Determining the burden of *Plasmodium falciparum* transmissible stages in sub-Saharan African settings:

Indices of transmission in Burkina Faso

André Lin Ouédraogo

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Indices of transmission in Burkina Faso

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"What we have done for ourselves alone dies with us; what we have done for others and the world remains and is immortal."

Albert Pike

Chapter 1

General introduction

1.1. The global epidemiology and burden of malaria

Malaria remains the most important parasitic disease affecting humans, with approximately one fourth of the world population at risk of infection. *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae* are the four species causing disease in humans. *P. knowlesi*, which naturally infects macaques, is also transmissible to humans in whom it may cause disease[1-5]. *P. falciparum* is the most pathogenic species. In 2008, an estimated 243 million clinical malaria cases occurred worldwide, with 863,000 deaths[6], the great majority (89%) falling on poor rural communities of sub-Saharan Africa[6-8]. In the same area, in addition to the human cost, malaria causes every year an average loss of 1.3% of economic growth [9], and represents a major obstacle to the development of disease-endemic countries[10].

1.2. Malaria in Burkina Faso

Burkina Faso is a landlocked Sahel country in West Africa lying between the Sahara Desert and the Gulf of Guinea (Figure 1). Its vegetation ranges from forest in the south to semi-desert, Sahel, in the north. The central part has the ecological characteristics of Sudan savannah. Annual rainfall varies from about 1,000 mm in the south to less than 250 mm in the extreme north[11]. The country has two distinct seasons, dry from November to May and wet from June to October.

Malaria represents a leading public health problem in Burkina Faso[12,13]. *P. falciparum* is the predominant plasmodial species and accounts for 90% of the infections; the remainder is attributed to either *P. malariae* (8%) or *P. ovale* (2%). The main malaria vectors are *Anopheles gambiae s.l.* and *An. funestus*[14]. Children and pregnant women are the most vulnerable groups at risk of malaria-related morbidity and mortality.

Rural families, that account for 80% of the population, are the least likely to have access to malaria control measures, since they live far from the nearest health facility and less able to afford treatment [15].

The climatic features during the wet season (high temperature and humidity) allow for excellent synchronization between the vector's multiplication and long life-span [16-18] with the parasite's replication in both vector and human hosts, together conferring a high

reproduction rate to *P. falciparum* [17] in the country. Malaria transmission differs, nevertheless, characterized as unstable in the extreme north but perennial in the south. In the central plateau, where it is holoendemic [19], transmission is stable and seasonal with an entomological inoculation rate (EIR) with peaks of 300-500 infective bites/person/year[20] during the wet season but effectively close to zero during the dry season. The prevalence of *P. falciparum* infection has been estimated to be approximately 90% in children below 5 years of age in the year of 2004[21,22].

Figure 1: Malaria transmission characteristics in Burkina Faso

1.3. The biology of *P. falciparum*

The asexual blood forms of *P. falciparum* are responsible for malaria-related morbidity and mortality, whilst transmission of the parasite depends on the presence of sexual forms (gametocytes) in human blood that can infect anopheline vectors.

The life cycle (Figure 2) of *P. falciparum* can be depicted as starting when an infected female *Anopheles* mosquito bites a human being, when sporozoites are inoculated and can enter the blood stream. 30% of the sporozoites leaving the skin bite area are estimated to invade the lymph nodes [23-25] whilst the remainder migrates to the liver. There, they invade and mature within hepatocytes, each sporozoite undergoing asexual reproduction to produce several thousand merozoites. The hepatocyte bursts and the merozoites thereby released invade red blood cells (erythrocytes) where they feed on haemoglobin and mature into trophozoites. Nuclear division ensues in an asexual reproductive cycle (erythrocytic schizogony) that leads to the formation of schizonts that comprise a collection of individual merozoites. After subsequent release and reinvasion of an erythrocyte, a fraction of these merozoites develop into individual male and female gametocyte sexual forms that are ingested by a female *Anopheles* mosquito taking a blood meal. In the mosquito midgut, the gametocytes mature into gametes that, after fertilization, form a motile zygote that matures into an ookinete and, ultimately, an oocyst on the mosquito midgut wall. Within each oocyst, hundreds of sporozoites develop, eventually rupturing the oocyst and migrating to the mosquito salivary glands from where they can be inoculated into the skin of the next human on whom the mosquito feeds.

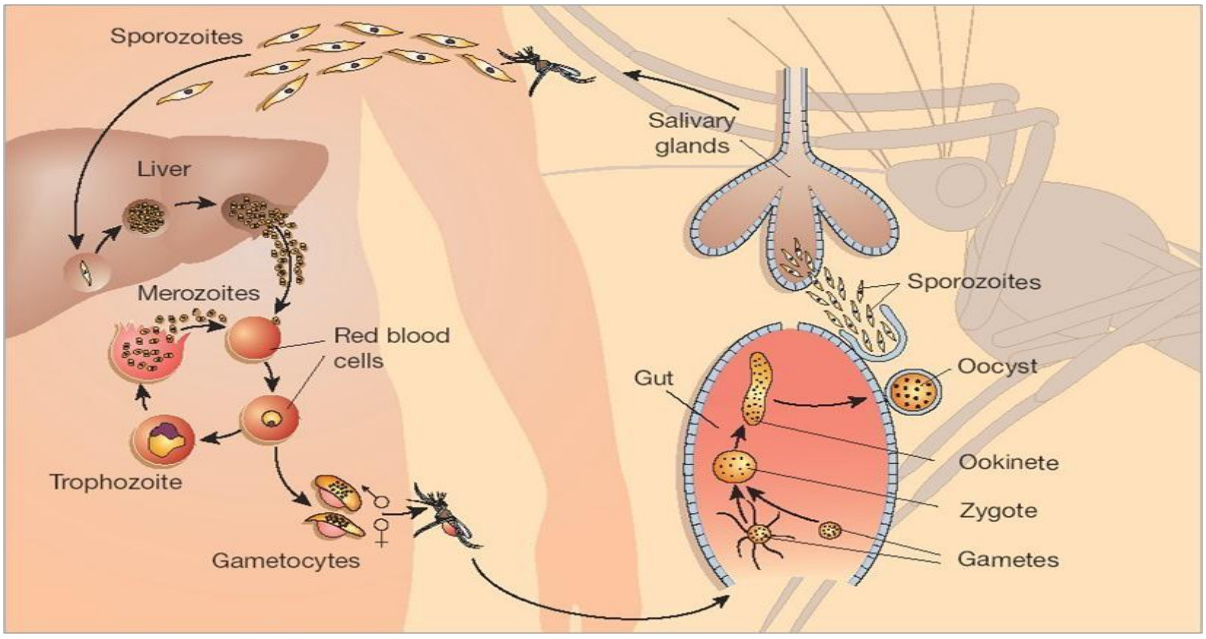

Figure 2: Life cycle of *P. falciparum*

1.4. Malaria control and elimination

1.4.1. History of malaria control

Early initiatives aimed at malaria control in the 1950s and 1960s, coordinated by the Global Malaria Eradication Campaign, recommended malaria case management and the use of dichlorodiphenyltrichloroethane (DDT) for indoor spraying of dwellings against the mosquito vector as major strategies to reduce the burden of malaria. The programme was successful in low and unstable transmission areas of North America and Europe[26]. Sub-Saharan Africa, which needed a long-term integrated programme, was excluded from this campaign because of economic instability and various other issues. In some areas like Sri Lanka, India and Cambodia, attempts to eliminate malaria were followed by disastrous increases in transmission once enthusiasm was lost and interventions were abandoned[27]. After this period, efforts to control malaria have been purely initiated on a bilateral basis or through international organizations. In 1992, the Global Malaria Control Strategy was adopted in Amsterdam and focused on prompt diagnosis, treatment and preventive measures. This was followed in 1996 by establishment of the Multilateral Initiative on Malaria that aimed to link the global malaria research community and strengthen research capacity in malaria endemic countries. In 1997, the Organization for African Unity called upon international organizations to give malaria greater priority.

1.4.2. Current malaria control strategies

In 1998 the Roll Back Malaria (RBM) Partnership was initiated to coordinate efforts in malaria control. The main goal of the RBM was to ensure a reduction in the burden of malaria of at least 50% by 2010 and of 75% by 2015, moving towards elimination in some countries [28].

The global strategy of the RBM Partnership is based on prevention and treatment through i) the use of long-lasting insecticide-treated nets (LLINs), ii) early diagnosis and timely seeking of appropriate treatment with artemisinin-based combination therapies (ACTs), iii) intermittent preventive treatment (IPT) during pregnancy and iv) indoor residual spraying (IRS) with insecticides to target indoor-resting mosquitoes.

1.4.3. Current effective tools for malaria prevention and treatment

LLINs are currently one of the most important tools for vector control. With a lifespan exceeding 3 years[6], they can not only reduce vector-human contact, but mosquitoes are also killed by the insecticide. LLINs have been shown to significantly reduce childhood malaria-related morbidity and mortality in malaria endemic areas[29,30]. Despite calls for a rapid scaling up of LLINs coverage by RBM in 2002, only an estimated 20% of children at risk in sub-Saharan Africa had received an LLIN by 2007[31].

IRS consists of the application of insecticides to the inner surfaces of dwellings, where endophilic anopheline mosquitoes often rest after taking a blood meal. IRS can be effective in reducing malaria transmission by killing / repelling mosquitoes and, subsequently, reducing morbidity and mortality[32]. However, caution is needed as resistance to insecticides may develop, especially pyrethroids[6]. In 2008, nineteen countries in the African Region, reported implementing IRS[6].

Intermittent preventive treatment (IPT) is recommended for women during pregnancy to prevent malaria that can lead to maternal anemia and placental infection that itself is associated with low birth weight babies[33-37]. WHO currently recommends that at least 2 doses of SP be administered as a prophylaxis after the first trimester during antenatal care[6].

1.4.4. Malaria diagnosis

Because preventive measures are not fully effective, early malaria diagnosis and effective treatment form important components of malaria control.

Although clinical diagnosis of malaria is imprecise, it remains the basis of therapeutic care for the majority of febrile patients in malaria endemic areas, where laboratory facilities are often limited. To avoid over prescription of ACTs, the WHO has recommended the use of a parasitological test before treating[38]. Rational treatment of malaria is essential both to avoid non-target effects, to delay the advent of resistance, and to save cost on alternative drugs.

For the diagnosis of clinical malaria attacks and quantification of clinically relevant parasite densities in the blood, microscopy is the most affordable and widely-used technique [39]. Microscopy can be used to detect and quantify malaria parasites in a thick blood smear by examining multiple (commonly 100) high power fields. Most often, parasites

are quantified by simultaneously enumerating the number of leukocytes per microscope field and then converting to parasite numbers per µl by assuming a standard 8000 leukocytes/µl blood. Depending on the number of leucocytes counted the detection limit of microscopy ranges between 5-20 asexual stage parasites/µl of blood and 8-16 gametocytes/µl of blood. Methodological studies of malaria microscopy have documented that the frequency of false-positive and false-negative results is remarkably high, increasesing markedly at lower parasite densities[40,41], and estimates of parasite densities may differ between individual microscopists by as much as an order of magnitude[42].

Rapid Diagnostic Tests (RDTs) are immuno-chromatographic tests with plasmodial antigen specificity[43-45]. RDTs were introduced as an alternative to microscopy in health settings lacking laboratory facility. Although RDTs have an important impact on clinical decisions[46], they have limitations including low sensitivity[6,47], absence of quantification, lack of parasite stage specificity, as well as cost.

1.4.5. Artemisinin-based combination therapies (ACTs)

Chloroquine (CQ) and sulphadoxine-pyrimethamine (SP), long considered the most effective and useful anti-malarial drugs, have become largely ineffective as monotherapy for the treatment of *P. falciparum* malaria in sub-Saharan Africa, with increases in malaria-related morbidity and mortality thought to be one of the primary consequences[48]. In recent years, following the RBM partnership recommendations, malaria treatment policies have thus shifted in most sub-Saharan countries to ACTs for the treatment of uncomplicated *P. falciparum* malaria[38].

Artemisinin-based combination therapy comprises the simultaneous use of an artemisinin component with one or more drugs that have independent modes of action and different biochemical targets in the parasite, thereby offering faster cure rates with the (theoretical) additional advantage of delaying the development of resistance to the partner drug for a much longer time period. Four combinations are currently recommended: artesunate-amodiaquine, artemether-lumefantrine, artsunate-mefloquine, and artesunate-sulfadoxine-pyrimethamine[45].

ACT acts as a double-sword by providing a complete cure of the infection, as well as reducing transmission[49-52], thereby eliminating the sources of new malarial infections[49,53]

However, ACTs are much more expensive than previous regimens and coverage with ACTs in sub-Saharan Africa is still extremely low. Only 3% of children under 5 years of age received ACTs in 2006 and 2007[54]. In Burkina Faso, ACTs were adopted as first-line treatment in 2005[55], after childhood mortality was found to have increased due to CQ resistance[56-62]. ACTs became widely available in 2007 in Burkina Faso[15] with artemether-lumefantrine (AL) and artesunate-amodiaquine (AS/AQ) available for treatment of uncomplicated malaria. Sulpfadoxine/pyrimethamine (SP) is used for intermittent preventive treatment during pregnancy.

1.4.6. Vaccines as additional tools for controlling malaria

History teaches us that malaria control interventions lead to often unmet expectations and that the real picture is one of increasingly more widespread drug-resistant parasites and insecticide-resistant vectors. In this context, and as part of the global efforts to control malaria, effective vaccines will be of great importance. The clinical protection provided by immunity acquired through natural repeated exposure[63] or by immunization with either irradiated[64] or viable sporozoites while under effective prophylaxis [65] suggest that a malaria vaccine is indeed feasible.

Vaccines against pre-erythrocytic stages or asexual blood stages aim to prevent infection or prevent/reduce (severe) clinical disease. Although several vaccine candidates are in the pipeline of clinical trials, the most advanced is the so-called RTS,S vaccine, developed originally by GSK and the US Army, which has shown up to 65% efficacy against *P. falciparum* infection and is now in multi-centre Phase 3 trials sub-Saharan across Africa[66-68].

Malaria vaccines can also target a third life stage, the transmission stage. The so-called transmission blocking vaccine is directed against the parasite's mosquito stages through induction of human immune responses that interfere with sporogonic development in the mosquito midgut, thereby reducing or interrupting transmission[69-76]. A transmission blocking vaccine will reduce the number of infected mosquitoes and is thus predicted to subsequently reduce population-wide malaria infection rates and ultimately malaria-associated morbidity/mortality. Its viability is supported by observations of naturally acquired transmission reducing immune responses in endemic situations.

1.4.7. Naturally-acquired and vaccine-induced transmission reducing activity (TRA)

Transmission reducing immunity was first reported in the 1970s in immunized chickens[77,78] and later using specific monoclonal antibodies [73,79-81] or naturally acquired antibodies[72,73,75,82-86] against sporogonic stages. Transmission reducing activity (TRA) in the mosquito midgut mostly depends on human humoral (antibody-mediated) immunity rather than cellular components[87,88]. Transmission reducing antibodies may prevent fertilization by blocking the fertilization receptors on gametes, by a complement-mediated lysis of gametes and zygotes or by preventing ookinete invasion of the midgut epithelium[89]. Transmission reducing antibodies are ingested as part of the blood meal of the anopheline vector, inhibiting the development of sporogonic stages resulting in the prevention of malaria transmission to another human. In endemic areas, natural transmission reducing immunity develops after exposure to gametocytes that have a finite lifespan and die if not transmitted to a mosquito. Effective immunity of this type is generally induced in individuals with little or no history of malaria, either young African children or travellers from non-endemic areas[69]. It is thought to be short-lived[90] in contrast to immune responses that protect against the pre-erythrocytic and/or asexual blood stages, immunity that is comparatively long-lived once acquired and that correlates positively with repeated exposure[91,92].

The most reliable methods to assess TRA are formed by experiments in which gametocytes are offered to mosquitoes. Different mosquito feeding assays can be used to measure transmission from man to mosquito: i) in the skin feeding assay[93-95], mosquitoes directly feed on a human volunteer but the subject's safety and outcomes of mosquito infection may be considered controversial if the mosquitoes used in the experiment are not reared under sterile conditions, raising ethical concerns; ii) in the direct membrane feeding assay[96,97], mosquitoes feed on whole blood sample offered through an artificial membrane; iii) in the standard membrane feeding assay[70,98,99], which is gold standard for transmission blocking activity measurement, gametocytes obtained from parasites cultured are mixed with test or control serum and offered to mosquitoes through an artificial membrane. By microscopic observation of the mosquito midgut 7 to 9 days later, the presence or absence of oocysts determines the human infectiousness.

In addition to mosquito feeding assays, serological tests (ELISA) can be used to measure gamete surface antigen-specific antibodies in serum as a surrogate marker for TRA[69,72-74,83].

