.2%)	1,903 (59.7%)
Age				
Infants, <6 mos	39 (1.5%)	60 (1.7%)	37 (1.1%)	47 (1.5%)
Young children, 6 mos - 5 yrs	613 (23.0%)	704 (20.3%)	736 (22.2%)	677 (21.2%)
School-aged children, 6 - 15 yrs	795 (29.8%)	1,083 (31.2%)	1,086 (32.7%)	1,065 (33.4%)
Adults, ≥16 yrs	1,155 (43.3%)	1,610 (46.3%)	1,460 (44.0%)	1,399 (43.9%)
PCR results				
Positive for *P. falciparum* [α]	363 (17.5%)	342 (12.4%)	605 (20.1%)	306 (10.5%)
Negative for *P. falciparum*	1,715 (82.5%)	2,418 (87.6%)	2,412 (80.0%)	2,622 (89.6%)
Not available	587	718	304	260
Microscopy results				

	Rainy season 2012 n (%)	Dry season 2012 n (%)	Rainy season 2013 n (%)	Dry season 2013 n (%)
Positive for any parasitemia	229 (11.4%)	158 (5.8%)	405 (13.3%)	182 (6.2%)
Negative for any parasitemia	1,789 (88.7%)	2,552 (94.2%)	2,637 (86.7%)	2,735 (93.8%)
Not available	647	768	279	271
EA transmission intensity [β]				
Low	885 (33.2%)	1,143 (32.9%)	1,055 (31.8%)	1,026 (32.2%)
Medium	839 (31.5%)	1,158 (33.3%)	1,094 (32.9%)	1,066 (33.4%)
High	941 (35.3%)	1,177 (33.8%)	1,172 (35.3%)	1,096 (34.4%)
SES Quartile [γ]				
Lowest	724 (27.4%)	912 (26.2%)	875 (26.5%)	865 (27.8%)
2nd	671 (25.4%)	875 (25.2%)	774 (23.5%)	766 (24.7%)
3rd	642 (24.3%)	874 (25.2%)	845 (25.6%)	749 (24.7%)
Highest	608 (23.0%)	814 (23.4%)	804 (24.4%)	727 (23.4%)
Household characteristics [δ]				
Unfinished	1,299 (48.9%)	1,667 (48.0%)	1,538 (46.3%)	1,438 (45.1%)
Finished	1,355 (51.1%)	1,805 (52.0%)	1,783 (53.7%)	1,750 (54.9%)
Eaves				
Open	423 (15.9%)	870 (25.1%)	762 (22.9%)	810 (25.4%)
Closed	2,232 (84.1%)	2,600 (74.9%)	2,559 (77.1%)	2,378 (74.6%)
Bednet use				
Slept under a net previous night	913 (35.4%)	2,014 (58.0%)	2,270 (68.4%)	1,783 (55.9%)
Net available but not used	513 (19.9%)	960 (27.6%)	658 (19.8%)	910 (28.5%)
No nets in household	1,153 (44.7%)	501 (14.4%)	393 (11.8%)	495 (15.5%)
IRS in previous 12 months				
Yes	470 (17.7%)	376 (10.9%)	867 (26.2%)	698 (22.4%)
No	2,185 (82.3%)	3,081 (89.1%)	2,444 (73.8%)	2,424 (77.6%)
Fever in the previous 2 weeks				
Yes	1,008 (46.1%)	749 (21.6%)	666 (20.1%)	548 (17.5%)
No	1,181 (54.0%)	2,721 (78.4%)	2,654 (79.9%)	2,575 (82.5%)
Any treatment or advice for illness sought in the previous 2 weeks				
Yes	923 (34.6%)	799 (23.0%)	753 (22.7%)	615 (19.3%)
No	1,051 (39.4%)	2,672 (76.8%)	2,567 (77.3%)	2,510 (78.7%)
Unknown	691 (25.9%)	7 (0.2%)	1 (0.0%)	63 (2.0%)

	Rainy season 2012 n (%)	Dry season 2012 n (%)	Rainy season 2013 n (%)	Dry season 2013 n (%)
Days of fever until treatment sought, mean (SD)	2.15 (2.29)	1.85 (1.62)	2.09 (1.92)	1.75 (1.77)
Source of treatment sought				
Government or private facility	387 (41.9%)	365 (45.7%)	347 (46.1%)	317 (51.5%)
Shop, traditional healer, community health worker, other	499 (54.1%)	311 (38.9%)	256 (34.0%)	244 (39.7%)
Source unknown	37 (4.0%)	123 (15.4%)	150 (19.9%)	54 (8.8%)
Antimalarial taken in the previous 2 weeks				
LA	165 (6.2%)	113 (3.3%)	194 (5.8%)	113 (3.5%)
Other antimalarial $^\varepsilon$	36 (1.4%)	21 (0.6%)	26 (0.8%)	11 (0.4%)
None	2,464 (92.5%)	3,344 (96.2%)	3,101 (93.4%)	3,064 (96.1%)

EA   Enumeration area
ICEMR   International Center of Excellence for Malaria Research
IRS   Indoor residual spraying
LA   Lumefantrine artemether
LDH   Lactate dehydrogenase
PCR   Polymerase chain reaction
SD   Standard deviation
SES   Socioeconomic status

α   Parasitemia as detected by PCR for *P. falciparum* LDH gene
β   Tertiles of parasite prevalence established for all 30 EAs from the first survey (rainy season 2012) data were used as a proxy estimate of transmission intensity in the EA. See Methods for details.
γ   SES indicator calculated using technique based on Filmer & Pritchett (2001).[23] See Methods section for details
δ   Roof, wall, and floor materials were each classified as finished or unfinished (rudimentary/natural); 'finished' households were those with 2 or 3 finished materials, 'unfinished' households were those with 0 or 1
ε   'Other' antimalarials included chloroquine, quinine, or sulfadoxine-pyrimethamine

Additional details about the relationships between different treatment-seeking variables are presented in Figure 4.1. Subjects that did not report a fever in the previous two weeks were less likely to seek treatment than those that were febrile. While some individuals who were reportedly afebrile had received an antimalarial in the previous two weeks, most subjects that had received an antimalarial had been febrile. In the study population, there were only 25 (<0.2%) subjects who took an antimalarial drug without experiencing fever in the previous two weeks. Among febrile individuals whose

treatment source was known, just over half sought care at a government or private facility as opposed to at a shop, community health worker, traditional healer, or 'other' source. Individuals who were febrile and sought treatment at either a government or a private health care facility were most frequently given an antimalarial. LA was the most commonly dispensed antimalarial for individuals from all treatment sources. At government or private health care facilities, LA was given to 38.0% of febrile individuals while only other antimalarials were given to 4.5%. At other treatment sources, only 5.8% of febrile individuals received LA, and 2.1% received another antimalarial. Of 585 subjects who reported taking LA, 11 had also taken another antimalarial. Three had taken SP/fansidar in addition to LA: one who had gone to a government or private facility, one who had gone to another source, and one whose source of treatment was unknown. The other eight had taken quinine with LA, all of whom had gone to a government or private facility. Of 94 individuals who only took antimalarials other than LA, 59 (62.8%) took SP/fansidar alone, 33 (35.1%) took quinine alone, and one (1.1%) took both SP/fansidar and quinine. Only one individual in the study population reported taking chloroquine, the use of which has been rare following concerns about chloroquine resistance in the early 1990s, though recent evidence indicates a return of chloroquine-sensitivity.[24]

Figure 4.1. Relationship of treatment-seeking variables in the ICEMR Cross-Sectional Study population across four surveys from rainy and dry seasons in 2012 and 2013
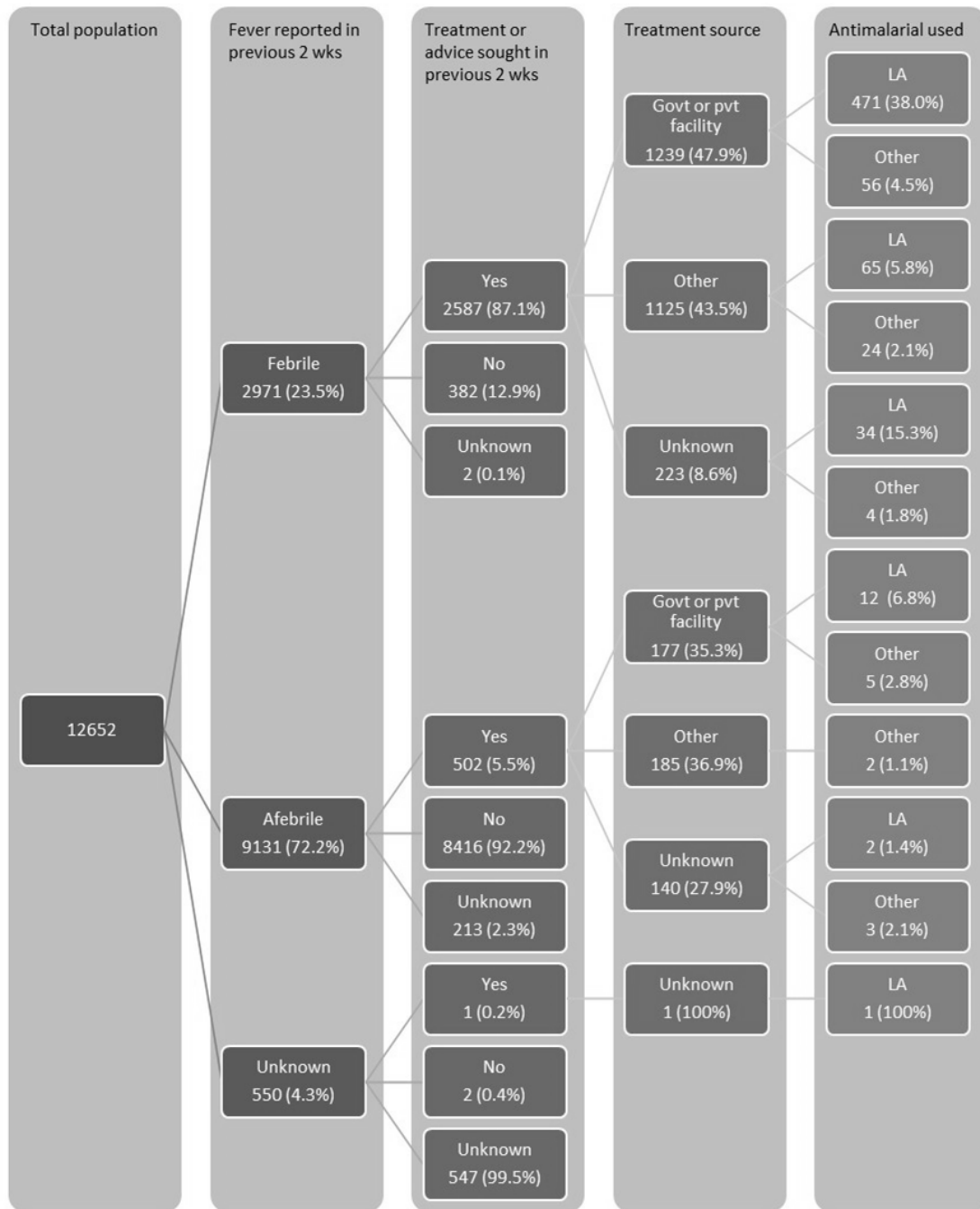
4.4.2 Treatment-seeking in study EAs

Each survey included a compact segment of approximately 30 households from 10 EAs in each of the three Districts (Blantyre, Thyolo, Chikhwawa). Among individuals with reported fever in the previous two weeks in a given EA, the proportion that sought

treatment tended to be high across the three Districts, though there was some variation among specific EAs within each District. A higher proportion of individuals sought treatment from the EAs in Blantyre than in Thyolo or Chikhwawa on average, and there was a slight increase in the average proportion seeking treatment over time since the surveys began, though the increase was not consistent for all EAs (Figure 4.2). There did not appear to be a clearly distinguishable pattern in the proportion of febrile individuals seeking treatment during dry seasons compared to rainy seasons.

