THE IMPACT OF LOW-DENSITY AND SUB-PATENT *PLASMODIUM FALCIPARUM* INFECTIONS ON TRANSMISSION AND DISEASE

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ABSTRACT

Erica E. Zeno: The impact of low-density and sub-patent *plasmodium falciparum* infections on transmission and disease.
(Under the direction of Emily W. Gower)

Low parasite density *Plasmodium falciparum* infections are often not detectable by conventional diagnostics. The natural history and clinical consequences of untreated low-density infection has not been fully described. These infections also may be contributing to the infectious reservoir of parasites in humans, which sustains transmission. However, the relationship between parasite density and onward transmission is poorly understood.

Using a 54-month longitudinal cohort of 757 people from 75 households across five villages in Western Kenya, this dissertation aimed to (1) estimate the effect of having a sub-patent infection on subsequent clinical episodes and (2) determine the relationship between parasite density in humans and successful human-to-mosquito transmission.

With inverse probability weighted Kaplan Meier curves, aim 1 found that over 54 months, 1,128 symptomatic episodes of suspected malaria were RDT-negative, of which 400 (35.5%) harbored sub-patent *P. falciparum* infections. Overall, the risk of developing clinical malaria within 60 days was low (7.7% (95% Confidence Interval (CI): 6.0%, 9.4%)). Transmission season modified the relationship between sub-patent infections and risk of clinical malaria (RD low season: 2.3%, CI: 0.4%, 4.2%; RD high season: -4.8%, CI: -9.53%, -0.05%). Next, adapting a previously published probabilistic model to estimate transmission, Aim 2 identified that compared to high-parasite density infections, low-parasite density infections had almost 80% higher odds of human to mosquito transmission (OR: 1.92, CI: 1.54, 2.42). Infections during the high transmission season were also more likely to transmit to mosquitos.

These findings indicate that sub-patent infections have a slightly elevated risk in the low-transmission season, which may merit alternate management but RDT's diagnose the majority of clinically relevant infections in the high transmission season. Low-density infections, which are often sub-patent, are also an important source for mosquito infections. Taken together, this work highlights the public health importance of low-density infections and provides rationale to specifically target low-density infections in order to progress malaria elimination efforts.

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LIST OF ABBREVIATIONS

ACT	Artemisinin-based Combination Therapies
AIDS	Acquired Immunodeficiency Syndrome
AL	Artemether-Lumefantrine
CI	Confidence Interval
CSP	Circumsporozoite Protein
DBS	Dried Blood Spot
DAG	Directed Acyclic Graph
DP	Dihydroarteminisin-Piperaquine
GEE	Generalized Estimating Equations
HIV	Human Immunodeficiency Virus
HRP	Histidine Rich Protein
HS-RDT	High Sensitivity Rapid Diagnostic Test
IPTi	Intermittent Preventative Treatment During Infancy
IPTp	Intermittent Preventative Treatment During Pregnancy
IPW	Inverse Probability Weight
IRS	Indoor Residual Spraying
ITN	Insecticide Treated Bed Nets
MDA	Mass Drug Administration
MOI	Multiplicity of Infection
OR	Odds Ratio
PCR	Polymerase Chain Reaction
RBCs	Red Blood Cells
RD	Risk Difference
RDT	Rapid Diagnostic Test
SMC	Seasonal Malaria Chemoprevention

SSA	Sub-Saharan Africa
WHO	World Health Organization

CHAPTER 1: SPECIFIC AIMS

Malaria case reductions have stalled under current interventions, in part due to sustained transmission from humans to mosquitoes.[1,2] In 2019 there were an estimated 229 million malaria episodes compared to 228 million in 2018, and about 94% of cases worldwide occur in sub-Saharan Africa.[3] The burden of disease, however, is not evenly distributed in the population. In many areas, about 20% of individuals experience 80% of malaria episodes and contribute disproportionately to human-to-mosquito transmission.[4] A fundamental question is, who is harboring parasites, transmitting malaria to mosquitoes, and fueling further transmission in the community? Failure to target the human "infectious reservoir" likely contributes to the infection's persistence. Understanding which infections are infectious, the determinants of infectiousness, and the contribution of low-density infections to the human malaria reservoir is essential for developing targeted interventions aimed at disrupting transmission.

Low parasite density and asymptomatic infections have been shown to be infectious and often go undetected and untreated.[5,6] These infections are frequently sub-patent, meaning below the limit of detection for the rapid diagnostic tests used in the field.[2,7,8] The impact of missing sub-patent infections on the risk of future clinical illness is not fully understood. Common control strategies such as test-and-treat or mass drug administration (MDA) can be optimized to target frequently missed infections, which can improve clinical outcomes and reduce onward transmission.

Data come from a longitudinal cohort in Western Kenya, which captured *Plasmodium falciparum* human-to-mosquito transmission by sampling people and naturally-collected indoor-resting Anopheles mosquitoes from 75 households over 54 months of follow-up. Using samples from the first 14 months, we matched parasites in human and mosquito infections using

amplicon deep sequencing and haplotype inference of the circumsporozoite protein (CSP). We matched infected humans and mosquitoes based on parasite genotypes and adapted a previously published, probabilistic modelling approach based on time, distance, and haplotype sharing to estimate successful human-to-mosquito transmission for each pair.[9] We aimed to:

Aim 1: Estimate the effect of having a sub-patent infection on subsequent clinical episodes. We used Kaplan-Meier curves with inverse probability weights (IPW) to compare the average 60-day risk of a future symptomatic infection for sub-patently infected participants and uninfected participants. We used weighted Kaplan-Meier curves to estimate an adjusted risk difference for symptomatic rapid diagnostic test (RDT)-positive malaria. We hypothesized that the average two-month risk of clinical malaria would be the same for sub-patently infected participants compared to symptomatic but uninfected participants. We also used parasitic genomic information to describe whether RDT positive infections following a sub-patent episode represents a new or recurring infection.

Aim 2: Determine the relationship between parasite density in humans and successful human-to-mosquito malaria transmission. We used logistic regression with generalized estimating equations (GEE) to a) estimate the association between parasite density and transmission at the infection pair-level, b) investigate modification by age, and c) evaluate the demographic, behavioral, clinical, and parasitological factors associated with successful transmission of *P. falciparum* from humans to mosquitoes. We hypothesized that low parasite density infections, which can be symptomatic or asymptomatic, are an important contributor to the infectious reservoir and will be associated with an increased probability of human-to-mosquito malaria transmission compared to higher density infections in this population.

Understanding the contribution of parasite density and other factors to successful human-to-mosquito transmission helps to identify and characterize the infectious reservoir. This research 1) explains the clinical implications of not treating sub-patent infections and 2) enables

better population-based estimates of transmission potential, which is imperative to malaria control.

CHAPTER II: BACKGROUND

Malaria Epidemiology

Malaria is a leading cause of illness and death among children under 5 years in sub-Saharan Africa. Malaria is caused by *Plasmodium spp.* parasites, which are spread to people through the bites of infected female *Anopheles* mosquitoes. Of the five *Plasmodium* species that cause malaria in humans, *P. falciparum* is the deadliest and most prevalent on the African continent.[10] Initial symptoms are fever, headache, and chills, which typically occur 10-15 days after an infectious mosquito bite and can be difficult to recognize as malaria. *P. falciparum* malaria can progress to severe illness and death within 24 hours if left untreated.[10]

In 2020, the WHO Africa region accounted for about 95% of malaria cases and 96% of malaria deaths globally[10]. Malaria incidence and mortality rates in the WHO Africa region had been consistently declining since 2000 but have plateaued in recent years[3]. About 80% of deaths in this region occurred in children under five.[10] Infants, children, pregnant women, and people with HIV/AIDS are extremely vulnerable to developing severe disease or death.[3,11] Kenya is one of 29 countries that contributed to 96% of global malaria deaths, despite increases of key malaria reducing interventions. About 3.5 million new clinical cases and 10,700 malaria-related deaths are reported in Kenya each year.[12] Vector control and improved clinical management have reduced morbidity and mortality in areas like Nairobi where the malaria prevalence in 2020 was estimated to be <1%. However, in western Kenya, particularly in areas near Lake Victoria, malaria prevalence was around 19% in 2020.[13]

Biology of *P. falciparum*

P. falciparum has a complex life cycle that involves cyclical infection of humans and female *Anopheles* mosquitoes. When an infected mosquito takes a blood meal from a human, it

transmits sporozoites into the bloodstream. The sporozoites travel to the liver and invade hepatocytes.[14,15] Within the hepatocytes, the sporozoites undergo asexual replication and form schizonts. The schizonts rupture, releasing merozoites into the bloodstream. Merozoites infect red blood cells (RBCs) and become trophozoites, which asexually reproduce to form more merozoites. This ruptures the RBCs, releasing merozoites back into the bloodstream to infect more erythrocytes. This blood stage during the lifecycle is responsible for the development of clinical symptoms in the human host.[14]

Some parasites enter new RBCs and sexually differentiate into sexual erythrocytic stages (gametocytes). Most gametocytes are cleared by the immune system, but male and female gametocytes can be ingested by an *Anopheles* mosquito during a blood meal[16]. Symptoms may prompt infected individuals to get tested and treated with artemisinin combination therapy (ACT) such as artemether/lumefantrine, which targets the asexual blood stage parasites. Since gametocytes take 8-12 days to fully mature, treatment during this blood stage will help to prevent gametocytes from reaching infectious levels[17]. However, if gametocytes are allowed to mature, human to mosquito transmission is more likely to occur.

Finally, the male and female gametocytes that are ingested by a mosquito develop into gametes, which combine to form a zygote which then develops into an oocyst. The fully developed oocyst ruptures, releasing sporozoites that travel to the salivary glands of the mosquito where they can be transmitted to a human.[14,18]

Clinical Manifestations of Malaria

The incubation period for *P. falciparum* malaria is about 10-15 days after an infectious bite from an *Anopheles* mosquito[19]. Commonly reported symptoms are fever, sweats, headache, chills, malaise, muscle aches, nausea, and vomiting. In some cases, malaria can become severe and might cause kidney failure, seizures, coma, and death[20]. A substantial number of people infected with *P. falciparum* are asymptomatic[15]. *Asymptomatic infections* are defined as people who are infected with parasites but do not have clinical symptoms and,

therefore, have not been treated with antimalarials.[21] While some asymptomatic infections are pre-symptomatic and will progress to clinical disease, others will not, due to partial immunity to parasites[21,22]. Repeated *P. falciparum* infections can cause exposure-related immunity. This can allow parasites to persist without the development of symptoms or can suppress parasite density altogether[22].

Prevention, Detection, and Treatment

Prevention

In the last two decades, much of sub-Saharan Africa achieved high levels of intervention coverage. Between 2000 and 2015, the prevalence of *P. falciparum* infection in endemic areas of Africa halved and the incidence of clinical malaria decreased by 40%[23]. Vector control and preventive chemotherapies had a major impact in reducing the burden of malaria. Vector control, including insecticide-treated nets (ITNs) and indoor residual spraying (IRS), is highly effective in reducing transmission.[10] As a whole, interventions between 2000 and 2015 prevented an estimated 663 million cases of malaria, and ITNs alone were responsible for 68% of all cases averted[23]**.**

Preventive chemotherapy includes chemoprophylaxis, intermittent preventive treatment of infants (IPTi) and pregnant women (IPTp), seasonal malaria chemoprevention (SMC) and mass drug administration (MDA).[10] These types of interventions are usually targeted to specific groups at risk for severe disease, like children and pregnant women.[24] These strategies aim to prevent *P. falciparum* infections and to complement ongoing malaria diagnosis and treatment of confirmed cases. Additionally, as of October 2021, the WHO also recommends the use of the RTS,S/AS01 malaria vaccine for children in regions with moderate to high *P. falciparum* transmission.[1]

Detection

Microscopy is considered the gold standard of malaria detection and can provide information on the severity of disease.[8,25] The sensitivity and specificity of microscopy can vary

greatly depending on a number of factors. For example, a highly-trained microscopist could detect parasites at 5 parasites per microliter of blood while an average laboratory worker might only be able to detect a positive case of malaria at 50-100 parasites/microliter of blood.[25] Rapid diagnostic tests (RDTs) are commonly used in the field and do not require trained personnel to administer. RDTs detect a high proportion of clinical malaria. However, the limit of detection is about 100-200 parasites/microliter and there is much evidence showing that *P. falciparum* infections below that limit can still transmit to mosquitoes.[7,8,25] The vast majority of RDTs for *P. falciparum* detect histidine rich protein (HRP) 2 or the related HRP3 protein. Mutations in the gene that encodes HRP2 causes some *P. falciparum* parasites not to express it[26]. First discovered in the Peruvian Amazon about 14 years ago, deletion of *P.* falciparum HRP2 genes has since been detected in many malaria-endemic countries[27]. HRP2 deletions threaten the utility of RDTs as they increase the number of false negative RDT results[28]**.**

Detection by polymerase chain reaction (PCR) requires trained personnel and more expensive equipment so it is not appropriate for widespread use in clinical care. However, it can detect much lower density infections and is an important tool for research.[29,30] A systematic review of asexual *P. falciparum* prevalence by microscopy and PCR estimated that microscopy detected about 50% of all PCR-detectable infections. This proportion, however, varied by location, demographics, transmission intensity, and seasonality. For example, in areas with high transmission (PCR prevalence > 75%), microscopy detected about 75% of infections[31]. In contrast, in areas with low transmission (PCR prevalence <10%), microscopy only detected 12% of infections.

Newer HS-RDTs have a lower limit of detection compared to conventional RDTs. It is unclear whether using these more sensitive diagnostic tests will improve patient care[32,33]. Few studies have directly investigated the clinical implications of using an HS-RDT compared to a conventional RDT, and those that have did not find any benefits for clinical diagnosis.[33,34]

However, there may be population-level benefits for human-to-mosquito transmission by detecting more low-density infections with a more sensitive diagnostic tool.

Treatment

The WHO's core principles of case management include early diagnosis and treatment, rational use of antimalarial agents, combination therapy, and appropriate weight-based dosing.[35] Previously, treatment policies recommended monotherapy with drugs including chloroquine, amodiaquine, and sulfadoxine-pyrimethamine. The growing threat of drug resistance jeopardized the effectiveness of these therapies[35] Malaria-endemic countries have since adopted artemisinin-based combination therapies (ACTs), which are more effective and more likely to kill a parasite that develops resistance to one of the drugs[36]. Guidelines are country specific, but in sub-Saharan Africa WHO strongly recommends treating children and adults with uncomplicated malaria with a combination of artemether + lumefantrine or artesunate + amodiaquine.[35]

Malaria Elimination

The WHO has set ambitious targets of reducing global malaria case incidence and mortality rates by at least 90% by 2030 compared to the 2015 baseline, eliminating malaria in at least 35 countries, and preventing it's re-establishment in all malaria-free countries.[10,37] The WHO defines malaria elimination as "the interruption of local transmission of a specified malaria parasite species in a defined geographical area as a result of deliberate activities."[10] Elimination also requires ongoing measures to prevent re-establishment of transmission. This is distinct from eradication, which is when all countries have achieved elimination with zero cases globally[38]. In 2020, 26 formerly-endemic countries reported fewer than 100 indigenous cases of malaria. After three consecutive years of zero indigenous cases, a country is eligible to apply for the WHO certification of malaria elimination.[10] Countries that have been certified as malaria free include United Arab Emirates, Morocco, Turkmenistan, Armenia, Argentina, Kyrgyzstan,

Uzbekistan, Paraguay, Sri Lanka, and Algeria. Between 2000 and 2019, none of the certified malaria-free countries reported transmission reestablishment[37].

Low Parasite Density and Sub-Patent Infections

Acquired immunity in infected individuals helps to control parasite density[15]. People with repeated exposures to parasites experience lower parasite densities compared to people with fewer exposures to parasites[39]. Adults, for example, are more likely to have low density infections than children because they have had more opportunities for exposure to parasites. Many low-density infections are considered sub-patent, defined as those that are present by molecular detection but absent by clinical diagnostics like microscopy or RDT[5]. Although sub-patent infections are mostly asymptomatic, people with symptoms suggestive of malaria can also harbor sub-patent *P. falciparum* infections. Since sub-patent infections, even if symptomatic, are not diagnosed as malaria with conventional diagnostics, they are untreated. The impact of missing sub-patent infections on the risk of future clinical illness is not fully understood. These untreated sub-patent infections also provide more opportunities for a mosquito to ingest gametocytes and serve as a reservoir for onward transmission.

The Human Infectious Reservoir

Progress toward malaria elimination has stalled, in part, due to an infectious reservoir of parasites in humans who transmit malaria parasites to mosquitoes[2,40–43]. The understanding of demographic, human behavioral, clinical, and parasitological factors associated with successful transmission to mosquitoes in a natural setting is incomplete. It is essential to identify the people contributing to this infectious reservoir since it helps sustain the parasite reservoir in mosquitoes and malaria transmission in the community.[2] Malaria persists in areas with high levels of vector control and treatment availability, which has led to increased efforts to describe and target the reservoir in different settings[2,44,45]. One priority is to determine the relative contribution of symptomatic malaria, asymptomatic malaria detectable with microscopy, and low-density infection detectable by molecular methods to the infectious reservoir[2].

Advances in methods to quantify malaria transmission have helped to characterize the human infectious reservoir. Mosquito feeding experiments use a variety of different techniques to measure the transfer of parasites to mosquitoes. During skin feeding assays, mosquitoes are put in contact directly with an infected human's skin and allowed to feed. Membrane feeding techniques involve allowing reared anopheline mosquitoes to feed through membrane feeders.[46] Of these artificial techniques, skin feeding assays provide more realistic conditions, but neither method replicates natural biting patterns. Skin feeding is also not always ethically justifiable, particularly when it involves young children. Membrane feeding assays are a more accepted ethical solution.

Parasite Density and the Infectious Reservoir

The relationship between parasite density in human infections and transmissibility to mosquitoes is poorly understood. Sub-patent *P. falciparum* infections that occur below the parasite density threshold of microscopy and RDTs are more likely to be asymptomatic and untreated, and can still be infectious to mosquitoes.[2,5] These low-density infections contribute to the infectious reservoir and human-to-mosquito transmission.