The targets of transmission reducing immunity are the extracellular (surface membrane-expressed) antigens of the sporogonic stages. These include, for example, *Pfs48/45 ,* a pre-fertilisation protein expressed on *P. falciparum* gametocytes and gamete surfaces that has a central role in male gamete fertility[100]. Recently, expression of a truncated form of Pfs48/45 (Pfs48/45-10C) produced in *E. coli* was shown to be able to induce antibody-mediated transmission blocking immunity in mice. In a standardized laboratory assay, the antibodies reduced *P. falciparum* oocyst numbers by up to 100% in laboratory-bred female *An. stephensi*[101].

Pfs230 is another protein expressed on the surface of gametocytes that induces natural transmission blocking antibodies that act by lysing gametes in the presence of complement[80,85,102].

Pfs25 is a post-fertilization protein expressed on the surface of zygote/ookinete. Antibodies against Pfs25 can also reduce transmission[103,104] but are not naturally induced in humans. The protein is not expressed in gametocytes and therefore not encountered by the human immune system. Pfs25 is one of the leading candidates for development of a transmission blocking vaccine. A Phase 1 human trial of a Pfs25-based vaccine showed that functional antibodies coud be elicited[105] but the trial was interrupted due to adverse events induced by the vaccine adjuvant.

Antibodies directed to Pfs48/45 detected in Cameroonian sera have been shown to be associated with TRA[75,83] while TRA observed in sera from Papua New Guinea correlated with the amount of anti-Pfs230 antibodies. In general, many questions remain about the functionality of sexual stage immune responses and their acquisition in relation to exposure to (low density) infections.

1.4.8. Microscopical and sub-microscopical parasite densities and malaria control and elimination

At any given moment, only a small fraction of the asexual blood-stage parasites of *P. falciparum* will generate gametocytes[106], and as a result only a fraction of infected individuals will carry gametocytes[106,107] that are estimated to survive from 1 week[107-109] to several months[108,110] in asymptomatic individuals. In areas where malaria is highly endemic, the prevalence of gametocytes circulating in peripheral blood is higher in children, the age-group that also carries the highest densities of their precursors, the asexual blood-stage

parasites[93,111,112]. In areas of low endemicity, the distribution of gametocytes is evenly spread across age-groups[107,113]. In both cases, the patterns of gametocytes reflect those of asexual blood-stage parasites that are themselves a reflection of transmission intensity and naturally-acquired immunity. The low occurrence of gametocytes in endemic areas is also partly a reflection of the low sensitivity of microscopy for their detection especially since they often circulate at very low densities[114-117]. More recently, molecular detection methods have been developed to detect gametocyte densities below the microscopical threshold.

Molecular techniques based on RNA quantification are more sensitive than microscopy. The reverse transcriptase polymerase chain reaction (RT-PCR) can detect different developmental stages of *P. falciparum* blood stage parasites based on stage-specific gene expression [118]. The use of RT-PCR has proved that submicroscopic gametocytaemia is common in endemic areas[119,120]. Gametocyte prevalence determined by RT-PCR in a low transmission area of Sudan was found to be between 2.5 and 4.5 fold higher than estimates by microscopy[121]. However, a limitation of RT-PCR is that the presence of DNA in malaria blood samples may interfere with RNA, negatively affecting amplification accuracy. An alternative molecular technique is quantitative nucleic sequence-based amplification (QT-NASBA). QT-NASBA[122] is based on the activity of three enzymes (AMV-RT, Rnase H and T7 RNA polymerase) and the use of two target-specific primers (one of which includes a T7 polymerase/promotor) to amplify RNA molecules at a low temperature of 41°C, restricting annealing to single strand RNA alone and not to DNA. QT-NASBA has been adapted for simple use in the field and parasitic ribosomal and messenger RNA has been found to be stable for at least two months when bound to silica and stored dry at -20° C. The technique allows parasite quantification to be performed using small-volume (50- 100 µl) finger prick blood samples, and has a lower limit of 10 asexual parasites/mL and 20-100 gametocytes/mL. Studies in malaria endemic areas of East and Central East Africa have shown that submicroscopic gametocyteamias quantified by QT-NASBA is common in asymptomatic children, but also at enrolment and after treatment of symptomatic children[123-125]. Sub-microscopic gametocyte carriers in both treated and asymptomatic groups were able to infect mosquitoes in membrane feeding experiments[49,126], highlighting the importance for malaria control efforts of more sensitive molecular techniques as potentially valuable tools in determining the true reservoir of infection.

Effectively attacking malaria would indeed benefit from better knowledge of the human infectious reservoir since the more precise the estimates, as well as their relative contribution to transmission, the more efficient will be the interventions, leading to more effective control and, ultimately, faster elimination.

2. Decline in the burden of malaria

In recent years a substantial decline in the burden of malaria has been reported by several countries in sub-Saharan Africa[127]. Most of these countries showed an overall 50–90% decline in the prevalence of malaria[128-135]. Although these findings have resulted in optimism about the possibilities to reduce the burden of malaria with currently available tools[136], caution is needed when considering the challenges of controlling or eliminating malaria. No reduction or even increases in the burden of malaria has been registered in countries such as Burkina Faso or Nigeria despite an increase in bed net coverage[137],[138] in countries with similar characteristics of malaria transmission like Nigeria. The observed decline in the burden of malaria in some of the countries is probably the result the WHO scaling up malaria control strategies. However, transmission reducing strategies would probably bring this much more down as new visions of eliminations highlight their importance.

3. Outline of this thesis

This thesis describes, in several steps, the prevalence of *P. falciparum* gametocytes and their infectiousness in an endemic area with seasonal malaria transmission: i) The within-host dynamics of *P. falciparum* gametocytes ii) The contribution of submicroscopic gametocytaemia to malaria transmission, iii) The relevance of sexual stage immunity to reducing malaria transmission.

The dynamics of gametocytes in the human host was studied through a series of cross-sectional surveys including individuals of all ages. Gametocytes were detected by standard microscopy on thick blood smears collected during distinct transmission seasons and the effect of age and season tested (Chapter 2). To get more insights in the effect of age on gametocytes, a more sensitive molecular technique for parasite quantification was further used (Chapter 3 & 4). As infectiousness of *P. falciparum* within the human host is a key

determinant for man-mosquito transmission, membrane feeding experiments were performed on malaria exposed individuals where whole blood samples were offered to locally reared *An. gambiae* mosquitoes to test the capacity of gametocyte-carrying humans to transmit malaria (Chapter 5). Specific immune responses against *P. falciparum* sexual stages are thought able to reduce man-mosquito transmission. In Chapter 6, the epidemiology of naturally acquired immune responses to sexual stages is studied. The implications of the overall results for malaria control and elimination are discussed.

References

1. Cox-Singh J, Singh B. Knowlesi malaria: newly emergent and of public health importance? *Trends Parasitol* 2008;**24**(9)**:**406-10.
2. Cox-Singh J, Davis TM, Lee KS, et al. *Plasmodium* knowlesi malaria in humans is widely distributed and potentially life threatening. *Clin Infect Dis* 2008;**46**(2)**:**165-71.
3. Singh B, Kim Sung L, Matusop A, et al. A large focus of naturally acquired *Plasmodium* knowlesi infections in human beings. *Lancet* 2004;**363**(9414)**:**1017-24.
4. Daneshvar C, Davis TM, Cox-Singh J, et al. Clinical and laboratory features of human *Plasmodium* knowlesi infection. *Clin Infect Dis* 2009;**49**(6)**:**852-60.
5. Ta Tang TH, Salas A, Ali-Tammam M, et al. First case of detection of *Plasmodium* knowlesi in Spain by Real Time PCR in a traveller from Southeast Asia. *Malar J*;**9**(1)**:**219.
6. WHO World Malaria report. *http://www.who.int/malaria/wmr* 2009.
7. Breman JG, Alilio MS, Mills A. Conquering the intolerable burden of malaria: what's new, what's needed: a summary. *Am J Trop Med Hyg* 2004;**71**(2 Suppl)**:**1-15.
8. Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI. The global distribution of clinical episodes of *Plasmodium falciparum* malaria. *Nature* 2005;**434**(7030)**:**214-7.
9. Gallup JL, Sachs JD. The economic burden of malaria. *Am J Trop Med Hyg* 2001;**64**(1-2 Suppl)**:**85-96.
10. Sachs J, Malaney P. The economic and social burden of malaria. *Nature* 2002;**415**(6872)**:**680-685.
11. Direction Nationale de la Météorologie. *Ministère des Transports du Burkina Faso*.
12. Muller O, Becher H, van Zweeden AB, et al. Effect of zinc supplementation on malaria and other causes of morbidity in west African children: randomised double blind placebo controlled trial. *Bmj* 2001;**322**(7302)**:**1567.
13. Sirima SB, Konate A, Tiono AB, Convelbo N, Cousens S, Pagnoni F. Early treatment of childhood fevers with pre-packaged antimalarial drugs in the home reduces severe malaria morbidity in Burkina Faso. *Trop Med Int Health* 2003;**8**(2)**:**133-9.
14. Petrarca V, Petrangeli G, Rossi P, Sabatinelli G. [Antimalarial campaign program in Ouagadougou (Burkina Faso): the Anopheles gambiae complex in the city of Ouagadougou and surrounding villages]. *Ann Ist Super Sanita* 1986;**22**(1)**:**189-91.
15. Tipke M, Louis VR, Ye M, et al. Access to malaria treatment in young children of rural Burkina Faso. *Malar J* 2009;**8:**266.
16. Ye Y, Hoshen M, Kyobutungi C, Louis VR, Sauerborn R. Local scale prediction of *Plasmodium falciparum* malaria transmission in an endemic region using temperature and rainfall. *Glob Health Action* 2009;**2**.
17. Paaijmans KP, Blanford S, Bell AS, Blanford JI, Read AF, Thomas MB. Influence of climate on malaria transmission depends on daily temperature variation. *Proc Natl Acad Sci U S A*.
18. Greenwood B, Mutabingwa T. Malaria in 2002. *Nature* 2002;**415**(6872)**:**670-672.

19. Muller O, Garenne M, Kouyate B, Becher H. The association between protein-energy malnutrition, malaria morbidity and all-cause mortality in West African children. *Trop Med Int Health* 2003;**8**(6)**:**507-11.
20. Cuzin-Ouattara N, Van den Broek AH, Habluetzel A, et al. Wide-scale installation of insecticide-treated curtains confers high levels of protection against malaria transmission in a hyperendemic area of Burkina Faso. *Trans R Soc Trop Med Hyg* 1999;**93**(5)**:**473-9.
21. Ouedraogo AL, Schneider P, de Kruijf M, et al. Age-dependent distribution of *Plasmodium falciparum* gametocytes quantified by Pfs25 real-time QT-NASBA in a cross-sectional study in Burkina Faso. *Am J Trop Med Hyg* 2007;**76**(4)**:**626-30.
22. Habluetzel A, Cuzin N, Diallo DA, et al. Insecticide-treated curtains reduce the prevalence and intensity of malaria infection in Burkina Faso. *Trop Med Int Health* 1999;**4**(8)**:**557-64.
23. Amino R, Giovannini D, Thiberge S, et al. Host cell traversal is important for progression of the malaria parasite through the dermis to the liver. *Cell Host Microbe* 2008;**3**(2)**:**88-96.
24. Menard R, Amino R, Thiberge S, Gueirard P. [A new view of malaria provided by parasite imaging]. *Bull Acad Natl Med* 2007;**191**(7)**:**1261-70; discussion 1271.
25. Menard R, Heussler V, Yuda M, Nussenzweig V. *Plasmodium* pre-erythrocytic stages: what's new? *Trends Parasitol* 2008;**24**(12)**:**564-9.
26. World Health Organization. Global malaria control and elimination: report of a technical review. Geneva. 2008.
27. Kidson C, Indaratna K. Ecology, economics and political will: the vicissitudes of malaria strategies in Asia. *Parassitologia* 1998;**40**(1-2)**:**39-46.
28. World Health Organization RBM. The global malaria action plan. Geneva. *http://www.rollbackmalaria.org/ Gmap* 2008.
29. D'Alessandro U, Leach A, Drakeley CJ, et al. Efficacy trial of malaria vaccine SPf66 in Gambian infants. *Lancet* 1995;**346**(8973)**:**462-7.
30. Lengeler C. Insecticide-treated bed nets and curtains for preventing malaria. *Cochrane Database Syst Rev* 2004(2)**:**CD000363.
31. Noor AM, Mutheu JJ, Tatem AJ, Hay SI, Snow RW. Insecticide-treated net coverage in Africa: mapping progress in 2000-07. *Lancet* 2009;**373**(9657)**:**58-67.
32. World Health Organization. Indoor residual spraying_Use of indoor residual spraying for scaling up global malaria control and elimination. *Geneva,* 2006.
33. Steketee RW, Nahlen BL, Parise ME, Menendez C. The burden of malaria in pregnancy in malaria-endemic areas. *Am J Trop Med Hyg* 2001;**64**(1-2 Suppl)**:**28-35.
34. Brabin B. An assessment of low birthweight risk in primiparae as an indicator of malaria control in pregnancy. *Int J Epidemiol* 1991;**20**(1)**:**276-83.
35. Menendez C, Ordi J, Ismail MR, et al. The impact of placental malaria on gestational age and birth weight. *J Infect Dis* 2000;**181**(5)**:**1740-5.
36. Tiono AB, Ouedraogo A, Bougouma EC, et al. Placental malaria and low birth weight in pregnant women living in a rural area of Burkina Faso following the use of three preventive treatment regimens. *Malar J* 2009;**8:**224.
37. Sirima SB, Sawadogo R, Moran AC, et al. Failure of a chloroquine chemoprophylaxis program to adequately prevent malaria during pregnancy in Koupela District, Burkina Faso. *Clin Infect Dis* 2003;**36**(11)**:**1374-82.
38. WHO World Health Organization. Guidelines for the treatment of malaria. *[http://www.who.int/malaria/docs/ TreatmentGuidelines].* 2006.
39. Warhurst DC, Williams JE. ACP Broadsheet no 148. July 1996. Laboratory diagnosis of malaria. *J Clin Pathol* 1996;**49**(7)**:**533-8.
40. Durrheim DN, Becker PJ, Billinghurst K. Diagnostic disagreement--the lessons learnt from malaria diagnosis in Mpumalanga. *S Afr Med J* 1997;**87**(8)**:**1016.

41. McKenzie FE, Sirichaisinthop J, Miller RS, Gasser RA, Jr., Wongsrichanalai C. Dependence of malaria detection and species diagnosis by microscopy on parasite density. *Am J Trop Med Hyg* 2003;**69**(4)**:**372-6.
42. Kilian AH, Metzger WG, Mutschelknauss EJ, et al. Reliability of malaria microscopy in epidemiological studies: results of quality control. *Trop Med Int Health* 2000;**5**(1)**:**3-8.
43. Shiff CJ, Minjas J, Premji Z. The ParaSight-F test: a simple rapid manual dipstick test to detect *Plasmodium falciparum* infection. *Parasitol Today* 1994;**10**(12)**:**494-5.
44. Moody A. Rapid diagnostic tests for malaria parasites. *Clin Microbiol Rev* 2002;**15**(1)**:**66-78.
45. World Health Organization. Malaria Microscopy Quality Assurance Manual. 2009.
46. Bisoffi Z, Gobbi F, Angheben A, Van den Ende J. The role of rapid diagnostic tests in managing malaria. *PLoS Med* 2009;**6**(4)**:**e1000063.
47. Mills CD, Burgess DC, Taylor HJ, Kain KC. Evaluation of a rapid and inexpensive dipstick assay for the diagnosis of *Plasmodium falciparum* malaria. *Bull World Health Organ* 1999;**77**(7)**:**553-9.
48. Trape JF. The public health impact of chloroquine resistance in Africa. *American Journal of Tropical Medicine and Hygiene* 2001;**64**(1-2 Suppl)**:**12-17.
49. Bousema JT, Schneider P, Gouagna LC, et al. Moderate effect of artemisinin-based combination therapy on transmission of *Plasmodium* falciparum. *J Infect Dis* 2006;**193**(8)**:**1151-9.
50. Drakeley CJ, Jawara M, Targett GA, et al. Addition of artesunate to chloroquine for treatment of *Plasmodium falciparum* malaria in Gambian children causes a significant but short-lived reduction in infectiousness for mosquitoes. *Trop Med Int Health* 2004;**9**(1)**:**53-61.
51. Sutherland CJ, Ord R, Dunyo S, et al. Reduction of malaria transmission to Anopheles mosquitoes with a six-dose regimen of co-artemether. *PLoS Med* 2005;**2**(4)**:**e92.
52. Oesterholt MJ, Alifrangis M, Sutherland CJ, et al. Submicroscopic gametocytes and the transmission of antifolate-resistant *Plasmodium falciparum* in Western Kenya. *PLoS One* 2009;**4**(2)**:**e4364.
53. Price RN, Nosten F, Luxemburger C, et al. Effects of artemisinin derivatives on malaria transmissibility. *Lancet* 1996;**347**(9016)**:**1654-8.
54. World Health Organization. World Malaria Report. Geneva. *http://www.who.int/malaria/wmr* 2008.
55. Ministry of Health of Burkina Faso. Application de la nouvelle politique de traitement du paludisme au Burkina Faso. 2005;**No:0932**.
56. Korenromp EL, Williams BG, Gouws E, Dye C, Snow RW. Measurement of trends in childhood malaria mortality in Africa: an assessment of progress toward targets based on verbal autopsy. *Lancet Infect Dis* 2003;**3**(6)**:**349-58.
57. Roll Back Malaria. Key malaria facts. *http://www.rollbackmalaria.org/keyfacts.html*.
58. Gbary AR, Guiguemde TR, Ouedraogo JB. [Emergence of chloroquine-resistant malaria in West Africa: the case of Sokode (Togo)]. *Trop Med Parasitol* 1988;**39**(2)**:**142-4.
59. Muller O, Traore C, Kouyate B. Clinical efficacy of chloroquine in young children with uncomplicated *falciparum* malaria--a community-based study in rural Burkina Faso. *Trop Med Int Health* 2003;**8**(3)**:**202-3.
60. Guiguemde TR, Aouba A, Ouedraogo JB, Lamizana L. Ten-year surveillance of drug-resistant malaria in Burkina Faso (1982-1991). *Am J Trop Med Hyg* 1994;**50**(6)**:**699-704.
61. Tinto H, Zoungrana EB, Coulibaly SO, et al. Chloroquine and sulphadoxine-pyrimethamine efficacy for uncomplicated malaria treatment and haematological recovery in children in Bobo-Dioulasso, Burkina Faso during a 3-year period 1998-2000. *Trop Med Int Health* 2002;**7**(11)**:**925-30.
62. Sirima SB, Tiono AB, Konate A, et al. Efficacy of artesunate plus chloroquine for the treatment of uncomplicated malaria in children in Burkina Faso: a double-blind, randomized, controlled trial. *Trans R Soc Trop Med Hyg* 2003;**97**(3)**:**345-9.