Figure 4.2. Proportion of febrile individuals that sought treatment in the previous two weeks in each enumeration area (EA) across four surveys
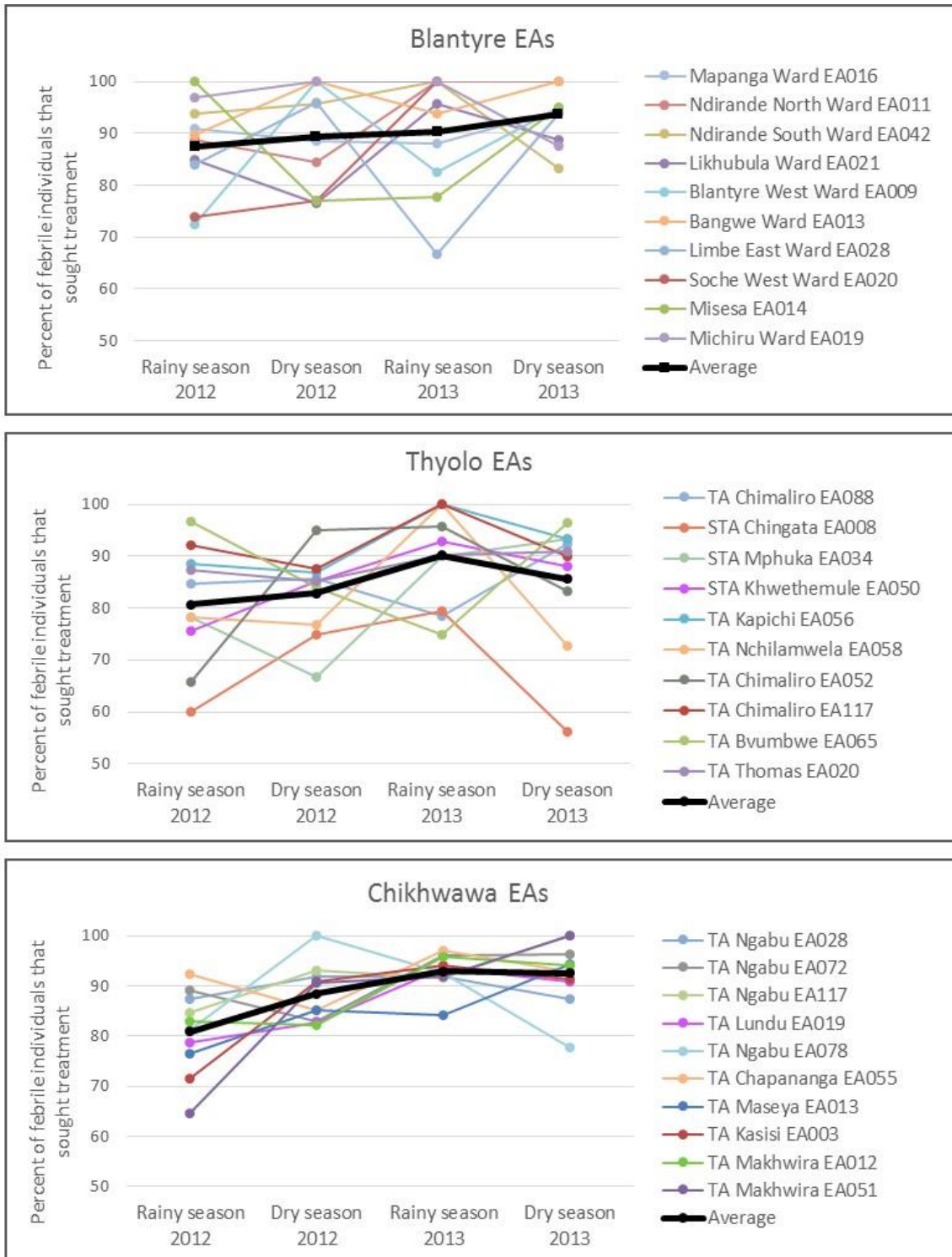
TA   Traditional authority
STA   Sub-traditional authority

EAs were divided into quartiles by the proportion of febrile individuals who reportedly sought treatment in the previous two weeks averaged across all four surveys and mapped with the location of health facilities (Figure 4.3). There were a large number of health facilities in both Blantyre and Thyolo Districts, with fewer in Chikhwawa. Most health facilities were classified as either dispensaries or maternities. In Blantyre, the distance from each compact segment to the nearest maternity care facility or dispensary ranged from 0.37 km to 2.46 km, with an average of 1.53 km (standard deviation (SD): 0.76) and a median of 1.37 km. The distance to the nearest health facility of another type (local and District health offices and hospitals) ranged from 0.72 km to 4.46 km, with an average of 2.83 km (SD: 1.45) and a median of 2.33 km. The average distances to either type of health facility were longer for both Thyolo and Chikhwawa than Blantyre. In Thyolo, the distance from each compact segment to the nearest maternity or dispensary ranged from 0.72 km to 5.30 km, with an average of 2.78 km (SD: 1.22) and a median of 2.38 km. The distance to the nearest health facility of another type ranged from 4.40 km to 16.81 km, with an average of 12.01 km (SD: 3.88) and a median of 13.73 km. In Chikhwawa, the distance from each compact segment to the nearest maternity or dispensary ranged from 0.55 km to 9.30 km, with an average of 6.24 km (SD: 2.69) and a median of 6.87 km. The distance to the nearest health facility of another type ranged from 2.35 km to 23.67 km, with an average of 12.32 km (SD: 5.51) and a median of 11.91 km. None of the 10 EAs in Blantyre was within the lowest quartile for proportion of febrile individuals who sought treatment. Most of the EAs from the lowest quartile were in the Thyolo District, and a few were in Chikhwawa; however, Thyolo and Chikhwawa both contained EAs from the highest quartile as well. Despite some spatial heterogeneity, the range of proportions was fairly small, with more than 80% of febrile individuals seeking treatment in all EAs except one in Thyolo. In that EA, only 68.9% of febrile individuals had sought treatment in the two weeks prior to survey.

Figure 4.3. Map of the proportion of febrile individuals who sought treatment in the previous two weeks in each EA, averaged over four surveys, 2012 - 2013
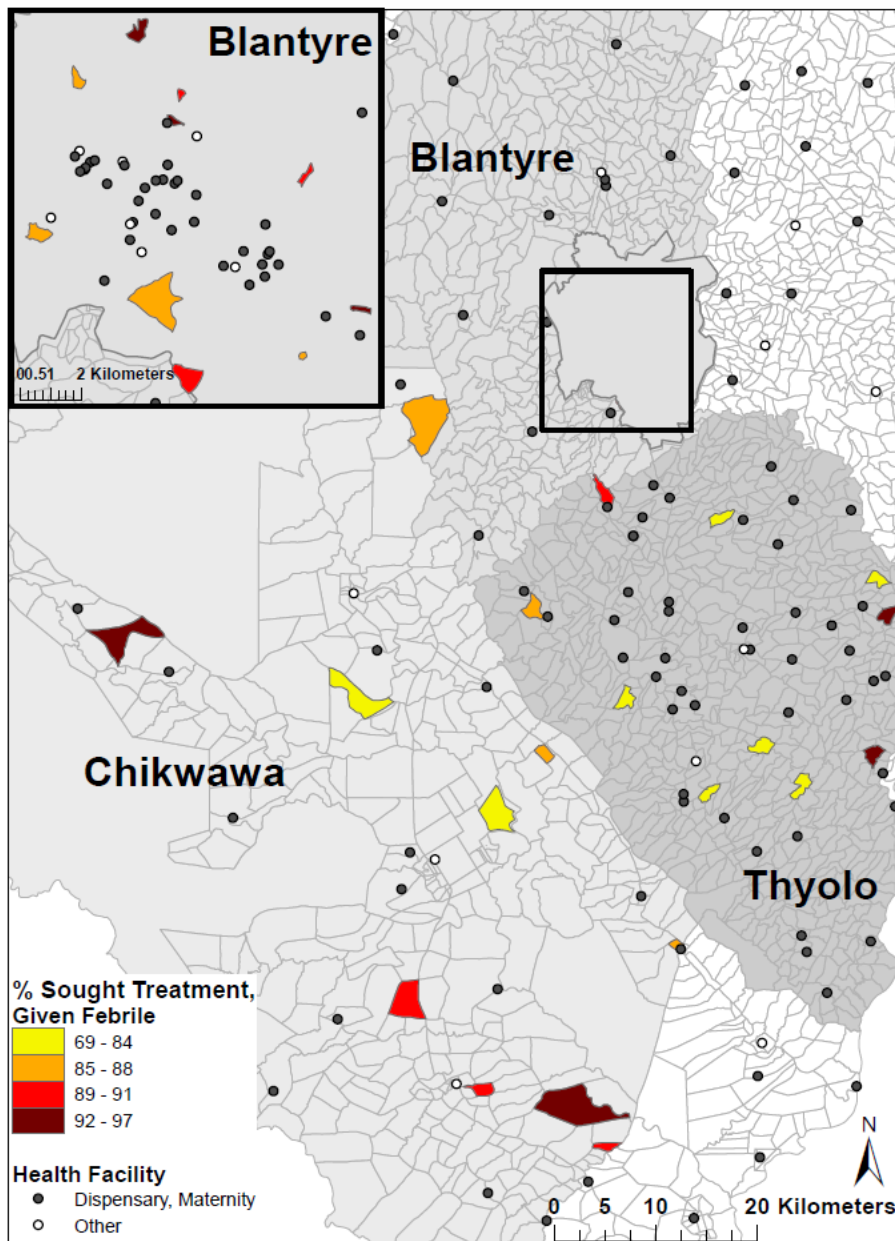
4.4.3   *Predictors of treatment-seeking among individuals*

We also analyzed predictors of treatment-seeking following reported fever in the previous two weeks using multilevel logistic regression models with random intercepts to account for potential household- and EA-level clustering (Table 4.2). These random intercepts were statistically significant in all models, suggesting further unexplained variation at both levels. As previously stated, febrile individuals had the highest odds of

seeking treatment in Blantyre District, and Thyolo District had the lowest (crude odds ratio (OR) using Blantyre as reference = 0.61, 95% confidence interval (CI): 0.41 – 0.93). The odds of seeking treatment when febrile in the previous two weeks were also lower in Chikhwawa District than Blantyre (crude OR = 0.82, 95% CI: 0.54 – 1.24), but the difference was not statistically significant. Preliminary analyses indicated that the associations between other potential predictors and treatment-seeking varied by District, so the final, adjusted logistic regression models were built separately for each District, although the same predictors were retained in all final models for comparisons.

Febrile subjects had lower odds of seeking treatment during the rainy season than the dry season (crude OR = 0.77, 95% CI: 0.61 – 0.97), but this may have been attributable to a general increase in the odds of treatment over time, and the low proportion of febrile subjects that sought treatment during the first survey (82.3%), which was during the rainy season, compared to the later three surveys (all 87-92%). Collinearity limited the ability to control for both season and survey in an adjusted model; survey was retained as it better fit the data. Febrile individuals from each survey had higher odds of seeking treatment than those from the preceding survey in the final adjusted models, though the ratios were only statistically significant in Blantyre and Chikhwawa. The odds ratios by survey in Thyolo did not consistently increase over time.

Distance from compact segment of residence to health facilities was not associated with the odds of seeking treatment when febrile in crude analysis, but there was strong evidence for interaction with District on the multiplicative scale (Table 4.2). A one km increase in distance from the compact segment of households to the nearest dispensary/maternity ward was associated with statistically significantly lower odds of seeking treatment when febrile in Blantyre (OR = 0.63, 95% CI: 0.45 – 0.88) and Thyolo (OR = 0.74, 95% CI: 0.63 – 0.86). In Chikhwawa, however, where the distances to health facilities tended to be farther (ranging from 0.55 km to 9.30 km), there was no association between a one km change in distance to a dispensary/maternity ward and the odds of seeking treatment when febrile (OR = 1.00, 95% CI: 0.91 – 1.09). After adjusting for the distance to a dispensary/maternity ward, the distance to other types of health care facilities was not associated with the odds of seeking treatment in any of the three Districts.