There is evidence that gametocyte density in a human infection has a strong positive association with the proportion of infected mosquitoes.[42,47] Despite the higher mosquito infection rates among those with high gametocyte density, submicroscopic gametocyte carriers are estimated to be responsible for about 15-24% of human-to-mosquito transmission.[42,48–50]

Multiple studies have investigated the association between asexual parasite density and infectivity to mosquitoes, but findings have not been consistent[40,51–53]. For example, a study in Ethiopia of people with microscopy- and PCR-detected infections used membrane feeding to measure contributions to the infectious reservoir across parasite densities and found no association between the proportion of infected mosquitoes and asexual parasite density[52]. In contrast, a membrane feeding study in children in Burkina Faso and Kenya observed an association between asexual parasite density and infectivity. Children with submicroscopic

infections were more infectious to mosquitoes than children with densities between 1 and 1000 parasites per microliter, but were less infectious to mosquitoes than those with over 1000 parasites per microliter[40].

Although there is some conflicting evidence about the association between total parasite density and infectivity to mosquitoes, studies have shown that low density infections that are not detectable by microscopy are responsible for a substantial amount of mosquito infections[5,54]. *P. falciparum* infections with low densities account for a significant proportion of all malaria infections, and in areas with low endemicity, they can make up the majority of infections, which highlights the scope of this source of transmission[15,21]. Rural areas like western Kenya often lack the advanced diagnostic tools and trained personnel necessary to detect low parasite density infections using microscopy or molecular methods. Improving diagnostic capabilities and access will allow for rapid detection of infections across different parasite densities. There is an urgent need for more sensitive diagnostic tests that can be easily integrated into rural and low resource settings.

Asymptomatic Infections and the Infectious Reservoir

It is well known that asymptomatic human infections can generate gametocytes and infect mosquitoes.[55] In malaria-endemic areas, chronic infections often lead to partial immunity to parasites and asymptomatic infections.[21,56] Asymptomatic *P. falciparum* infections are important contributors to this reservoir as they often go undetected but can still be infectious.[2,6,21,57,58] Individuals with asymptomatic infections are unlikely to seek treatment and, therefore, are missed by passive surveillance systems, which rely on symptomatic testing and treatment.[59] This allows parasites to persist in the population at low densities and fuels onward transmission.

Not only are asymptomatic human infections capable of infecting a mosquito, but there is also evidence that these infections are more likely to transmit to a mosquito compared to symptomatic infections. Fever, for instance, has been strongly associated with transmission

failure.[60] A Ugandan longitudinal study quantified the contribution of asymptomatic and symptomatic infections to the reservoir through membrane feeding assays. Based on the proportion of mosquitos infected, they observed that asymptomatic microscopy-detected infections were responsible for 83% of the reservoir and asymptomatic submicroscopic infections were responsible for 15.6%, while symptomatic infections were only responsible for 0.6%.[50] There is still debate on the exact contribution of asymptomatic malaria to onward transmission and on the best method to find and treat these infections. Some advocate for testing and treating asymptomatic individuals while others believe that mass drug administration is the most effective measure.[59]

Age and the infectious reservoir

Age is associated with infectiousness to mosquitoes; however, there is a lack of understanding about the relative contribution of different age groups to onward transmission. Individual children are generally thought to be more infectious to mosquitoes compared to adults and make up a substantial proportion of the infectious reservoir, with children under 15 contributing to over half of all infectious mosquitoes[47–50,61]. While some studies determined that children aged 5-15 years were more likely to infect mosquitoes[40,62], another found that children under 5 had similar levels of infectivity to school-age children [48]. Adults also contribute to the infectious reservoir. While children have a higher individual risk of transmission compared to adults, adults have higher exposure to mosquitoes due to the larger surface area on their bodies. Accounting for this difference in exposure balances their contribution to the reservoir on the population level[48,63].

Seasonality of Malaria Transmission

Malaria incidence often varies seasonally; however, this variation is not consistent each year or between different locations. Infections and symptomatic episodes are more common during the high transmission season, which usually occurs following seasonal rains when mosquitoes are most abundant[64]. Although the drivers of seasonality are complex and not fully

understood, many studies use proxies for seasonality to incorporate this variation into their research and to define different transmission seasons. Mosquito abundance, malaria incidence, and climate factors including temperature and rainfall are all common proxies for seasonality[65]. In areas of seasonal transmission, asymptomatic infections during the dry season seed outbreaks when mosquito vectors reappear during the rainy season.[66] Knowledge of the seasonality of malaria transmission can also inform interventions that are more effective if implemented at seasonally optimal times. SMC was an effective strategy at reducing malaria morbidity and mortality in areas of west and central Africa[67].

Summary

In rural sub-Saharan Africa, where high quality microscopy is not always available, symptomatic people undergo testing with conventional RDTs, which adequately detect parasites at densities associated with clinical disease.[68] However, there is evidence that some symptomatic infections are not detectable by RDTs.[69,70] There is limited information describing the natural course and clinical implications of sub-patent *P. falciparum* infections among people with symptoms suggestive of malaria. Findings from this dissertation study will inform decision-making in high-transmission settings for treatment of people with suspected malaria and for the choice of diagnostics to evaluate suspected malaria.

While mosquito feeding studies are useful for understanding the biological mechanisms that govern transmission, they do not capture natural mosquito behavior and the complex dynamics of infection in high transmission settings.[2] Factors that are critical to transmission such as variance in behaviors among mosquito vectors, parasite replication rates, and participant exposure to vectors are not captured in such mosquito feeding experiments. [2,48,61] Interrupting human-to-mosquito transmission by identifying the stable and modifiable risk factors for membership in the infectious reservoir is essential to move toward malaria elimination. The infectious reservoir can be targeted by interventions like the scale up of long-lasting insecticide treated nets, indoor residual spraying, improved diagnostics, and broad use of artemisinin-

based combination therapy.[71] Efficient and effective interventions might help overcome the plateau in progress over the last few years and result in a significant decrease in malaria morbidity and mortality.[2] The results of these analyses will provide new insight into mosquito-human interactions that enhance parasite transmission and enabled us to more precisely define contributions to the infectious reservoir across the spectrum of parasite densities. With this understanding, we will enable better population-based estimates of transmission potential and will provide evidence for the rational targeting of malaria control interventions.

CHAPTER III: METHODS

Data Source

Data from this study come from a longitudinal prospective cohort in Webuye, a town in Western Kenya in Bungoma East sub-county. Webuye is a rural community midway between Eldoret and the Ugandan frontier. Malaria transmission is perennial, with a seasonal peak following the rains in May-June and is primarily transmitted by *Anopheles gambiae s. l* (89.9%) and *An. Funestus* (6.2%)[72]. Ninety percent of homes have at least one insecticide-treated net, yet prevalence of infection in children less than 10 years old approaches 50% during the rainy season.[73]

Study Population

The cohort first enrolled in 2017 with 38 households in three villages. In 2020, the cohort expanded to 75 households across 5 villages. Cohort households were selected by randomly identifying a starting household using a village roster and then enrolling all members of surrounding households until 12 households had been enrolled in that village. Enrollment was based on household membership, irrespective of gender or age, so the cohort demographics resemble that of the surrounding community. Study staff offered enrollment to all members over one year of age in participating households. All adult participants provided written informed consent and participants aged 1-18 years were included if their parent or legal guardian provided written informed consent for them. We also obtained verbal assent from children older than eight years.

Specimen and data collection

Data for these analyses were collected from June 2017 to November 2021. On a monthly basis, the field team collected dried blood spots and administered demographic and

behavioral questionnaires for each participant. Information was collected about sleeping location, bed net usage, bedtimes, and travel. Participants experiencing symptoms contacted study staff as needed; staff collected dried blood spots (DBS) and tested for *P. falciparum* infection using an RDT (Carestart © Malaria HRP2 *Pf*, Accessbio)[74]. If the RDT results were positive, participants were treated with Artemether-Lumefantrine.

Field entomologists collected resting mosquitoes weekly from each household by vacuum aspiration with a Prokopack[75]. Collection occurred in the morning before doors and windows were opened. Mosquitoes were killed with chloroform and sorted by genus and sex. Female *Anopheles* mosquitoes were dissected and preserved.

Participant and Mosquito Sample Processing

Human dried blood spots and dissected mosquito abdomens were shipped to Duke University for molecular detection and quantification of *P. falciparum.* Genomic DNA (gDNA) from each sample was tested in duplicate for *P. falciparum* using a real-time quantitative polymerase chain reaction assay targeting the *P. falciparum pfr364* motif[76,77]. Parasite densities were estimated using plate-specific standard curves generated from amplifications of templates with known parasite density[6]. Parasite density was expressed as the number of parasites per microliter of blood. For the first 14 months of the study,[78] *P. falciparum*-positive samples were genotyped at *Plasmodium falciparum* circumsporozoite protein (*pfcsp)* using PCR amplification and sequencing on an Illumina MiSeq[78–80] . Following quality-filtering[80–82] haplotypes were identified using DADA1 (version 1.8) in R (version 4.1.3)[83,84], and haplotypes were filtered to mitigate false discovery risk using previously validated criteria[85].

Estimating Transmission

The outcome for Aim 2 of this dissertation, the probability that a human-mosquito pairing represented a transmission event, was calculated for a previously published analysis of this cohort.[9] In that study, human participants and mosquitos were matched based on time and distance. Mosquitoes were matched to a particular participant and DBS time point if they were

caught between 7 days before and 14 days after the DBS was collected and caught within or near (0.55 km) of a participant's home. Genotyping using next generation amplicon deep sequencing techniques allowed us to identify shared haplotypes between human and mosquito infections and to estimate the probability that each match represented human-to-mosquito transmission. This probability was estimated as a function of the degree of haplotype sharing. This was computed as:

$$P(TE_h) = (1 - \prod_{n=1}^{s} PopPrev_n{}^{1/3})(\frac{s}{MOI_i})$$

Where s=the number of haplotypes shared between a human and mosquito pair, PopPrev = the prevalence of the genotype across the entire population, and MOI_i= the person's multiplicity of infection (MOI), which refers to the number of unique haplotypes in each participant's sample.

Data Analysis

Aim 1 Analysis

The analysis for Aim 1 was conducted using data from the full 54-month follow-up period. This cohort includes 757 participants across 75 households and 5 villages (Kinesamo, Maruti, Sitabicha, Nangili, and Lurare). Our primary analysis population included symptomatic episodes experienced by cohort participants that were RDT-negative. We also defined two secondary sub-populations. The *febrile* population included episodes during which the participant's measured temperature exceeded 37.4C, they reported a recent history of fever, or both. The *low-density* population comprised episodes in which the parasite density in the RDT-negative infection was ≤100 parasites per microliter. We conducted stratified analyses by age group (<5, 5-15, >15 years) and transmission season (low and high), categorized by the number of female Anopheline mosquitos collected across the study site in the 14 days prior to evaluation into low (≤75) or high (>75).

We divided the analytic population into exposed and unexposed episodes based on real time PCR positivity for *P. falciparum*. RDT negative episodes with PCR positivity were

considered sub-patent (exposed), while those with PCR negativity were considered uninfected (unexposed). For all analyses, the outcome was clinical malaria, defined as symptomatic, RDT-positive *P. falciparum* infection that was observed within 60 days of the index RDT-negative event.

We used stabilized inverse probability (IP) weighted Kaplan-Meier curves to compare the average 60-day risk of clinical malaria for sub-patent and uninfected episodes and to calculate adjusted risk differences. We used logistic regression to calculate IP weights to account for confounding by age, sex, bed net use, and transmission season (see **Supplementary Information)**. We also used logistic regression to calculate IP weights to account for informative censoring by age and transmission season (see **Supplementary Information)**. For both sets of weights, the minimally-sufficient adjustment set of covariates for inclusion was determined by directed acyclic graph (DAG) analysis (**Figures S4.2 and S4.3)**. Weights were multiplied together and applied to the Kaplan Meier curves.[86] We calculated weighted risk differences between groups using IP-weighted Kaplan Meier curves and used bootstrapped standard error estimation to calculate 95% confidence intervals. We repeated these statistical methods in our secondary febrile and low-density populations and in stratified analyses to assess modification by age and transmission season.

Aim 2 Analysis

The analysis for Aim 2 was conducted among 198 participants across 38 households who contributed 3,727 human and mosquito pairs during the first 14 months of follow-up. The main exposure was parasite density, which was defined as the number of parasites detected per microliter in participant dried blood spots. We dichotomized parasite density into low (≤200 parasites per microliter) and high (>200 parasites per microliter) based on common limits of detection for diagnostic tests in the field[7,68]. The outcome for this aim was the probability that a human and mosquito pairing represented a transmission event based on the degree of haplotype sharing, which was calculated for a previously published analysis[78]. The distribution

of the probability of human-to-mosquito transmission due to haplotype sharing is shown in **Supplementary Figure S5.8.**

We considered a few different approaches to model the probability that a pairing represented transmission in order to account for the uncertainty in our estimation. The first approach was modeling the probability continuously using logistic regression. Next, we modeled transmission as a binary outcome using the probability as a weight. In this model, we duplicated the data set and assigned one copy as events and the other as nonevents. We then gave episodes with events a weight equal to the probability of transmission, and gave those without an event a weight of one minus the probability of transmission. Finally, in the third model, we used a quantitative bias analysis approach. We imputed transmission for each person and ran the analysis 5000 times, plotted the distribution of estimates, and calculated the median point estimate of these replicates. The results from these models are in **Table 3.1.**

The estimates from all three models were the same and standard errors were very similar. Therefore, we chose to use the continuous outcome approach. Due to convergence issues when fitting a multilevel model, we used logistic regression with generalized estimating equations (GEE) to account for repeated measures at the participant level. (**Table 3.1**).

Table 3.1. Results comparing different modeling approaches for the probability of transmission

Modeling Method	Odds Ratio for Low vs High Parasite Density**	SE
Modeling Transmission Variable		
Continuous outcome logistic regression	1.61	0.12
Binary outcome, weighted logistic regression	1.61	0.12
Quantitative Bias Analysis	1.61	0.10
Accounting for clustering		
Multilevel logistic regression model*	--	--
Continuous outcome logistic regression with GEE	1.79	0.11

*Model did not converge
**Adjusted for age, sex, transmission season, infection type, and village

For the primary analysis, we compared the odds of that a pairing represented human-to-mosquito transmission between low and high parasite density infections using logistic regression with generalized estimating equations to account for repeated measures. We controlled for confounding by age, sex, village, infection type, and transmission season as indicated by a direct acyclic graph (DAG) (**Figure S5.2**). Effect measure modification by age was assessed by including an interaction term in the model and calculating stratum specific estimates. We next evaluated sex, age, transmission season, bed net use, and infection persistence as risk factors for transmission. We restricted the analysis population for the infection persistence model to human-mosquito pairings with asymptomatic human infections, which are more likely to be persistent. For each risk factor, we used a DAG to identify appropriate covariates to control for confounding (**Figures S5.3 – S5.7),** and fit a separate logistic regression model with GEE.

CHAPTER IV: RISK OF MALARIA FOLLOWING UNTREATED SUB-PATENT *PLASMODIUM FALCIPARUM* INFECTIONS: RESULTS OVER 4 YEARS FROM A COHORT IN A HIGH TRANSMISSION AREA IN WESTERN KENYA

Introduction

WHO recommends parasitologic confirmation before treatment for malaria to enhance rational use of antimalarials [87]. Testing options include smear microscopy and rapid diagnostic tests (RDT), which have different lower limits of detection. RDTs for *Plasmodium falciparum*, which typically detect histidine rich protein (HRP) 2, have largely replaced microscopy as the standard diagnostic tool for malaria [88]: In 2021, over 400 million RDTs were sold globally[3]. While conventional RDTs can detect quantities greater than 100-200 parasites per microliter of blood, many *P. falciparum* infections are below the limit of detection of conventional RDTs and missed in routine testing [68,89,90]. More sensitive diagnostics, such as high-sensitivity RDTs and molecular detection methods, can detect lower parasite densities, [32].but these methods are not in wide clinical use. Some sub-patent *P. falciparum* infections, defined as those that are present by molecular test detection but absent by clinical diagnostic tests, could progress to clinical malaria.

It is unclear whether detecting lower parasite density infections would enhance the management of suspected malaria. The natural history is incompletely understood of low-density infections in different endemic settings[69,70,89]. Clear evidence exists that some infections in symptomatic people remain undetected by conventional RDTs [69,70], but few studies have directly investigated the natural course and clinical consequences in symptomatic patients of these *P. falciparum* infections. One study of febrile children found no difference in negative clinical outcomes between those with untreated low-density infections and no infections[6]. Longitudinal studies of the natural history of low-density infections and the clinical

consequences of untreated low-density infections are needed [91]. Estimating the risk of future clinical sequalae for those whose infections are not detected by RDTs can inform treatment decision-making in high-transmission settings for people with suspected malaria and for the choice of diagnostics to evaluate suspected malaria.

We investigated the clinical consequences of untreated sub-patent *P. falciparum* malaria infection among symptomatic patients with negative RDT tests. Using data from a 54-month longitudinal cohort in a high-transmission setting in Western Kenya, we compared between those with sub-patent *P. falciparum* infections and those uninfected the risk of subsequent clinical malaria, defined as symptomatic, RDT-positive infection. We hypothesized that, because RDTs are believed to adequately detect parasites at densities that routinely cause clinical symptoms [68,89], the 60-day risk of symptomatic RDT-positive malaria would be similar between sub-patently infected and uninfected people.

Methods

Ethical statement

The study protocol was approved by the ethical review committees of Duke University (Pro0008200) and Moi University (2017/36). University of North Carolina at Chapel Hill's Institutional Review Board (IRB) deemed the analyses presented in this paper exempt. We obtained written informed consent from all participants or their parent for those under 18 years, who also provided assent if greater than 8 years.

Study site and participants

We analyzed data collected from a cohort of people aged 1 – 85 years living in 75 households in Webuye, Western Kenya. The cohort first enrolled in 2017 with 38 households in three villages selected by radial sampling in an area of high malaria transmission [6,72]. In 2020, we expanded to 75 households across 5 villages. Throughout the study, when participants experienced symptoms of suspected malaria, they contacted study staff who administered an RDT (Carestart © Malaria HRP2 *Pf*, Accessbio) using capillary blood [74]. RDT-positive

participants were treated with Artemether-Lumefantrine. Participants also provided dried blood spots (DBS) at the time of RDT testing. Additionally, as previously described [78], households were visited weekly for morning collections by vacuum aspiration of mosquitos, which were morphologically graded for genus and sex. Demographic and behavioral questionnaires were administered monthly.