63. Baird JK. Host Age as a determinant of naturally acquired immunity to *Plasmodium* falciparum. *Parasitol Today* 1995;**11**(3)**:**105-11.
64. Egan JE, Hoffman SL, Haynes JD, et al. Humoral immune responses in volunteers immunized with irradiated *Plasmodium falciparum* sporozoites. *Am J Trop Med Hyg* 1993;**49**(2)**:**166-73.
65. Roestenberg M, McCall M, Hopman J, et al. Protection against a malaria challenge by sporozoite inoculation. *N Engl J Med* 2009;**361**(5)**:**468-77.
66. Alonso PL, Sacarlal J, Aponte JJ, et al. Duration of protection with RTS,S/AS02A malaria vaccine in prevention of *Plasmodium falciparum* disease in Mozambican children: single-blind extended follow-up of a randomised controlled trial. *Lancet* 2005;**366**(9502)**:**2012-8.
67. Alonso PL, Sacarlal J, Aponte JJ, et al. Efficacy of the RTS,S/AS02A vaccine against *Plasmodium falciparum* infection and disease in young African children: randomised controlled trial. *Lancet* 2004;**364**(9443)**:**1411-20.
68. Abdulla S, Oberholzer R, Juma O, et al. Safety and immunogenicity of RTS,S/AS02D malaria vaccine in infants. *N Engl J Med* 2008;**359**(24)**:**2533-44.
69. Bousema JT, Roeffen W, van der Kolk M, et al. Rapid onset of transmission-reducing antibodies in javanese migrants exposed to malaria in papua, indonesia. *Am J Trop Med Hyg* 2006;**74**(3)**:**425-31.
70. Drakeley CJ, Bousema JT, Akim NI, et al. Transmission-reducing immunity is inversely related to age in *Plasmodium falciparum* gametocyte carriers. *Parasite Immunol* 2006;**28**(5)**:**185-90.
71. Graves PM, Doubrovsky A, Sattabongkot J, Battistutta D. Human antibody responses to epitopes on the *Plasmodium falciparum* gametocyte antigen PFS 48/45 and their relationship to infectivity of gametocyte carriers. *Am J Trop Med Hyg* 1992;**46**(6)**:**711-9.
72. Healer J, McGuinness D, Carter R, Riley E. Transmission-blocking immunity to *Plasmodium falciparum* in malaria-immune individuals is associated with antibodies to the gamete surface protein Pfs230. *Parasitology* 1999;**119 (Pt 5):**425-33.
73. Roeffen W, Geeraedts F, Eling W, et al. Transmission blockade of *Plasmodium falciparum* malaria by anti-Pfs230-specific antibodies is isotype dependent. *Infect Immun* 1995;**63**(2)**:**467-71.
74. Roeffen W, Lensen T, Mulder B, et al. Transmission blocking immunity as observed in a feeder system and serological reactivity to Pfs 48/45 and Pfs230 in field sera. *Mem Inst Oswaldo Cruz* 1994;**89 Suppl 2:**13-5.
75. Roeffen W, Mulder B, Teelen K, et al. Association between anti-Pfs48/45 reactivity and *P. falciparum* transmission-blocking activity in sera from Cameroon. *Parasite Immunol* 1996;**18**(2)**:**103-9.
76. Sauerwein RW. Malaria transmission-blocking vaccines: the bonus of effective malaria control. *Microbes Infect* 2007;**9**(6)**:**792-5.
77. Carter R, Chen DH. Malaria transmission blocked by immunisation with gametes of the malaria parasite. *Nature* 1976;**263**(5572)**:**57-60.
78. Gwadz RW. Successful immunization against the sexual stages of *Plasmodium* gallinaceum. *Science* 1976;**193**(4258)**:**1150-1.
79. Rener J, Carter R, Rosenberg Y, Miller LH. Anti-gamete monoclonal antibodies synergistically block transmission of malaria by preventing fertilization in the mosquito. *Proc Natl Acad Sci U S A* 1980;**77**(11)**:**6797-9.
80. Rener J, Graves PM, Carter R, Williams JL, Burkot TR. Target antigens of transmission-blocking immunity on gametes of *Plasmodium* falciparum. *J Exp Med* 1983;**158**(3)**:**976-81.
81. Vermeulen AN, Ponnudurai T, Beckers PJ, Verhave JP, Smits MA, Meuwissen JH. Sequential expression of antigens on sexual stages of *Plasmodium falciparum* accessible to transmission-blocking antibodies in the mosquito. *J Exp Med* 1985;**162**(5)**:**1460-76.
82. Mulder B, Lensen T, Tchuinkam T, et al. *Plasmodium* falciparum: membrane feeding assays and competition ELISAs for the measurement of transmission reduction in sera from Cameroon. *Exp Parasitol* 1999;**92**(1)**:**81-6.

83. Roeffen W, Lensen T, Mulder B, et al. A comparison of transmission-blocking activity with reactivity in a *Plasmodium falciparum* 48/45-kD molecule-specific competition enzyme-linked immunosorbent assay. *Am J Trop Med Hyg* 1995;**52**(1)**:**60-5.
84. Targett GA. *Plasmodium* falciparum: natural and experimental transmission-blocking immunity. *Immunol Lett* 1988;**19**(3)**:**235-40.
85. Healer J, McGuinness D, Hopcroft P, Haley S, Carter R, Riley E. Complement-mediated lysis of *Plasmodium falciparum* gametes by malaria-immune human sera is associated with antibodies to the gamete surface antigen Pfs230. *Infect Immun* 1997;**65**(8)**:**3017-23.
86. Mendis KN, Munesinghe YD, de Silva YN, Keragalla I, Carter R. Malaria transmission-blocking immunity induced by natural infections of *Plasmodium vivax* in humans. *Infect Immun* 1987;**55**(2)**:**369-72.
87. Lensen AH, Bolmer-Van de Vegte M, van Gemert GJ, Eling WM, Sauerwein RW. Leukocytes in a *Plasmodium* falciparum-infected blood meal reduce transmission of malaria to Anopheles mosquitoes. *Infect Immun* 1997;**65**(9)**:**3834-7.
88. Healer J, Graszynski A, Riley E. Phagocytosis does not play a major role in naturally acquired transmission-blocking immunity to *Plasmodium falciparum* malaria. *Infect Immun* 1999;**67**(5)**:**2334-9.
89. Carter R, Graves PM, Keister DB, Quakyi IA. Properties of epitopes of Pfs 48/45, a target of transmission blocking monoclonal antibodies, on gametes of different isolates of *Plasmodium* falciparum. *Parasite Immunol* 1990;**12**(6)**:**587-603.
90. Bousema JT, Drakeley CJ, Sauerwein RW. Sexual-stage antibody responses to *P. falciparum* in endemic populations. *Curr Mol Med* 2006;**6**(2)**:**223-9.
91. Baird JK. Age-dependent characteristics of protection v. susceptibility to *Plasmodium* falciparum. *Ann Trop Med Parasitol* 1998;**92**(4)**:**367-90.
92. Baird JK, Snow RW. Acquired immunity in a holoendemic setting of *Plasmodium falciparum* and *P. vivax* malaria. *Am J Trop Med Hyg* 2007;**76**(6)**:**995-6.
93. Githeko AK, Brandling-Bennett AD, Beier M, Atieli F, Owaga M, Collins FH. The reservoir of *Plasmodium falciparum* malaria in a holoendemic area of western Kenya. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 1992;**86:**355-358.
94. Graves PM, Burkot TR, Carter R, et al. Measurement of malarial infectivity of human populations to mosquitoes in the Madang area, Papua, New Guinea. *Parasitology* 1988;**96 (Pt 2):**251-63.
95. Toure YT, Doumbo O, TOURE A, et al. Gametocyte infectivity by direct mosquito feeds in an area of seasonal malaria transmission: implications for Bancoumana, Mali as a transmission-blocking vaccine site. *Am. J. Trop. Med. Hyg.* 1998;**59**(3)**:**481–486.
96. Boudin C, Olivier M, Molez JF, Chiron JP, Ambroise-Thomas P. High human malarial infectivity to laboratory-bred Anopheles gambiae in a village in Burkina Faso. *Am J Trop Med Hyg* 1993;**48**(5)**:**700-6.
97. Bonnet S, Gouagna C, Safeukui I, Meunier JY, Boudin C. Comparison of artificial membrane feeding with direct skin feeding to estimate infectiousness of *Plasmodium falciparum* gametocyte carriers to mosquitoes. *Trans R Soc Trop Med Hyg* 2000;**94**(1)**:**103-6.
98. Ponnudurai T, Lensen AH, Van Gemert GJ, Bensink MP, Bolmer M, Meuwissen JH. Infectivity of cultured *Plasmodium falciparum* gametocytes to mosquitoes. *Parasitology* 1989;**98 Pt 2:**165-73.
99. Bousema JT, Drakeley CJ, Kihonda J, et al. A longitudinal study of immune responses to *Plasmodium falciparum* sexual stage antigens in Tanzanian adults. *Parasite Immunol* 2007;**29**(6)**:**309-17.
100. van Dijk MR, Janse CJ, Thompson J, et al. A central role for P48/45 in malaria parasite male gamete fertility. *Cell* 2001;**104**(1)**:**153-64.

101. Outchkourov NS, Roeffen W, Kaan A, et al. Correctly folded Pfs48/45 protein of *Plasmodium falciparum* elicits malaria transmission-blocking immunity in mice. *Proc Natl Acad Sci U S A* 2008;**105**(11)**:**4301-5.
102. Quakyi IA, Carter R, Rener J, Kumar N, Good MF, Miller LH. The 230-kDa gamete surface protein of *Plasmodium falciparum* is also a target for transmission-blocking antibodies. *J Immunol* 1987;**139**(12)**:**4213-7.
103. Kubler-Kielb J, Majadly F, Wu Y, et al. Long-lasting and transmission-blocking activity of antibodies to *Plasmodium falciparum* elicited in mice by protein conjugates of Pfs25. *Proc* Natl Acad Sci U S A 2007;104(1):293-8.
104. Miura K, Keister DB, Muratova OV, Sattabongkot J, Long CA, Saul A. Transmission-blocking activity induced by malaria vaccine candidates Pfs25/Pvs25 is a direct and predictable function of antibody titer. Malar J 2007;6:107.
105. Wu Y, Ellis RD, Shaffer D, et al. Phase 1 trial of malaria transmission blocking vaccine candidates Pfs25 and Pvs25 formulated with montanide ISA 51. PLoS One 2008;3(7):e2636.
106. Taylor LH, Read AF. Why so few transmission stages? Reproductive restraint by malaria parasites. Parasitol Today 1997;13(4):135-40.
107. Drakeley C, Sutherland C, Bousema JT, Sauerwein RW, Targett GA. The epidemiology of *Plasmodium falciparum* gametocytes: weapons of mass dispersion. Trends Parasitol 2006;22(9):424-30.
108. Bousema T, Okell L, Shekalaghe S, et al. Revisiting the circulation time of *Plasmodium falciparum* gametocytes: molecular detection methods to estimate the duration of gametocyte carriage and the effect of gametocytocidal drugs. *Malar J*;**9:**136.
109. Bousema JT, Gouagna LC, Drakeley CJ, et al. *Plasmodium falciparum* gametocyte carriage in asymptomatic children in western Kenya. *Malar J* 2004;**3**(1)**:**18.
110. Miller MJ. Observations on the natural history of malaria in the semi-resistant West African. *Trans R Soc Trop Med Hyg* 1958;**52**(2)**:**152-68.
111. Akim NIJ, Drakely C, Kingo T, Simon B, Senkoro K, Sauerwein RW. Dynamics of *P. falciparum* gametocytemia in symptomatic patients in an area of intense perennial transmission in Tanzania. *American Journal of Tropical Medicine and Hygiene* 2000;**63**(3-4)**:**199-203.
112. Muirhead-Thomson RC. The malarial infectivity of an African village population to mosquitoes (Anopheles gambiae); a random xenodiagnostic survey. *Am J Trop Med Hyg* 1957;**6**(6)**:**971-9.
113. Baird JK, Jones TR, Danudirgo EW, et al. Age-dependent acquired protection against *Plasmodium falciparum* in people having two years exposure to hyperendemic malaria. *Am J Trop Med Hyg* 1991;**45**(1)**:**65-76.
114. Eichner M, Diebner HH, Molineaux L, Collins WE, Jeffery GM, Dietz K. Genesis, sequestration and survival of *Plasmodium falciparum* gametocytes: parameter estimates from fitting a model to malariatherapy data. *Trans R Soc Trop Med Hyg* 2001;**95**(5)**:**497-501.
115. Snounou G, Viriyakosol S, Zhu XP, et al. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. *Mol Biochem Parasitol* 1993;**61**(2)**:**315-20.
116. Sinden RE. Sexual development of malarial parasites. *Adv Parasitol* 1983;**22:**153-216.
117. Kain KC, Harrington MA, Tennyson S, Keystone JS. Imported malaria: prospective analysis of problems in diagnosis and management. *Clin Infect Dis* 1998;**27**(1)**:**142-9.
118. Babiker HA, Abdel-Wahab A, Ahmed S, et al. Detection of low level *Plasmodium falciparum* gametocytes using reverse transcriptase polymerase chain reaction. *Molecular and Biochemical Parasitology* 1999;**99:**143-148.
119. Abdel-Wahab A, Abdel-Muhsin AA, Ali E, et al. Dynamics of gametocytes among *Plasmodium falciparum* clones in natural infections in an area of highly seasonal transmission. *Journal of Infectious Diseases* 2002;**185**(12)**:**1838-1842.