Sex was associated with the odds of seeking treatment in a crude model, but not after controlling for other relevant predictors. Age category, however, was a statistically significant predictor after adjustments for other key predictors in all three Districts (Table 4.2). Young children (≤5 yrs) had the highest odds of seeking treatment when febrile in all Districts, with nearly two times the odds of adults (≥16 yrs) in both Thyolo and Chikhwawa and nearly four times the odds of adults in Blantyre, though the Blantyre estimate was less precise than those of Thyolo and Chikhwawa. The odds for school-aged children (6 - 15 yrs) were also higher than those for adults in all Districts, but the magnitudes of the associations were smaller than for young children, and were not statistically different from those of adults in any of the Districts.

There was a positive crude association between the odds of seeking treatment and both SES quartile and household construction, but only household construction was significantly associated in the adjusted models, with an association that varied by District (Table 4.2). Having a finished household was associated with increased odds of seeking treatment when febrile only in Blantyre and Thyolo, though the estimate was imprecise and not statistically significant in Blantyre. In the most rural District of Chikhwawa, having a finished household was associated with lower odds of seeking treatment relative to individuals who lived in unfinished houses, though the ratio was not statistically significant.

Previous analyses in this study population found that bednet ownership was highest among people with the highest prevalence of *Plasmodium* infections. The current analysis also found that individuals living in households that owned at least one bednet had higher odds of seeking treatment when febrile than individuals in households with no bednets. After controlling for other key predictors, the association was significant only in Chikhwawa. In Thyolo, the directions of association were the same as Chikhwawa, but did not reach statistical significance. In Blantyre, subjects who lived in a household that owned bednets but did not report sleeping under one the previous night actually had lower odds of seeking treatment than individuals in households that did not own any nets, but neither this association, nor the increased odds of seeking treatment among those that did report using a net compared to non-owners was statistically significant.

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Table 4.2. Predictors of treatment-seeking among individuals with reported fever in the previous two weeks by District using multilevel models with random intercepts for household and EA, ICEMR-Malawi Cross-Sectional Study 2012 - 2013

	n	Sought treatment in past two wks n (%)	Unadjusted OR, all Districts (95% CI)	Adjusted* OR, Blantyre (95% CI)	Adjusted* OR, Thyolo (95% CI)	Adjusted* OR, Chikhwawa (95% CI)
District						
Blantyre	805	722 (89.7%)	1.00 (ref)			
Thyolo	949	797 (84.0%)	**0.61 (0.41 – 0.93)**			
Chikhwawa	1215	1068 (87.9%)	0.82 (0.54 – 1.24)			
Season						
Dry	1297	1152 (88.8%)	1.00 (ref)			
Rainy	1672	1435 (85.8%)	**0.77 (0.61 – 0.97)**			
Survey						
Rainy season 2012	1006	828 (82.3%)	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)
Dry season 2012	749	652 (87.1%)	**1.45 (1.09 – 1.94)**	1.27 (0.69 – 2.33)	0.92 (0.54 – 1.59)	1.45 (0.86 – 2.45)
Rainy season 2013	666	607 (91.1%)	**2.27 (1.62 – 3.17)**	1.51 (0.77 – 2.99)	1.46 (0.79 – 2.69)	**2.43 (1.33 – 4.46)**
Dry season 2013	548	500 (91.2%)	**2.25 (1.57 – 3.22)**	**2.59 (1.15 – 5.84)**	1.18 (0.63 – 2.18)	**2.50 (1.32 – 4.73)**
EA transmission intensity ^{α}						
Low	940	821 (87.3%)	1.00 (ref)			
Medium	954	821 (86.1%)	0.93 (0.60 – 1.44)			
High	1075	945 (87.9%)	1.07 (0.69 – 1.66)			
Distance to nearest health facilities (km)						
Dispensary/maternity			0.97 (0.91 – 1.03)	**0.63 (0.45 – 0.88)**	**0.74 (0.63 – 0.86)**	1.00 (0.91 – 1.09)

	n	Sought treatment in past two wks n (%)	Unadjusted OR, all Districts (95% CI)	Adjusted* OR, Blantyre (95% CI)	Adjusted* OR, Thyolo (95% CI)	Adjusted* OR, Chikhwawa (95% CI)
Other facilities			1.00 (0.97 – 1.03)			
Age						
Young children, ≤5 yrs	862	790 (91.6%)	**2.10 (1.58 – 2.80)**	**3.68 (1.82 – 7.44)**	**1.86 (1.16 – 3.00)**	**1.99 (1.25 – 3.17)**
School-aged children, 6-15 yrs	649	573 (88.3%)	**1.43 (1.07 – 1.91)**	1.20 (0.68 -2.11)	1.64 (0.97 – 2.79)	1.56 (0.95 – 2.56)
Adults, ≥16 yrs	1428	1200 (84.0%)	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)
Sex						
Male	1094	975 (89.1%)	**1.33 (1.05 – 1.70)**			
Female	1875	1612 (86.0%)	1.00 (ref)			
Household characteristics $^\beta$						
Finished	1433	1273 (88.8%)	**1.34 (1.03 – 1.74)**	2.10 (0.87 – 5.08)	**1.59 (1.05 – 2.40)**	0.85 (0.52 – 1.38)
Unfinished	1529	1309 (85.6%)	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)
Bednet use						
Slept under net previous night	1631	1472 (90.3%)	**2.09 (1.60 – 2.73)**	1.31 (0.71 – 2.39)	1.63 (0.99 – 2.69)	**1.95 (1.20 – 3.16)**
Net available but not used	569	495 (87.0%)	**1.54 (1.11 – 2.14)**	0.61 (0.31 – 1.20)	1.14 (0.633 – 2.03)	**2.30 (1.21 – 4.37)**
No nets in household	736	601 (81.7%)	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)
SES Quartile $^\gamma$						
Lowest	893	756 (84.7%)	1.00 (ref)			
2nd	702	613 (87.3%)	1.25 (0.91 – 1.71)			
3rd	754	667 (88.5%)	**1.39 (1.01 – 1.91)**			
Highest	597	531 (88.9%)	1.38 (0.95 – 1.98)			

CI   Confidence interval
EA   Enumeration area
OR   Odds ratio

	n	Sought treatment in past two wks n (%)	Unadjusted OR, all Districts (95% CI)	Adjusted* OR, Blantyre (95% CI)	Adjusted* OR, Thyolo (95% CI)	Adjusted* OR, Chikhwawa (95% CI)

SES   Socioeconomic status

Bolded values are those where the 95% CI does not contain 1.0.
*Each OR adjusted for the other variables listed in the column.
α   Tertiles of parasite prevalence established for all 30 EAs from the first survey (rainy season 2012) data were used as a proxy estimate of transmission intensity in the EA. See Methods for details.
β   Roof, wall, and floor materials were each classified as finished or unfinished (rudimentary/natural); 'finished' households were those with 2 or 3 finished materials, 'unfinished' households were those with 0 or 1
γ   SES indicator calculated using technique based on Filmer & Pritchett (2001).[23] See Methods section for details

4.4.4 *Sources of treatment*

Multilevel level logistic regression was used to assess predictors of seeking treatment at a government or private facility compared to other sources among the febrile individuals who sought treatment (Table 4.3). Random intercepts were again included to account for potential clustering at the household- and EA-levels. Interactions by District were explored, but none proved to be significant for retention in the final adjusted model. The OR for Thyolo relative to Blantyre was statistically significant in the final adjusted model when only fixed effects were included; incorporation of random intercepts for multilevel modeling led to loss of precision for the estimate such that p=0.08, but District was retained as a predictor in the final model.

Just as a relatively small proportion of febrile individuals sought treatment during the two weeks prior to the first survey in the rainy season 2012 compared to later surveys, a relatively low percentage of those who did seek treatment chose to do so at a government or private facility over a shop, traditional healer, community health worker, or 'other' source during the first survey (44.1%) compared to the later three surveys (54.3 – 59.5%).

When young children (≤5 yo) sought treatment for fever, they had significantly higher odds of doing so at a government or private facility than did either school-aged children (6 – 15 yo) or adults (≥16 yo), for whom the odds were nearly equal. The OR for febrile young children seeking treatment compared to adults was 1.70 (95% CI: 1.37 – 2.10), whereas for school-aged children compared to adults the OR was 1.02 (95% CI: 0.80 – 1.30).

Few of the evaluated predictors were significant after adjustment for survey and age. Increased distance of EA households from the nearest health facilities (dispensary/maternity) was associated with lower odds of using a government or private facility among those that sought any treatment for fever in the two weeks prior to survey in both crude and adjusted models. A one km increase in distance was associated with 0.88 times the odds of seeking care at a government or private facility instead of a shop, traditional healer, community health worker, or other source.

Table 4.3. Predictors of seeking treatment at a government or private health facility instead of at a shop, traditional healer, community health worker, or 'other' source among febrile individuals who sought treatment at a known source

	n	Treated at govt/pvt facility n (%)	p-value	Unadjusted OR (95% CI)	Adjusted OR,* multilevel (95% CI)
Season					
Dry	1041	**577 (55.4%)**		1.00 (ref)	
Rainy	1323	**662 (50.0%)**	**0.01**	**0.81 (0.68 – 0.95)**	
Survey					
Rainy season 2012	814	**359 (44.1%)**		1.00 (ref)	1.00 (ref)
Dry season 2012	576	**313 (54.3%)**		**1.51 (1.22 – 1.87)**	**1.54 (1.19 – 1.99)**
Rainy season 2013	509	**303 (59.5%)**		**1.86 (1.49 – 2.33)**	**2.03 (1.55 – 2.67)**
Dry season 2013	465	**264 (56.8%)**	**<0.001**	**1.67 (1.32 – 2.10)**	**1.67 (1.27 – 2.20)**
District					
Blantyre	661	**411 (62.2%)**		1.00 (ref)	1.00 (ref)
Thyolo	732	**337 (46.0%)**		**0.52 (0.42 – 0.64)**	0.63 (0.37 – 1.06)
Chikhwawa	971	**491 (50.6%)**	**<0.001**	**0.62 (0.51 – 0.76)**	1.12 (0.53 – 2.35)
EA transmission intensity [α]					
Low	762	420 (55.1%)		1.00 (ref)	
Medium	733	385 (52.5%)		0.90 (0.74 – 1.10)	
High	869	434 (49.9%)	0.11	**0.81 (0.67 – 0.99)**	
Age					
Young children, ≤5 yrs	733	**441 (60.2%)**		**1.59 (1.32 – 1.92)**	**1.70 (1.37 – 2.10)**
School-aged children, 6-15 yrs	520	**256 (49.2%)**		1.02 (0.83 – 1.26)	1.02 (0.80 -1.30)
Adults, ≥16 yrs	1087	**530 (48.8%)**	**<0.001**	1.00 (ref)	1.00 (ref)
Sex					

	n	Treated at govt/pvt facility		Unadjusted OR (95% CI)	Adjusted OR,* multilevel (95% CI)
		n (%)	p-value		
Male	885	460 (52.0%)		0.97 (0.82 – 1.15)	
Female	1479	779 (52.7%)	0.74	1.00 (ref)	
Distance to nearest health facilities (km)					
Dispensary/maternity	n/a	Median (govt/pvt): 2.46			
		Median (other): 2.49	**<0.001**	**0.92 (0.90 – 0.95)**	**0.88 (0.79 – 0.99)**
Other facilities	n/a	Median (govt/pvt): 9.56			
		Median (other): 11.37	**<0.001**	**0.98 (0.97 – 0.99)**	
Household characteristics $^\beta$					
Finished	1173	**680 (58.0%)**		1.00 (ref)	
Unfinished	1186	**559 (47.1%)**	**<0.001**	**0.65 (0.55 – 0.76)**	
Bednet use					
Slept under net previous night	1335	**715 (53.6%)**		**1.26 (1.04 – 1.54)**	
Net available but not used	437	**243 (55.6%)**		**1.37 (1.07 – 1.76)**	
No nets in household	574	**274 (47.7%)**	**0.02**	1.00 (ref)	
SES Quartile $^\gamma$					
Lowest	688	**335 (48.7%)**		1.00 (ref)	
2nd	560	**274 (48.9%)**		1.01 (0.81 – 1.26)	
3rd	614	**322 (52.4%)**		1.16 (0.94 – 1.45)	
Highest	486	**298 (61.3%)**	**<0.001**	**1.67 (1.32 – 2.12)**	

CI   Confidence interval
EA   Enumeration area
km   Kilometers
OR   Odds ratio
SES   Socioeconomic status

	n	Treated at govt/pvt facility		Unadjusted OR (95% CI)	Adjusted OR,* multilevel (95% CI)
		n (%)	p-value		

Bolded values are those where the 95% CI does not contain 1.0 and p<0.05.