Sample processing procedures

Sample processing has been previously described [6]. Briefly, each DBS was tested for *P. falciparum* using a real-time quantitative PCR assay, which consistently detects parasite densities down to 0.1 parasites per microliter of whole blood [92]. For the first 14 months of the study, *P. falciparum*-positive samples were genotyped using amplicon deep sequencing to identify haplotypes [78].

Exposure, outcome, and covariate assessment

After excluding symptomatic RDT-positive episodes which resulted in treatment, we divided the analytic population of symptomatic RDT-negative episodes into exposed (sub-patent) and unexposed (uninfected) episodes based on positivity for *P. falciparum* by real time PCR. We excluded 40 episodes (6% of the data) with either inconclusive RDTs (n=2) or missing DBS (n=38). For all analyses, the outcome was clinical malaria, defined as symptomatic, RDT-positive *P. falciparum* infection observed within 60 days after the index RDT-negative episode. We included age, sex, transmission season, and bed net use as covariates. We categorized age (<5, 5-15, and >15 years) using standard categories [24,55]. Transmission season was categorized by the number of female Anopheline mosquitos collected across the study site in the 14 days prior to evaluation into low (<=75) or high (>75). From May-July 2020, mosquito collection was interrupted owing to the COVID-19 pandemic, and these months were categorized as high season based on historical patterns. Bed net use was assessed during a monthly behavioral survey, and regular use was defined as reporting sleeping under a bed net

more than five nights in the week preceding the study visit. If bed net use data were missing, we used information from the previous month's behavioral questionnaire.

Analysis population

Our primary analysis population consisted of all symptomatic, RDT-negative episodes experienced by cohort participants who had not received antimalarials for their current illness. We also defined two secondary sub-populations. The febrile population comprised episodes during which the participant's measured temperature exceeded 37.4C or they reported a recent history of fever, or both. The low-density population comprised all uninfected episodes, and sub-patent episodes in which the parasite density in the infection was ≤100 parasites per microliter, in order to account for potential RDT technical failures.

Statistical Analysis

To compare the incidence of subsequent clinical malaria between symptomatic episodes with and without sub-patent *P. falciparum*, we used stratified Kaplan Meier survival curves for clinical malaria after exposed and unexposed episodes. We used stabilized inverse probability (IP) weights to account for confounding by age, sex, bed net use, and transmission season. In addition, we used stabilized IP weights to account for informative censoring by age and transmission season. For both sets of IP weights, directed acyclic graph (DAG) analyses determined the minimally sufficient adjustment set of covariates. We estimated both sets of weights using logistic regression, multiplied them together and applied them to the Kaplan Meier function [86]. We calculated weighted risk differences between groups using IP-weighted Kaplan Meier curves and used bootstrapped standard error estimation to estimate Wald-type 95% confidence intervals. We repeated these statistical methods in our secondary febrile and low-density sub-populations and in all sub-analyses.

The unit of analysis was an episode; thus, participants could contribute more than one RDT-negative episode to the analysis. Participants were followed until they developed clinical malaria or were censored at 60 days, whichever came first. If a participant had another RDT-

negative episode within 60 days of an index episode, the index episode was censored, and they re-entered the analysis as a new RDT-negative episode. For those who developed clinical malaria within 60 days, we calculated summary statistics for the number of days in between an index episode and clinical malaria event. We repeated these analyses within subgroups defined by transmission season (high and low) and age group (under 5, 5-15, and over 15 years) to assess modification. We also conducted sensitivity analyses to test the effects of different censoring criteria (**see Supplemental Materials and Table S4.1**).

Using parasite genotype data from the first 14 months of the study, we compared the unique *pfcsp* haplotypes detected in sub-patent index infections and future RDT-positive infections.

Results

Index episode characteristics

Within our main cohort of 757 participants, we observed at least one symptomatic, RDT-negative episode in 347 participants (**Table 4.1**). This subgroup experienced 1,128 RDT-negative episodes, of which 59.9% were in females, 56.0% were among participants older than 15 years, 69.9% occurred during low transmission season, and 80.3% occurred in people reporting regular bed net use. The most commonly reported symptoms prompting RDT testing were aches (41.0%) and fever (39.0%).

Across these 1,128 symptomatic, RDT-negative episodes, 400 (35.5%) were real-time PCR positive for *P. falciparum* (**Table 4.1**). The proportion of PCR-positive episodes was significantly higher in the high compared to low transmission season (43.2% vs. 30.5%; $p<0.0001$) and was negatively associated with fever ($p=0.03$), but was not associated with village, sex, age, or other covariates. Among these 400 sub-patent infections, the median parasite density was 1.02 parasites per microliter of blood (IQR: 0.34 – 8.24), consistent with the negative result by RDT (**Figure 4.1**).

Clinical malaria events

Clinical malaria occurred following 7.7% of RDT-negative episodes (n=87). The median time to event was 25 days (IQR: 15 – 41), the median parasite density for outcome events was 864 p/μL (IQR 45.1, 6840.2), and the most common symptoms prompting repeat RDT testing were fever (82.8%) and aches (46.0%). Using weighted Kaplan-Meier survival analysis, the overall 60-day risk of clinical malaria following a symptomatic, RDT-negative evaluation was 7.7% (95% CI: 6.0%, 9.4%).

Table 4.1. Characteristics of symptomatic RDT-negative episodes

		P. falciparum Real-time PCR result		
Variable	**Overall**	**Positive**	**Negative**	**p-value***
N (%)	1,128	400 (35.5)	728 (65.5)	
Village, N (%)				0.07
Kinesamo	353 (31.3%)	123 (30.8%)	230 (31.6%)	
Lurare	77 (6.8%)	20 (5.0%)	57 (7.8%)	
Maruti	263 (23.3%)	98 (24.5%)	165 (22.7%)	
Nangili	81 (7.2%)	21 (5.2%)	60 (8.2%)	
Sitabicha	354 (31.4%)	138 (34.5%)	216 (29.7%)	
Sex, N (%)				0.42
Female	676 (59.9%)	246 (61.5%)	430 (59.1%)	
Male	452 (40.1%)	154 (38.5%)	298 (40.9%)	
Age, years, N (%)				0.79
<5	176 (15.6%)	59 (14.8%)	117 (16.1%)	
5-15	632 (56.0%)	229 (57.2%)	403 (55.4%)	
>15	320 (28.4%)	112 (28.0%)	208 (28.6%)	
Transmission Season‡, N (%)				<0.001
Low	788 (69.9%)	253 (63.2%)	535 (73.5%)	
High	340 (30.1%)	147 (36.8%)	193 (26.5%)	
Regular Bed Net Use‡, N (%)				0.72
No	222 (19.7%)	81 (20.2%)	141 (19.4%)	
Yes	906 (80.3%)	319 (79.8%)	587 (80.6%)	
Duration of Illness, days, median (IQR)	2.0 (1.0 – 3.0)	2.0 (1.0 – 3.0)	2.0 (1.0 – 3.0)	0.61
Symptom§, N (%)				
Fever	687 (60.9%)	227 (56.8%)	460 (63.2%)	0.03
Aches	459 (40.7%)	158 (39.5%)	301 (41.3%)	0.55
Chills	201 (17.8%)	68 (17.0%)	133 (18.3%)	0.59
Cough	181 (16.0%)	59 (14.8%)	122 (16.8%)	0.38
Congestion	131 (11.6%)	44 (11.0%)	87 (12.0%)	0.63
Vomiting	63 (5.6%)	27 (6.8%)	36 (4.9%)	0.21
Diarrhea	42 (3.7%)	18 (4.5%)	24 (3.3%)	0.31
Other‖	444 (39.4%)	148 (37.0%)	296 (40.7%)	0.23

*Pearson's Chi-squared test for categorical variables or Wilcoxon rank sum test for continuous values
†Based on the number of female *Anopheles* mosquitoes collected in the two weeks following the index episode.
‡Regular bed net use was defined as sleeping under a net more than five nights in the previous week. Data on bed net usage were available from the current monthly behavioral questionnaire for 34.4% of the episodes while the remaining 65.6% came from a previous monthly behavioral questionnaire.
§Participants could experience more than one symptom per episode
‖Other reported symptoms include headache, stomach ache, nausea, loss of appetite, fatigue, back pain, weakness, joint pain, and chest pain.

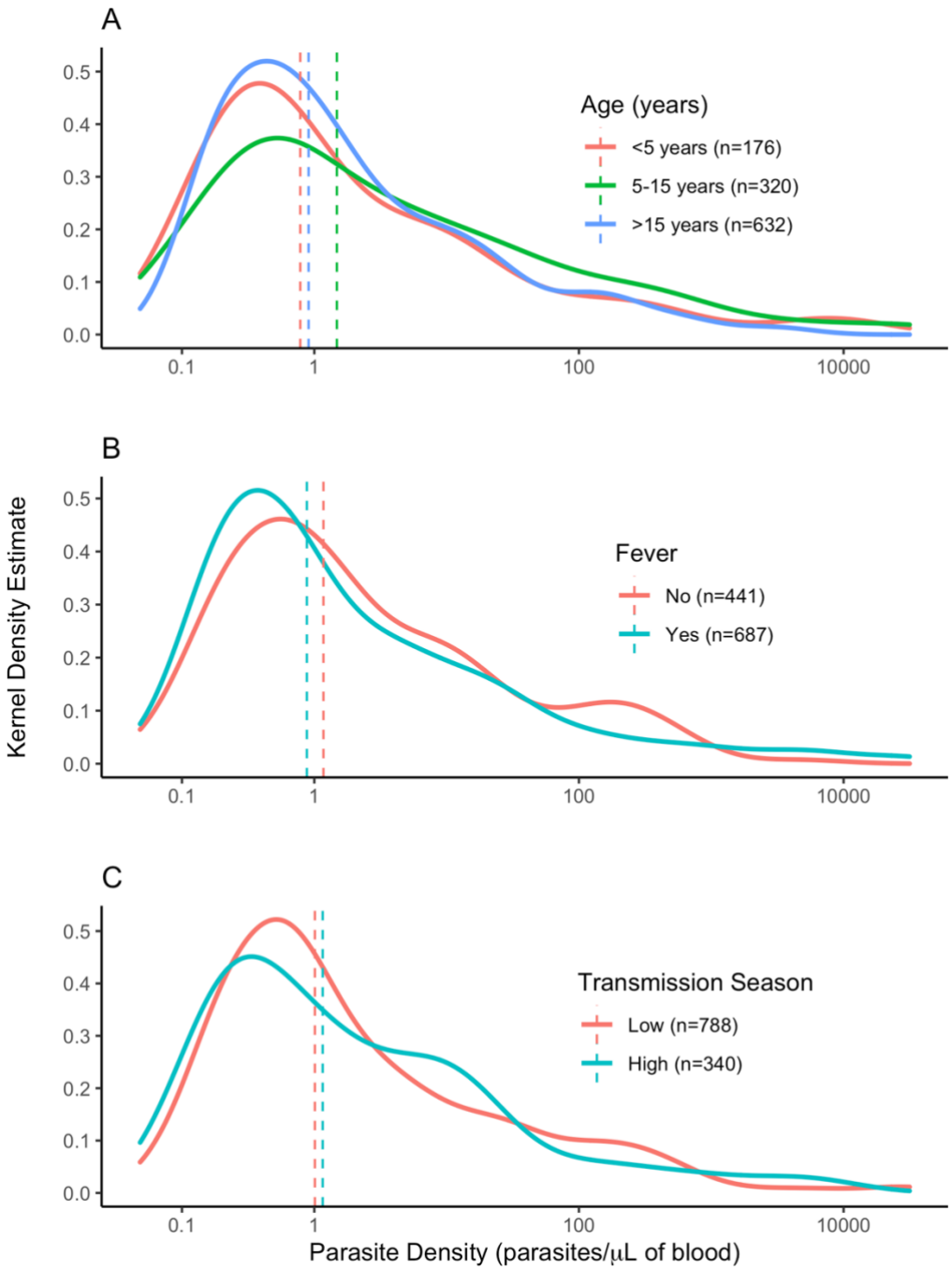


Figure 4.1. Distribution of parasite densities in RDT-negative sub-patent infections. Kernel density curves show the distribution of parasite densities across A) age groups, B) febrile status, and C) transmission seasons. The vertical lines represent the median parasite density in each group. Febrile episodes were defined as episodes during which the participant's measured temperature exceeded 37.4C or they reported a recent history of fever, or both. Transmission season was categorized by the number of female Anopheline mosquitos collected across the study site in the 14 days prior to evaluation into low (<=75) or high (>75).

Associations of sub-patent infections with clinical malaria

We recorded 34 clinical malaria events following the 400 sub-patent episodes and 53 events following the 728 uninfected episodes. In survival analyses, the risk of clinical malaria over 60 days was similar between infected (9.0%) and uninfected (8.7%) groups (Risk Difference (RD): 0.3%; 95% Confidence Interval (CI): -1.9%, 2.6%). (**Figure 4.2**), suggesting that the presence of parasites in symptomatic people who test negative by RDT does not increase the risk of clinical malaria.

We next estimated the risk of clinical malaria in the febrile and low-density sub-populations. Among 440 febrile, RDT-negative episodes, 155 (35.2%) were sub-patent episodes and 285 (64.8%) uninfected episodes. We observed 38 clinical malaria episodes, 14 following sub-patent episodes and 24 following uninfected episodes. The overall 60-day risk of malaria following febrile RDT-negative episodes was 7.6% (95% CI: 5.4%, 9.7%). Similar to the primary analysis, the risk of subsequent clinical malaria following febrile RDT-negative episodes was similar between the sub-patent (7.7%) and uninfected (9.3%) groups (RD: -1.6%, CI: -3.9%, 0.8%). We also did not observe a significant risk difference in the low parasite density sub-population (N=1,089) (RD: -0.9%, CI: -2.8%, 1.0%). In an additional analysis stratified by age, risk differences for clinical malaria between infected and uninfected episodes were minimal for children under age 5 years (RD: -2.3%, CI -8.4%, 3.1%), school-age children (RD: 1.8%, 95% CI: -2.8%, 6.3%) and adults (RD: 0.6%, 95% CI: -1.6%, 2.8%).

Surprisingly, we observed contrasting risk differences between transmission seasons. During the low transmission season, the risk of malaria was significantly higher following infected than uninfected episodes (7.1% vs 4.7%; RD: 2.3%, CI: (0.4%, 4.2%). In contrast, during the high transmission season, the risk of malaria was significantly lower following an infected than an uninfected episode (13.0% vs 17.8%; RD: -4.8%, CI: (-9.53%, -0.05%) (**Figure 4.3**).

We conducted additional analyses stratified by transmission season across our pre-defined sub-groups. Although power was limited in these stratified sub-populations, we observed that the risk of clinical malaria was consistently lower following a sub-patent episode during the high transmission season and slightly higher during the low transmission season across febrile and low parasite density sub-populations (**Figure 4.3**) and age groups (**Figure 4.4**) (**Table 4.2**).

The results of the sensitivity analyses can be found in **Supplementary Table S4.1.** We observed the same pattern of lower risk of clinical malaria following a sub-patent infection compared to an uninfected episode during the high transmission season, and a slightly increased risk during the low transmission season. Broadly, the risk differences were similar to those in the main analyses, although most were not statistically significant. Collectively, these analyses suggest that the risk of clinical malaria following a sub-patent infection is highly influenced by parasite exposure during the high and low transmission seasons.

Parasite genotypes in index and outcome infections

Parasite genotypes were available for 83 sub-patent infections, among which followed. seven clinical malaria outcomes. In five of the seven sub-patent infections, the subsequent episode of malaria shared a parasite haplotype with the initial infection (**Figure 4.5**), indicating that some malaria events following sub-patent infections were genetically related to the index infection.