120. Menegon M, Severini C, Sannella A, et al. Genotyping of *Plasmodium falciparum* gametocytes by reverse transcriptase polymerase chain reaction. *Molecular and Biochemical Parasitology* 2000;**111**(1)**:**153-161.
121. Nassir E, Abdel-Muhsin AM, Suliaman S, et al. Impact of genetic complexity on longevity and gametocytogenesis of *Plasmodium falciparum* during the dry and transmission-free season of eastern Sudan. *Int J Parasitol* 2005;**35**(1)**:**49-55.
122. Schneider P, Schoone G, Schallig H, et al. Quantification of *Plasmodium falciparum* gametocytes in differential stages of development by quantitative nucleic acid sequence-based amplification. *Mol Biochem Parasitol* 2004;**137**(1)**:**35-41.
123. Schneider P, Bousema T, Omar S, et al. (Sub)microscopic *Plasmodium falciparum* gametocytaemia in Kenyan children after treatment with sulphadoxine-pyrimethamine monotherapy or in combination with artesunate. *Int J Parasitol* 2006;**36**(4)**:**403-8.
124. Shekalaghe S, Drakeley C, Gosling R, et al. Primaquine clears submicroscopic *Plasmodium falciparum* gametocytes that persist after treatment with sulphadoxine-pyrimethamine and artesunate. *PLoS One* 2007;**2**(10)**:**e1023.
125. Shekalaghe SA, Bousema JT, Kunei KK, et al. Submicroscopic *Plasmodium falciparum* gametocyte carriage is common in an area of low and seasonal transmission in Tanzania. *Trop Med Int Health* 2007;**12**(4)**:**547-53.
126. Schneider P, Bousema JT, Gouagna LC, et al. Submicroscopic *Plasmodium falciparum* gametocyte densities frequently result in mosquito infection. *Am J Trop Med Hyg* 2007;**76**(3)**:**470-4.
127. O'Meara WP, Mangeni JN, Steketee R, Greenwood B. Changes in the burden of malaria in sub-Saharan Africa. *Lancet Infect Dis* 2010;**10**(8)**:**545-55.
128. Yeshiwondim AK, Gopal S, Hailemariam AT, Dengela DO, Patel HP. Spatial analysis of malaria incidence at the village level in areas with unstable transmission in Ethiopia. *Int J Health Geogr* 2009;**8:**5.
129. Otten M, Aregawi M, Were W, et al. Initial evidence of reduction of malaria cases and deaths in Rwanda and Ethiopia due to rapid scale-up of malaria prevention and treatment. *Malar J* 2009;**8:**14.
130. van der Werff ten Bosch JE, Demanet C, Balduck N, et al. The use of the anti-malaria drug Fansidar (pyrimethamine and sulphadoxine) in the treatment of a patient with autoimmune lymphoproliferative syndrome and Fas deficiency. *Br J Haematol* 1998;**102**(2)**:**578-81.
131. O'Meara WP, Bejon P, Mwangi TW, et al. Effect of a fall in malaria transmission on morbidity and mortality in Kilifi, Kenya. *Lancet* 2008;**372**(9649)**:**1555-62.
132. Okiro EA, Hay SI, Gikandi PW, et al. The decline in paediatric malaria admissions on the coast of Kenya. *Malar J* 2007;**6:**151.
133. Barnes KI, Durrheim DN, Little F, et al. Effect of artemether-lumefantrine policy and improved vector control on malaria burden in KwaZulu-Natal, South Africa. *PLoS Med* 2005;**2**(11)**:**e330.
134. Ceesay SJ, Casals-Pascual C, Erskine J, et al. Changes in malaria indices between 1999 and 2007 in The Gambia: a retrospective analysis. *Lancet* 2008;**372**(9649)**:**1545-54.
135. Bouyou-Akotet MK, Mawili-Mboumba DP, Kendjo E, et al. Evidence of decline of malaria in the general hospital of Libreville, Gabon from 2000 to 2008. *Malar J* 2009;**8**(1)**:**300.
136. Griffin JT, Hollingsworth TD, Okell LC, et al. Reducing *Plasmodium falciparum* malaria transmission in Africa: a model-based evaluation of intervention strategies. *PLoS Med*;**7**(8).
137. Muller O, Ye M, Louis VR, Sie A. Malaria in sub-Saharan Africa. *Lancet* 2009;**373**(9658)**:**122.
138. Orimadegun AE, Fawole O, Okereke JO, Akinbami FO, Sodeinde O. Increasing burden of childhood severe malaria in a Nigerian tertiary hospital: implication for control. *J Trop Pediatr* 2007;**53**(3)**:**185-9.

Chapter 2

Seasonal patterns of *Plasmodium falciparum* gametocyte prevalence and density in a rural population of Burkina Faso

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Summary

Gametocytes are the malaria parasite stages that secure the transmission from the human host to the mosquito. Identifying natural parameters that influence gametocyte carriage contribute to a better understanding of the dynamics of the sexual stage parasites for transmission reducing strategies. A total of 3400 blood slide readings were done during 4 cross sectional surveys (2002-2003) including all age groups to determine the effect of season on *Plasmodium falciparum* gametocytes in a seasonal malaria transmission area of Burkina Faso. Entomological data were collected to determine the malaria transmission intensity in relation to seasons. Transmission intensity was estimated by monthly EIRs, averaging 28 and 32 infective bites/person/month in the wet seasons of 2002 and 2003 respectively. The EIR in the dry seasons was below one infective bite/person/ month.

The gametocyte prevalence was significantly higher at the start and peak of the wet season compared to the dry season when corrected for asexual parasite density and age. Gametocyte density significantly increased during the wet season after correction for asexual parasite density and age. In this study, season appears to be an independent parameter that determines gametocyte prevalence and density and should be considered to be included in epidemiological studies on malaria transmission.

Introduction

Plasmodium falciparum is the most pathogenic specie of human malaria and an important cause of childhood morbidity and mortality in sub Saharan Africa. Gametocytes are the parasite stages that do not cause clinical disease, but are responsible for the transmission from the human host to the mosquito. In malaria transmission areas, the effect of climatic features on vectorial capacity may lead to a variation of transmission intensity and subsequently on the epidemiology of the malaria infections. In some areas, season-related patterns were demonstrated in prevalence of asexual parasites[1,2] and in gametocytes[3-5]. The general goal of these studies was to identify natural factors that are associated with changes in the malaria parasite prevalence as they serve as base-line data for possible interventions. The only and little detailed study conducted in Burkina Faso on transmission showed a slight variation in gametocyte prevalence between the wet and the dry season[6] and concluded that this variation could not be used for transmission control. The objective of the present study was to determine *P. falciparum* gametocyte prevalence and density in Burkina Faso in relation to season. We have thus analyzed the relationship between entomological and parasitological parameters in two different seasons (wet and dry) in a rural area.

Material and Methods

Study area and population

The study took place in 2002 and 2003 in two rural zones of Burkina Faso. Three villages were selected from each zone based on their proximities and a recent evidence of high malaria endemicity. The distance between zones is about 30 km. Both zones are located 30 km north and northwest of Ouagadougou, the capital. The distance between villages inside each zone varies from 1 to 5 km. All together, these villages are situated in a Sudanese savannah area with a marked wet season from June to October. Malaria transmission in the area is stable and markedly seasonal. The average entomological inoculation rate (EIR) is estimated 300-500 infective bites/person/year[7] peaking during the rainy season. *P. falciparum* is the predominant malaria species in the region, accounting for 90% of the infections; the remaining 8% and 2% are attributed to *P. malariae* and *P. ovale*, respectively[8].

Populations belong to the Mossi tribe and are predominantly subsistence farming communities. We first performed a complete census, which showed that there were 2767 residents in the six villages, each of comparable size and with similar age distributions. The census sizes were 348, 333, 494, 488, 680 and 424 in village 1 to 6, respectively. Participation of villagers to the Study followed an enrolment procedure at which the first subjects that came were enrolled in the study. We performed a sample sizes calculation using STATA 9.0 (Stata Corporation, Texas, USA) with pre-existing data on gametocyte prevalence in Burkina Faso[6]. A sample size of 76 subjects per age group (1-4, 5-9, 10-14, 15-24 and 25+ years) would allow over 85% power to detect a decrease from 23% of gametocyte prevalence in children under 5 to 5% in adults above 25 years with a type 1 error of 0.05. To correct for unexpected missing data, a minimum number of 120 individuals / age group (pool of 20 individuals / age group / village) was thus included in the sample at each cross sectional survey to allow a statistically robust testing between age groups. Furthermore, we combined the five age groups into three age groups to increase the power of the study.

The study was approved by the Ministry of Health of Burkina Faso. Villagers were informed about the purpose of the study and their consent was obtained. Benefits for the participants were free treatment for malaria and other common infections. In addition, protective means (insecticide treated bed nets) were given to volunteers that allowed the indoor mosquitoes collection from their houses. All participants with fever (axillary temperature $\geq$37.5°C) were treated with chloroquine according to the current national policy during the study period (2002-2003). Participants diagnosed with severe infections other than malaria were treated with antibiotics or offered transport to a medical centre when needed.

Entomologic data collection

The main malaria vector in the study area is the complex *Anopheles gambiae s.l.,* with *An. funestus* contributing less to transmission[9]. Two men per village were recruited and trained for assistance in mosquito collection. CDC light trap captures were carried out within the six villages to estimate the entomological inoculation rate (EIR) as described by Cuzin-Ouattara and collaborators[7]. This design of indoor mosquito collection covered all time points of the parasitological studies. One cross sectional survey was carried out in the dry season of 2002

from 19th to 30th March in 16 randomly selected houses of each village. Two longitudinal surveys were carried out from June to November 2002 and from April to September 2003. Each month, the mosquito collections were carried out in 16 randomly selected houses per village. These houses were each made of one room and inhabited by one person, a male adult or teenager, who slept under a non-impregnated bed net that we supplied. The CDC light trap was placed close to the bed. A monthly mosquito collection was completed per village within 4 weeks at the rate of 4 house captures per week. Field workers always returned to the same 16 houses, every month. Indoor mosquito capture was done on one day per house from 7:00 pm to 6:00 am. Mosquito species were identified morphologically, counted and stored in tubes with silica gel. The total density of *An. gambiae* was divided by the number of days of capture to define the daily biting rate per village assuming that each female captured by CDC light trap had a drive for biting. The monthly biting rate was estimated by multiplying the daily biting rate with the number of days in the month. A representative sample of *An. gambiae* mosquito thoraces and heads were examined for *P. falciparum* circumsporozoite protein (CSP) positivity index using CSP 2-site ELISA[10]. The monthly sporozoite rate was estimated, testing a maximum of randomly selected specimens of *An. gambiae* from each village. The monthly sporozoite rate for each year was obtained by dividing the monthly number of positive mosquitoes in ELISA on the number of mosquitoes tested. The monthly Entomological Inoculation Rate (number of infectious bites per person-time unit) was calculated as the product of the monthly biting rate and the monthly sporozoite rate. The uninfected biting rate was obtained by subtracting the number of infective bites per person-month from the monthly biting rate.

Data on seasons and rainfall were obtained from the Direction de la Météorologie Nationale, Ministère des Transports, Burkina Faso.

Parasitological data collection

Cross sectional surveys were performed at 3 monthly intervals, at the start of the wet season (May 27th -June 05th 2002), the peak wet season (August 19-24th 2002), the end of the wet season (December 2 -13th 2002) and the dry season (April 14-19th 2003). At each cross sectional survey, all adults and children of both sexes initially recorded in the census file that arrived at our location (a usual gathering place, not a health centre) were systematically included in the study. Thick and thin blood films were both made on the same

slide from finger prick blood, and air-dried. Villagers with fever (axillary temperature ≥37.5°C) were immediately treated with chloroquine according to the national policy. The four surveys conducted by our technical staff resulted in 3400 blood samples.

At the CNRFP laboratory, the thick smear was stained with 5% Giemsa for 35 minutes. One hundred high power fields per thick smear were examined for malaria parasites. Two microscopists read each slide sample independently and the mean density was considered. A third reader was involved when the difference between the readers exceeded 30% and in such a case the median reading was used. Trophozoite and gametocyte densities were assessed by counting against 500 leukocytes of blood and converted to counts per µl by assuming a standard count of 8000 leukocytes/µl blood. A slide was considered negative if no parasite stages were found after examination of 100 fields.

Data analysis

Data were double entered by 2 independent data clerks and were compared for typing errors. The number of missing values was very low (< 0.5%). Analyses of data were performed using SPSS version 12.01 (SPSS Inc., Chicago, IL, USA). Parasite densities of positive individuals were analyzed after log-transformation. Geometric mean densities and medians were calculated. The distribution of positive densities of log-transformed densities of gametocyte- and asexual parasite was tested for normality using the Kolmogorov-Smirnov test.

Seasonal patterns were analyzed using the dry season survey as a reference category for the other three surveys. Unadjusted (crude) values of odds ratios and of the regression coefficient determine the effect of season on gametocytes dependent of age and asexual parasite density as selected potential confounding factors. Gender was left out as variable, because it did not influence the analysis outcome. Gametocyte prevalence and gametocyte density were used as dependent variables. Season, age and log asexual parasite density were used as independent variables. Adjusted values of odds ratios and regression coefficient (β) were calculated using a logistic and linear regression model to determine the specific effect of season on gametocyte prevalence and log density, independent of age and asexual parasite density. In these models, the effect of age was assessed after categorization into groups 1-4 years, 5-14 years and ≥15 years. Odds ratios and regression

coefficients were calculated with 95% confidence intervals (95% CI). Comparison of parasite densities was performed using the Student's *t*-test for normally distributed data and the Mann-Whitney U Test in case of data not conforming to a normal distribution.

Contrarily to gametocyte prevalence and density and asexual parasite density measured at 3 monthly intervals time points (cross sectional surveys) at an individual based level, entomological data were longitudinally collected from a representative set of houses to describe seasonality of malaria transmission in the area. Consequently, our data on entomology were not included in regression analyses for predicting gametocyte prevalence, but were used to support effects of seasons on gametocytes independently of individual based variables as age and asexual parasite density.

Results

Entomological and parasitological parameters

The weather in our study area was characterized by a clear seasonality with peak rainfall between June and September (figure 1A). The biting rate, the sporozoite rate and EIR calculations were based on *A. gambiae*. Representative samples of 4,593 and 3,569 female *An. gambiae* (approximately half numbers of total mosquitoes collected) were tested to estimate transmission intensity in 2002 and 2003 respectively. All entomological parameters are shown in Figure 1B. Transmission intensity quantified by the number of infectious bites (EIR) peaked one month after the peak rainfall (Figure 1A, 1B).

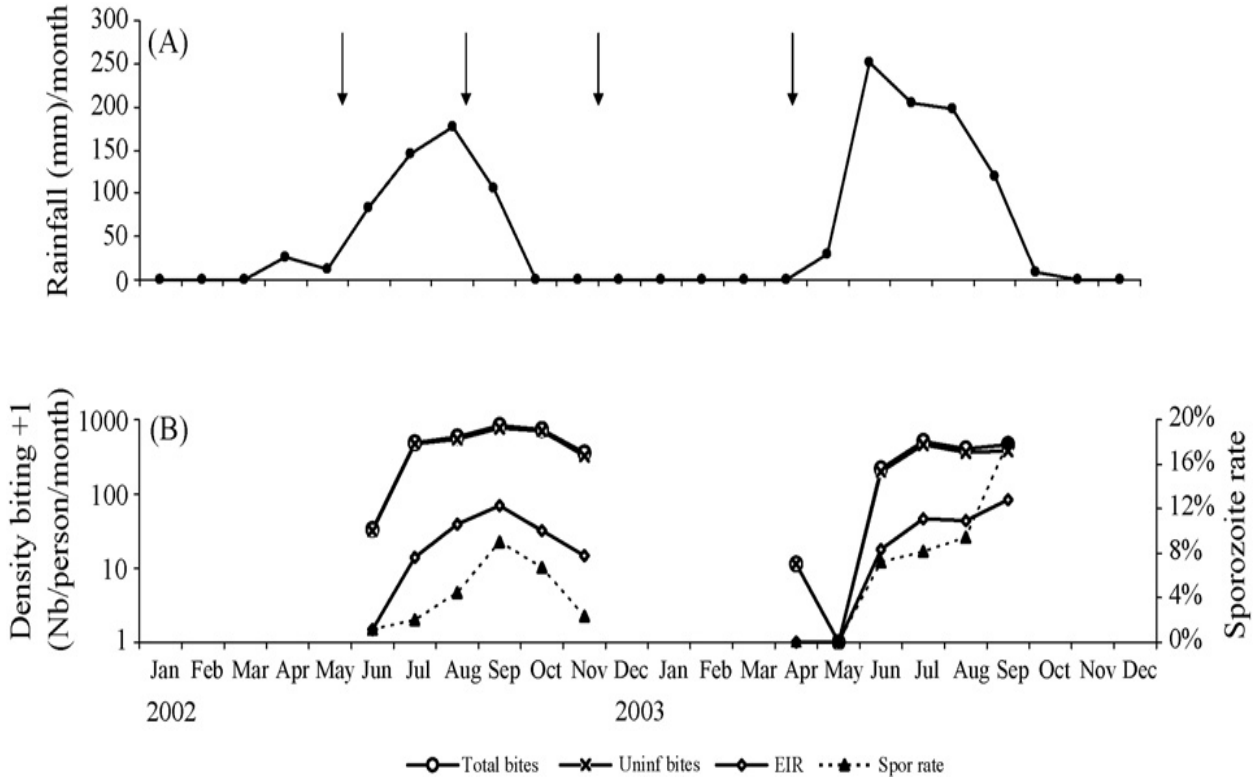

Figure 1. A: Mean monthly rain fall in 2002 and 2003. B: Mean number of bites /person/month (total bites), mean number of infective bites /person/month (EIR), mean number of uninfected bites /person/month (Uninf bites), monthly sporozoite rate (Spor rate) in 2002 and 2003. *Arrows indicate cross-sectional surveys for parasitological data collection. A total of 10,262 mosquitoes were longitudinally captured from the start to the end of the wet season 2002 and showed 9,785 An. gambiae versus 477 An. funestus. From the dry season to the peak wet season 2003; 6,044 An. gambiae were captured versus 445 An. funestus.*

The entomological cross sectional survey carried out during the dry season in March 2002 resulted in the capture of 8 *An. gambiae* mosquitoes. A total of 68 uninfected *An. gambiae* were captured in April in the dry period of 2003 and 0 in May 2003. Infectious mosquitoes were not collected in both dry seasons. While the exposure to infectious bites at the start of the wet season was low, exposure to uninfected mosquito bites was elevated to 32 and 200 mosquito bites per person per month at the start of the wet season (June) in 2002 and 2003, respectively (Figure 1B).