*All ORs adjusted for the full set of variables listed in the column. Final model included random intercepts at the EA- and household-levels.

α   Tertiles of parasite prevalence established for all 30 EAs from the first survey (rainy season 2012) data were used as a proxy estimate of transmission intensity in the EA. See Methods for details.

β   Roof, wall, and floor materials were each classified as finished or unfinished (rudimentary/natural); 'finished' households were those with 2 or 3 finished materials, 'unfinished' households were those with 0 or 1

γ   SES indicator calculated using technique based on Filmer & Pritchett (2001).[23] See Methods section for details

4.4.5   *Predictors of antimalarial use among treatment-seeking individuals*

The proportion of treatment-seeking individuals who received an antimalarial varied by EA, ranging from 11.0% to 43.1% on average across all four surveys. The EAs were divided into quartiles by the proportion of treatment-seeking individuals who received antimalarial treatment and mapped with the locations of health facilities in Figure 4.4. Nearly all of the EAs from the highest quartile were in Chikhwawa, while the other quartiles were more distributed among Thyolo and Blantyre EAs. In the four cross-sectional surveys, 1,416 (45.8%) individuals went to a government or private health care facility, 1,310 (42.4%) went to a shop, community health worker, traditional healer, or other source only, and 364 (11.8%) went to an unknown source. Of these, 544 (38.4%), 91 (7.0%), and 44 (12.1%), respectively, reported taking an antimalarial. The frequencies of antimalarial use by other study variables for the two categories of known treatment sources are presented in Table 4.4.

Figure 4.4. Proportion of individuals in each study EA that received an antimalarial, given that they sought treatment in the two weeks prior to any of four cross-sectional surveys, 2012 - 2013
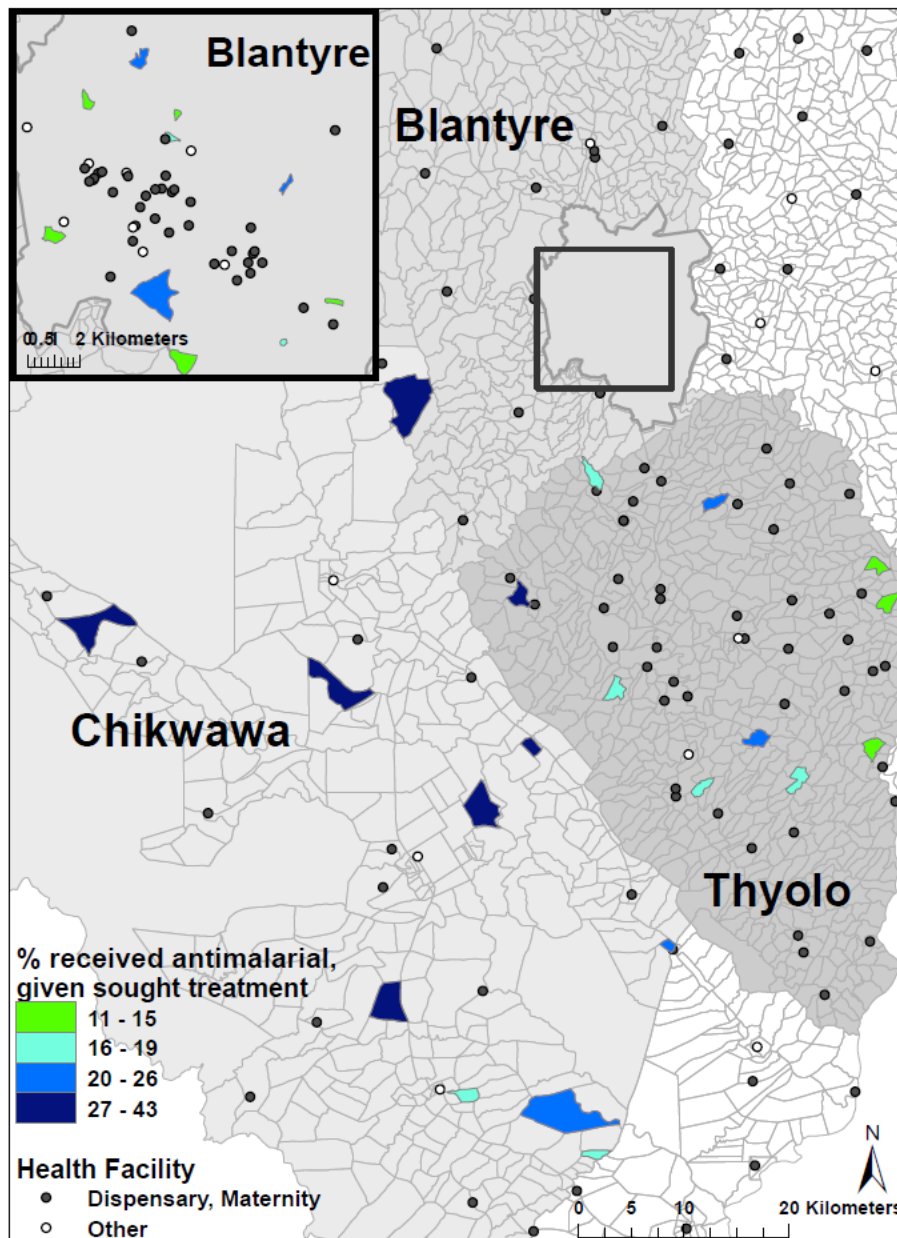
There were many statistically significant predictors of taking an antimalarial given that treatment was sought in univariate analysis (Table 4.4). Febrile individuals were more likely to receive an antimalarial than afebrile individuals from both treatment sources, though a small proportion of reportedly afebrile individuals did receive an antimalarial. Subjects from whom a finger- or heel-stick blood sample was taken to test for parasite presence were more likely to be given an antimalarial at both treatment

sources than untested individuals, though few of those who sought treatment at sources other than government and private facilities were known to have been tested. Even though the pretreatment PCR and microscopy status were not available, subjects who had been treated with an antimalarial were still more likely to be parasitemic by either detection method at the time of survey. The amount of time since initiating treatment was not recorded. A higher proportion of subjects received antimalarials during the rainy season than the dry season from either category of treatment source, and the proportion was also higher for EAs with greater estimated transmission intensity than lesser estimated intensities. The proportion of people who received antimalarials in each District varied by the source of treatment. The association with sex also varied by treatment source, with males being more likely than females to receive an antimalarial at a government or private facility, but a greater proportion of females than males receiving antimalarials from other sources of treatment. A higher proportion of young and school-aged children than adults received an antimalarial by either treatment source, though the relationship had only borderline significance in 'other' sources of treatment (p=0.06). Individuals with lower SES quartile and unfinished houses were more likely to receive antimalarials when treated at a government or private facility, but these variables were not statistically significantly associated with antimalarial treatment among those that frequented 'other' sources of treatment. Finally, there was not strong evidence for an association beyond chance between net use and availability and antimalarial use from government or private facilities, though the proportion of individuals who received an antimalarial was weakly higher for non-owners of nets when obtained from other treatment sources (p=0.08).

Table 4.4. Frequencies of antimalarial use among individuals that sought any treatment in the previous two weeks from four surveys by source of treatment,[α] 2012 - 2013

	Government or private facility			Shop, traditional healer, community health worker, other		
		Antimalarial used			Antimalarial used	
	n	n (%)	p-value	n	n (%)	p-value
Total	1416	544 (38.4%)		1310	91 (7.0%)	
Fever in the previous two wks						
Yes	1239	**527 (42.5%)**		1125	**89 (7.9%)**	
No	177	**17 (9.6%)**	**<0.001**	185	**2 (1.1%)**	**<0.001**
Finger or heel stick performed?						
Tested	580	**334 (57.6%)**		30	**4 (13.3%)**	
Untested	640	**189 (29.5%)**		1034	**83 (8.0%)**	
Unknown	196	**21 (10.7%)**	**<0.001**	246	**4 (1.6%)**	**<0.001**
Microscopy results at time of survey						
Positive for any parasitemia	91	**54 (59.3%)**		141	**16 (11.4%)**	
Negative for any parasitemia	1186	**449 (37.9%)**	**<0.001**	1061	**68 (6.4%)**	**0.03**
PCR results at time of survey						
Positive for *P. falciparum*	146	**92 (63.0%)**		212	21 (9.9%)	
Negative for *P. falciparum*	1148	**417 (36.3%)**	**<0.001**	1010	65 (6.4%)	0.07
Season						
Dry	682	**219 (32.1%)**		555	**27 (4.9%)**	
Rainy	734	**325 (44.3%)**	**<0.001**	755	**64 (8.5%)**	**0.01**
Survey						
Rainy season 2012	387	**159 (41.1%)**		499	**35 (7.0%)**	
Dry season 2012	365	**113 (31.0%)**		311	**14 (4.5%)**	
Rainy season 2013	347	**166 (47.8%)**		256	**29 (11.3%)**	
Dry season 2013	317	**106 (33.4%)**	**<0.001**	244	**13 (5.3%)**	**0.009**
District						
Blantyre	464	**126 (27.2%)**		308	**10 (3.3%)**	
Thyolo	406	**131 (32.3%)**		456	**49 (10.8%)**	
Chikhwawa	546	**287 (52.6%)**	**<0.001**	546	**32 (5.9%)**	**<0.001**
EA transmission intensity [β]						
Low	481	**146 (30.4%)**		417	**12 (2.9%)**	

	Government or private facility			Shop, traditional healer, community health worker, other		
		Antimalarial used			Antimalarial used	
	n	**n (%)**	**p-value**	**n**	**n (%)**	**p-value**
Medium	447	**144 (32.2%)**		397	**21 (5.3%)**	
High	488	**254 (52.1%)**	**<0.001**	496	**58 (11.7%)**	**<0.001**
Sex						
Male	518	**217 (41.9%)**		499	31 (6.2%)	
Female	898	**327 (36.4%)**	**0.04**	811	60 (7.4%)	0.41
Age						
Young children, ≤5 yrs	472	**206 (43.6%)**		328	26 (7.9%)	
School-aged children, 6 -15 yrs	286	**136 (47.6%)**		300	27 (9.0%)	
Adults, ≥16 yrs	646	**195 (30.2%)**	**<0.001**	670	35 (5.2%)	0.06
SES Quartile γ						
Lowest	376	**170 (45.2%)**		397	26 (6.6%)	
2nd	327	**139 (42.5%)**		337	24 (7.1%)	
3rd	363	**131 (36.1%)**		326	24 (7.4%)	
Highest	339	**101 (29.8%)**	**<0.001**	244	15 (6.2%)	0.94
Household characteristics δ						
Unfinished	629	**302 (48.0%)**		696	50 (7.2%)	
Finished	787	**242 (30.8%)**	**<0.001**	608	41 (6.7%)	0.76
Bednet use						
Slept under a net previous night	811	312 (38.5%)		710	47 (6.6%)	
Net available but not used	286	106 (37.1%)		248	11 (4.4%)	
No nets in household	309	121 (39.2%)	0.86	339	31 (9.1%)	0.08

EA Enumeration area
PCR Polymerase chain reaction
SES Socioeconomic status

Bolded p-values are those <0.05.
α Antimalarials included lumefantrine artemether, chloroquine, quinine, or sulfadoxine-pyrimethamine. Individuals whose treatment source was unknown (n=364) were excluded.
β Tertiles of parasite prevalence established for all 30 EAs from the first survey (rainy season 2012) data were used as a proxy estimate of transmission intensity in the EA. See Methods for details.
γ SES indicator calculated using technique based on Filmer & Pritchett (2001).[23] See Methods section for details
δ Roof, wall, and floor materials were each classified as finished or unfinished (rudimentary/natural); 'finished' households were those with 2 or 3 finished materials, 'unfinished' households were those with 0 or 1

There were 872 individuals who sought treatment at a government or private health facility in the previous two weeks that did not report taking any antimalarial. Of these, 311 (35.7%) had received an antibiotic (amoxicillin, penicillin, or Bactrim/cotrimoxazole), an additional 13 (1.5%) received oral rehydration salts, and 338 (38.8%) received only an analgesic (acetaminophen/paracetamol, aspirin, ibuprofen, or Indocin/indomethacin). The treatment was unknown or uncategorized for the remaining 210 individuals (24.1%). Of these 872 individuals, 785 provided a blood sample for PCR testing during one of the cross-sectional surveys, and 54 (6.9%) tested positive for *P. falciparum* parasitemia. Nine (9) of the PCR-positive subjects were known to have been tested by a finger or heel stick at the health facilities, despite not receiving an antimalarial: five (5) of these were sub-microscopically parasitemic, but four (4) had microscopically detectable parasitemia, with the parasitemia of one of the subjects estimated to be 12,164 parasites/µL, one estimated at 715 parasites/µL, and the other two estimated at <100 parasites/µL.