Table 4.2. Results from stratified analyses by transmission season

	High Transmission Season					Low Transmission Season				
	N	**Risk in sub-patent episodes**	**N**	**Risk in uninfected episodes**	**Adjusted risk difference (95% CI)**	**N**	**Risk in sub-patent episodes**	**N**	**Risk in uninfected episodes**	**Adjusted risk difference (95% CI)**
All episodes	147	13.0%	193	17.8%	-4.8% (-9.5%, -0.05%)	253	7.1%	535	4.7%	2.3% (0.4%, 4.2%)
Fever	95	9.3%	126	19.0%	-9.8% (-15.3%, -4.2%)	132	6.4%	334	4.7%	1.7% (-0.8%, 4.3%)
Low parasite density	228	11.5%	535	17.6%	-6.0% (-10.8%, -1.3%)	133	5.9%	193	4.7%	1.2%, (-0.7%, 3.1%)
Age (years)										
<5	18	0%	31	18.3%	-18.3% (-34.1%, -2.6%)	41	8.3%	86	6.3%	2.0% (-8.2%, 12.2%)
5-15	37	19.5%	46	26.2%	-6.7% % (-26.6%, 13.2%	75	11.5%	162	6.6%	4.9% (-3.9%, 13.7%)
>15	92	13.1%	116	14.0%	-0.09% (-11.3%, 9.5%).	137	4.3%	287	3.0%	1.2% (-2.9%, 5.4%)

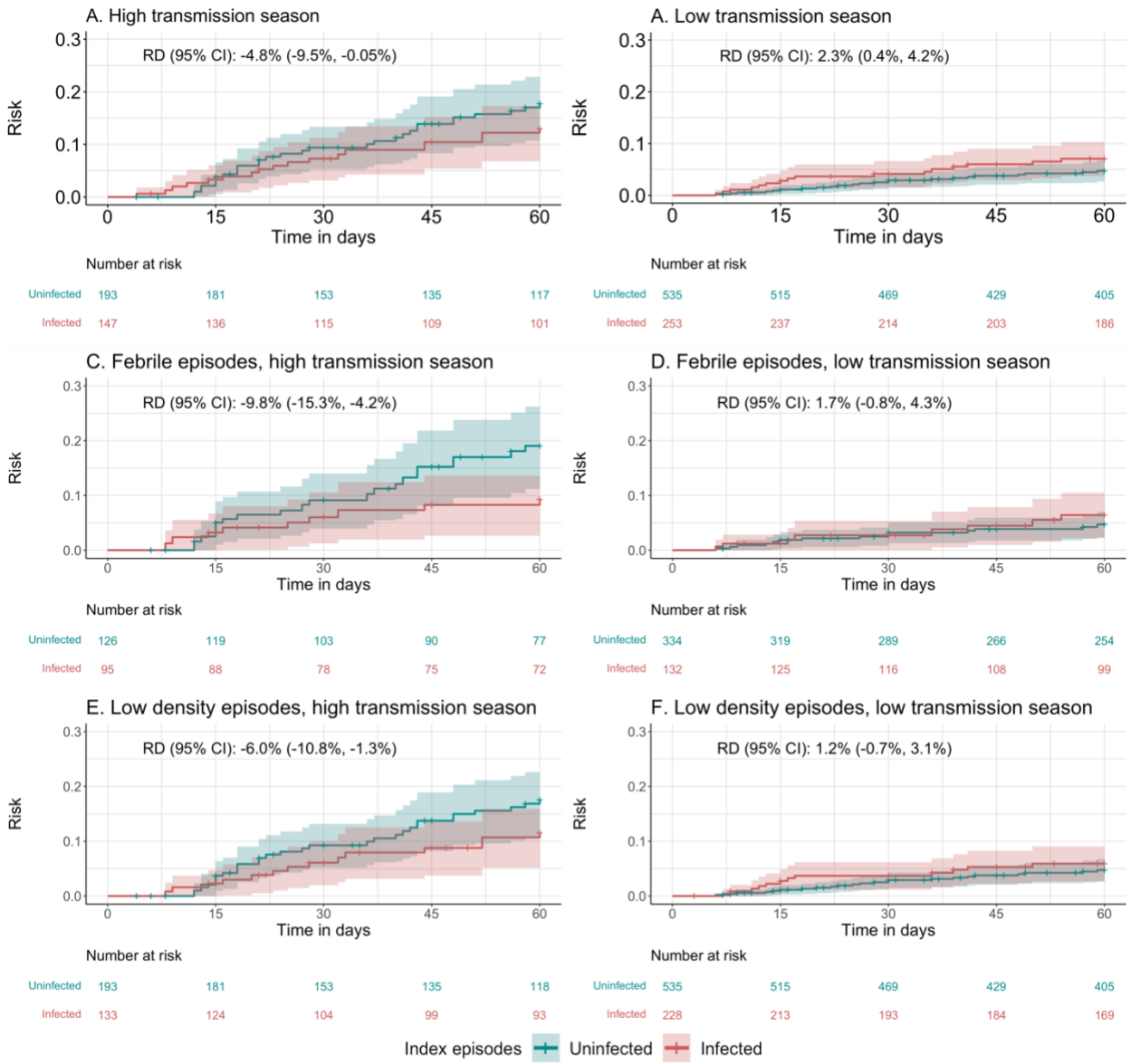


Figure 4.2. Risk of clinical malaria following a symptomatic, index RDT-negative episode among the total population and defined sub-populations stratified by transmission season. Cumulative incidence functions from inverse probability weighted Kaplan-Meier estimation indicating time to clinical malaria following symptomatic RDT-negative episodes. Crosses indicate censoring on either the date of the next RDT-negative episode or at the end of the follow-up period (60 days). The shaded areas indicate the 95% confidence intervals (CI). Sixty-day risk differences (RD) were calculated using the weighted Kaplan-Meier curves.

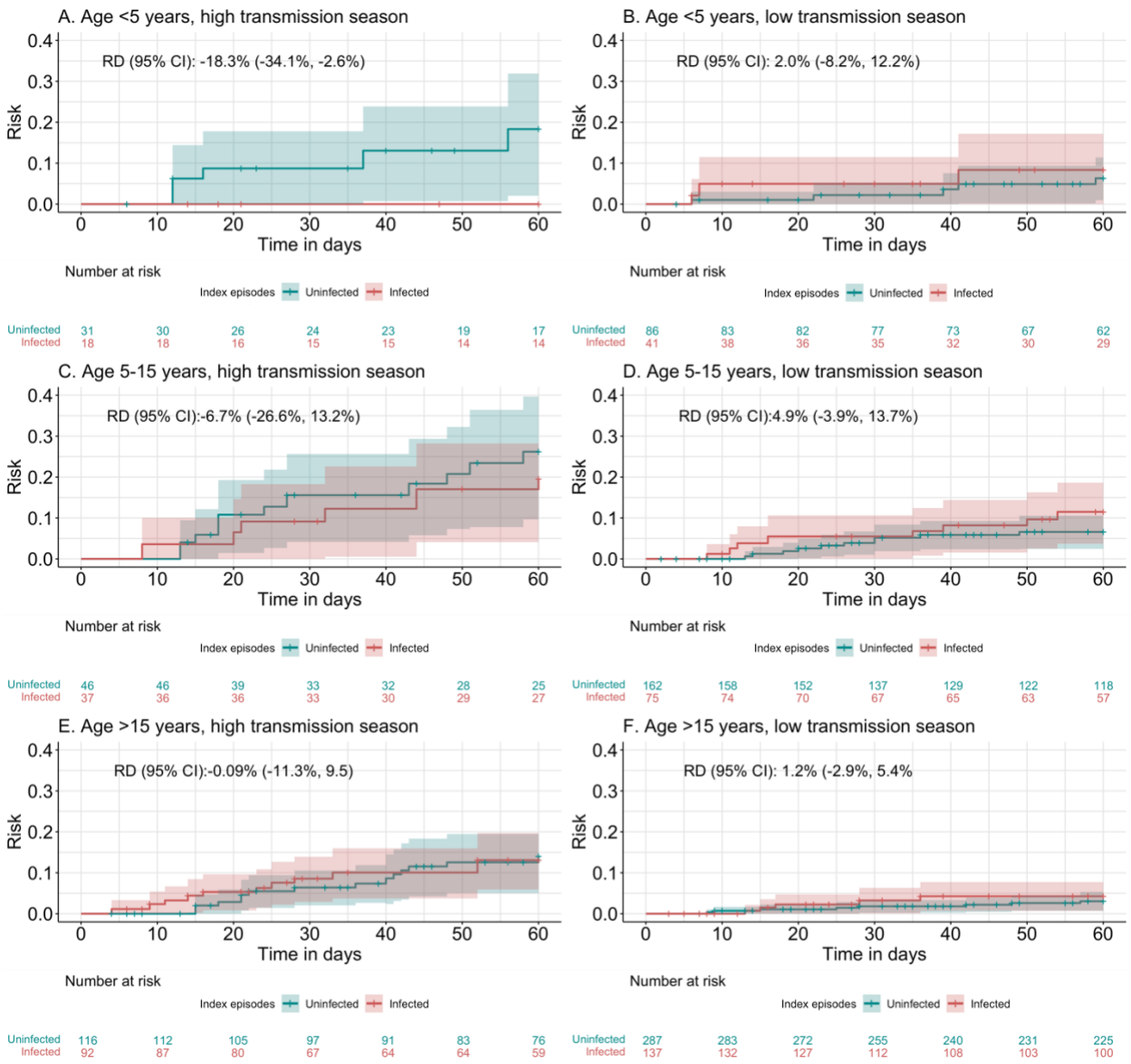


Figure 4.3. Risk of clinical malaria following a symptomatic RDT-negative episode stratified by age group and transmission season. Cumulative incidence functions from inverse probability weighted Kaplan-Meier estimation indicating time to clinical malaria following symptomatic RDT-negative episodes. Crosses indicate censoring on either the date of the next RDT-negative episode or at the end of the follow-up period (60 days). The shaded areas indicate the 95% confidence intervals (CI). Sixty-day risk differences (RD) were calculated using the weighted Kaplan-Meier curves.

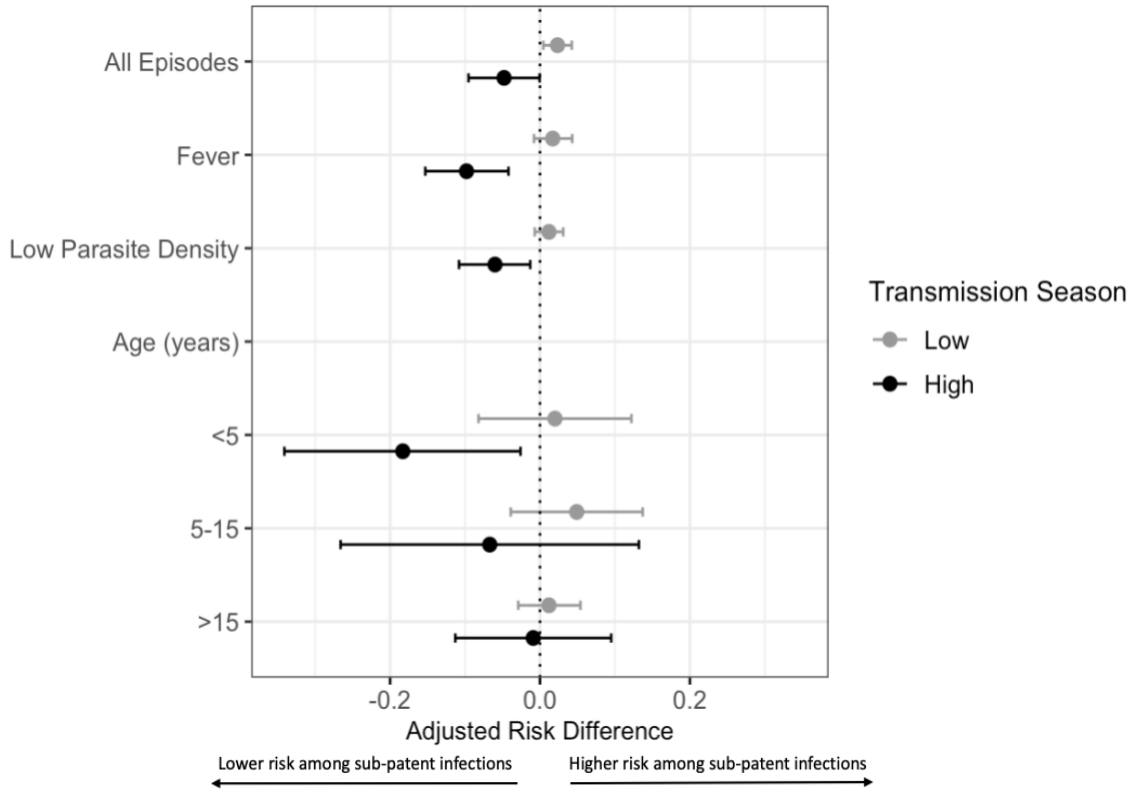


Figure 4.4. Risk of clinical malaria following a sub-patent, RDT-negative infection stratified by transmission season. IP weighted risk differences and 95% confidence intervals of clinical malaria between people with and without sub-patent infection. Sub-group analyses were conducted among febrile RDT-negative episodes and low parasite density infections, while stratified analyses were conducted for different age groups. All analyses were stratified by transmission season. Dots indicate the risk difference and the lines indicate the 95% confidence intervals. In the primary and sub-group analyses, IP weights for confounding included age, sex and bed net use, while IP weights for informative censoring included age. In the analyses stratified by age group and transmission season, IP weights for confounding included sex, and bed net use.

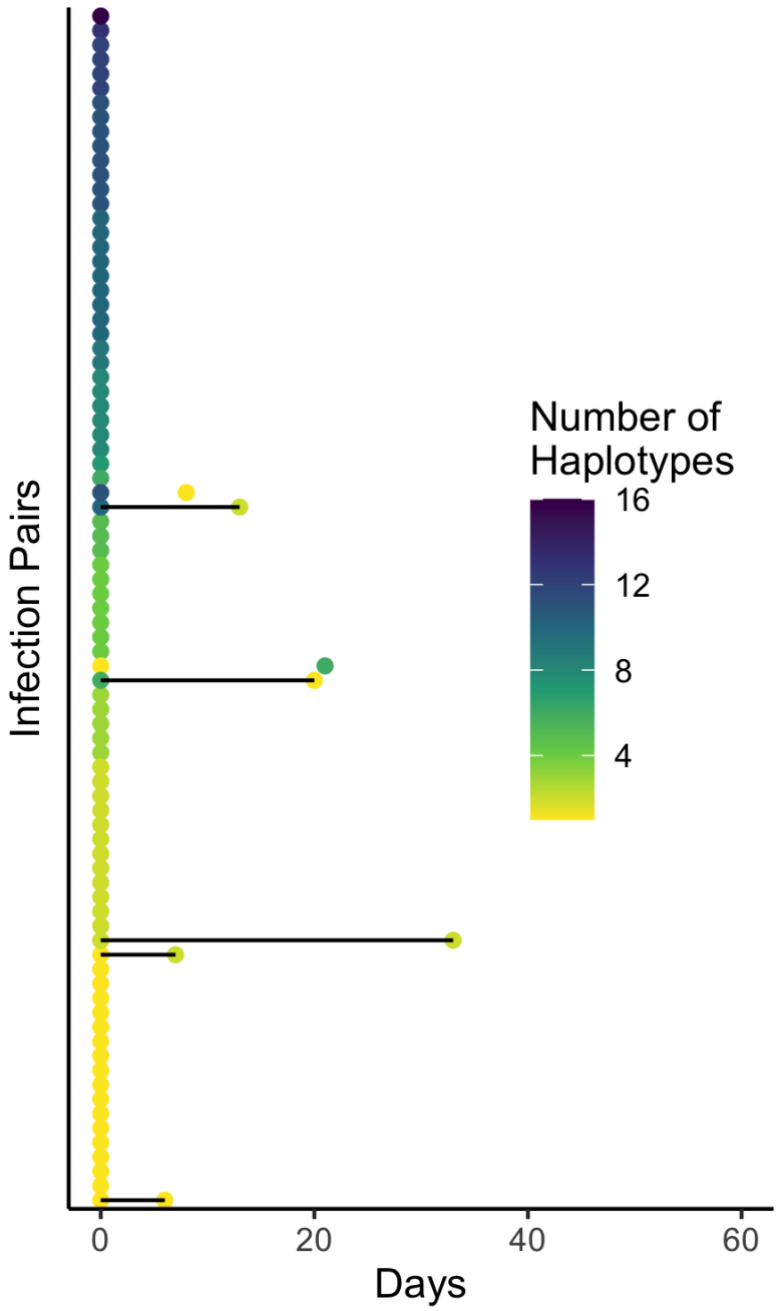


Figure 4.5. Identified haplotypes in index and future infections. The dots at day 0 represent index infections and dots at day X represent subsequent clinical malaria events. The color of the dot corresponds to the number of unique *P. falciparum* circumsporozoite protein haplotypes detected in each sample. A line between the dots indicates at least one shared haplotype between the index and subsequent infection.

Discussion

We used a 54-month longitudinal cohort to investigate the association between symptomatic sub-patent *P. falciparum* infections and subsequent clinical malaria. We observed that following episodes of suspected malaria during which people tested negative for *P. falciparum* with an RDT, the risk of subsequent clinical malaria among those with a sub-patent *P. falciparum* infection was low. Additionally, the comparative risk with uninfected people was modified by transmission season: sub-patent infections were associated with a slightly increased risk of subsequent clinical malaria during the low transmission season and a reduced risk during the high transmission season. Taken together, these findings suggest that though the slightly elevated risk in the low transmission season may merit alternate management, RDTs diagnose the majority of clinically relevant infections.

We observed that transmission season influenced the risk of malaria following a sub-patent infection. Compared to those that were uninfected, those with sub-patent infections had a slightly elevated risk of clinical malaria during the low season and a reduced risk during the high season, a pattern that was also observed in the febrile and low-density populations, and in children under 5. To our knowledge, our study is the first to analyze clinical outcomes for sub-patent infections in a longitudinal cohort across multiple transmission seasons in a high transmission setting. One explanation of our findings could be that sub-patent symptomatic infections do indeed confer some mildly increased risk of malaria during low transmission season, when exposure to incident infections is limited owing to the paucity of vectors. This could be counteracted during high transmission seasons by some protective benefit that prevents or forestalls malaria, consistent with our prior observation that the presence of persistent parasites limits the symptomaticity of newly acquired, superinfecting parasites[93] and with evidence that blood-stage infections enhance adaptive immune responses [94]. Because the high transmission season is characterized by the exposure to many infectious bites with diverse

parasites, undetected and untreated sub-patent infections may attenuate the clinical impact of newly- acquired parasites or enhance immunity among people with parasitemia [95,96].

The clinical significance is unclear of the increased risk during the low transmission season. Given the low risk of malaria among people with sub-patent infections during the low season (7.1%), the two-percentage point increase in risk might be minimal. Alternate strategies during the low transmission season, including more sensitive clinical diagnostics and presumptive prescribing of antimalarials for later use, could be useful to detect and treat sub-patent infections that may progress to clinical malaria.

Clinical malaria following a sub-patent infection associated with symptoms was rare, and our investigation into haplotype sharing between index and subsequent infections suggests that some of these rare events occurred following index sub-patent infections that were “pre-patent.” Of the 83 sub-patent index infections with available haplotype data, only seven were followed by clinical malaria episodes, of which five shared at least one haplotype between index and secondary infections. Although we cannot make decisive conclusions from this limited analysis, the short time to clinical malaria and presence of shared haplotypes between index and secondary infections suggest that some of these sub-patent infections may represent a pre-clinical phase. Such infections, not yet above the density threshold for RDTs at the time of testing, could have progressed to be detectable shortly thereafter.

This study has several strengths. The availability of a comparator group consisting of symptomatic *P. falciparum*-negative episodes allowed us to form a sample representative of people with untreated suspected malaria, which made our results more generalizable to our target population. Additionally, our study design used identical mechanisms for the ascertainment of exposures and outcomes, namely self-reported symptoms. As a result, only participants in the overall cohort who utilized this care-seeking were able to enter the analysis, enhancing the ability to rigorously capture outcome events. Finally, using IP weighted Kaplan Meier survival curves takes advantage of our longitudinal study design and unequal follow-up

time between participants. This approach is more interpretable and does not have the methodological issues of using a Cox proportional hazards model [97]. IPW standardizes the population such that one survival curve represents the entire sample if all episodes were sub-patent infections and the other represents the entire sample if all were uninfected episodes [86], allowing us to interpret our findings as the average effect in the population[98], which is more interpretable compared to the conditional estimates produced by other methods.