During the entire study period, *P. falciparum* asexual or sexual stages were microscopically detected in 61.8% (2,100 of 3,400 blood slides). Only asexual parasites were detected in 43.4% (n = 1477); asexual parasites and gametocytes in 14.8% (n = 503) and only

gametocytes in 3.5% (n = 120). Of the children under 5 years of age 71.3% (452/634) and 27.9% (177/634) carried asexual parasites and gametocytes, respectively. In subjects >30 years only 23.5% (160/682) and 5.3% (36/682) carried asexual parasites and gametocytes, respectively. The negative association between age in years and parasite prevalence was statistically significant for both asexual parasites (OR = 0.94; 95% CI 0.94 – 0.95) and gametocytes (OR = 0.95; 95% CI 0.95 – 0.96).

Season and asexual parasites

Asexual parasite prevalence showed a marked seasonality (Figure 2A). Compared to the dry season, asexual parasite prevalence was significantly elevated at the peak (OR = 3.90, 95% CI 3.12-4.86; p<0.001) and end (OR = 2.31, 95% CI 1.84-2.91; p<0.001) of the wet season, after adjustment for age. The geometric mean of positive parasite densities was markedly different between the age groups at any period of the year (Figure 2B). The parasite density was highest at the peak of the wet season and decreased towards the end of the wet season. This lower asexual parasite density at the end compared to the peak of the wet season was significant for children aged 5-14 years (t = 3.889; df = 521; p<0.001) but not for adults of ≥15 years (t = 1.814, df= 219; p = 0.07) and children below five years of age (t=1.05; df= 228; p = 0.3).

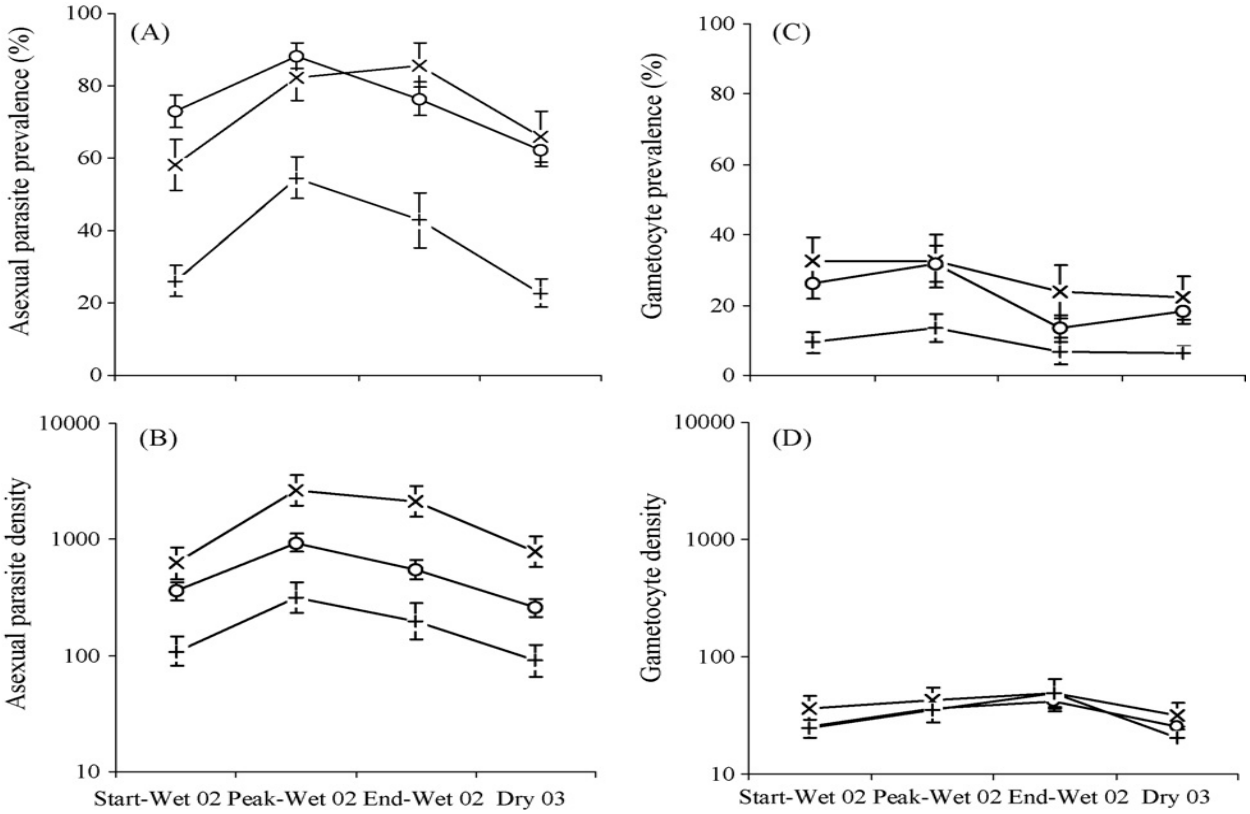

Figure 2: *Plasmodium falciparum* asexual parasite prevalence (A), density (B) and gametocyte prevalence (C) and density (D) in relation to season in different age group, (x) = 1-4 years; (o) = 5-14 years; (+) = 15+ years. *The error bars show the ± limits of the 95% confidence intervals. The number of blood samples collected at the start, peak and end of the wet season in 2002 was 968, 749 and 599, respectively. During the dry season 2003, 1084 samples were collected. Densities concern positive cases only.*

Season and gametocytes

Gametocyte prevalence was positively associated with log-transformed asexual parasite density (OR 1.17; 95% CI 1.05 – 1.31) and was also elevated in the wet season (Figure 2C). Gametocyte prevalence was significantly higher at the beginning and the peak of the wet season, as compared to the dry season, after adjustment for age and asexual parasite density. In contrast, gametocyte prevalence appeared to be somewhat lower at the end of the wet season (Table 1).

Densities of gametocytes were 1-2 logs lower than asexual parasites with no consistent difference between age groups (Figure 2D). The median of gametocyte density was 24 (IQR

16-40) gametocytes/µl at the start of the wet season, 32 (IQR 16-64) at the peak of the wet season, 40 (IQR 36-56) at the end of the wet season and 24 (IQR 16-32) in the dry season.

There was no difference in the gametocyte density between the start of the wet season and the dry season (p = 0.48, Mann-Whitney U Test). It was significantly higher at the peak and end of the wet season compared to the dry season (p<0.001 and p<0.001 respectively, Mann-Whitney U Test).

After adjustment for age and asexual parasite density, gametocyte density was significantly higher at the peak and end but not at the beginning of the wet season, when compared to the dry season (Table1). Despite these seasonal differences in low gametocyte densities, the prevalences are more robust**,** particularly at the start of the transmission season.

Table1: Crude (unadjusted) and adjusted effect of season on gametocyte prevalence and density

Gametocyte prevalence	**n/N**	**Crude OR (95% CI)**	**P-value**	**Adjusted OR (95% CI)[a]**	**P-value**
Start wet season	201/968	1.62 (1.28 – 2.04)	<0.001	1.81(1.36-2.42)	<0.001
Peak wet season	188/749	2.07 (1.63 – 2.62)	<0.001	1.75 (1.30-2.35)	<0.001
End wet season	83/599	0.99 (0.75 – 1.33)	0.97	0.68 (0.48-0.96)	0.03
Dry season	151/1084	1.0[b]		1.0[b]	
Gametocyte density	**Mean[c] (n)**	**Crude β (95% CI)**	**P-value**	**Adjusted β (95% CI)[a]**	**P-value**
Start wet season	28.3 (201)	0.04 (-0.027 – 0.107)	0.24	0.014 (-0.064 – 0.092)	0.73
Peak wet season	37.6 (188)	0.164 (0.10 – 0.23)	<0.001	0.109 (0.028 – 0.189)	0.008
End wet season	45.1 (83)	0.243 (0.16 – 0.33)	<0.001	0.152 (0.053 – 0.251)	0.003
Dry season	25.8 (151)	0.0[b]	0.0[b]		

OR = Odds ratio; β = regression coefficient; CI = confidence interval; n=number of positive slides; N=total number of slides read

[a]Values are adjusted for age and log transformed individual asexual parasite density

[b]Reference group

[c]Geometric mean of positive samples

Discussion

This study shows the parasitological characteristics in a rural area in Burkina Faso during distinct seasons. An important finding is that season appears to be a factor independent of age and asexual parasite density that drives gametocyte prevalence and density. The prevalence and density of asexual parasites and gametocytes increase during the wet season. Malaria transmission varies between villages but is intense in the entire study area. Estimated sporozoite rates are in line with results from previous studies performed in the same area[7] as such studies described higher prevalences of infected mosquitoes reaching 20 % during peak transmission period.

Seasonal patterns of *P. falciparum* gametocyte prevalence have been described before[4,5,11-13]. Gametocyte prevalence at the start and peak of the wet season is higher than in the dry season. In addition, gametocyte density is also elevated in the wet season, although only at the peak and end of the wet season. The elevated gametocyte prevalence and density in the wet season generally coincides with an increased asexual parasite density.

Gametocytes are derived from asexual parasites and asexual parasite density is strongly associated with gametocyte prevalence and density[14-16]. It is therefore not surprising that a strong correlation is found between asexual parasite density and gametocyte prevalence. The same argumentation may explain the negative association between gametocyte prevalence and age[5,14,15]. In addition, increased gametocyte density may be the result of a partial loss of immunity against gametocytes during the long dry season[6], although evidence for the existence of specific gametocyte immunity remains inconclusive[17].

Though gametocyte prevalence is found higher in younger children, the contribution of older children and adults to the infectious reservoir should be taken into consideration as they represent a big part of the whole population and contribute considerably to malaria transmission[18-21].

Our findings suggest that gametocyte prevalence and density may have an independent relationship with season. This follows from our finding that the effect of season on gametocyte prevalence and -to a lesser extent- density remains apparent after adjustment for age and asexual parasite density at the individual level. The development of

gametocytes from asexual parasites takes 8-11 days[22], and variation in asexual parasite carriage in the weeks prior to the start of the wet season may (partly) explain the observed effect on gametocyte prevalence.

Secondly, the elevated gametocyte prevalence that precedes the rise in sporozoite exposure and asexual parasite density may due to a sudden increase in uninfected mosquito bites in this period, as suggested by Paul and collaborators[23]. Parasites would indeed have a substantial fitness advantage if gametocytogenesis would be upregulated by uninfected mosquito bites. Our study design did not allow us to directly determine the relation between uninfected mosquito bites and gametocytogenesis since mosquito catches were not conducted throughout the dry season. The hypothesis, which gives equivocal results in rodent malaria studies[24,25] require future field studies with a longitudinal design[26] in combination with more sensitive molecular detection of gametocytes[16,27].

Studies using highly sensitive molecular methods have recently revealed that the vast majority of gametocytes remain undetected by microscopy[16,27]. Therefore our microscopical data only reflect the patterns of the relatively higher gametocyte densities, starting to increase at the beginning of the transmission season and continuing during the remainder of the wet season. The increased infection rate during the wet season may be the result of a higher complexity of infections, which may lead to an increased gametocyte production because of interclonal competition[28]. Extended duration of illness[29] and/or the use of anti-malarial drugs[30] may further stimulate gametocyte carriage during the wet season. Finally, more efficient asexual parasite immunity halfway acquired through the wet season because of accumulated recent parasite exposure may also drive parasites towards sexual stage development[6,11].

In conclusion, linked parasitological and entomological data from an area of highly seasonal transmission in Burkina Faso, shows that microscopic gametocyte prevalence and density is independently determined by age, asexual parasite density and season. The mechanisms behind the seasonal component remain elusive and will be subject to further studies.

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References

1. Baird JK, Owusu Agyei S, Utz GC, et al. Seasonal malaria attack rates in infants and young children in northern Ghana. *Am J Trop Med Hyg* 2002;**66**(3)**:**280-6.
2. Sissoko MS, Dicko A, Briet OJ, et al. Malaria incidence in relation to rice cultivation in the irrigated Sahel of Mali. *Acta Trop* 2004;**89**(2)**:**161-70.
3. Barnish G, Maude GH, Bockarie MJ, Eggelte TA, Greenwood BM, Ceesay S. Malaria in a rural area of Sierra Leone. I. Initial results. *Ann Trop Med Parasitol* 1993;**87**(2)**:**125-36.
4. Nacher M, Carrara VI, Ashley E, et al. Seasonal variation in hyperparasitaemia and gametocyte carriage in patients with *Plasmodium falciparum* malaria on the Thai-Burmese border. *Trans R Soc Trop Med Hyg* 2004;**98**(5)**:**322-8.
5. van der Kolk M, Tebo AE, Nimpaye H, Ndombol DN, Sauerwein RW, Eling WM. Transmission of *Plasmodium falciparum* in urban Yaounde, Cameroon, is seasonal and age-dependent. *Trans R Soc Trop Med Hyg* 2003;**97**(4)**:**375-9.
6. Boudin C, Lyannaz J, Bosseno MF, Carnevale P, Ambroise-Thomas P. Epidemiology of *Plasmodium falciparum* in a rice field and a savanna area in Burkina Faso: seasonal fluctuations of gametocytaemia and malarial infectivity. *Ann Trop Med Parasitol* 1991;**85**(4)**:**377-85.
7. Cuzin-Ouattara N, Van den Broek AH, Habluetzel A, et al. Wide-scale installation of insecticide-treated curtains confers high levels of protection against malaria transmission in a hyperendemic area of Burkina Faso. *Trans R Soc Trop Med Hyg* 1999;**93**(5)**:**473-9.
8. Habluetzel A, Cuzin N, Diallo DA, et al. Insecticide-treated curtains reduce the prevalence and intensity of malaria infection in Burkina Faso. *Trop Med Int Health* 1999;**4**(8)**:**557-64.
9. Petrarca V, Petrangeli G, Rossi P, Sabatinelli G. [Chromosomal study of Anopheles gambiae and Anopheles arabiensis in Ouagadougou (Burkina Faso) and various neighboring villages]. *Parassitologia* 1986;**28**(1)**:**41-61.
10. Beier JC, Perkins PV, Wirtz RA, Whitmire RE, Mugambi M, Hockmeyer WT. Field evaluation of an enzyme-linked immunosorbent assay (ELISA) for *Plasmodium falciparum* sporozoite detection in anopheline mosquitoes from Kenya. *Am J Trop Med Hyg* 1987;**36**(3)**:**459-68.
11. Abdel-Wahab A, Abdel-Muhsin AA, Ali E, et al. Dynamics of gametocytes among *Plasmodium falciparum* clones in natural infections in an area of highly seasonal transmission. *Journal of Infectious Diseases* 2002;**185**(12)**:**1838-1842.
12. Molineaux L, Storey J, Cohen JE, Thomas A. A longitudinal study of human malaria in the West African Savanna in the absence of control measures: relationships between different *Plasmodium* species, in particular *P. falciparum* and *P. malariae*. *Am J Trop Med Hyg* 1980;**29**(5)**:**725-37.