There were 1,219 individuals who did not report taking an antimalarial after visiting only a shop, traditional healer, community health worker, or other source of treatment. Of these, 184 (15.1%) received an antibiotic, an additional 7 (0.6%) received oral rehydration salts, and 882 (72.4%) received only an analgesic. The treatment was unknown or uncategorized for the remaining 146 individuals (12.0%). Of these 1,219 individuals, 1,136 provided a blood sample for PCR testing during one of the cross-sectional surveys, and 191 (16.8%) tested positive for *P. falciparum* parasitemia. Nine (9) of the PCR-positive subjects were known to have been tested by a finger- or heel-stick blood sample at the health care source: only three (3) of these were sub-microscopically parasitemic. The estimated parasitemia for the remaining six (6) individuals ranged from 154 – 60,999 parasites/µL.

Multilevel logistic regression modeling was used to explore the predictors of antimalarial use among febrile individuals who sought treatment, with random intercepts to adjust for clustering at the household and EA levels (Table 4.5). Both random intercepts accounted for a significant component of the unexplained variance, and thus were retained in the final adjusted models. Few afebrile people sought treatment, and of these a very small proportion received an antimalarial. As the causal pathways leading to

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antimalarial use were thought to potentially differ between afebrile and febrile individuals, and because the small sample size precluded effective adjustment or assessment of interaction in regression modeling, afebrile individuals were excluded from the regression analysis. Because microscopy and PCR results were collected after treatment-seeking, and parasite infection status at the time of treatment-seeking could not be properly classified for all individuals, neither was considered as a potential 'predictor' of antimalarial use in regression modeling.

According to Malawi MoH recommendations, a finger- or heel-stick should be used to obtain blood samples to diagnostically test for *Plasmodium* infection by microscopy or RDT in any febrile, treatment-seeking person before they should be prescribed an antimalarial.[18] Many febrile individuals in this study population who sought care were not reportedly tested, even among those who sought care at government or private health care facilities, yet antimalarials were more likely to be used by the individuals who were tested for infection. Subjects who had a blood sample tested were more likely to have been treated according to their actual infection status than subjects who were untested, in whom the infection status would be unknown and therefore the antimalarial use presumptive. For this reason, 'testing' a finger- or heel-stick blood sample was explored as an interaction term in the adjusted regression models. As the preliminary analyses also indicated differences in the frequencies of antimalarial use among different categories of subjects by source of treatment, separate models were built for individuals who sought treatment at a government or private facility versus those who sought treatment at other sources. Subjects whose source of treatment was unknown were excluded from these adjusted models. Few subjects that had sought treatment at a non-government or private facility were tested, so models using blood testing as an interaction term failed to converge for the 'other' treatment source model and were ultimately excluded.

In the final model for government or private facilities, febrile individuals had higher odds of receiving an antimalarial during the rainy season than the dry season, and in Thyolo or Chikhwawa than Blantyre, although the ratio between Thyolo and Blantyre was not statistically significant (Table 4.5). Young children (≤5 yo) and school-aged children (6 – 15 yo) had higher odds of receiving antimalarials than adults. There was an

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association between SES quartile and odds of antimalarial treatment, but the direction of association varied by whether or not the individual had a blood test. Household characteristics were not significantly associated with the odds of antimalarial treatment after adjustment for SES quartile, and were not included in the final model.

There were several important interactions between the reported associations and blood testing status at the government or private health care facility. Season and District had the same direction of association with antimalarial use among those that were blood tested as those that were untested and treated presumptively, though the magnitude of the associations was larger among the untested subjects (Table 4.5). This was not the case for school-aged children or for SES quartile. For school-aged children the magnitude of the association with antimalarial use (relative to adults) was lower among those that were untested for parasitemia than among those that were tested. Individuals who had a higher SES quartile, even after controlling for District, had significantly lower odds of receiving antimalarial treatment if they had been tested by a finger or heel stick than individuals in a lower SES quartile, but had slightly higher odds of receiving presumptive treatment when untested for parasitemia (though the difference was not statistically significant).

Only 91 of the 1,310 individuals who sought treatment at a shop, community health worker, traditional healer, or other source received an antimalarial (Table 4.4), and none of the considered variables proved to be a statistically significant predictor of antimalarial use in this group. The significance of the random intercepts at the EA (p<0.0001) and household (p=0.02) levels suggest unexplained variation at both levels. Various results are presented for comparison to the association at government or private facilities; the directions of the associations are generally similar, though not significant (Table 4.5). The odds of antimalarial treatment from 'other' sources are very low compared to the odds at government or private facilities (crude OR for antimalarial treatment at government/private facility vs. 'other' sources = 8.62, 95% CI: 6.75 – 11.00).

Table 4.5. Predictors of the use of any antimalarial$^\alpha$ in the previous two weeks among febrile individuals who had sought treatment, 2012-2013

	Unadjusted OR (95% CI)	Government or private facility, Adjusted* OR (95% CI)	'Other' health care source, Adjusted* OR (95% CI)
Treatment source			
Government or private facility	**8.62 (6.75 – 11.00)**		
Shop, traditional healer, community health worker, other	1.00 (ref)		
Unknown	**2.39 (1.59 – 3.61)**		
Finger or heel stick performed			
Tested	**6.57 (5.38 – 8.03)**	NR	
Untested	1.00 (ref)		
Season			
Dry	1.00 (ref)	1.00 (ref)	1.00 (ref)
Rainy	**1.41 (1.18 – 1.70)**	Tested: 1.20 (0.82 -1.75)	1.36 (0.78 – 2.36)
		Untested: 1.77 (1.19 – 2.64)	
District			
Blantyre	1.00 (ref)	1.00 (ref)	1.00 (ref)
Thyolo	1.19 (0.93 – 1.53)	Tested: 1.55 (0.86 – 2.80)	1.57 (0.42 – 5.95)
		Untested: 1.59 (0.74 – 3.40)	
Chikhwawa	**1.74 (1.39 – 2.18)**	**Tested: 2.31 (1.34 – 4.00)**	1.17 (0.28 – 4.88)
		Untested: 3.69 (1.74 – 7.83)	
EA transmission intensity $^\beta$			

	Unadjusted OR (95% CI)	Government or private facility, Adjusted* OR (95% CI)	'Other' health care source, Adjusted* OR (95% CI)
Low	1.00 (ref)		1.00 (ref)
Medium	1.06 (0.84 – 1.35)		1.58 (0.45 – 5.62)
High	**2.07 (1.66 – 2.57)**		2.21 (0.57 – 8.54)
Sex			
Male	1.05 (0.87 – 1.30)		
Female	1.00 (ref)		
Age			
Young children, ≤5 yrs	**1.76 (1.43 – 2.17)**	Tested: 1.37 (0.91 – 2.08)	1.68 (0.92 – 3.06)
		Untested: 1.56 (1.01 – 2.43)	
School-aged children, 6 - 15 yrs	**1.72 (1.37 – 2.17)**	**Tested: 2.22 (1.35 – 3.64)**	1.72 (0.94 – 3.17)
		Untested: 1.67 (0.99 – 2.82)	
Adults, ≥16 yrs	1.00 (ref)	1.00 (ref)	1.00 (ref)
SES Quartile [γ]			
Lowest	1.00 (ref)	1.00 (ref)	1.00 (ref)
2nd	1.05 (0.82 – 1.33)	Tested: 1.02 (0.58 – 1.80)	1.09 (0.55 – 2.18)
		Untested: 1.06 (0.61 – 1.85)	
3rd	0.86 (0.68 – 1.09)	**Tested: 0.54 (0.32 – 0.91)**	0.90 (0.44 – 1.82)
		Untested: 1.07 (0.61 – 1.86)	
Highest	**0.76 (0.59 – 0.99)**	Tested: 0.54 (0.28 – 1.02)	1.23 (0.51 – 2.97)
		Untested: 1.28 (0.65 – 2.53)	
Household characteristics [δ]			
Unfinished	**1.26 (1.06 – 1.51)**		

	Unadjusted OR (95% CI)	Government or private facility, Adjusted* OR (95% CI)	'Other' health care source, Adjusted* OR (95% CI)
Finished	1.00 (ref)		
Bednet use			
Slept under a net previous night	0.97 (0.78 – 1.20)		
Net available but not used	0.94 (0.72 – 1.24)		
No nets in household	1.00 (ref)		

CI   Confidence interval
EA   Enumeration area
NR   Not reported
OR   Odds ratio
SES   Socioeconomic status

Bolded values are those for which p<0.05.
*Adjusted models included all variables listed in their respective column and random intercepts for EA- and household-level. The model for government/private facilities was also adjusted for whether or not a finger/heel stick test was performed, though the independent effect of testing was not recorded in consideration of the large number of interacting variables.

α   Antimalarials included lumefantrine artemether, chloroquine, quinine, or sulfadoxine-pyrimethamine. Individuals for whom treatment source was unknown (n=364) were excluded from the two final, adjusted models by treatment source.
β   Tertiles of parasite prevalence established for all 30 EAs from the first survey (rainy season 2012) data were used as a proxy estimate of transmission intensity in the EA. See Methods for details.
γ   SES indicator calculated using technique based on Filmer & Pritchett (2001).[23] See Methods section for details
δ   Roof, wall, and floor materials were each classified as finished or unfinished (rudimentary/natural); 'finished' households were those with 2 or 3 finished materials, 'unfinished' households were those with 0 or 1

4.4.6 *Parasitemia and fever among subjects that did not seek treatment*

Of the 8,800 individuals from the study population over all four surveys that did not seek treatment in the two weeks prior to the survey, 7,686 provided samples for PCR testing and answered the question about experiencing fever in the previous two weeks. There were 1,188 individuals who tested positive for *P. falciparum* by PCR (15.5%); only 56 of these (4.7%) reported experiencing a fever in the previous two weeks. As 395 subjects who had recently sought treatment were PCR-positive for *P. falciparum*, this meant that 75.0% of all prevalent *P. falciparum* infections at the times of the cross-sectional surveys had not recently pursued antimalarial treatment.

4.5   Discussion

Plasmodium infection is endemic in southern Malawi, despite high coverage (>80%) with bednets.[12] Chapter 3 of this dissertation showed that gametocyte carriage is common among prevalent infections in both urban and rural areas in southern Malawi, suggesting potential contributions to ongoing transmission of the parasite. Other studies have indicated that untreated or inadequately treated *Plasmodium* infections can persist chronically for months, and effective antimalarial treatment of chronic infections may be necessary to effectively interrupt transmission.[15–17] To explore the implications of inadequate treatment on transmission, the present study assessed treatment-seeking behaviors and antimalarial use in three Districts of southern Malawi, and identified several key ways in which the current practices are inadequate for clearing chronic *Plasmodium* infections. Our results may have implications for future policy changes and intervention efforts to reduce the burden of malaria.