These analyses are subject to limitations. Parasitic genetic data were unavailable for the majority of the study period, which precluded a comprehensive investigation of haplotype sharing between index and subsequent infections. However, we still observed evidence of identical parasite haplotypes in index and outcome infections, demonstrating an ability to observe some expected “pre-patent” infections. Additionally, some exposures may have been mis-classified owing to parasites which do not express the HRP2 antigen that is detected by RDTs [28]. We did not assess HRP2 deletion among parasites in this study, though HRP2 deletions have proven to be rare in western Kenya [99] and the multiplicity of parasite clones we have observed in this cohort would also “mask” the effect of individual parasites lacking HRP2 within complex infections.

In our longitudinal study of follow-up after symptomatic RDT-negative episodes, clinical malaria was less likely following a sub-patent *P. falciparum* infection than following an uninfected, symptomatic episode during the high transmission season. Although clinical malaria was slightly more likely following a sub-patent infection compared to an uninfected episode in the low transmission season, this difference was minimal. The absence of a clinically-significant increased risk following undetected, untreated infections supports the notion that current malaria RDTs adequately diagnose the large majority of clinically-relevant *P falciparum* infections. In areas without substantial HRP2-deleted parasites, negative testing on conventional RDT should prompt evaluation for alternate etiologies of symptoms.

CHAPTER V: LOW PARASITE DENSITY AND OTHER PREDICTORS OF HUMAN-TO-MOSQUITO *PLASMODIUM FALCIPARUM* TRANSMISSION IN A HIGH TRANSMISSION AREA OF WESTERN KENYA

Introduction

Progress toward malaria elimination has stalled, owing in part, to an infectious reservoir of parasites in humans who disproportionately transmit malaria parasites to mosquitoes[40–43]. It is essential to identify the people contributing to this infectious reservoir since it sustains the parasite reservoir in mosquitoes and malaria transmission in the community[2].In malaria-endemic areas, chronic infections often lead to partial immunity to parasites and asymptomatic infections[21,56] Studies have suggested that asymptomatic *Plasmodium* falciparum infections are important contributors to this reservoir, as they often go undetected and untreated but can still be infectious[2,6,21,58,100]. Partial immunity to parasites can cause low parasite density infections, where the number of parasites per microliter of blood (parasites /μL) is too small to be detectable by rapid diagnostic tests (RDTs)[2,56]

The relationship between parasite density in human infections and transmissibility to mosquitoes is poorly understood. Studies of transmission and gametocytes, the sexual stage parasites responsible for transmission to mosquitoes, have shown that submicroscopic gametocyte carriers contribute to human-to-mosquito transmission [5,49,61]. However, there are conflicting findings about the association between asexual parasites, the blood stage precursor to gametocytes, which cause symptoms of malaria, and infectivity to mosquitoes. Investigations found a positive association[48], no association[52], and a non-linear association between asexual parasite density and infectivity to mosquitoes[40]. In addition, limited research exists identifying other demographic, human behavioral, clinical, and parasitological factors associated with successful transmission to mosquitoes in a natural setting. Many studies have analyzed risk

factors for transmission using skin or artificial membrane feeding of laboratory-reared mosquitoes[47,48,50,61] and others have used mathematical modeling[56,101]. Age is a commonly identified risk factor. Children are considered more infectious to mosquitoes compared to adults because they tend to have higher gametocyte density infections[47,48,50]. Other studies, however, have accounted for higher levels of exposure to infectious bites among adults, which balances their contribution to the infectious reservoir[48,101]. While these studies are useful for understanding the biological mechanisms that govern transmission, they incompletely capture natural processes and the complex dynamics of infection in high transmission settings. For example, differences in mosquito feeding behavior and in human behavior are not captured by mosquito feeding experiments, but are important to better understand malaria epidemiology.

Therefore, we investigated the impact of parasite density and other risk factors on human-to-mosquito transmission using data from a cohort of humans and naturally fed mosquitoes. We used human and mosquito pairings and adapted probabilistic modeling from a previously published analysis, which found that asymptomatic infections had more than double the odds of transmission to a mosquito compared to symptomatic infections [9]. In this study, we estimated the odds that shared haplotypes in human and mosquito pairings represented a transmission event and compared the odds between low- and high-parasite density infections. We hypothesized that lower parasite density infections, white are more likely to persist undetected, are an important contributor to the infectious reservoir and would be associated with an increased odds of human-to-mosquito malaria transmission in this population compared to high-parasite density infections.

Methods

Ethical statement

The study protocol was approved by the ethical review committees of Duke University (Pro0008200) and Moi University (2017/36). University of North Carolina at Chapel Hill's Institutional Review Board (IRB) deemed the analyses presented in this paper exempt. We

obtained written informed consent from all participants or their parent for those under 18 years, who also provided assent if greater than 8 years.

Study site, participants, and sample processing

As described previously, we analyzed data collected from June 2017 to July 2018 from a cohort of people aged 1 – 85 years living in 38 households in Webuye, Western Kenya[6,72]. Human and mosquito sample collection details have been previously reported[72]. Dried blood spot (DBS) samples were collected, and demographic and behavioral questionnaires were administered each month. Between visits, participants experiencing symptoms consistent with malaria contacted study staff, provided a DBS, and were tested using a rapid diagnostic test (RDT) (Carestart © Malaria HRP2 *Pf* from Accessbio)[74]. Field entomologists collected resting mosquitoes weekly from each household by vacuum aspiration with a Prokopack[102]. Female *Anopheles* mosquitoes were immediately dissected and preserved for parasite detection[78].

Genomic DNA (gDNA) from each DBS and mosquito abdomen was tested for *P. falciparum* using a real-time quantitative polymerase chain reaction assay[23,24]. Parasite densities were estimated using standard curves generated from amplifications on each plate of templates of known parasite density[6]. *P. falciparum*-positive DBS and mosquito abdomen samples were genotyped at *Plasmodium falciparum* circumsporozoite protein (*pfcsp)* using amplicon deep sequencing to identify haplotypes [78–83,85]

Analysis population

Our primary analysis population consisted of all human-mosquito pairs for which transmission was possible based on proximity in time and space. A previous study of this cohort paired mosquitoes with human participants based on time and distance[78]. Mosquitoes were paired with a particular participant and DBS time point if they were caught between 7 days before and 14 days after the DBS was collected and caught within or near (<0.55 km) a participant's home. Mosquitoes could be paired with more than one participant and participants could be paired with more than one mosquito.

Exposure, outcome, and covariate assessment

We defined the main exposure, parasite density in human infections, as the number of parasites detected per microliter of blood. We dichotomized parasite density to 200 or fewer parasites per microliter of blood and over 200 parasites per microliter based on the limit of detection for conventional rapid diagnostic tests (RDT)[68]. We expressed our primary outcome, the probability of transmission between each human and mosquito pair, as the degree of haplotype sharing between pairs. Calculated for a previously published analysis[78], the probability of haplotype sharing is a function of the number of shared genotypes between infections, the prevalence of the genotype across the study population, and multiplicity of infection (MOI), which refers to the number of unique haplotypes in each participant's sample.

We included age, sex, bed net use, infection type (symptomatic or asymptomatic), infection persistence, and transmission season as covariates. We categorized age (<5, 5-15, and >15 years) for comparability to the literature, which has established differences in risk of malaria between young children, school aged children, and adults[24,55]. Bed net use was collected during monthly behavioral surveys, and we defined regular use as reporting sleeping under a bed net more than five nights in the week preceding the study visit. For symptomatic episodes outside monthly visits, we used information from the monthly behavioral questionnaire (n=497). We defined infection type as asymptomatic if the infection was detected by PCR during active case detection in a participant with no symptoms. Symptomatic infections were defined as those that were detectable by both RDT and PCR during active or passive case detection with at least one malaria-like symptom (ex. fever, nausea, headache). Transmission season was expressed as mosquito abundance defined as ≤75 (low) or >75 mosquitoes (high) collected across the study site in the following week, as previously reported[78].

Among asymptomatic infections, we defined persistent infections as the presence of an asymptomatic PCR-positive infection within 30 days preceding the current infection, regardless of haplotype. We removed three infections that were preceded by and shared haplotypes with a

symptomatic infection. Infections that were preceded by a month with no infection (PCR negative) were also classified as not persistent. Each participant's first instance of testing for malaria in the study was excluded from the model evaluating infection persistence as a risk factor as we did not have their infection status for the previous month.

Statistical analysis

To estimate the odds of a human-to-mosquito transmission events, we compared the probability that shared haplotypes observed in a human and a mosquito represented a transmission event between human infections with parasite densities of ≤200, and >200 parasites/μL using logistic regression models with generalized estimating equations (GEE) to account for repeated measures for participants that experienced multiple *P. falciparum* infections during the study. We assessed age as an effect modifier by including an interaction term in the model and calculating stratum-specific odds ratios. We controlled for the following confounders: sex, age, transmission season, bed net use, and infection type based on evaluating potential confounders in a directed acyclic graph (DAG). We also conducted a risk factor analysis by fitting logistic regression models with GEE for age, sex, bed net use, transmission season, and infection persistence separately to evaluate each as a potential risk factor for transmission. We restricted the analysis population for the infection persistence model to human-mosquito pairings with asymptomatic human infections, which are more likely to be persistent. For each model, we chose the minimally sufficient adjustment set of covariates to control for confounding based on separate DAGs.

Results

Characteristics of participants and human-mosquito pairings

The final analytic population consisted of 3,727 human-mosquito pairings that were paired based on temporal and spatial overlap matching the aforementioned criteria. These pairings comprised 198 malaria-infected participants in 37 households and 182 infected

mosquitoes. On average, each malaria-infected participant was paired with 17.6 infected mosquitoes (range 1-36), while malaria-infected mosquitoes were paired with to 25.5 participants (1-44). Fourteen percent of participant-mosquito infection pairings occurred in the same household structure. Among infected participants, 57% were females, 12% were children less than 5 years, 43% were children 5-15 years, and 45% were over 15 years (**Table 5.1**).

Across these 3,727 human-mosquito pairings representing potential human-to-mosquito transmission, the median DBS parasite density for human infections was 43.5 (IQR: 1.8, 733.6) parasites per microliter of blood, and 60% of human infections within human-mosquito pairings had low parasite densities (≤200 parasites per microliter) (**Table 5.1**). The vast majority of low-parasite density infections were asymptomatic (94%). Some people were infected at their first study visit. For these individuals, infection persistence could not be ascertained because the previous month's infection status was unknown. Among 1,532 asymptomatic human infections paired with mosquitoes that could be classified as persistent vs. not persistent, 15.5% (n=237) were persistent. Additionally, compared to those including high parasite density human infections, pairings including low parasite density infections were more likely to occur in participants 5-15 years and over 15 years (*p* value: <0.001), females (*p* value: <0.001), and during the low transmission season (*p* value: <0.001). Low parasite density infections were also more likely to occur in participants who reported regular bed bet use (*p* value: <0.001), and among infections classified as not persistent (*p* value: <0.001). (Table 5.1).

Outcomes

Of the 3,727 human-mosquito pairings, 38.9% (n=1,449) had no shared haplotypes between the human and mosquito infections and, thus, zero probability of transmission. The balance of the infection pairings (n=2,278) shared at least one haplotype between human and mosquito hosts, and across these pairings, the median probability that a pairing represented a transmission event based on haplotype sharing was 0.20 (IQR: 0.10-0.32).

Associations of parasite density with human-to-mosquito transmission

We used logistic regression with GEE to estimate the odds that human-mosquito pairings with shared parasite haplotypes represented a transmission event. Compared to infections with high parasite density, the likelihood was over 90% higher (Adjusted Odds Ratio (aOR): 1.92 95% Confidence Interval (CI): 1.54, 2.42) that shared parasite haplotypes between a mosquito and an infection with low parasite density represented a transmission event. We examined this association separately by age group and did not identify any meaningful variation in the stratified estimates.

Other risk factors for human-to-mosquito transmission

We next utilized separate logistic regression models with GEE to analyze each risk factor (**Figure 5.1**). Fitting separate models allowed us to estimate and correctly interpret the total association between the risk factor and the odds that an infection pairing that shared parasite haplotypes represented a transmission event. We observed that infection pairings were more likely to represent a transmission event when the human infection occurred during the high transmission season (compared to low transmission; OR: 1.29, CI: 1.17, 1.41) (Figure 2)), and were less likely to occur for persistent human infections (compared to not persistent; aOR: 0.67, CI: 0.53, 0.85[23] We did not observe an association between transmission and younger age groups compared to people over 15 years (OR <5: 1.06, CI: 0.92, 1.22; OR 5-15: 0.95, CI: 0.86, 1.05). We also found no association between transmission and sex (OR male: 0.99 CI: 0.83, 1.18) or bed net use (aOR: 1.00, CI: 0.76, 1.31) (**Table 5.2**).

Table 5.1 Characteristics of participants and human-mosquito pairs

Variable	Participants N = 198	Human-Mosquito Pairs Overall, N = 3,727	Parasite Density		p-value[2]
			High, N = 1,466 (39.3%)	Low, N = 2,261 (60.7%)	
Participant-level characteristic					
Village					<0.001
Kinesamo	62 (31.3%)	824 (22.1%)	427 (29.1%)	397 (17.6%)	
Maruti	68 (34.3%)	2,678 (71.9%)	931 (63.5%)	1,747 (77.3%)	
Sitabicha	68 (34.3%)	225 (6.0%)	108 (7.4%)	117 (5.2%)	
Sex					<0.001
Female	113 (57.1%)	2,236 (60.0%)	712 (48.6%)	1,524 (67.4%)	
Male	85 (42.9%)	1,491 (40.0%)	754 (51.4%)	737 (32.6%)	
Age					<0.001
<5 years	23 (11.6%)	438 (11.8%)	238 (16.2%)	200 (8.8%)	
5-15 years	86 (43.4%)	1,806 (48.5%)	1,004 (68.5%)	802 (35.5%)	
>15 years	89 (44.9%)	1,483 (39.8%)	224 (15.3%)	1,259 (55.7%)	
Human-mosquito pair-level characteristics					
Transmission Season					<0.001
High		1,936 (51.9%)	870 (59.3%)	1,066 (47.1%)	
Low		1,791 (48.1%)	596 (40.7%)	1,195 (52.9%)	
Infection Type					<0.001
Asymptomatic		3,012 (80.8%)	883 (60.2%)	2,129 (94.2%)	
Symptomatic		715 (19.2%)	583 (39.8%)	132 (5.8%)	
Regular Bed Net Use					<0.001
No, <5 nights		986 (28.5%)	570 (46.6%)	416 (18.6%)	
Yes, >5 nights		2,478 (71.5%)	653 (53.4%)	1,825 (81.4%)	
Missing		263	243	20	
Infection Persistence[3]					<0.001
Not Persistent		1,295 (84.5%)	349 (75.2%)	946 (88.6%)	
Persistent		237 (15.5%)	115 (24.8%)	122 (11.4%)	
Missing		1,480	419	1,061	

[1]n (%)
[2]Pearson's Chi-squared test
[3]Defined among asymptomatic human infections (N=3,012)

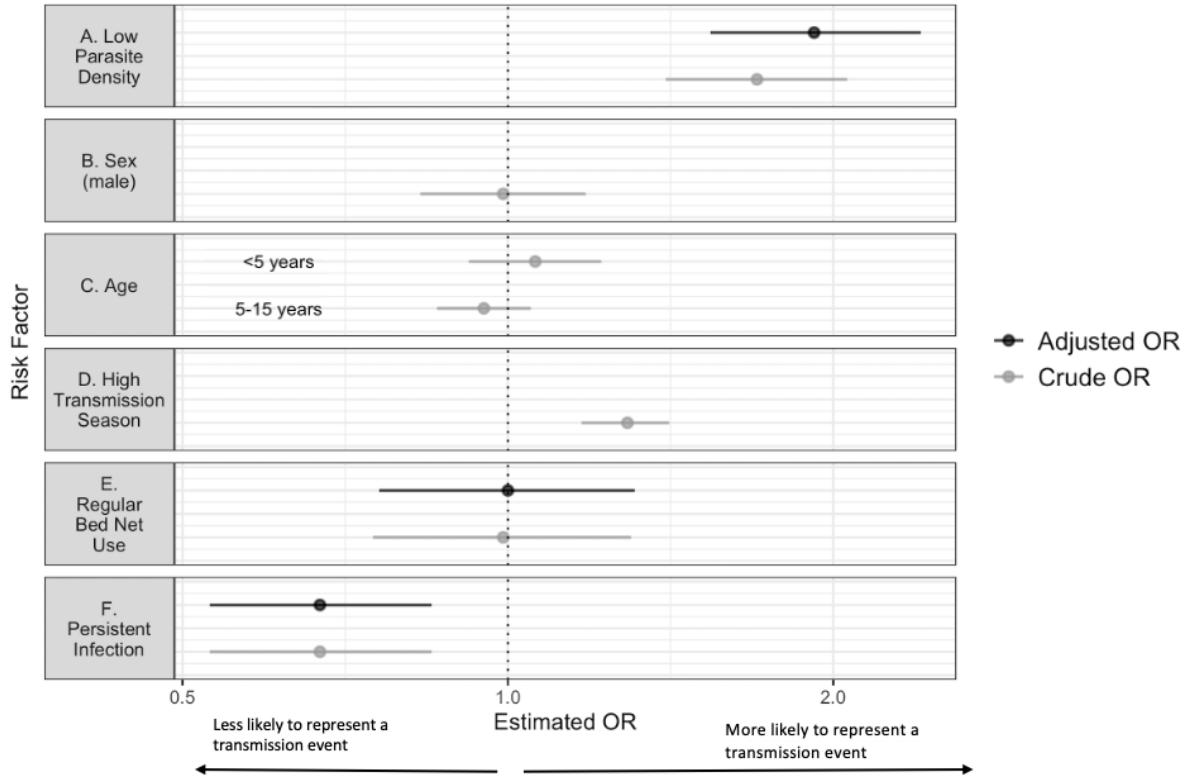


Figure 5.1. Estimates of associations between infection characteristics and an elevated likelihood that the infection was transmitted to a collected mosquito. Odds ratios of the probability of malaria transmission from human participants to mosquitoes. Dots indicate the odds ratio and the lines indicate the 95% confidence intervals. ORs were computed using logistic regression models with generalized estimating equations (GEE). The probability of transmission outcome was coded continuously. The model for parasite density was adjusted for village, age, for sex, transmission season. Infection type was found to be highly collinear with parasite density and was removed from the final model for parasite density. The model for bed net use was adjusted for age, transmission season, and village. The model for infection persistence was adjusted by age. Models for age, sex, and transmission season did not require adjustment to estimate associations as indicated by directed acyclical graph (DAG) analyses.