13. Rosenberg R, Andre RG, Ketrangsee S. Seasonal fluctuation of *Plasmodium falciparum* gametocytaemia. *Trans R Soc Trop Med Hyg* 1990;**84**(1)**:**29-33.
14. Akim NIJ, Drakely C, Kingo T, Simon B, Senkoro K, Sauerwein RW. Dynamics of *P. falciparum* gametocytemia in symptomatic patients in an area of intense perennial transmission in Tanzania. *American Journal of Tropical Medicine and Hygiene* 2000;**63**(3-4)**:**199-203.
15. Bousema JT, Gouagna LC, Drakeley CJ, et al. *Plasmodium falciparum* gametocyte carriage in asymptomatic children in western Kenya. *Malar J* 2004;**3**(1)**:**18.
16. Ouedraogo AL, Schneider P, de Kruijf M, et al. Age-dependent distribution of *Plasmodium falciparum* gametocytes quantified by Pfs25 real-time QT-NASBA in a cross-sectional study in Burkina Faso. *Am J Trop Med Hyg* 2007;**76**(4)**:**626-30.
17. Taylor LH, Read AF. Why so few transmission stages? Reproductive restraint by malaria parasites. *Parasitol Today* 1997;**13**(4)**:**135-40.
18. Drakeley CJ, Akim NI, Sauerwein RW, Greenwood BM, Targett GA. Estimates of the infectious reservoir of *Plasmodium falciparum* malaria in The Gambia and in Tanzania. *Trans R Soc Trop Med Hyg* 2000;**94**(5)**:**472-6.
19. Muirhead-Thomson RC. Factors determining the true reservoir of infection of *Plasmodium falciparum* and Wuchereria bancrofti in a West African village. *Trans R Soc Trop Med Hyg* 1954;**48**(3)**:**208-25.
20. Toure YT, Doumbo O, TOURE A, et al. Gametocyte infectivity by direct mosquito feeds in an area of seasonal malaria transmission: implications for Bancoumana, Mali as a transmission-blocking vaccine site. *Am. J. Trop. Med. Hyg.* 1998;**59**(3)**:**481–486.
21. Muirhead-Thomson RC. The malarial infectivity of an African village population to mosquitoes (Anopheles gambiae); a random xenodiagnostic survey. *Am J Trop Med Hyg* 1957;**6**(6)**:**971-9.
22. Sinden RE. Sexual development of malarial parasites. *Adv Parasitol* 1983;**22:**153-216.
23. Paul RE, Diallo M, Brey PT. Mosquitoes and transmission of malaria parasites - not just vectors. *Malar J* 2004;**3:**39.
24. Billingsley PF, Snook LS, Johnston VJ. Malaria parasite growth is stimulated by mosquito probing. *Biol Lett* 2005;**1**(2)**:**185-9.
25. Shutler D, Reece SE, Mullie A, Billingsley PF, Read AF. Rodent malaria parasites *Plasmodium* chabaudi and P. vinckei do not increase their rates of gametocytogenesis in response to mosquito probing. *Proc Biol Sci* 2005;**272**(1579)**:**2397-402.
26. Dunyo S, Milligan P, Edwards T, Sutherland C, Targett G, Pinder M. Gametocytaemia after drug treatment of asymptomatic *Plasmodium* falciparum. *PLoS Clin Trials* 2006;**1**(4)**:**e20.
27. Schneider P, Wolters L, Schoone G, et al. Real-time nucleic acid sequence-based amplification is more convenient than real-time PCR for quantification of *Plasmodium* falciparum. *J Clin Microbiol* 2005;**43**(1)**:**402-5.
28. Nassir E, Abdel-Muhsin AM, Suliaman S, et al. Impact of genetic complexity on longevity and gametocytogenesis of *Plasmodium falciparum* during the dry and transmission-free season of eastern Sudan. *Int J Parasitol* 2005;**35**(1)**:**49-55.
29. Adedeji AA, Fehintola FA, Fateye BA, et al. *Plasmodium falciparum* malaria in Nigerian children during high and low transmission seasons: gametocyte carriage and response to oral chloroquine. *J Trop Pediatr* 2005;**51**(5)**:**288-94.
30. Price R, Nosten F, Simpson JA, et al. Risk factors for gametocyte carriage in uncomplicated *falciparum* malaria. *Am J Trop Med Hyg* 1999;**60**(6)**:**1019-23.

Chapter 3

Age-dependent distribution of *Plasmodium falciparum* gametocytes quantified by Pfs25 Real-Time QT-NASBA in a cross sectional study in Burkina Faso

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Summary

Sexual stages of *Plasmodium falciparum* play a key role in the transmission of malaria. Studies on gametocytes are generally based on microscopical detection but more sensitive detection methods for *P. falciparum* gametocytes frequently detect sub-patent gametocytes. We have used Pfs25 mRNA QT-NASBA to quantify gametocytes in 412 samples from a cross-sectional study in Burkina Faso, covering all age groups, to determine age-related patterns in gametocyte carriage and gametocyte density. The more sensitive QT-NASBA technique gave estimates of gametocyte prevalence 3.3 fold higher than microscopy (70.1% versus 21.4% respectively). Prevalence of gametocytes significantly decreased with age. Our data suggest that asexual parasite densities are primarily responsible for the age-related decrease of gametocyte prevalence, possibly due to developing asexual stage immunity. Gametocyte densities decrease also with age, primarily due to decreasing asexual parasite densities; only a small but significant age effect on gametocyte density may be due to developing sexual stage-specific immunity.

Introduction

Malaria is caused by protozoan parasites of the genus *Plasmodium*. The life cycle of these parasites consists of both asexual and sexual phases occurring in two hosts. Sexual reproduction occurs in the invertebrate vector, *Anopheles* mosquitoes for human malaria, while reproduction in the vertebrate host is solely asexual. However, sexual stage development starts in the vertebrate host where a proportion of the asexual parasites transform into sexual stages, called gametocytes. The gametocytes can infect mosquitoes, reproduce sexually and are responsible for ongoing transmission of malaria to the next host. Despite the importance of gametocytes for the spread of malaria, relatively little is known about sexual stage development in comparison to the asexual stages that cause disease symptoms. Until recently, studies on gametocytes were based on microscopy, which is rather insensitive and inaccurate in quantification of gametocytes in blood samples (Ouédraogo AL and others, unpublished data). Individuals without microscopically detectable gametocytes can infect mosquitoes[1] and higher gametocyte prevalences are found when larger volumes of blood are used for analysis.[2--4] More sensitive detection methods for *Plasmodium falciparum* gametocytes such as the Pfs25 or Pfg377 reverse transcriptase polymerase chain reaction[5,6,7] are able to detect sub-patent gametocytes,[8,9] which can be quantified by Pfs25 mRNA QT-NASBA.[10,11] The Pfs25 QT-NASBA has a detection limit of 20-100 gametocytes per ml of blood and the high throughput format allows its use in large epidemiological studies. A previous study with Pfs25 QT-NASBA showed very high prevalence of gametocytes in symptomatic children in Kenya.[11]

The objective of this study is to determine submicroscopic levels of gametocytes in a different epidemiological setting. We have used Pfs25 QT-NASBA to quantify gametocytes in 412 samples from a cross-sectional study in Burkina Faso, covering all age groups and determined age-related patterns in gametocyte carriage and density.

Materials and Methods

Field study. The study took place in 6 small rural villages 30 kilometers north of Ouagadougou, Burkina Faso. These villages are situated in a Sudanese savannah area with a marked wet season from June to October. The distance between villages varies from 1 to 5 km. Malaria transmission is highest in the wet season and peaks around September.

P. falciparum is responsible for 90% of malaria infections with Anopheles gambiae and A. funestus as major vectors.[12] Health care facilities are equally distributed in the six villages and residents live by subsistence farming.

A cross-sectional survey was carried out at the end of the transmission season (December 2003) at which time 412 villagers of all ages were enrolled. Adults and children of both sexes and exclusively of the Mossi ethnic were randomly included in the study by order of arrival until a minimum number of 10 individuals per age group with informed consent were included. A finger prick blood sample was taken from all participants. Thick and thin blood films were made, air-dried and stained with 5% Giemsa. For collection of nucleic acids, 100 μl of blood was mixed with 900 μl of L6 lysis buffer and stored for RNA extraction. Most participants were asymptomatic, only 7% had a body temperature of over 37.5 °C and were treated with chloroquine according to the national policy after blood samples for the study had been obtained. The study received ethical approval of the Ministry of Health of Burkina Faso.

Microscopic detection of P. falciparum parasites. Samples were considered negative if no parasites were detected in 100 fields (10x100 magnification). Both asexual stage and gametocyte densities were simultaneously assessed by counting against 500 leucocytes in the thick smear. The lower limit of microscopy for gametocyte quantification was therefore evaluated to 16 gametocytes/ μl of blood. Parasite counts were converted to numbers of parasites per μl by assuming a standard count of 8000 leucocytes/μl of blood. Each sample was read independently by two microscopists. A third reader was involved when the difference between the readers exceeded 30% and in such a case the median reading was used (CNRFP Ouagadougou).

Real-time Pfs25 QT-NASBA and nucleic acid extraction. Nucleic acids were extracted from blood samples using the Guanidiumisothiocyanate (GuSCN)/silica procedure[13] and 18S rRNA real-time QT-NASBA[14] and *Pfs25* real time mRNA QT-NASBA[11] were performed as described elsewhere. Briefly, real-time QT-NASBA for Pfs25 mRNA (Genbank accession

number AF193769.1) was performed on a Nuclisens EasyQ analyser (bioMérieux) using the Nuclisens BasicKit for amplification according to manufacturer's instructions at a KCl concentration of 80mM. Reactions were performed in a total reaction volume of 10 μl per reaction. Forward primer: 5'-gactgtaaataaaccatgtggaga-3'; reverse primer: 5'-aattctaatacgactcactatagggagaaggcatttaccgttaccacaagtta-3'; Pfs25 molecular beacon: 5'-TexasRed-cgatcgcccgtttcatacgcttgtaacgatcg-DABSYL-3'. For quantification, time to positivity is calculated, i.e. the time point during amplification at which the fluorescence detecting target amplicons becomes higher than the mean fluorescence of three negative controls + 20 standard deviations (SD). The use of a standard gametocytes stage V dilution series allows exact calculation of the number of gametocytes present in unknown samples.[10,11]

Data analysis. Statistical analyses were performed in SPSS 12.0.1. Because parasitological parameters, including total parasite and gametocyte densities, were not different between the 6 villages (data not shown), samples were pooled for analyses. Spearman correlation was used to determine correlation between results of microscopy and QT-NASBA. A ^{10}Log(x+1) transformation was applied to both asexual parasite and gametocyte counts to allow negative samples in the analysis. Geometric mean of gametocyte density was calculated for gametocyte positive samples and for all samples, including the negatives. To determine the relation to age group and asexual parasite density, logistic regression was used for gametocyte prevalence and linear regression for gametocyte density. The age range was very large and therefore we have analyzed age group as a categorical variable in comparison to the oldest age group of 25+ years (adults). With such analyses, a decrease of the correlation coefficient with increasing age group indicates a negative relation between age group and the parameter under investigation.

Results

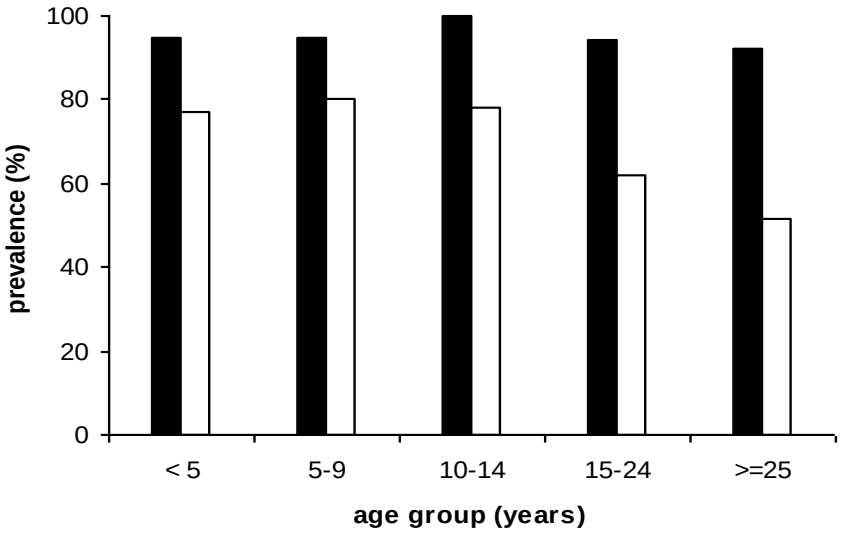
Comparison QT-NASBA and microscopy for gametocyte detection. Blood samples for QT-NASBA analysis were collected randomly from 412 individuals with mean age of 17.5 yrs (range 2--83 years) after informed consent. All participants were assigned to age groups <5 years (n = 79), 5--9 years (n = 97), 10--14 years (n = 78), 15--24 years (n = 71) and ≥ 25 years (n = 87).

Pfs25 mRNA QT-NASBA confirmed 92% (81/88) of samples gametocyte positive by microscopy with a geometric mean QT-NASBA gametocyte density of $3.90*10^3$ per ml blood (IQR $5.11*10^2$--$2.72*10^4$). In general, gametocyte densities detected by microscopy were close to the microscopical detection limit with 1 gametocyte /500 leucocytes counted in 51.1% of these samples. The more sensitive QT-NASBA detected gametocytes in an additional 208 samples with a geometric mean density below the detection limit of microscopy ($1.98*10^3$ per ml blood; IQR $3.95*10^2$--$9.60*10^3$).

Parasite prevalence. Detection of gametocytes by Pfs25 real-time QT-NASBA considerably increased gametocyte prevalence from 21.4% (microscopy) to 70.1%. Total parasite and gametocyte prevalence as detected by the two methods are shown for all age groups in figure 1. The detection of total parasite prevalence and gametocyte prevalence were higher by QT-NASBA compared to standard microscopy. A decrease of *P. falciparum* prevalence with age was found by microscopy but not by QT-NASBA, indicating that parasite densities in adults were merely reduced to submicroscopic levels. Gametocyte prevalence was negatively associated with age and 3.2--3.8 fold higher in the 3 youngest age groups (0--15 years) compared to that in adults (25+ years) by QT-NASBA analyses (table 1A). For microscopy this was 1.7--1.9 fold higher (data not shown).

Asexual parasite densities detected by microscopy decrease significantly with age (Ouédraogo and others, unpublished data). Because gametocytes are derived from their asexual progenitors, it is important to separately detect the effects of age on asexual parasites and on gametocytes. Therefore, the asexual parasite density was included as an explanatory variable in the analyses of age-related decrease of gametocyte prevalence. After adjustment for asexual parasite density, no significant decrease of gametocyte prevalence, detected by either QT-NASBA (table1A) or microscopy (data not shown) was found with increasing age.

A



B

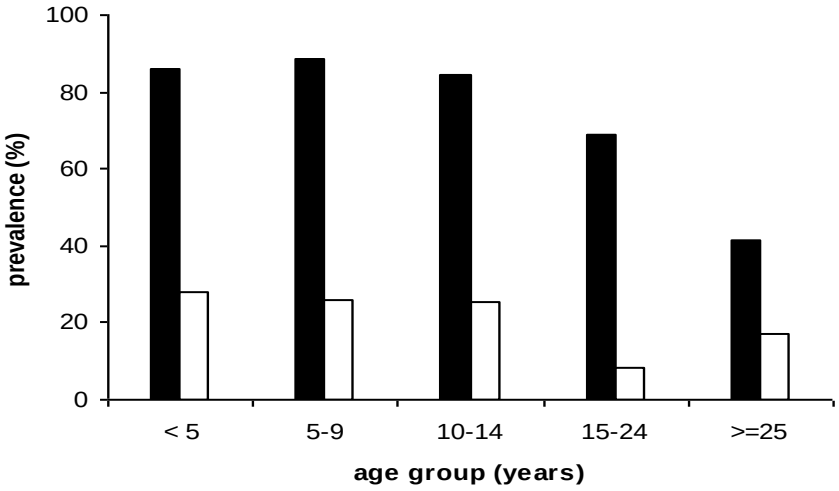


Figure 1: Parasite prevalence for the different age groups, calculated by QT-NASBA (A) and microscopy (B). *Solid bars represent total parasite prevalence, including asexual parasites and gametocytes. Open bars represent gametocyte prevalence.*

A significant positive association was shown between asexual parasite density and gametocyte prevalence (OR = 1.27; 95% CI = 1.16--1.38; p<0.001 for QT-NASBA (table 1A); OR = 1.12; 95% CI = 1.01--1.25; p = 0.040 for microscopy). These data suggest that the age-

related decrease of gametocyte prevalence is probably the result of decreasing asexual parasite densities.

Parasite density. Figure 2 shows that gametocyte densities as well as the asexual parasite densities decrease with age. Linear regression analyses showed a significant negative effect of age on gametocyte density detected by both QT-NASBA (Table1B) and microscopy (data not shown). Individuals over 15 years of age carried lower gametocyte densities than children aged 0--15 years.