A high proportion (~87%) of people who reported having a fever in the previous two weeks reported seeking some kind of treatment outside the home. Subjects who did not report experiencing a fever in the two weeks prior to survey were unlikely to seek treatment, and unlikely to receive any antimalarial if they did seek treatment. Prevalent *Plasmodium* infections are frequently afebrile in endemic populations, though symptom status is not associated with gametocytemia/potential infectiousness (Chapter 3). In this community-based sample, 75% of prevalent infections had not recently sought any treatment, and less than 5% of these individuals reported a fever in the previous two

weeks. Since these afebrile infections can be gametocytemic, interruption of transmission will require special efforts to detect and treat them with effective gametocytocidal drugs.

Although the proportion of febrile individuals who sought treatment was high overall, there were important differences in treatment-seeking patterns of different groups of people. A major finding of both the gametocyte analysis in Chapter 3 and general parasitemia analysis in Walldorf *et al.*[12] was the high prevalence of any parasite infection and gametocytemic infection in school-aged children, 6 – 15 yo. As most of the mortality due to malaria is concentrated in children under 5 yo, a majority of intervention efforts have focused on preventing and treating infections in these young children. Most people in this malaria-endemic region of Malawi seem to be aware of the elevated risk to young children, as the odds of seeking treatment for a febrile child under 5 yo were roughly twice those for febrile adults (≥16 yo). The odds of treatment-seeking for school-aged children fell between that of young children and adults, though they were not statistically significantly different from either category in the final adjusted models by District. The same pattern by age category existed in the analysis of time to treatment-seeking after fever onset.

Interestingly, school-aged children were more similar to adults in terms of the source of treatment sought, with young children having significantly greater odds of being taken to a government or private facility than the two older age categories. Just over half of febrile school-children and adults sought treatment at a shop or other source, with no evidence of a difference between the two groups. Since people who frequented a shop or other source had a much lower probability of being treated with an antimalarial than people who attended a government or private clinic, these treatment-seeking behaviors may contribute to the observed high prevalence of infection in school-aged children. These older children are still viewed as having enough risk to prompt more frequent treatment than adults when febrile, but the type of treatment sought may be inadequate since they are perceived as being at lower risk than younger children. Thus, infections in school-aged children may be of longer duration than those of younger children, since they are less likely to receive prompt and adequate antimalarial treatment. Adults, who were treated less frequently and typically at inadequate sources, do not have the highest prevalences of infection, so it is likely that many years of previous infection

eventually offers some degree of protective natural immunity from the risks of developing new *Plasmodium* infections, and/or some immune protection that can limit the natural duration of their infections relative to young and school-aged children.

Treatment-seeking behaviors had a complex relationship with urbanicity. People from the mostly urban Blantyre District were more likely to seek treatment than their more rural counterparts in Thyolo and Chikhwawa. On the other hand, the odds of seeking treatment in Chikhwawa, the most rural District with the fewest health-care facilities, were actually higher than in Thyolo, a semi-rural District with a higher density of health facilities, though this difference was not statistically significant. Given that significant variation remained unexplained at the EA-level in the final multilevel model, further research is needed to determine the etiology of the observed between-District relationships and treatment-seeking beyond basic categorizations of urban vs. rural.

One potential EA-level explanatory variable that was considered in this analysis, the distance from the households to the nearest health care facilities, had a complex interaction with the District. The accuracy of this distance variable is unknown, as the health care facilities were mapped by a separate JICA research project, and the specific locations of health facilities where study participants sought treatment were unknown. The health facility data from JICA is also unlikely to have captured locations for all the types of treatment sources reported by participants of this study, such as local shops, traditional healers, or community health workers. Nonetheless, the JICA spatial data were compared to the locations of study households to give a rough approximation of the general accessibility of healthcare as a potential barrier to seeking treatment.

Curiously, in the more rural Chikhwawa District, where the health facilities were sparser and the distances greater, proximity to the nearest health facility was not associated with the odds of seeking treatment when febrile. Conversely, in both Blantyre and Thyolo Districts, which are less rural and have a greater density of health facilities, greater distance from the nearest health facility was associated with significantly lower likelihood that febrile people sought treatment. This may have been because individuals in remote Chikhwawa EAs were more likely to visit unmapped local shops when ill, but the subsequent analysis of treatment seeking at a government or private facility versus other more local sources, did not provide unequivocal support for this interpretation.

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After adjustment for other predictors, residents of Chikhwawa did not have lower odds of seeking treatment at government or private facilities than residents of the other two Districts. Though the absolute percent of individuals who used such a facility instead of another type of source was lower in Chikhwawa than in Blantyre, it was actually higher than in Thyolo. Another potential explanation for the observed associations could involve the relative magnitudes of distances. In Blantyre, all study EAs were less than 2.5 km from the nearest dispensary or maternity ward. In Chikhwawa, however, people lived, on average, more than 6.2 km from the nearest dispensary or maternity ward. A distance of one km may have a stronger impact on perceived access to care when the difference is between one and two km, than when it is between six and seven km, and a long journey is already required. Future studies of treatment-seeking may need to consider differences in how people perceived access to health care in addition to the actual distances from health care facilities.

As malaria disease and *Plasmodium* infection are seasonal in the region, treatment-seeking behaviors were also compared between dry and rainy season surveys. Interpretation of these findings was limited by differences in data from the first survey, which occurred during the rainy season of 2012. During that survey, the proportion of participants that sought treatment when febrile and the proportion of those that went to government or private facilities instead of another type of source were both much lower than for all subsequent surveys. Without additional data points from before 2012, it is difficult to determine whether these findings represent a true change in treatment-seeking behaviors after May 2012, whether the comparatively low rates were random, or whether they were artefacts of the differences in data collection methods, participation, and survey completion in the initial run of the survey. Since the low proportions of the first survey (rainy season 2012) represented one of the two rainy season samples, rainy season was crudely associated with lower odds of seeking treatment when febrile, lower odds of seeking treatment at a government or private facility, and longer times to seeking treatment after fever onset; however, it is unclear whether this reflects a true seasonal difference in individual treatment-seeking behaviors. Additional data from subsequent surveys during the rainy and dry seasons of 2014 may offer insight into this interpretation.

There was a very slight tendency for people with lower SES or who lived in houses made of more 'unfinished' materials to seek treatment less often and less quickly when febrile, and to go to government or private facilities less often than those in higher SES quartiles or those living in finished houses. These associations tended to be weak, and were rarely statistically significant after adjusting for other key predictors. The lack of a large treatment gap by SES may be attributable to the free provision of most health services by the MoH in Malawi. Differences in perception of risk and access to health care facilities by SES may explain the observed crude associations. There was, however, an interesting interaction between District and house materials in the model for the odds of seeking any treatment when febrile. Consistent with the other SES-treatment relationships, people who lived in finished houses were more likely to seek treatment than those who lived in unfinished houses in both Blantyre and Thyolo; however, individuals in finished houses in Chikhwawa were less likely to seek treatment than those in unfinished houses. The difference in Chikhwawa may have been due to chance, as the confidence interval included the null value, but it bears considering that high-risk individuals in this District with high transmission intensity may potentially be more aware of their risks and respond accordingly.

This interpretation seems to be consistent with the association of bednet use and treatment-seeking in Chikhwawa. Earlier research in this study population found that individuals with high prevalence of *Plasmodium* infection were more likely to live in households that owned at least one bednet than households without one, even if they themselves did not use the bednet the night before the survey.[12] Individuals from the households in Chikhwawa who owned any nets, both those that had slept under them on the previous night and those who had not, were significantly more likely to seek treatment when febrile, and to do so more quickly after the onset of fever, than individuals who did not own nets. Treatment-seeking and bednet ownership likely share a common underlying cause. People who own bednets may have more risk averse personalities, causing them to seek out both bednets and treatments, or they may be aware of their own greater risk and exposure to mosquito bites. There is somewhat more support for the latter explanation, as those people who owned but did not use bednets had similarly high odds of seeking treatment in Chikhwawa as individuals that used the

bednet on the previous night, despite seemingly lower concern for risk aversion. Furthermore, the relationships were strongest in Chikhwawa, the district with the highest transmission intensity. After adjusting for other key predictors, net ownership and use were not associated with the source of treatment sought.

Although treatment-seeking is largely driven by the perceptions of illness among people in the communities, the odds of receiving an antimalarial given that treatment is sought are largely determined by the health care providers. Analyses of antimalarial treatment patterns are limited by the lack of data from said providers regarding each of the reported treatment-seeking visits. Patient self-reporting of medication use may be open to misclassification, and when antimalarials were not provided it is unknown whether this was due to the provider's choice or medication shortages. A further limitation is the lack of longitudinal blood samples from the time pre-treatment to accompany the samples taken at the time of survey. Subjects whose samples were positive by either PCR or microscopy could potentially have been infected subsequent to seeking treatment, and uninfected individuals could have cleared a previous infection since seeking treatment, whether naturally or through the use of antimalarials. One useful indicator to address this limitation involves whether or not sick people were tested for *Plasmodium* infection when they sought treatment, as per the Malawian MoH recommendations.[18] Antimalarial use in individuals that had been tested for *Plasmodium* offers insight into actual infection status at the time of treatment-seeking, despite potential misclassification of low-density/submicroscopic infections enabled by the absence of more sensitive detection methods. Antimalarial use among untested individuals, on the other hand, reflects presumptive treatment by the health care professionals. Although this is not a measure of the true infection status of study participants, it is informative as to the beliefs and practices of health care workers. A much larger proportion of patients (~41%) who attended a government or private facility were known to have been tested than of those who went to a shop or other source (~2%), so essentially all treatment at 'other' sources was presumptive.

Among individuals who were tested for malaria at a government or private facility, antimalarial treatment conformed to the patterns of risk factors for *Plasmodium* infection that were detected in earlier analyses (Chapter 3 and Walldorf *et al.*[12]).

Individuals had slightly higher odds of receiving an antimalarial during the rainy season than the dry season, if they lived in Thyolo or Chikhwawa Districts than in Blantyre District, if they were young or school-aged children than adults, and if they were from lower SES quartiles than higher SES quartiles. Febrile people who were blood tested were more likely to receive an antimalarial than those who were not, but inferences from this finding were limited by lack of knowledge about the presence and severity of other relevant symptoms at the time of seeking treatment that may have prompted testing and treatment, the lack of information about whether *Plasmodium* testing was available when treatment was sought, and the lack of knowledge about the actual results of the blood testing to determine whether antimalarial treatments were dispensed appropriately. The results do appear suggestive of general compliance with the MoH recommendations, but we can also conclude that the process is not infallible for the detection and treatment of *Plasmodium* infection.

Previous research on malaria treatment-seeking behaviors in sub-Saharan Africa has typically taken a more clinical perspective of treatment-seeking in terms of its impact on malaria survival for symptomatic cases, as opposed to the potential role of treatment in reducing transmission from the human infectious reservoirs. This is reflected in a frequent focus on vulnerable subpopulations, such as children[25–31] and pregnant women.[32–35] A critical review also pointed out that previous studies using Knowledge, Attitudes, and Practices (KAP) surveys have provided insight as to perceptions of risk and barriers to treatment, but that such knowledge and attitude did not necessarily correspond with actual behaviors.[36] This review also noted heterogeneity across different settings, and even over time within the same settings, with differences in study methodology limiting the ability for comparisons.[36] The current study encompassed reports of actual recent treatment-seeking behaviors among an all-ages study population across several different time points and a variety of eco-epidemiologic settings with various transmission intensities. This breadth enabled direct assessment of the heterogeneity of studied associations in different transmission contexts. As previously analyzed parasitological data from each survey were also available, we were able to consider the treatment-seeking results in terms of their relevance to ongoing transmission.