Table 5.2. Logistic regression results for the odds of a human-to-mosquito *P. falciparum* transmission event from participants with low parasite density infections compared to high parasite density infections

Risk Factor[1]	N	Odds Ratio (95% CI)	Adjusted OR (95% CI)
Parasite density, p/μL	3,727	==	--
≤200		1.70 (1.40, 2.06)	1.92 (1.54, 2.41)
>200		Ref	Ref
Sex	3,727		
Female		Ref	--
Male		0.99 (0.83, 1.18)	--
Age, years	3,727		
< 5		1.06 (0.92, 1.22)	--
5 – 15		0.95 (0.86, 1.05)	--
> 15		Ref	--
Transmission season	3,727		
Low		Ref	--
High		1.29 (1.17, 1.41)	--
Infection type	3,727		
Symptomatic		Ref	Ref
Asymptomatic		1.40 (1.22, 1.61)	1.42 (1.23, 1.63)
Bed Net Use	3,464		
No		Ref	Ref
Yes		0.99 (0.75, 1.30)	1.00 (0.76, 1.31)
Infection persistence[2]	1,532		
Persistent		0.67 (0.53, 0.85)	0.67 (0.53, 0.85)
Not Persistent		Ref	Ref

[1]ORs were computed using separate logistic regression models with generalized estimating equations. Models were adjusted by covariates if needed based on directed acyclic graph analysis.
[2]Defined among asymptomatic human infections

Discussion

In this 14-month cohort of people and naturally-fed mosquitoes, we found that compared to high-density infections, low-density human infections over 90% increased odds (OR: 1.92 (1.54, 2.41) of transmission to mosquitoes. We investigated this relationship using a previously published probabilistic model[78] to estimate the likelihood that the presence of identical parasite genotype in and human and a mosquito infection represented human-to-mosquito transmission. Several previous studies have described the transmission potential of low-density infections[5,40,42,48–50,52,53,103,104], but few have statistically compared the infectivity of high- versus low-density infections. Our observations could be explained by the high prevalence of asymptomatic infections among those with low parasite density (94%). Low parasite density infections are a marker for asymptomatic and often chronic infections that have time to generate gametocytes[17]. Given that low-parasite density infections are often undetectable, our findings suggest that alternative strategies are needed to target these infections and reduce transmission. Some possible strategies include using more sensitive diagnostic tools with active case detection, and mass drug administration (MDA).

Several previous studies have established that low-density infections can transmit to mosquitoes but these have largely focused on the infectivity of infections with sub-microscopic gametocytes and determined that these infectious contribute substantially to the infectious reservoir[40,52,53]. Few studies have statistically compared the infectivity of high- and low-density infections. Our findings are in contrast with two studies that found no association between total parasite density and infectivity[52,53]. This is likely due to differences in the study populations and the measurement of transmission events. Those studies also used mosquito feeding laboratory experiments to observe transmission, which allowed them to directly measure whether parasite density correlated with infectivity to mosquitoes, but does not capture natural mosquito feeding behaviors or transmission dynamics[2]. Our study builds upon this previous work by investigating

the contribution of low-density infections to transmission using a longitudinal study design and naturally-fed mosquitoes[30].

We also observed that infections during the high transmission season had higher odds of transmission from humans to mosquitoes. Age, sex, and regular bed net usage were not associated with transmission. Additionally, the association between parasite density and human-to-mosquito transmission did not vary by age. Age is commonly used as a proxy for acquired immunity to parasites, which governs parasite density and whether a person develops symptoms[17]. However, there is a lack of understanding about the relative contribution of different age groups to onward transmission. While some studies determined that children aged 5-15 years contribute the most to the infectious reservoir[40,62], another found that children under age five had similar levels of infectivity to school-age children [48]. That study also concluded that higher exposure to mosquitoes among adults balanced their contribution to the infectious reservoir[48]. In our cohort, both symptomatic and low-density infections were more common in older participants compared to younger participants. This supports the idea that even though children individually may be more likely to transmit parasites, the number of adults, higher potential for exposure to mosquitoes, and higher potential for more chronic low-density infections balanced their contribution on a population level. Children under five also only represented about 12% of infection pairs; therefore, their contribution to transmission was likely reduced by the high number of older children and adults.[48]

Surprisingly, human-mosquito pairings with persistent human infections were less likely to represent transmission events. This could be explained by our observation that the majority of asymptomatic persistent infections had higher parasite densities. The timing of human DBS sampling was too coarse to estimate infection duration, which would allow for a more robust estimate of persistence and the timing of when mosquitoes can ingest gametocytes.

Despite two-thirds of participants reporting bed net use more than five nights per week, regular bed net use was not associated with lower human-to-mosquito transmission. Several

other studies have established the importance of bed nets in preventing transmission from mosquitoes to humans[105–108], but to our knowledge, none have estimated the impact on human-to-mosquito transmission. One possible explanation for this finding is that participants are overreporting bed net usage. This potential social desirability bias could be masking the effect of bed nets on transmission. Despite the availability of bed nets, potential underutilization highlights the need for combinations of transmission reduction strategies that interrupt transmission at multiple potential points of contact between humans and mosquitoes. The Malaria Eradication Research Agenda initiative identified this as a priority for elimination efforts[109]. Proposed strategies include combining interventions like bed nets with enhanced vector control through insecticide treated clothing and blankets, topical repellants, insecticide treated livestock, or housing improvements[110].

Our ability to detect transmission events was limited by our mosquito collection method, which provided a less direct way of observing transmission compared to mosquito feeding experiments. The probability of transmission based on haplotype sharing is a proxy for human-to-mosquito transmission. There is the potential for differential misclassification of transmission events because non-events were more likely to be misclassified as events if they shared haplotypes with a mosquito that did not bite them. This could happen particularly if that haplotype is common in the population; however, the probabilistic model for haplotype sharing that we employed penalizes common haplotypes[78]. In addition, at about 50% of study visits, multiple participants were infected within the same household and paired to the same mosquito based on timing and distance. This could also lead to misclassification because we allowed participants to be paired with more than one mosquito, and mosquitoes to be paired with more than one participant. If participants shared haplotypes with each other, and were paired with the same mosquito, we could not determine if the mosquito acquired parasites from one or multiple participants, which would inflate the number of human-to-mosquito transmission events captured in this study. Despite this, our collection method is a better reflection of mosquito

feeding behaviors in a natural setting and allowed for a relative estimate of human-to-mosquito transmission in this population.

This study has some further limitations. We sampled households for mosquitoes weekly because daily collection was not feasible. Therefore, we missed mosquitoes that fed on participants outside of their homes or on days between mosquito collections. Despite this, the frequent collection of mosquitoes allowed for relative estimates of inferred transmission. Additionally, all symptomatic RDT-positive infections in our cohort were treated with artemether-lumefantrine (AL). Therefore, our classification of persistent infections labeled infections as not persistent if they were preceded by a symptomatic infection. It is possible that after treatment, some infections were not fully cleared by the next test date. We classified as not persistent 10 asymptomatic infections that occurred within a month of a symptomatic infection, allowing that incompletely cleared recrudescent parasites may be included. These contributed to a total of 125 infection pairs in the analysis. The efficacy of AL, however, is high if there is full adherence[111,112] and this likely did not impact the results. Finally, we did not specifically measure gametocyte densities, the parasite stages that are responsible for human to mosquito transmission, which do not always correlate with asexual parasite density.

In this study of paired human and mosquito *P. falciparum* infections, human infections with < 200 parasite/uL were almost twice as likely to be observed in collected mosquitoes compared to high-density infections. Future work should investigate the impact of detecting and treating low-density infections on human-to-mosquito transmission, and continue to characterize the infectious reservoir to inform targeted intervention development.

CHAPTER VI: CONCLUSIONS

Summary of Findings

This dissertation aimed to better understand the natural history of sub-patent *P. falciparum* infections and the impact of low-density infections on onward transmission. We used data from a longitudinal cohort of participants followed from June 2017 to November 2021 in a high-transmission area in Western Kenya. We hypothesized that among symptomatic RDT-negative episodes, the risk of clinical malaria for sub-patent infections would be similar compared to uninfected episodes. We also anticipated that low parasite density infections would be more likely to transmit to mosquitos compared to high parasite density infections.

In Aim 1, we observed that the overall 60-day risk of clinical malaria was minimal. Only 7.7% of RDT-negative episodes were followed by a clinical malaria episode within 60 days. We detected effect measure modification of the relationship between sub-patent infections and clinical malaria by transmission season. In the high-transmission season, the risk of clinical malaria was 4.8 percentage points lower following sub-patent episodes compared to uninfected episodes. During the low transmission season, the risk of malaria was 2.3 percentage points higher following sub-patent episodes compared to uninfected episodes. Our non-significant findings in the overall, febrile, and low-density populations and in the age-stratified analyses are due to these opposing effects by transmission season. We conclude that a slightly elevated risk in the low season may merit alternate management, but RDTs diagnose the majority of clinically relevant infections in the high transmission season.

In Aim 2, after adjusting for village, age, sex, infection type, and transmission season, we found that compared to high parasite density infections, low-density infections were 75% more likely to transmit to mosquitoes. In the risk factor analysis, asymptomatic infections,

persistent infections, and infections during the high transmission season all had increased odds of human-to-mosquito transmission. We did not observe an association between transmission and age, sex, or bed net use. We conclude that low-density infections are an important contributor to the infectious reservoir of parasites and should be targeted with more sensitive diagnostics and improved interventions.

Although the clinical significance of the increased risk of malaria following sub-patent infections during the low season is unclear, sub-patent and low-density infections have important implications for transmission reduction. RDTs detect most clinically significant cases of malaria during the high transmission season, but do not detect low parasite density infections that are capable of transmitting to mosquitos, thereby fueling onward transmission.

Public Health Implications and Future Directions

Our results highlight the role of low-density and sub-patent infections in clinical disease and transmission from humans to mosquitoes. Sub-patent infections are often missed because the parasite density is below the limit of detection for rapid diagnostic tests or microscopy. We observed a pattern across our stratified analyses of a slightly increased risk in the low transmission season paired with a decreased risk in the high transmission season. The implications of this are that alternate strategies could be useful to detect and treat sub-patent infections. Additionally, conventional RDTs adequately diagnose clinically-relevant *P. falciparum* infections during the high transmission season and negative testing on a conventional RDT should prompt evaluation for alternate etiologies of symptoms.

We also found an increased likelihood of transmission among low density infections. Low-density infections are marker for asymptomatic and chronic infections, which are often untreated, allowing for gametocyte development[17]. The implications of our findings are that current strategies are insufficient to identify and target the infectious reservoir and that control and elimination efforts need to consider using more sensitive diagnostics. Together, these dissertation findings suggest that sub-patent and low parasite density *P.* falciparum infections

have different implications for individual health versus public health. Low density infections are often sub-patent. While the clinical consequences for individuals whose infections are missed by conventional diagnostics are unclear, these undiagnosed and untreated infections provide opportunities for a mosquito to ingest parasites.[2,5] Our findings provide clear rational for the targeting of low-density infections to progress elimination efforts.

A major challenge is the most efficient and effective way to detect low parasite density infections to reduce their contribution to the infectious reservoir. Strategies to target low parasite density and sub-patent episodes include using improved diagnostic methods and mass treatment. More sensitive diagnostics, such as high sensitivity RDTs (HS-RDT), are capable of detecting a greater range of parasite densities and can find infections that otherwise would go untreated[32]. Despite no clear clinical benefit to using HS-RDTs, there could be a public health benefit to detecting low density infections and depleting the infectious reservoir. However, replacing conventional RDTs with HS-RDTs presents challenges, particularly in resource-limited settings. HS-RDTs have colder storage requirements and a shorter shelf life compared to conventional RDTs[32,113]. Additionally, mass drug administration can successfully interrupt transmission especially when combined with other interventions[114], although there is the risk of increased drug resistance. Future research should investigate whether detecting and treating more low-density infections through active case detection with HS-RDTs or mass drug administration reduces onward transmission.

Additional longitudinal cohort studies that sample naturally caught mosquitoes should expand upon our work and continue to characterize the infectious reservoir using genetic approaches to estimate transmission. Improved estimates of infection persistence and duration using genetic approaches would be especially beneficial to our understanding of what groups harbor parasites for long periods of time. Interrupting human-to-mosquito transmission by identifying the stable and modifiable risk factors for membership in the infectious reservoir is essential to move toward malaria elimination.

Strengths

This dissertation study has several strengths. In Aim 1 we leveraged the longitudinal study design and used a robust analytic approach to describe the natural history of sub-patent infections and to estimate the risk of clinical malaria. The availability of a comparator group consisting of symptomatic *P. falciparum*-negative episodes allowed us to form a sample representative of people with untreated suspected malaria. This allowed us to hone in on the study question of interest and made our results more generalizable to our target population of people with untreated suspected malaria. Additionally, using IP weighted Kaplan Meier survival curves took advantage of our longitudinal study design and unequal follow-up time between participants. This approach is more interpretable and does not have the methodological issues of using a Cox proportional hazards model to estimate hazard ratios (HR), namely the proportional hazards assumption [97] that would be violated given that our survival curves crossed. Instead, Kaplan Meier curves with IPW allowed for the visualization of the changes in survival over time and the estimation of absolute risks that were weighted to mitigate bias due to confounding and informative censoring.[86] Assuming no unmeasured confounding, IPW standardized the population such that one survival curve represented the entire sample if all episodes were sub-patent infections and the other represented the entire sample if all were uninfected episodes.[86] Therefore, we could interpret our findings as the average effect in the population[98], which is more interpretable compared to the conditional estimates produced by other methods.

For Aim 2, we used highly dimensional genetic data to infer human-to-mosquito transmission under natural conditions. Previous work has measured predictors of transmission through direct or membrane feeding of mosquitoes, which does not capture natural mosquito feeding behaviors and transmission dynamics. Our large sample size included a high proportion of low parasite density infections, which allowed us to investigate the association between parasite density and transmission from humans to mosquitoes. Finally, we considered each

potential risk factor using separate DAGs to identify the minimally sufficient adjustment sets of covariates to mitigate confounding bias, and to assume exchangeability between exposure groups in each analysis. Fitting separate logistic regression models controlling for covariates identified in a DAG allowed us to avoid the Table 2 Fallacy[115] where adjusted effect estimates for covariates from the same model are presented and interpreted incorrectly as total effect estimates.

Limitations

Missingness

For both Aim 1 and Aim 2, we may have missed some symptomatic episodes since episodes were only detected by participants alerting study staff to their symptoms. This was likely mitigated by the availability of free testing and frequent contact with study personnel during weekly mosquito collection who were well-known to long-term study participants. In Aim 2, asymptomatic infections were only captured during monthly visits so any asymptomatic infection that occurred between these visits was missed. Given that the majority of asymptomatic infections in our cohort had low parasite densities, asymptomatic infections that were missed in between monthly study visits may have been low-density infections.

In Aim 1 there was some missing exposure data. We excluded about 6% of the data due to 2 inconclusive RDTs and 38 missing PCR results, which we believe are the result of dried bloodspots being lost. This small percentage was unlikely to have biased our findings. Additionally, parasite DNA was only sequenced for the first 14 months of cohort follow up, and not for the remaining 40 months of follow up, which precluded a comprehensive investigation of haplotype sharing between index and subsequent infections. However, we still observed evidence of haplotype sharing, which strengthens our assertion that we could be observing “pre-patent” infections.

In Aim 2, we sampled households for mosquitoes weekly because daily collection was not feasible. Therefore, we missed mosquitoes that fed on participants outside of their homes or

on days between mosquito collections, which could lead to missing data bias for our human-to-mosquito transmission outcome. Missing mosquitoes reduces the number of haplotype sharing events used to infer transmission. This could mean that we are underestimating the odds of transmission and the effect might be greater than what we observed. Our mosquito collection method provides a less direct way of observing transmission compared to mosquito feeding experiments, but is a better reflection of mosquito feeding behaviors in a natural setting and allowed for a relative estimate of human-to-mosquito transmission

Measurement

For Aim 1, we are not concerned about exposure misclassification for sub-patent and uninfected episodes because PCR is highly reliable for the detection and quantification of parasites. The lower limit of quantitation is around 1 parasite per microliter[29,30] and anything below that threshold is stochastic, but still a positive finding indicating a very low parasite density. For Aim 2, we are not concerned about misclassification of parasite density due to the reliability of PCR results. Additionally, we did not assess HRP2 deletion among parasites in this study, which could explain the false negative RDT results[28]**.** However, HRP2 deletions have proven to be rare in western Kenya so we do not anticipate a high prevalence in this sample of parasites[99].

In Aim 2, the probability of transmission based on haplotype sharing is a proxy for the human-to-mosquito transmission, which could lead to misclassification of transmission events. We cannot know for certain from these data if a mosquito bit a particular participant. Therefore, differential outcome misclassification may have occurred if some non-events were incorrectly classified as transmission events. Another related limitation is that we only have information about total parasite density and do not have gametocyte density. Male and female sexual stage gametocytes are required for human-to-mosquito transmission. We will be unable to directly measure the transfer of gametocytes with this data and instead must infer transmission from the genetic information from other parasite stages.

Generalizability

This research used data from a longstanding cohort of participants of all ages in a high malaria transmission area. Our observations may be useful to other high transmission settings in sub-Saharan Africa. There is the potential for lack of generalizability of these results to other regions due to differences in ecology, vector distribution, and underlying immunity in the population. Transmission dynamics vary on a local scale which may affect the transportability of these results. Generally, these robust methods could be applied in other settings to estimate contributions to the infectious reservoir.