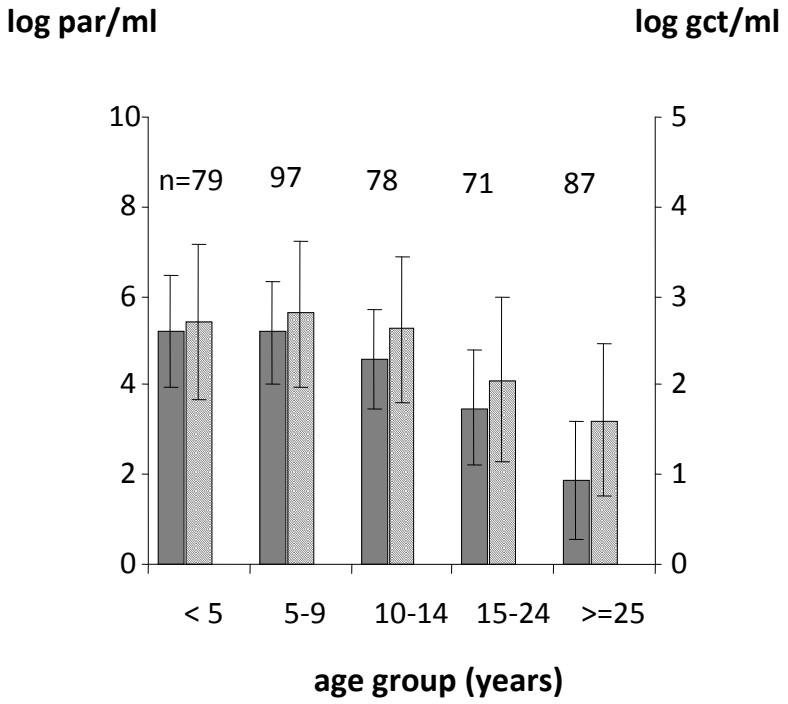


Figure 2: Mean parasite densities for different age groups, calculated for all samples. Solid bars represent asexual parasite density measured by microscopy. *Striped bars represent gametocyte density measured by QT-NASBA. Numbers of individuals per group are presented on top of bars. Error bars indicate standard deviations.*

When asexual parasite density was included as a covariate, a significant positive relation was found with gametocyte density by both QT-NASBA (β = 0.18; se (β) = 0.033; p<0.001 (table 1B) and microscopy (β = 0.076; se (β) = 0.036; p = 0.036).

Table1: Effect of asexual parasite density on age distribution of gametocyte carriage and gametocyte density measured by QT-NASBA

A. Gametocyte prevalence					
Age group	N	OR [a] (95% CI)	P-value	OR [b] (95% CI)	P-value
<5	79	3.16 (1.61-6.20)	0.001	1.53 (0.73-3.24)	0.262
5-9	97	3.83 (2.00-7.37)	0.000	1.86 (0.90-3.83)	0.094
10-14	78	3.35 (1.69-6.63)	0.001	1.85 (0.89-3.88)	0.101
15-24	71	1.52 (0.80-2.88)	0.197	1.05 (0.53-2.08)	0.884
≥25	*87*	*1.0*[c]		*1.0* [c]	
B. Gametocyte density [d]					
Age group	N	β [a] (se (β))	P-value	β [b] (se(β))	P-value
<5	79	1.11 (0.26)	0.000	0.50 (0.28)	0.071
5-9	97	1.19 (0.25)	0.000	0.59 (0.27)	0.027
10-14	78	1.02 (0.27)	0.000	0.53 (0.27)	0.053
15-24	71	0.45 (0.27)	0.098	0.16 (0.27)	0.556
≥25	87	0.0 [c]		0.0 [c]	
C. Gametocyte density [e, f]					
Age group	N	β [a] (se (β))	P-value		
<5	61	0.406 (0.19)	0.037		
5-9	78	0.369 (0.19)	0.046		
10-14	61	0.248 (0.19)	0.201		
15-24	44	0.213 (0.21)	0.309		
≥25	45	0.0 [c]			

[a] Crude values of odds ratio (OR) and β; [b] OR and β adjusted for [10]Log asexual parasite density. Asexual density OR = 1.27 (CI = 1.16--1.38, p<0.001) for gametocyte prevalence and β = 0.18 (se(β) = 0.03; p<0.001) for gametocyte density; CI confidence interval; se standard error; [c] age group ≥25 years was used as reference group; [d] All samples; [e] Samples from Pfs25 QT-NASBA positive gametocyte carriers; [f] Adjustment for asexual density was not significant with β = 0.023, se (β) = 0.026 and p = 0.378.

After this adjustment for asexual parasite density, the age-related difference in gametocyte density has mostly disappeared although a trend remained. These results suggest that age-

related decrease of gametocyte densities mainly depend on asexual density as shown for gametocyte prevalence.

Calculations of mean gametocyte densities are influenced by gametocyte prevalence if gametocyte-negative samples are included. Therefore, any factor that influences gametocyte prevalence may have been included in the analysis, without necessarily having a direct relation to gametocyte density. To avoid the risk of incorporating such indirect effects, the analyses were repeated in only QT-NASBA gametocyte-positive samples. Asexual density still tended to decrease with age, although the relation was not significant ($p = 0.378$). However, QT-NASBA gametocyte densities still decreased slightly with age. Table 1C shows that the 2 youngest age groups (<10 years) carry significantly higher gametocyte densities compared to adults. This effect of age is independent of asexual density. Such an age effect was not significant for microscopy, with or without asexual parasite density as a covariate.

Discussion

Pfs25 real-time QT-NASBA showed an overall gametocyte prevalence of 70.1% during a cross-sectional study at the end of the rainy season in Burkina Faso, which is higher than in studies based on microscopy[15,16]. Although the additionally detected gametocyte carriers in general have very low gametocyte densities, the potential contribution of this group to the infectious reservoir should not be ignored (Schneider and Bousema and others, unpublished data).[11] Therefore, transmission studies based on mosquito feeds with random population samples[16--18] will give a more adequate estimation of population-wide transmission potential than studies based on microscopy.[15,19--21]

Age-related decreases in both asexual parasites and gametocytes have been shown before.[15,16,18,22--24] Such relations may be the result of developing immunity to asexual and sexual stages of *Plasmodium falciparum* over time. In this study, the age-dependent decrease of asexual parasites suggests the development of asexual stage immunity. Gametocyte prevalence and densities also decreased with age. As these stages are formed from their asexual progenitors, this may be the result of a lower availability of asexual progenitors (asexual stage immunity), of cross-stage immunity[25] or of sexual stage-specific immunity. Our results show that the relation between age and both gametocyte prevalence and density depends primarily on asexual parasite density, suggesting that asexual or cross-stage immunity may be an important determinant. This effect may obscure direct influences of age, i.e. anti-gametocyte immunity. We have adjusted for this effect in two different ways. 1) By adjusting for asexual parasite density. Although a trend was seen, age-related decreases in gametocyte density were non-significant after this adjustment. However, as a result of adjustment with highly variable asexual parasite densities, larger samples sizes may be required to obtain significant relationships. 2) By selecting only *Pfs25* QT-NASBA positive samples. The influence of asexual parasite density was reduced to insignificant levels in this selection, while an age-related decrease in gametocyte density was still seen. This decrease of gametocyte densities with age, unaffected by asexual parasite densities, may be the result of anti-gametocyte immunity. However, the age-related, decrease of gametocytes is likely determined by immunity that results in primarily a decreased asexual parasite density rather than direct effect on gametocytes.

The influence of asexual parasite density on gametocytes, described above, was based on analyses including microscopically counted asexual parasites. Although

microscopical detection of asexual parasites is more robust than that of gametocytes, more sensitive methods like QT-NASBA may also detect sub-patent asexual parasites. Ideally we would have included asexual parasite density determined by QT-NASBA as a covariate. QT-NASBA is currently available for quantification of the total parasite load[14] or for gametocytes only, but not yet for specific quantification of asexual parasites as is the case for other molecular biology methods such as PCR[7,14].

Our results were obtained with samples collected at the end of the wet season. With a marked seasonal transmission in Burkina Faso, we cannot generalize these results to all seasons. It is possible that gametocyte prevalence and mean gametocyte densities as well as the relations with age vary over time. However, in a preceding study over various seasons, of which the present samples are a small part, we came to similar conclusions with microscopical parasite detection (Ouédraogo and others, unpublished data). QT-NASBA analysis of samples of cross-sectional surveys in other seasons will be performed.

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References

1. Muirhead-Thomson RC, 1954. Factors determining the true reservoir of infection of *Plasmodium falciparum* and *Wuchereria bancrofti* in a West African village*. Trans R Soc Trop Med Hyg 48:* 208--225.
2. Trape JF, 1985. Rapid evaluation of malaria parasite density and standardization of thick smear examination for epidemiological investigations*. Trans R Soc Trop Med Hyg 79:* 181--184.
3. Dowling MAC, Shute GT, 1966. A comparative study of thick and thin blood films in the diagnosis of scanty malaria parasitaemia. *Bull World Health Organ 34:* 249--267.
4. Greenwood BM, Armstrong JR, 1991. Comparison of two simple methods for determining malaria parasite density*. Trans R Soc Trop Med Hyg 85:* 186--188.

5. Babiker HA, Abdel-Wahab A, Ahmed S, Suleiman S, Ranford-Cartwright L, Carter R, Walliker D, 1999. Detection of low level *Plasmodium falciparum* gametocytes using reverse transcriptase polymerase chain reaction. *Mol Biochem Parasitol 99*: 143--148.
6. Menegon M, Severini C, Sannella A, Paglia MG, Sangare D, Abdel-Wahab A, Abdel-Muhsin AA, Babiker H, Walliker D, Alano P, 2000. Genotyping of *Plasmodium falciparum* gametocytes by reverse transcriptase polymerase chain reaction. *Mol Biochem Parasitol 111:* 153--161.
7. Paganotti GM, Palladino C, Modiano D, Sirima BS, Raberg L, Diarra A, Konaté A, Coluzzi M, Walliker D, Babiker HA, 2006. Genetic complexity and gametocyte production of *Plasmodium falciparum* in Fulani and Mossi communities in Burkina Faso. *Parasitol 132:* 607--614.
8. Abdel-Wahab A, Abdel-Muhsin AA, Ali E, Suleiman S, Ahmed S, Walliker D, Babiker HA, 2002. Dynamics of gametocytes among *Plasmodium falciparum* clones in natural infections in an area of highly seasonal transmission. *J Infect Dis 185:* 1838--1842.
9. Nassir E, Abdel-Muhsin AM, Suliaman S, Kenyon F, Kheir A, Geha H, Ferguson HM, Walliker D, Babiker HA, 2005. Impact of genetic complexity on longevity and gametocytogenesis of *Plasmodium falciparum* during the dry and transmission-free season of eastern Sudan. *Int J Parasitol 35:* 49--55.
10. Schneider P, Schoone G, Schallig H, Verhage D, Telgt D, Eling W, Sauerwein R, 2004. Quantification of *Plasmodium falciparum* gametocytes in differential stages of development by quantitative nucleic acid sequence-based amplification. *Mol Biochem Parasitol 137:* 35--41
11. Schneider P, Bousema T, Omar S, Gouagna L, Sawa P, Schallig H, Sauerwein R, 2006. (Sub)microscopic *Plasmodium falciparum* gametocytaemia in Kenyan children after treatment with sulphadoxine-pyrimethamine monotherapy or in combination with artesunate. *Int J Parasitol 36:* 403--408.
12. Petrarca V, Petrangeli G, Rossi P, Sabatinelli G, 1986. Chromosomal study of Anopheles gambiae and Anopheles arabiensis in Ouagadougou (Burkina Faso) and various neighboring villages. *Parassitologia 28:* 41--61.
13. Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-van-Dillen PM, Noordaa Jvd, 1990. Rapid and simple method for purification of nucleic acids. *J Clin Microbiol 28:* 495--503.
14. Schneider P, Wolters L, Schoone G, Schallig H, Sillekens P, Hermsen R, Sauerwein R, 2005. Real-time nucleic acid sequence-based amplification is more convenient than real-time PCR for quantification of *Plasmodium falciparum. J Clin Microbiol 43:* 402--405.
15. Boudin C, Lyannaz J, Bosseno MF, Carnevale P, Ambroise-Thomas P, 1991. Epidemiology of *Plasmodium falciparum* in a rice field and a savanna area in Burkina Faso: seasonal fluctuations of gametocytaemia and malarial infectivity. *Ann Trop Med Parasitol 85:* 377--385.
16. Boudin C, Olivier M, Molez JF, Chiron JP, Ambroise-Thomas P, 1993. High human malarial infectivity to laboratory-bred Anopheles gambiae in a village in Burkina Faso. *Am J Trop Med Hyg 48:* 700--706.
17. Muirhead-Thomson RC, 1957. The malarial infectivity of an African village population to mosquitoes (Anopheles gambiae); a random xenodiagnostic survey. *Am J Trop Med Hyg 6:* 971--979.
18. Githeko AK, Brandling-Bennett AD, Beier M, Atieli F, Owaga M, Collins FH, 1992. The reservoir of *Plasmodium falciparum* malaria in a holoendemic area of western Kenya. *Trans R Soc Trop Med Hyg 86:* 355--358.
19. Gouagna LC, Bonnet S, Gounoue R, Verhave JP, Eling W, Sauerwein R, Boudin C,

2004. Stage-specific effects of host plasma factors on the early sporogony of autologous *Plasmodium falciparum* isolates within Anopheles gambiae*. Trop Med Int Health 9:* 937--948.

20. Toure YT, Doumbo O, Toure A, Bagayoko M, Diallo M, Dolo A, Vernick KD, Keister DB, Muratova O, Kaslow DC, 1998. Gametocyte infectivity by direct mosquito feeds in an area of seasonal malaria transmission: implications for Bancoumana, Mali as a transmission-blocking vaccine site*. Am J Trop Med Hyg 59:* 481--486.
21. Drakeley CJ, Akim NI, Sauerwein RW, Greenwood BM, Targett GA, 2000. Estimates of the infectious reservoir of *Plasmodium falciparum* malaria in The Gambia and in Tanzania*. Trans R Soc Trop Med Hyg 94:* 472--476.
22. Akim NI, Drakeley C, Kingo T, Simon B, Senkoro K, Sauerwein RW, 2000. Dynamics of *P. falciparum* gametocytemia in symptomatic patients in an area of intense perennial transmission in Tanzania*. Am J Trop Med Hyg 63:* 199--203.
23. Kolk Mvd, Tebo AE, Nimpaye H, Ndombol DN, Sauerwein RW, Eling WM, 2003. Transmission of *Plasmodium falciparum* in urban Yaounde, Cameroon, is seasonal and age-dependent*. Trans R Soc Trop Med Hyg 97:* 375--379.
24. Bousema JT, Gouagna LC, Drakeley CJ, Meutstege AM, Okech BA, Akim IN, Beier JC, Githure JI, Sauerwein RW, 2004. *Plasmodium falciparum* gametocyte carriage in asymptomatic children in western Kenya*. Malar J 3:* 18. 25. Piper KP, Hayward RE, Cox MJ, Day KP, 1999. Malaria transmission and Naturally acquired immunity to PfEMP-1*. Infect Immun 67:* 6369--6374.

Chapter 4

The plasticity of *Plasmodium falciparum* gametocytaemia in relation to age in Burkina Faso

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Summary

Malaria transmission depends on the presence of gametocytes in the peripheral blood. In this study, the age-dependency of gametocytaemia was examined by microscopy and molecular tools.

A total of 5,383 blood samples from individuals of all ages were collected over six cross sectional surveys in Burkina Faso. One cross-sectional study used quantitative nucleic acid sequence based amplification (QT-NASBA) for parasite quantification (n=412). The proportion of infections with concurrent gametocytaemia and median proportion of gametocytes among all parasites were calculated.

Asexual parasite prevalence and gametocyte prevalence decreased with age. Gametocytes made up 1.8% of the total parasite population detected by microscopy in the youngest age group. This proportion gradually increased to 18.2% in adults ($p<0.001$). Similarly, gametocytes made up 0.2% of the total parasite population detected by QT-NASBA in the youngest age group, increasing to 5.7% in adults ($p<0.001$). This age pattern in gametocytaemia was also evident in the proportion of gametocyte positive slides without concomitant asexual parasites which increased from 13.4% (17/127) in children to 45.6% (52/114) in adults (OR 1.55, 95% CI 1.38-1.74, $p<0.001$).

The findings of this study suggest that although gametocytes are most commonly detected in children, the proportion of asexual parasites that is committed to develop into gametocytes may increase with age. These findings underscore the importance of adults for the human infectious reservoir for malaria.

Introduction

The malaria parasite's life- cycle is composed of several developmental stages, one of which is the transmissible sexual stage comprising male and female gametocytes. Mature gametocytes are apparently benign, causing no overt disease symptoms. They appear to be developmentally arrested at the G_0 phase of the cell cycle and circulate within erythrocytes in the peripheral blood of the human host until they are taken up by a feeding female mosquito. In the mosquito midgut, gametocyte activation and fertilization take place. The subsequent formation of sporogonic stages results in the development of thousands of sporozoites that migrate to and invade the salivary glands, rendering the mosquito infectious to humans.