While the assessment of heterogeneity was a strength of the study, there were limitations in the measurement and estimation of some of the study parameters. Symptom status is an important factor in treatment-seeking, but the classification of symptomatic individuals was based on only one dichotomous variable in this analysis. The only symptom measured in the time frame of relevance for the data on treatment seeking was fever. Classification of fever in the previous two weeks was based on self-report, and milder episodes of fever may have been underrecognized and underreported, while more severe febrile episodes were disproportionately likely to have been captured. However, since it is each individual's own perceptions of disease that influence their decisions to seek treatment, self-report is perhaps more relevant to the outcome of the analysis than a clinical threshold temperature measurement. Access to treatment was approximated only by Euclidean distance to health facilities, and did not consider any land features that might introduce additional challenges to accessing care, such as poor road conditions during the rainy season in remote areas. We also lacked information about the quality of health facilities and general availability of diagnostic testing and antimalarial treatment at the GPS-located facilities, which may have impacted individuals' perceptions of their access to care at the nearest facilities. Finally, as with many previous studies,[36] data on antimalarial use was only available dichotomously, and information on prescribed dose and actual use were unavailable for any analysis of 'appropriate' treatment.

There were a number of individuals who tested positive for *P. falciparum* infection at the time of survey, but had not been given an antimalarial when they sought treatments in the previous two weeks. We cannot rule out the possibility that some of these infections developed after the treatment visit, however that number is likely to be small. There were 54 PCR-positive *P. falciparum* infections among people who had recently visited a government or private facility and not received an antimalarial (6.9%) and 191 (16.8%) who had visited any other source and not received an antimalarial. It is unlikely that all of these were new infections since the time of seeking treatment, and not all were submicroscopic at the time of survey. Better detection at health care facilities could have potentially identified and treated these individuals to more quickly remove them from the population of infectious reservoirs.

As mentioned above, the single strongest predictor of receiving an antimalarial when seeking treatment was the source of the treatment. Individuals who sought treatment at a government or private facility had 8.6 times the odds of receiving an antimalarial as individuals who sought treatment at a shop or other source. Since only 30 individuals who sought treatment at these other sources were tested for fever, most antimalarial dispensation is likely presumptive treatment. There were no statistically significant predictors of antimalarial treatment at these sources, likely because the precision of the estimates was limited by the relatively small number of subjects who received an antimalarial (91 of 1,310, 7.0%).

Even among individuals who were untested for fever, the proportions that received an antimalarial as presumptive treatment were higher at government or private facilities (29.5%) than among all subjects that went to other treatment sources (7.0%). Health care providers seemed to be generally aware of the risk factors for *Plasmodium* infection, being more likely to provide presumptive treatment to the groups of individuals that were also more likely to be treated when infection status was determined by blood testing; however, the providers may overestimate the impact of some risk factors in determining whether they should provide antimalarials. For instance, after blood testing, there was only a weak association (OR=1.20) between rainy season and the odds of receiving an antimalarial, but antimalarials were significantly more likely to be dispensed presumptively to untested individuals during the rainy season than the dry season (OR=1.77). The magnitude of the association between receiving an antimalarial and residence in Chikhwawa relative to Blantyre was also greater for individuals who were presumptively treated than for individuals who had a blood test before receiving treatment. The same was true of the association between the odds of young children receiving an antimalarial relative to adults, which had a slightly larger magnitude among untested individuals treated presumptively than among individuals that had had a blood test. On the other hand, health care providers in the area may underestimate the prevalence of infection in school-aged children, for whom the magnitude of the association was *lower* among untested individuals than among those who had had a blood test. Health care professionals also had similar or even higher odds of providing antimalarials presumptively to individuals of higher SES quartiles than lower SES

quartiles, though the lower odds of treatment for higher SES individuals after diagnostic testing suggest that their actual risk of infection is lower than for individuals from lower SES quartiles. Additional studies of knowledge, attitudes, and practices about presumptive treatment among health care workers could help identify the underlying causes of such associations, but the results suggest potential undertreatment of infections in school-aged children, individuals of lower SES, and dry season infections when diagnostic testing is not performed.

Ultimately, while this study faces several limitations in developing causal inferences regarding treatment-seeking behaviors and antimalarial use, the findings about current practices highlight several needs for intervention to interrupt *Plasmodium* transmission. There is an urgent need to develop safe antimalarials with gametocytocidal properties to quickly limit potential infectiousness. These drugs should have low risks of side effects for use in the general population, and should be distinct from first-line antimalarials reserved for the clinical treatment of symptomatic cases in order to minimize the promotion of resistance to first-line drugs. Specific interventions and outreach will be necessary to treat asymptomatic, gametocytemic infections, as afebrile individuals in these populations are currently unlikely to seek any treatment that could lead to diagnosis and clearing of infection. Furthermore, in endemic areas, it may be useful to perform a blood test for *Plasmodium* infection in all individuals who come into contact with the health care system, even if they are asymptomatic and accompanying a sick individual or if they are symptomatic with symptoms that are not suggestive of malaria disease. This could help to detect some of the 'silent' reservoirs of infection in the population, but demands more sensitive diagnostic techniques and higher facility testing capacity than are currently available in order to maximize effectiveness.

When they did seek treatment, many individuals in this study population did not choose to visit government or private facilities, opting instead for other, local sources such as shops, community health workers, or traditional healers, despite the fact that most government-provided services are free. Government facilities may need to be more accessible to encourage use in rural areas with high prevalence of *Plasmodium* infection, but it may also be helpful to expand access to diagnostic testing, at least in the form of RDTs, and antimalarial treatments through shops in endemic areas, particularly those that

are far from government facilities. This may especially help reduce the contribution of school-aged children to ongoing transmission. The prevalence of gametocytemia was high in this age group, compared to both young children and adults, but they were less likely than young children to get treatment, and more likely to be taken to a shop or other source. These behaviors would contribute to relatively longer duration of infection and higher prevalence in school-aged children than young children, even if the incidence rates are similar between the two groups. Increased education and awareness of the high prevalence of infection in this age group, both among health care providers and the general population, and increased diagnostic and treatment capacity in local shops, could help increase treatment and reduce the prevalence of infection in these school-aged individuals with high odds of gametocyte carriage. Additional research in underway through ICEMR-Malawi to evaluate the potential of other school-based interventions in order to target this age-group directly in the hopes of efficiently reducing the number of infectious human reservoirs of *Plasmodium*.

In conclusion, current treatment practices in southern Malawi seem to be insufficient to reduce the ongoing *Plasmodium* transmission. New efforts should focus on treating infections in all afebrile people, who are frequently gametocytemic, but rarely seek antimalarial treatment that could help limit the duration of the potentially infectious period. Though treatment-seeking is common among febrile individuals, many do not receive adequate antimalarial treatment to clear infection, particularly individuals who do not attend government or private facilities. Low perceptions of the risks during the dry season, in individuals from low SES, and in school-aged children may demand informative outreach. The effectiveness of all such strategies is limited until cheap and sensitive diagnostic techniques are widely available to detect chronic, low-density infections in the community, and until safe gametocytocidal drugs can be incorporated with antimalarials that clear asexual stage parasites to quickly clear gametocytemia and end the infectious period.

4.6   References

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CHAPTER 5 Conclusions

5.1   Review of major findings

This dissertation used data from a repeated-sample, cross-sectional study to better characterize the human infectious reservoir for malaria in southern Malawi. The first analysis validated the use of a two-stage molecular testing process, using polymerase chain reaction (PCR) to select *Plasmodium* parasite-infected samples for testing with a novel quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay that assessed parasite stage composition, particularly presence of gametocytes (Chapter 2). This assay was used to analyze the epidemiology of gametocytemia in a community-based study population, by identifying the human reservoirs with the potential to contribute to ongoing transmission, and determining the predictors of gametocyte carriage at the population-level (Chapter 3). Finally, treatment-seeking behaviors in the study population were evaluated to better understand who receives appropriate antimalarial treatment, which can limit the duration of infectiousness, and identify gaps where additional outreach and intervention will be needed to reduce the infectious reservoir in the population (Chapter 4). Overall, these studies demonstrated that people with asymptomatic *Plasmodium* infections, even when the blood density of parasites is submicroscopic, are as likely to carry gametocytes as people reporting recent symptoms, and therefore do have the potential to contribute significantly to ongoing transmission, particularly as afebrile people are very unlikely to seek or receive any antimalarial treatment in this region. Since untreated asymptomatic infections may persist chronically for months, and have been shown to be often gametocytemic, any plans for malaria elimination will need to address the asymptomatic reservoir to achieve significant reductions in transmission.

5.1.1 *qRT-PCR Validation (Chapter 2)*

The qRT-PCR assay used for analysis of *P. falciparum* stage-composition was recently developed by collaborators at Harvard University, and initially tested in samples from clinical cases.[1] It had not been used to test samples from the general community, in whom the frequency and densities of infection are generally lower than that observed among clinically ill populations. Molecular testing can detect densities of ~1 gametocyte/µL, which captures the majority of gametocyte densities of potential relevance to transmission. However, the per sample cost of the qRT-PCR assay and the long testing process (two to three days to process a group of 10 samples) made it inefficient to test all samples from the cross-sectional study, in which the majority of people were expected to be aparasitemic. This demanded an alternative testing strategy, using PCR results to first screen the population for prevalent *P. falciparum* infections. This limited the threshold of detection of the testing to that of PCR for lactate dehydrogenase (LDH), which is ~2.7 parasites/µL at the Malawi ICEMR laboratory. Using qRT-PCR to test all samples would likely have detected a few additional people with very low parasitemia, but the cost of detecting each additional positive sample would have been prohibitively high. Regardless, this method significantly improved upon the sensitivity of microscopy. True validation of the test was complicated by the lack of a gold standard for comparison, but the evidence seems to suggest that the qRT-PCR results corresponded well with the PCR results, and that the findings of the combined testing strategy were consistent with significant improvement over microscopy in the sensitivity for the detection of gametocytes.

There were two particular benefits to using this qRT-PCR assay as opposed to testing for *Pfs25* or *Pfg377*, the previously used mRNA markers of mature gametocyte presence.[2,3] First, the primers were designed to span exon-exon junctions, such that the parasite genomic DNA would not contain the specific sequences. All positive test results should therefore reflect only mRNA expression by the specific stages of the parasite present, and reduce the chance for false positives (improved specificity).[1] Secondly, the assay allows the simultaneous detection and distinction of both immature and mature stage gametocytes relative to asexual stages. Immature gametocytes are particularly challenging to distinguish from asexual ring stages by morphology under microscopic

examination,[4] and the previously identified marker of immature gametocyte stages, *pfs16*,[5] has rarely been used, and not for population-based prevalence studies. Thus, this assay and testing strategy could be applied to a variety of questions about stage-specific dynamics of *P. falciparum* infection.

Ultimately, the use of this assay will allow for the quantification of each stage of the parasite relative to the others, but the model calculations are in development and did not correlate well with the qualitative detection of each stage of the parasite in this community-based sample. This may be attributable to the fact that the mathematical model for inferring relative quantities of each stage was developed based on stage-specific counts in clinical cases and *in vitro* parasites only, and may not extrapolate well to the parasite patterns of less severe and asymptomatic infections. For this reason, only the qualitative assessment of stage-specific presence was included in the present analysis.