Using robust analytic methods, we described the natural history of sub-patent *P. falciparum* infections and investigated the contribution of low-density infections to onward transmission. We found that the risk of clinical malaria among people with sub-patent infections was modified by transmission season, and that lower density infections had higher odds of transmitting to mosquitos. These results provide justification for targeting low-density infections in order to reduce onward transmission.

APPENDIX

Supplementary Information for Chapter IV

Inverse Probability of Treatment and Censoring Weights

$$\pi_i = \frac{P(A_i = a)}{P(A_i = a|Z_i)}$$

The formula to calculate inverse probability of treatment weights is above. Where A is the exposure group and Z is a set of covariates. To calculate the numerator of the stabilized weights, we fit an outcome-only logistic regression model to estimate the probability of having a sub-patent infection. To calculate the denominator, we fit a logistic regression model with sub-patent infection as the outcome and age, sex, transmission season, and bed net use as covariates to calculate the probability of having a sub-patent infection given these covariates. For the sub-patent group, the weights will be calculated as $\frac{numerator}{denominator}$. For the uninfected group, the weights will be calculated as $\frac{1-numerator}{1-denominator}$. We used bootstrapped standard errors to calculate 95% confidence intervals.

$$\pi_D = \frac{P(T_D > t|A_0)}{P(T_D > t|Z_t, A_0)} = \prod_{out=1}^{[t]} \frac{1 - P(D_{out} = 1|D_{in} = 0, A_0)}{1 - P(D_{out} = 1|\overline{D}_{in} = 0, Z_{in}, A_0}$$

Above is the formula used to calculate censoring weights. All episodes that resulted in clinical malaria received an outcome of one. We used logistic regression to estimate the numerator an denominator of the above equation for those who did not experience the outcome. T_D represents the time of censoring, *t* is time, A is the exposure group, Z is a set of covariates, and D(t) is an indicator of censoring at time *r.*. We multiplied the weights together and applied them to the Kaplan-Meier curves to estimate risks and calculate risk differences.

Sensitivity Analyses

During the low transmission season, participants with symptoms are more likely to have a non-malaria illness. Therefore, under the main approach, they are more likely to be censored in the analysis after a negative RDT within 60 days of the index episode. This created an imbalance of time at risk between transmission seasons that we mitigated by including transmission season in the inverse probability weight for informative censoring. We conducted sensitivity analyses to account for differences in censoring in the different transmission seasons. Specifically, we allowed episodes to remain in the analysis for the full 60 days or until they developed clinical malaria. Any subsequent RDT-negative episode within 60 days of the index episode was not considered a censoring event, but was treated as an index episode.

Figure S4.1. Study flowchart and application of inclusion criteria for Aim 1.

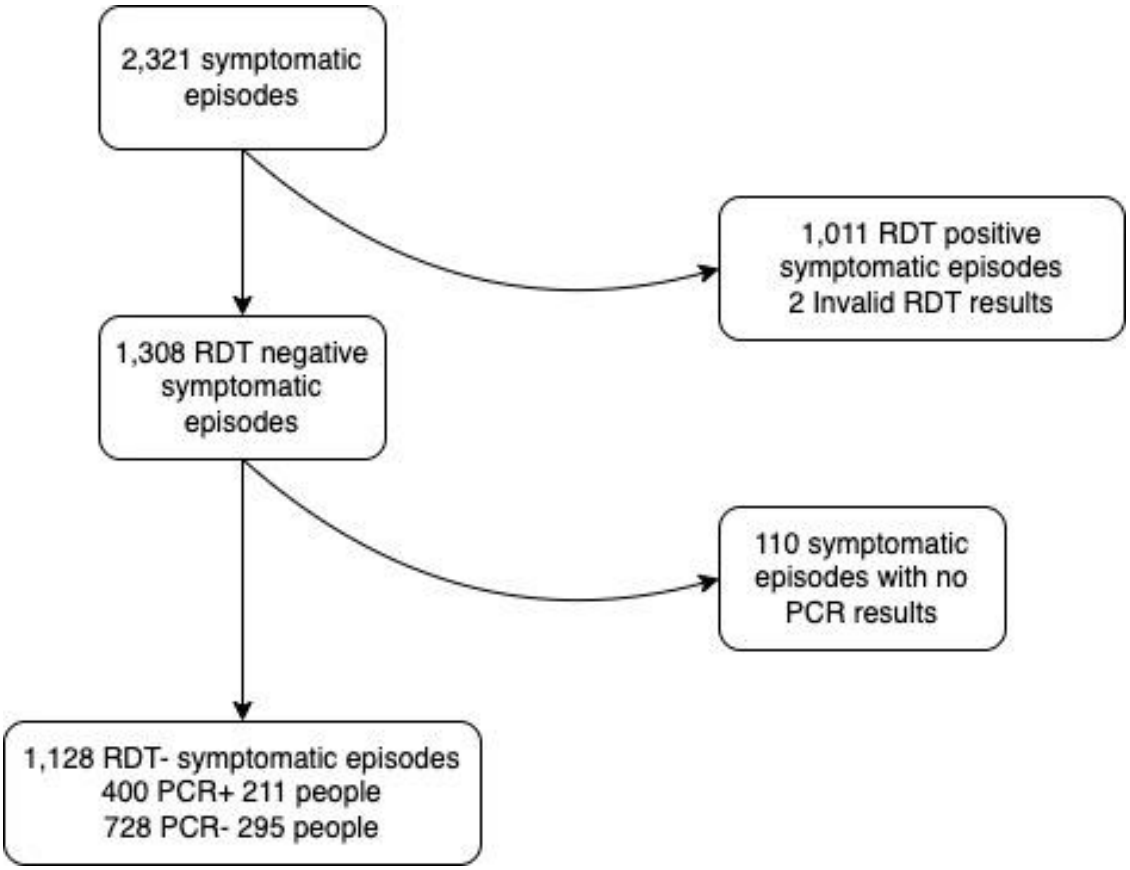


Figure S4.2. Directed acyclical graph of the association between sub-patent malaria and future RDT positivity.
The minimally sufficient adjustment set is age, sex, bed net use, and transmission season.

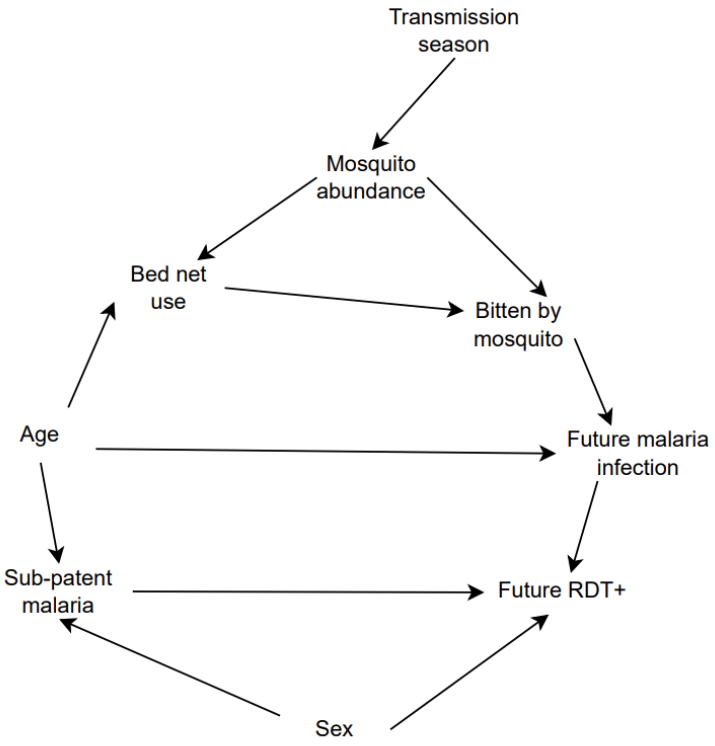


Figure S4.3. Directed acyclical graph investigating informative censoring of symptomatic RDT negative episodes.
We used a DAG to assess which variables influenced whether a participant had a negative RDT after their index infection, which would censor them at the time of testing.

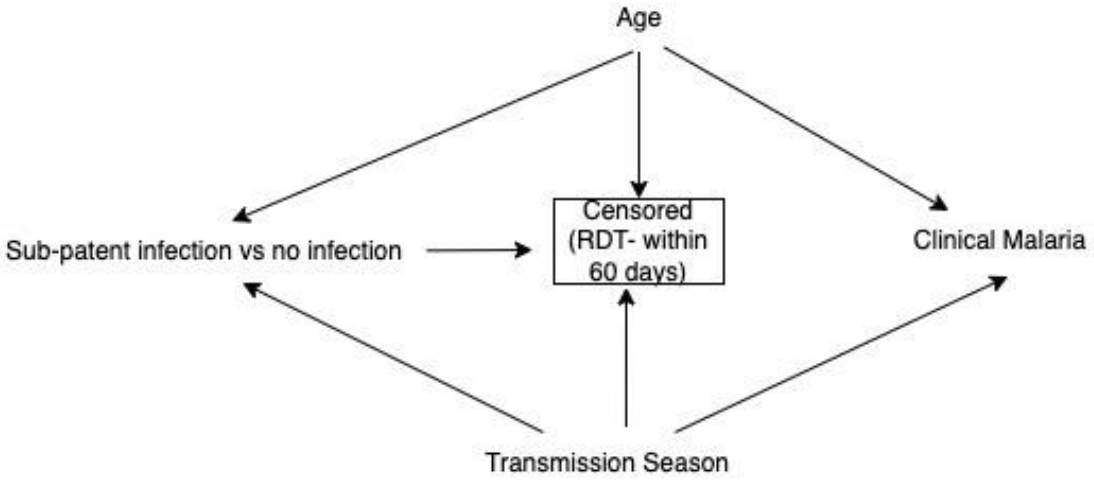


Table S4.1. **Results from sensitivity analysis using alternative censoring criteria**

	High Transmission Season					Low Transmission Season				
	N	**Risk in sub-patent episodes**	**N**	**Risk in uninfected episodes**	**Adjusted risk difference (95% CI)**	**N**	**Risk in sub-patent episodes**	**N**	**Risk in uninfected episodes**	**Adjusted risk difference (95% CI)**
All episodes	147	11.7%	193	15.7%	-4.0% (-11.4%, 3.5%)	253	6.5%	535	4.3%	2.2% (-1.2%, 5.6%)
Fever	95	8.5%	126	17.1%	-8.6 (-17.4%, 0.3%)	132	5.8%	334	4.3%	1.5% (-2.8%, 5.8%)
Low parasite density	228	10.3%	535	15.5%	-5.2 (-12.7%, 2.3%)	133	5.4%	193	4.3%	1.2% (-2.2%, 4.5%)
Age (years)										
<5	18	0%	31	15.7%	-15.7% (-28.2%, 3.2%)	41	7.7%	86	5.6%	2.1% (-6.9%, 11.0%)
5-15	37	18.8	46	23.1%	-4.3% (-22.8%, 14.2%)	75	11.0%	162	6.0%	5.0% (-3.3%, 13.2%)
>15	92	11.6%	116	12.2%	-0.6% (-9.7%, 8.5%)	137	3.9%	287	2.8%	1.1% (-2.7%, 4.9%)

Supplementary Information for Chapter V

Figure S5.1. Study flowchart and application of inclusion criteria for Aim 2.

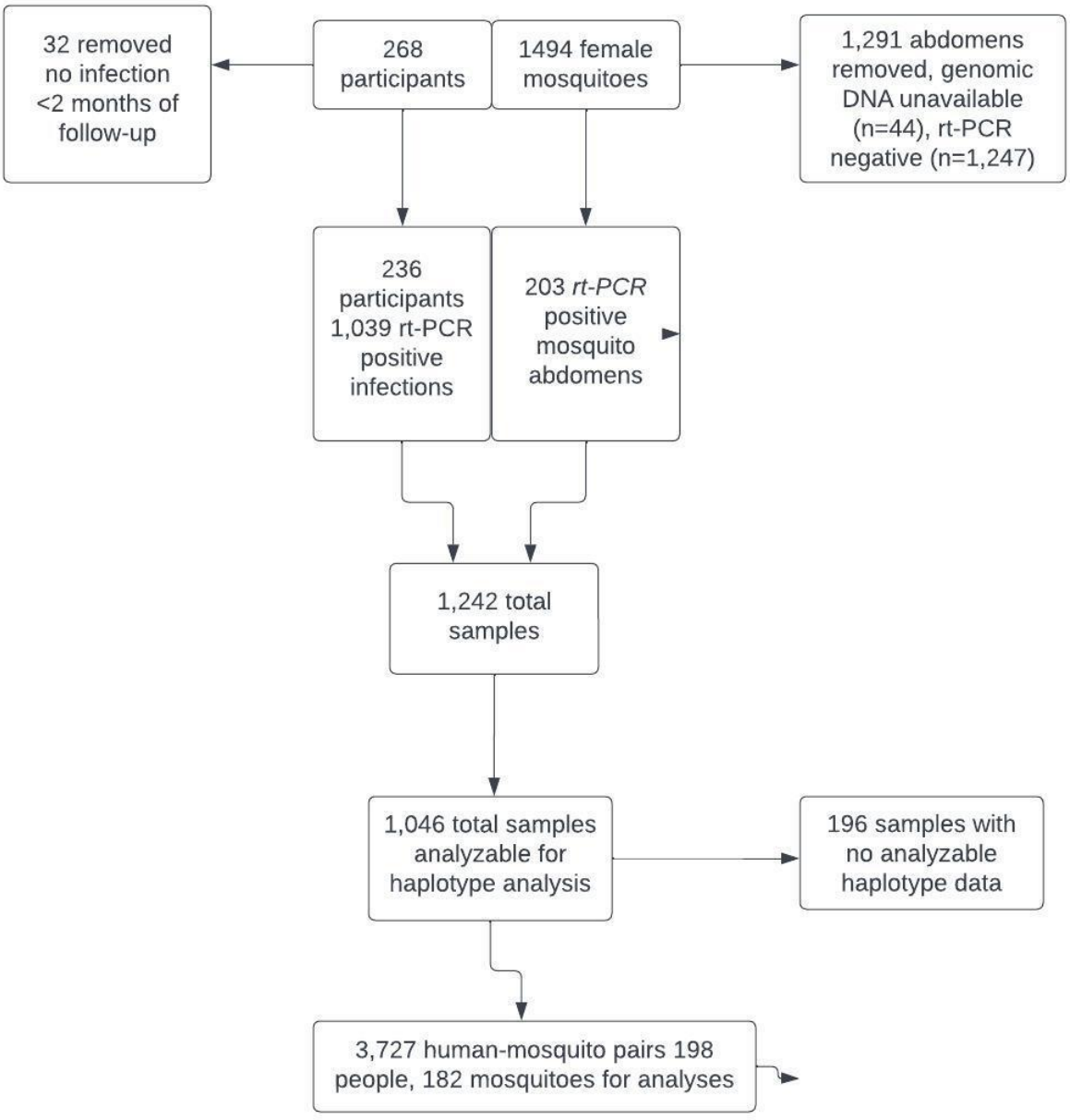


Figure S5.2. Directed acyclical graph illustrating the association between parasite density and transmission.
The minimally sufficient adjustment set is age, sex, village, infection type, and transmission season.

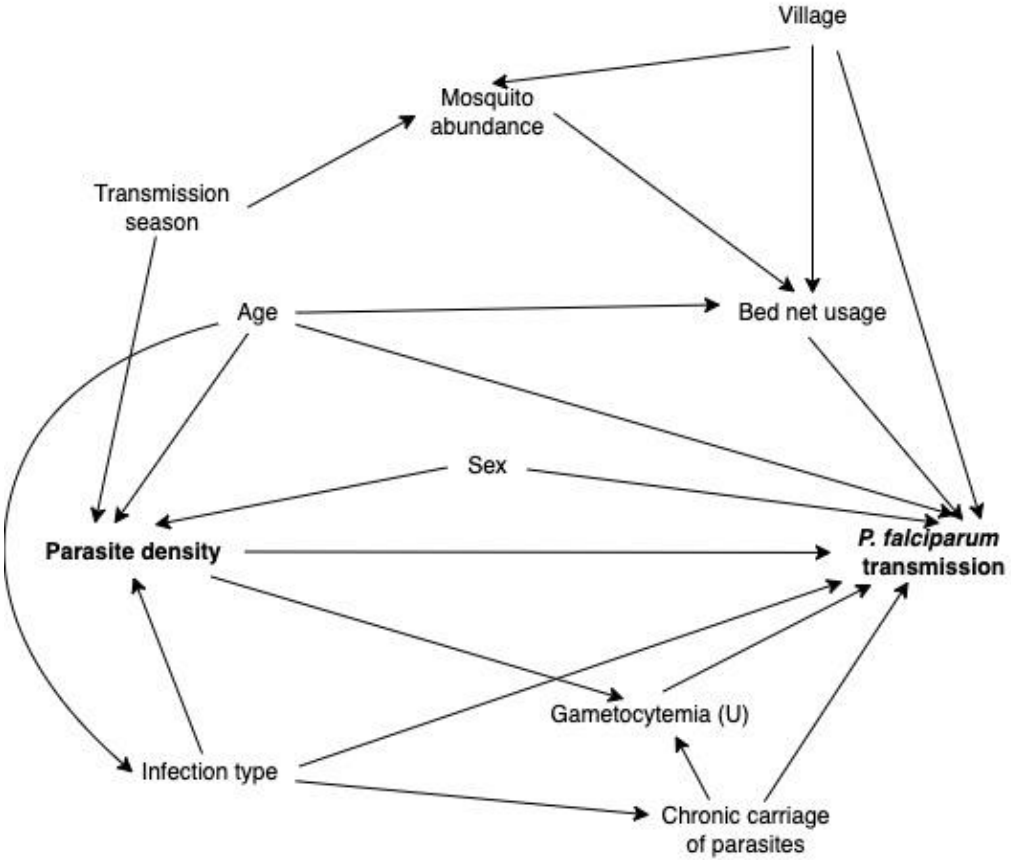


Figure S5.3. Directed acyclical graph illustrating association between age and transmission.
No adjustment is necessary to estimate the association between age and transmission.