5.1.2   *Predictors of gametocytemia (Chapter 3)*

The analysis of the predictors of gametocytemia in the total population generally corresponded to the predictors of any parasitemia in the total population. Among individuals who were parasitemic, the directions of the associations tended to be the same as for the predictors of any parasitemia in the population, suggesting that being at higher risk and/or more frequently exposed to mosquitoes and *P. falciparum* infection increases the likelihood of being gametocytemic when infected. The predictors of gametocytogenesis are very poorly understood, but it is thought that some 'stressors' to the parasite may stimulate investment in gametocytogenesis as a way to encourage transmission before elimination by the potential threat.[5] These 'stressors' could include immune responses to the parasite; however, when *P. falciparum* infected, adults had the lowest odds of carrying gametocytes compared to younger children and school-aged children, which suggests that the development of immunity can ultimately prevent gametocyte carriage. Previous research has documented the presence of transmission-blocking immunity (TBI) in sera from people in endemic areas, mediated through immune responses to sexual stage antigens Pfs48/45 and Pfs230, among other unknown factors.[5] It is purely speculative, given that no such conclusions can be drawn from these cross-sectional data, but possible that development of a specific immune response to gametocytes takes many years, while the development of immune responses to asexual

stages of the parasite is more immediate, and the immune system's attacks on asexual stages of the parasite prompt them to invest in gametocytogenesis, therefore leading to elevated rates of gametocyte carriage in people with frequent exposures in the short term. This dynamic would correspond well to previous hypotheses that the parasite invests in a relatively large proportion of asexual stages in order to overwhelm the immune system and allow gametocytes to evade immune detection and clearance,[5] which could delay development of TBI. The interpretation would also correspond to the tendency toward higher odds of gametocytemia when infected among individuals that also had higher odds of any parasitemia, but none of these individual associations was statistically significant. It is also possible that these people with more frequent exposures are also more likely to be asymptomatic after developing a non-sterilizing immune response, and may simply seem to have higher odds of gametocytemia because the duration of the infection has been longer than in lower risk individuals, who are more likely to show symptoms. However, little is known about the dynamics of gametocytemia over the course of an individual infection, so an older infection would not necessarily be more likely to be gametocytemic than a newer infection. This is especially difficult to study in high transmission areas where the Entomological Inoculation Rate (EIR) may be so high that a new infectious bite occurs almost daily. Alternatively, all of the observed associations may have simply been due to chance, given the imprecise estimates and weak associations observed, so it is difficult to draw any firm conclusions based on the data available. The most significant finding was that school-aged children have high odds of being gametocytemic when infected compared to adults and younger children, emphasizing their potential importance in propagating *P. falciparum* transmission. Overall, the lack of strong predictors, other than age, of gametocytemia among parasitemic individuals suggests that future studies and interventions may not need to specifically detect gametocyte carriage, and that simply identifying any-stage infections will be a sufficient approximation of gametocyte presence.

This study did confirm that people who are infected but asymptomatic are often gametocytemic, and therefore may potentially contribute to transmission. While they may exhibit a transmission blocking immune response that limits the success of transmission to the vector, or may have lower-density infections that have decreased likelihood of

infecting a mosquito in a single blood meal, gametocyte carriage is a prerequisite for transmission potential. If none of the individuals who were asymptomatically infected were carrying gametocytes, it would have suggested that these cases are irrelevant to population transmission dynamics. The evidence that they are frequently gametocytemic suggests the need for further research into the specific transmission potential of these 'silent' infections compared to symptomatic cases.

5.1.3   *Treatment-seeking (Chapter 4)*

The results of Chapter 4 are useful for understanding the implications of the findings of Chapter 3. First, differences in treatment-seeking by age help to explain the observed odds of parasitemia and gametocytemia in school-aged children, which were higher than those of younger children. When they are febrile, younger children are more likely to be taken to government or private health facilities, locations where patients are far more likely to receive an antimalarial than at the small shops and other treatment sources, which more often serve school-aged children. Additionally, school-aged children who were *P. falciparum*-infected by PCR were less likely to report a fever in the previous two weeks (~18%) than young children who were *P. falciparum* infected (~42%), and afebrile people were unlikely to seek or receive any treatment in this study. Both situations would allow for *Plasmodium* infections in school-aged children to go untreated more often than those in younger children. The prevalence of gametocytemia was nearly three times as high in school-aged children as in younger children. As a rough approximation, if the incidence rates were assumed to be approximately equal, this would suggest that the duration of gametocytemia in school-aged children is more than three times as long as that of younger children. Implementing interventions to encourage antimalarial treatment-seeking among school-aged children may be an efficient way to reduce the infectious reservoir, when these children do experience symptomatic disease.

Despite the availability of free services at government health facilities, a large proportion of the study population reported seeking treatment solely at a shop or other local source, even though it was rare to receive antimalarial treatment there. More than 15% of the people who had gone to these shops and did not receive an antimalarial were *P. falciparum*-infected by PCR when surveyed. Since these people sought treatment, they could potentially have been removed from the infectious reservoir population if they had

been properly treated. Efforts should be undertaken to either remove the additional barriers to seeking treatment at government or private facilities (greater number, larger capacity, less distance), or to expand malaria diagnostic testing and treatment capacity through local shops. The latter option might be more directly helpful in the short term, although the former is necessary to improve the long-term health conditions of the country.

Afebrile individuals rarely sought or received treatment. Given that asymptomatic *Plasmodium* infections can still be gametocytemic, and potentially infectious to vector mosquitoes, existing interventions and treatment patterns will be insufficient to clear these 'silent' infections and interrupt transmission in highly endemic areas. Active intervention efforts will be needed to reach all infected populations and test for current infection. Mass antimalarial drug administration has been suggested as a possible alternative to individual screening and treatment in order to surmount the challenge of detection with the limited and insensitive field tools that are currently available.[6] Both mass treatments and diagnostic screening follow by treatments would increase selective pressure and could promote the development and spread of antimalarial resistance in endemic populations, so the choice of treatments used will have to balance effectiveness in parasite clearance against the risks of developing resistance to those compounds.

5.2   Future research

One limitation of the present research is that the mere presence of mature gametocytes in people does not guarantee infectiousness to mosquitoes, only potential infectiousness. While these studies have proven that asymptomatic infections can be gametocytemic, it does not necessarily confirm that they are infectious. Questions have been raised about whether the low density infections and existence of TBI could limit the role of people with asymptomatic infections in population transmission dynamics.[7] Research on the likelihood of infecting mosquitoes from gametocytemic infections of different densities has shown that there is certainly an increase in the probability of infection with increasing gametocyte density, but that even very low densities of gametocytes are surprisingly infectious.[5,8] However, people can also develop transmission-blocking immune factors that are taken up with gametocytes in a mosquito

blood meal and hinder parasite development within the mosquito.[5,9–11] The immune response that protects from malaria symptoms in asymptomatic infections might be correlated with TBI, such that asymptomatically infected people would not necessarily be infectious when gametocytemic. The extent of TBI in asymptomatically infected people could be testing using membrane-feeding assays to feed mosquitoes on blood taken from asymptomatic and symptomatic human infections, matched on gametocyte density. We could then compare the proportion of mosquitoes that developed infectious sporozoites in the weeks following membrane feeding for those fed on blood from asymptomatically infected vs. symptomatically infected humans. Furthermore, the blood samples from asymptomatically and symptomatically infected people could be tested for the presence of sexual stage antigens Pfs48/45 and Pfs230, which contribute to TBI.[11] These experiments would provide further information about the potential infectiousness of asymptomatic human infections. It does not seem likely that all asymptomatic infections would have immune responses that completely block transmission, but it is possible that asymptomatically infected people are less infectious per mosquito blood meal than symptomatically infected people, and further research could clarify the relative degree of infectiousness.

Even with a reduced degree of infectiousness, people with asymptomatic infections may contribute significantly to population transmission dynamics if they have longer periods of untreated infectiousness. It would be both ethically and logistically challenging to obtain quality data on the duration of gametocytemia in symptomatically and asymptomatically infected individuals without providing antimalarial treatments. However, mathematical models could be used to explore the contributions of each group under a variety of different realistic assumptions and conditions, and this could be a guide for determining intervention priorities.

The results of this dissertation research suggested several potential community-level intervention trials. Given the high prevalence of infection and gametocytemia in school-aged children, school-based interventions may be a cost-effective way to prevent *Plasmodium* transmission. Expansion of the Malawi ICEMR cross-sectional study is now beginning to consider this question, by investigating whether school attendance is linked to infection risk, such that school-based interventions would not reach the most

vulnerable or highest prevalence groups of children. If schools could be used for interventions applied over a period of days or weeks, the limitation introduced by daily differences in attendance could be mitigated. Such interventions might include using schools as bases for distribution of long-lasting insecticide treated nets (LLINs), educational campaigns about the need for people of all ages to sleep under bed nets, or for periodic distribution of antimalarial treatments at key times during the year. Since the interventions would hopefully have both direct and indirect transmission-reducing effects on the incidence and prevalence of malaria, intervention trials should include many school catchment areas, and randomly assign the intervention to schools, with both passive and active detection of infections in the surrounding populations of intervention and non-intervention catchment areas. It would be helpful to select sites where surveillance data has already been collected to control for baseline prevalence variations. To increase the rates of adequate antimalarial treatment among *Plasmodium*-infected populations, interventions would either need to expand people's treatment-seeking at government or private facilities, or increase the availability of appropriate treatments through shops and community health workers. In either case, qualitative research would be a necessary first step. People who report using shops for recent treatment should be interviewed to obtain more information about the perceived barriers to seeking treatment at government facilities. Shop workers could also be interviewed to determine the reasons why antimalarials are rarely provided to customers. These qualitative results would help to identify specific actions that could be taken to change population perceptions of the barriers to care, or to increase access to antimalarial treatments among customers of shops.

5.3   Implications for malaria interventions

The findings that emerged from investigations in this dissertation strongly suggest that new tools, strategies, and research capacities are needed before malaria elimination can even be considered possible in areas of high endemicity like most of Malawi. Because they are often gametocyte carriers, asymptomatic infected people must be detected and treated with effective anti-gametocytocidal drugs in order to prevent ongoing transmission. However, asymptomatic infections are often submicroscopic, so

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traditional detection methods are insufficient. There is an urgent need for both increased access to molecular testing in malaria endemic settings, and for the development of more sensitive detection methods that are affordable and easy to use in both laboratory and field settings. Until such tools are widely accessible, mass screen and treat (MSAT) interventions are unlikely to identify enough of the infectious reservoir to effectively interrupt local transmission. This supposition has been borne out by the failures of recent MSAT trials.[12–15]

Furthermore, the antimalarial treatments like sulfadoxine pyrimethamine (SP) and artemisinin combination therapies (ACTs) that clear asexual parasite stages often have limited effects on gametocytes, whether mature or immature, and allow gametocytemia to persist for several weeks after clinical symptoms disappear. The safety concerns related to the gametocytocidal drug primaquine have prevented its widespread adoption, despite World Health Organization (WHO) recommendations to include a low dose (0.25 mg/kg) with the use of first-line ACTs for treatment of clinical malaria cases.[16,17] If there is to be any hope for malaria elimination in endemic areas with high EIRs, gametocyte clearance must become a priority in antimalarial treatment strategies. New gametocytocidal drug development is critical, whether for mass distribution, or to be taken in combination with first-line antimalarials in the treatment of clinical cases. An ideal drug would have rapid impacts on both immature and mature gametocytes, would not compete with the mechanism of action of the first-line treatment (thus minimizing selection for resistance), and would have limited side effects. In the absence of a new gametocytocidal drug, field testing methods to detect people with glucose-6-phosphate dehydrogenase (G6PD) deficiency could help to screen out those for whom the use of primaquine is contraindicated to enable safe mass drug administration with primaquine.

Until new treatments and sensitive detection methods are available to effectively address the challenge of asymptomatic human reservoirs, the best hope for interrupting transmission is to prevent human exposure to anopheline mosquito bites. Insecticide-treated bed net coverage in Malawi is already quite high among people with high prevalence of *Plasmodium* infection. However, school-aged children should be encouraged to sleep under bed nets more frequently to reduce ongoing transmission from this high prevalence group, even though distribution of bed nets alone will not eliminate

the 'silent' reservoir or completely interrupt transmission in these areas. Vector control strategies could be expanded and emphasized in zones of Malawi with high transmission. Indoor residual spraying attempts have been limited in the country thus far, and have had a restricted geographical scope.[18] Given the potentially long duration of untreated human infections, vector control efforts would need to occur at regular intervals and be combined with other efforts to treat the human infectious reservoir in order to achieve maximum effects on transmission levels.

The evidence provided in this dissertation suggests that achieving malaria elimination in the region will require a comprehensive plan to treat the 'silent' reservoirs of *Plasmodium* infection. While there may be some hope of targeting interventions at the school-aged children who comprise a large proportion of the gametocytemic reservoir in southern Malawi, limited capacity to detect and treat all asymptomatic infections means that elimination will remain unattainable. International efforts that go beyond malaria control and are aimed at the ultimate goal of eradication should support research into gametocytocidal drugs, transmission blocking vaccines, and sensitive field diagnostic techniques that can be used safely on a wide scale, in order to eliminate this debilitating and deadly disease.

5.4   References

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