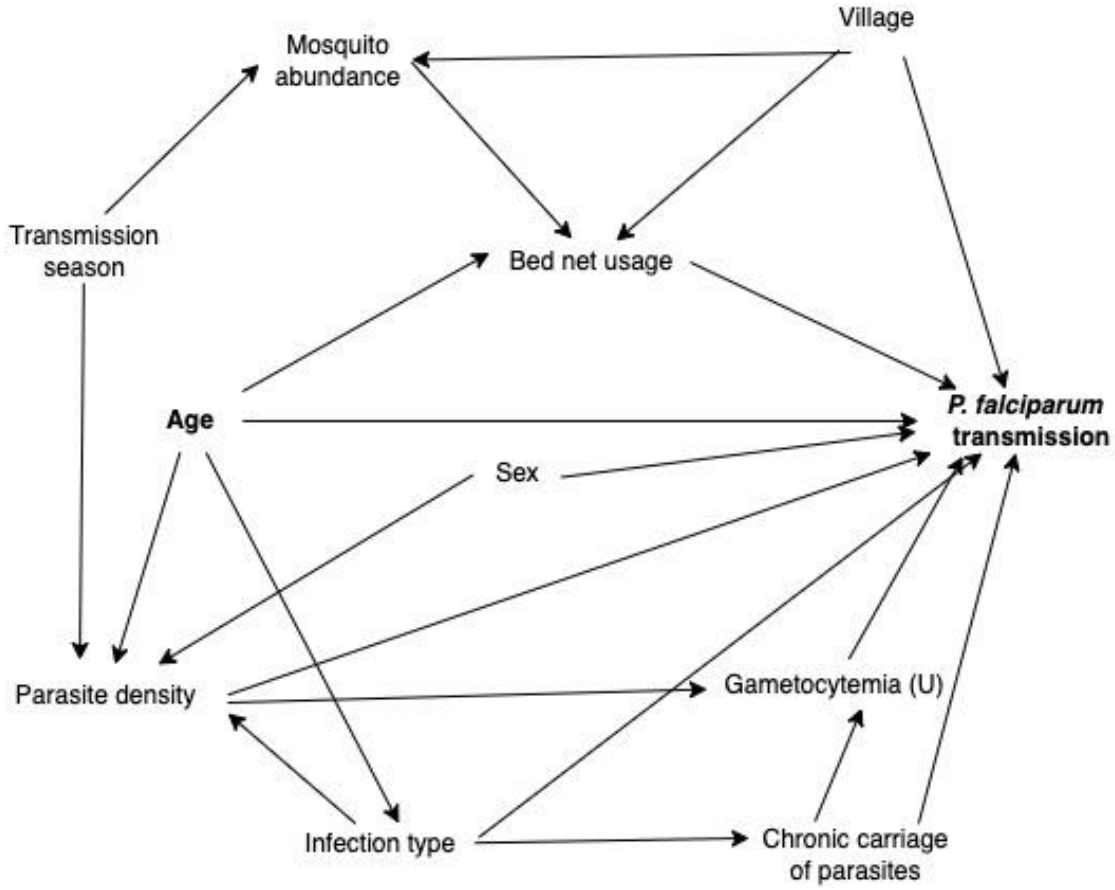


Figure S5.4. Directed acyclical graph illustrating the association between sex and transmission.
No adjustment is necessary to estimate the association between sex and transmission.

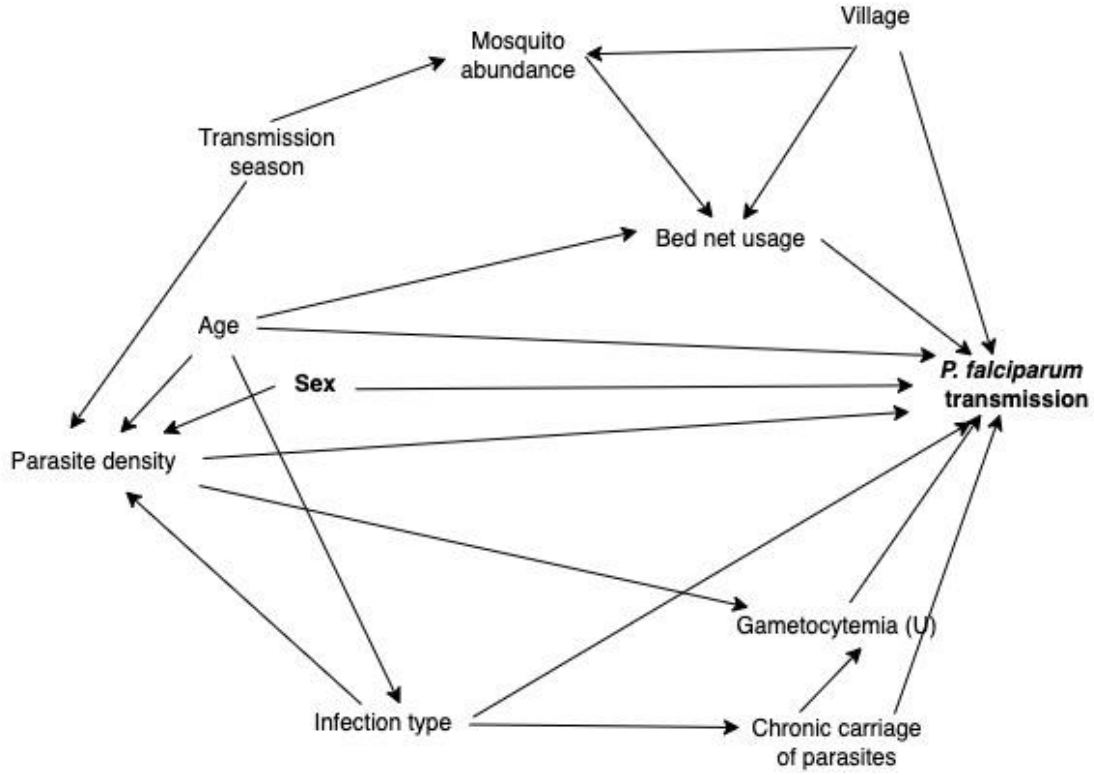


Figure S5.5. Directed acyclical graph illustrating the relationship between bed net use and transmission.
The minimally sufficient adjustment set is age, transmission season, and village.

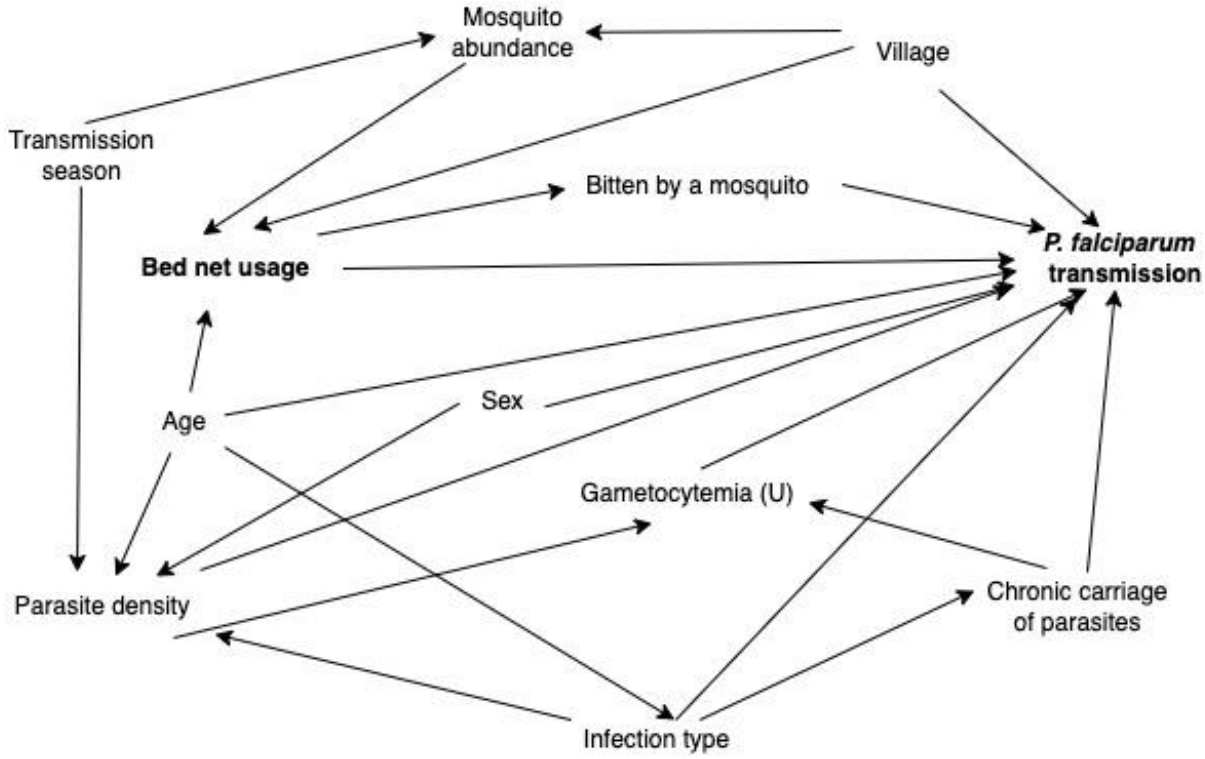


Figure S5.6. Directed acyclical graph illustrating the association between transmission season and transmission.
No adjustment is necessary to estimate the association between transmission season and transmission

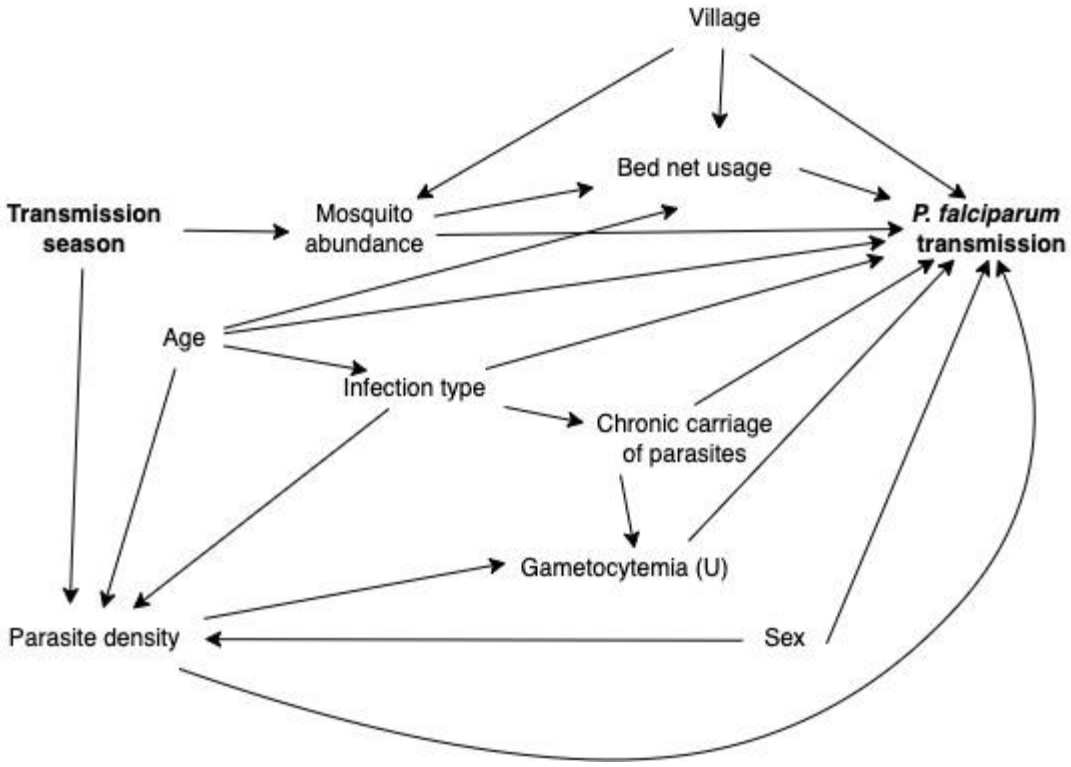


Figure S5.7. Directed acyclical graph illustrating the relationship between infection persistence and transmission.
The minimally sufficient adjustment set is age and infection type.

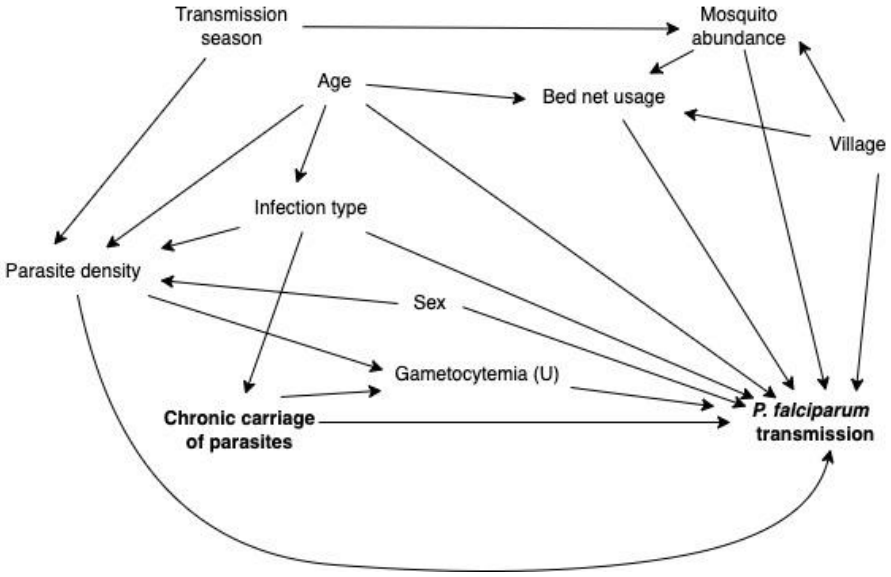


Figure S5.8 Distribution of outcome variable: probability that a human-mosquito pairing represents a transmission event.

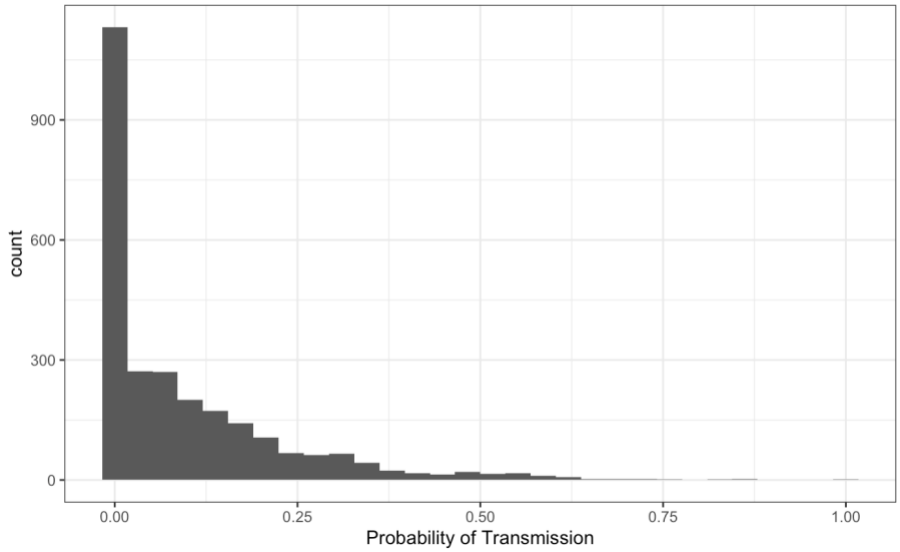
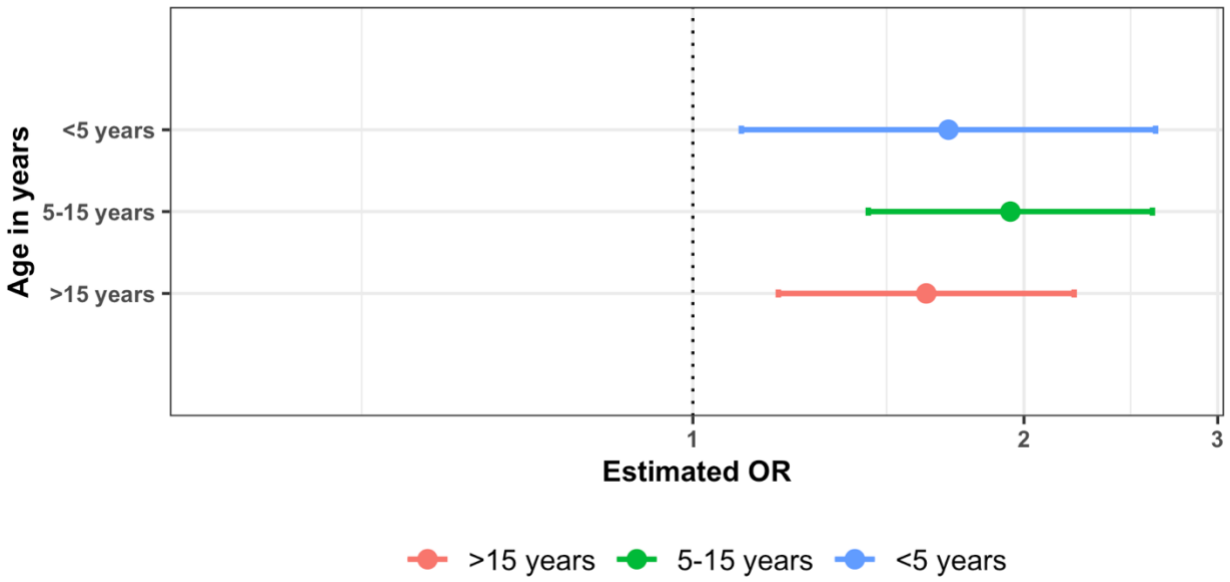


Figure 5.9. Logistic regression results for the odds of human-to-mosquito transmission for low parasite density infections compared to high parasite density infections stratified by age group.
Adjusted stratum specific odds ratios (ORs) of the probability of malaria transmission from humans to mosquitoes. Dots indicate the ORs and the lines indicate the 95% confidence intervals. ORs were computed using logistic regression models with generalized estimating equations (GEE) to account for the correlation between infections in the same participant. The probability of transmission outcome was coded continuously. ORs were adjusted for sex, transmission season, and infection type. The similarity in stratum specific odds ratios and overlapping confidence intervals indicates that age does not modify the association between low parasite density and transmission.



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