During the course of an infection with *Plasmodium falciparum*, gametocytes are generated from asexual stage parasites. Only a small fraction of the asexual parasites of *P. falciparum* commit to form gametocytes [1] and as a result only a fraction of infected individuals also harbour gametocytes [1,2]. It is now understood that this apparently low occurrence of gametocytes is partly a reflection of the low sensitivity of microscopy for the detection of gametocytes [2,3]. However, the fact remains that asexual parasitaemia is not always accompanied by gametocyte carriage [1-3], and that the relationship between asexual parasite density and gametocyte prevalence or density is not straightforward. Some studies report a positive association between asexual parasite densities and gametocyte prevalence [4-6] and density [4] while others observe inverse associations [7,8] or report that the association may be modified by age [2].

Factors that trigger and regulate the commitment of asexual stage parasites to gametocytes are largely unknown but are thought to include intrinsic parasite factors [9], anti-malarial treatment [4,6,10] and treatment outcome [4-6,11], fever [7,8], haematological disruptions [6,12,13] and the presence of competing parasite strains [14,15] or species [6,16]. In general the mechanism of sexual commitment appears to be highly plastic and environment sensitive [17,18]. The flexible gametocyte production can be interpreted as a response mechanism of the parasite to stressful situations: if the survival of the asexual stage parasite is challenged, the investment in transmission stages increases.

Here, we explore age-dependent variation in gametocytaemia in a series of cross-sectional surveys in an area of seasonal malaria transmission in Burkina Faso, using both microscopy and quantitative nucleic acid sequence based amplification (QT-NASBA).

Methods

Study site and population

The study was carried out in the vicinity of Ouagadougou, the capital of Burkina Faso. The area has the ecological characteristics of Sudan savannah. Participating populations from six villages (longitude: 1° 46'- 1°79'; latitude: 12°52'-12°61') were of the same ethnic group (Mossi) and had similar age distributions. Transmission intensity is intense and seasonal in this region. Study subjects were given detailed explanations of the procedures, risk and benefits involved in the study and their consent was obtained. The study protocol was viewed and approved by the Ministry of Health of Burkina Faso (Research's Authorization number 2000/3174/MS/SG/DEP).

Blood sample collection

Cross-sectional surveys were performed in January, May, August and December 2002 and in April and December 2003. Participants were randomly selected from previously determined age groups (0.5–4, 5–9, 10–14, 15–24 and 25+ years) based on census lists and computer generated randomization tables. Thick and thin blood smears were made from finger-prick blood. The body temperature was measured and febrile individuals who were parasitaemic were treated with chloroquine according to the national policy in 2002. In the cross-sectional survey of December 2003, a single finger prick sample was used for blood smears and the collection of nucleic acids for quantitative-nucleic acid sequence based amplification (QT-NASBA); 100µL blood samples were collected from 412 volunteers of all ages from the six villages. The first part of the RNA extraction was done in the field following the original guanidinium isothiocyanate (GuSCN) RNA extraction method [19] until the nucleic acids were bound to silica dioxide particles. At this point, samples were stored at -20°C and transferred to the laboratory for completion of the extraction and QT-NASBA analysis.

Microscopical detection of P. falciparum parasites

Samples were considered negative if no parasites were detected in 100 high-power fields of Giemsa-stained thick blood smears. Both asexual stage and gametocyte densities were assessed in the thick smear by counting against 500 and 1,000 leucocytes, respectively. Based on this approach, the lower limit of microscopy for gametocyte quantification was estimated at 8 gametocytes/μl of blood. Parasite counts were converted to numbers of parasites per μl by assuming a standard count of 8,000 leucocytes/μl of blood. Each sample was read independently by two microscopists and the mean density was used. A third reader was involved when the first two readers disagreed about the prevalence of gametocytes or their estimated densities differed ≥30%. In these cases the mean density of the two closest readings was used.

Real-time Pfs25 QT-NASBA and nucleic acid extraction

18S rRNA real-time QT-NASBA and Pfs25 real time mRNA QT-NASBA were performed as described elsewhere [20]. Briefly, real-time QT-NASBA for *Pfs*25 mRNA (Genbank accession number AF193769.1) was performed on a Nuclisens EasyQ analyser (bioMérieux) using the Nuclisens Basic Kit for amplification according to manufacturer's instructions at a KCl concentration of 80mM. Reactions were performed in a total reaction volume of 10 μl per reaction. For quantification, time to positivity is calculated, i.e. the time point during amplification at which the fluorescence detecting target amplicons becomes higher than the mean fluorescence of three negative controls + 20 standard deviations (SD). The use of a standard gametocyte stage V dilution series allows exact calculation of the number of gametocytes present in unknown samples [20]. The sensitivity of this method is 20-100 gametocytes/mL.

Statistical analysis

The age-dependency of gametocytaemia by microscopy was determined by categorizing the population into 0.5-2, 3-4, 5-9, 10-14, 15-19 and ≥20 year-old individuals [2]. For QT-NASBA data, numbers were smaller and age groups were combined to form 0.5-4, 5-9, 10-19 and ≥20 year-old individuals. The proportion of asexual stage parasite carriers that also harboured gametocytes was calculated. Similarly, the individual proportion of gametocytes among total parasites was calculated by dividing the individual gametocyte density by the

total parasite density (asexual parasite density plus gametocyte density). An identical approach was used for QT-NASBA where the *Pfs*25 QT-NASBA gametocyte prevalence or density was divided by the 18S QT-NASBA total parasite prevalence or density. Age-dependent trends in parasite carriage were determined by linear (density after log transformation) or logistic (prevalence) regression models and regression coefficients or odds ratios are presented, respectively. Estimates were adjusted for seasonality (dry, start wet, peak wet and end wet) where appropriate. The majority of individuals were only included in one of the surveys. Several individuals were included in more than one cross-sectional survey with a time-gap between repeated measures of two months (n=484) or ≥4 months (n=781). Adjusting for the correlation between observations from the same individual by generalized estimating equations (GEE) did not indicate a significant impact of autocorrelation (i.e. estimates and confidence intervals remained unaltered) and, therefore, conventional regression models were used. Trends in other variables were determined by χ^2-test for trend or non-parametric trend tests for continuous variables. All statistical analyses were performed using STATA 11 [Statacorp, Texas US].

Results

Overall, the six cross-sectional studies yielded a total of 5,383 observations: 1,216 observations from January 2002, 968 from June, 749 from August, 599 from December, 1,084 from April 2003 and 767 from December 2003. 58.1% of the participants were of the female gender (3,127/5,383). Details on the seasonality of parasite carriage were presented previously [21]. These findings were derived from 3154 individuals, 59.9% (1889/3154) of whom were seen once, 21.7% (685/3154) twice, 10.1% (320/3154) three times, 5.1% (161/3154) four times, 2.4% (75/3154) five times and 0.8% (24/3154) six times. The vast majority of individuals who donated a blood sample were afebrile: Only 5.1% of children below 15 years of age had a temperature ≥37.5°C (165/3,250) and 1.0% of older individuals (21/2,105).

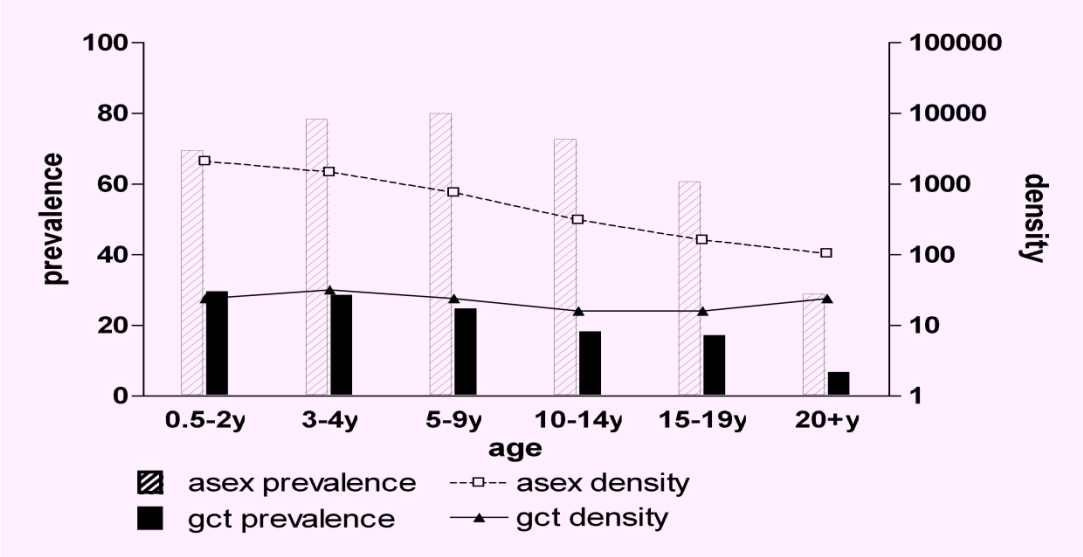

Figure 1. Parasite carriage in different age groups by microscopy. Asex = asexual parasite; gcyt = gametocyte. The number of asexual parasite carriers (with gametocytes) for the different age groups was 0.5-2y: 297 (127); 3-4y: 468 (171); 5-9y: 1053(326); 10-14y: 690(174); 15-19y: 254(72); 20+y: 285(114).

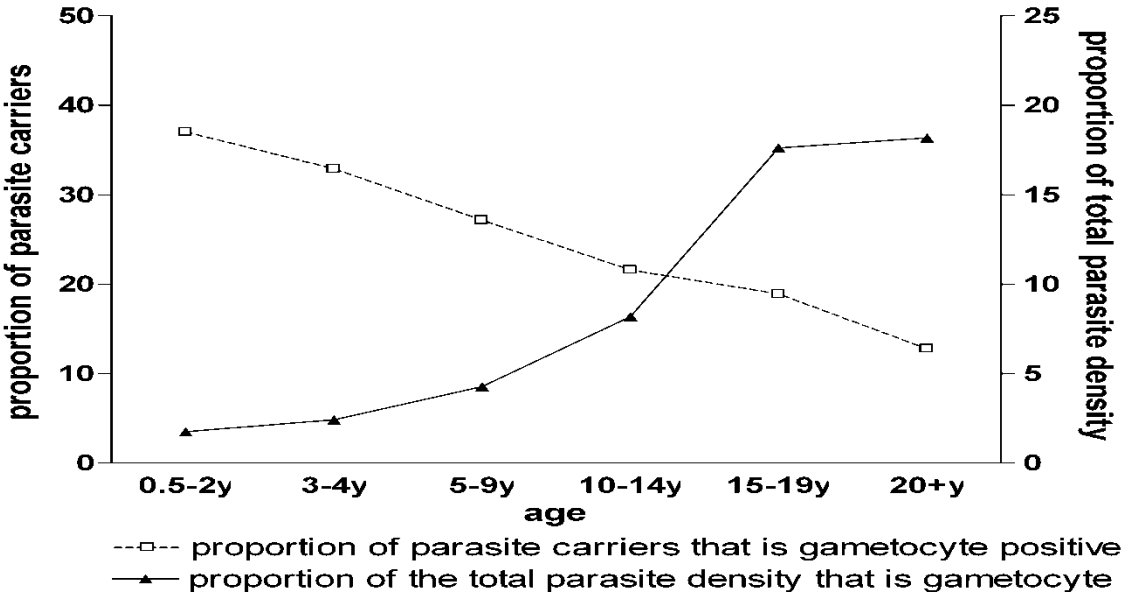

Figure 2. The prevalence and density of gametocytes relative to total parasite carriage by microscopy. The number of asexual parasite carriers (with gametocytes) for the different age groups was 0.5-2y: 297 (127); 3-4y: 468 (171); 5-9y: 1053(326); 10-14y: 690(174); 15-19y: 254(72); 20+y: 285(114). Note: data for those carrying gametocytes in the absence of asexual parasitaemia were excluded (see Figure 3).

The prevalence of asexual parasites (OR 0.59; 95% CI 0.57-0.62, p<0.001) and gametocytes (OR 0.70; 95% CI 0.67-0.74, p<0.001) decreased with age (Figure 1). In parasite positive individuals, the log-transformed density of asexual parasites also decreased over age categories (β=-0.23; 95% CI -0.24 – -0.21, p<0.001). The gametocyte density in gametocyte carriers also decreased significantly but to a lesser extent (β=-0.041; 95% CI -0.055 – -0.028, p<0.001). The proportion of infections with concomitant gametocytaemia decreased with age (Figure 2). Whilst 37.0% (110/297) of 0.5-2 year-old carriers of asexual stage parasites concurrently had gametocytes, this was only true for 12.8% (62/485) of the parasite carriers who were 20 years old and above (OR=0.76;95% CI 0.72-0.81, p<0.001). In contrast with this trend, the median proportion of gametocytes among all parasites increased with increasing age (Figure 2). Thus, whilst gametocytes only represented 1.8% of the density of the total parasite population in the youngest age group, this proportion gradually increased to a peak of 18.2% in adults (β=0.42; 95% CI 0.33-0.51, p<0.001). Although the majority of gametocyte carriers also harboured asexual parasites, microscopical evidence of concurrent asexual parasitaemia was lacking in 17.8% (175/984) of all gametocyte carriers. The proportion of gametocyte carriers without concurrent asexual parasites detected by microscopy was 13.4% (17/127) in children below two years of age and increased with age to 45.6% (52/114) in the oldest age group (Figure 3; trend for age in categories OR 1.55, 95% CI 1.38-1.74, p<0.001).

Patterns of gametocyte carriage and asexual parasite carriage were unaffected by the presence of fever and did not change when molecular methods were used for parasite detection instead of microscopy. For the limited number of samples (n=412) for which both 18S QT-NASBA (total parasite) and *Pfs25* QT-NASBA (gametocyte) prevalence and density data were available, total parasite prevalence (OR =0.85; 95% CI 0.63-1.15, p=0.29) and gametocyte prevalence (OR=0.73; 95% CI 0.63-0.85, p<0.001) both decreased with increasing age (Figure 4). Log-transformed total parasite density showed a decrease with age (β=-0.36; 95% CI -0.44 – -0.28, p<0.001) that was more pronounced than that of gametocyte density (β=-0.096; 95% CI -0.17 – -0.017, p=0.02). Densities of gametocytes detected by *Pfs25* QT-NASBA in gametocyte carriers were low with a median gametocyte

density of 2.3 gametocytes/μL (IQR 0.4-10). Similar to observations by microscopy, the proportion of parasite carriers with concurrent gametocytes decreased with age. While 81.1% (60/74) of parasite carriers in the youngest age group harboured gametocytes, this proportion gradually decreased to 62.0% (67/108) in the oldest age group (OR=0.75;95% CI 0.64-0.87, p<0.001). On the other hand, and again consistent with the pattern revealed by microscopy, the median proportion of gametocytes among total parasites increased from 0.2% in the youngest to 5.7% in the oldest age group (β=0.61; 95% CI 0.35-0.86, p<0.001, Figure 5).

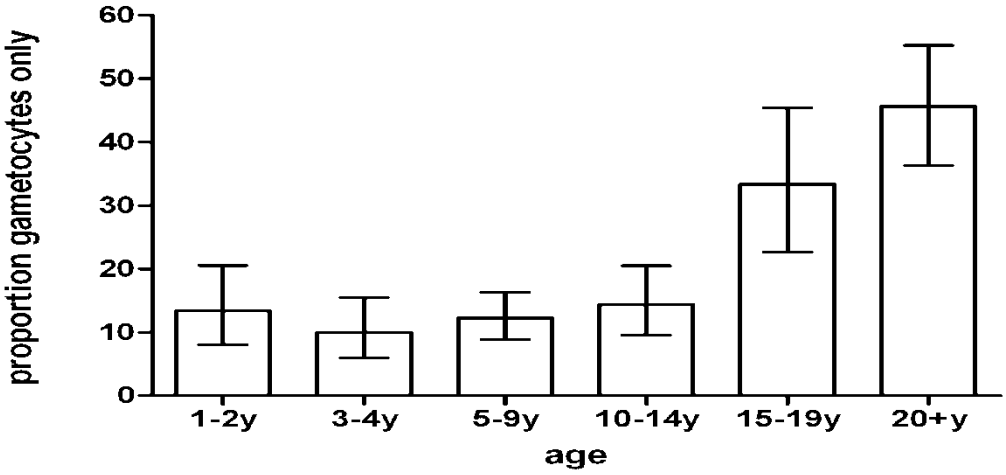

Figure 3. Gametocyte carriage in the absence of microscopically detectable asexual parasites. Bars indicate the proportion of microscopically detected gametocyte carriers in each age category without microscopically confirmed asexual parasites. Error bars indicate the upper and lower limit of the 95% confidence interval around the proportion. The number of gametocyte carriers (without concurrent asexual parasitaemia) for the different age groups was 0.5-2y: 127(17); 2-3y: 171(17); 5-9y: 326(40); 10-15y 174(25); 15-20y: 72(24); 20+y: 114(52).

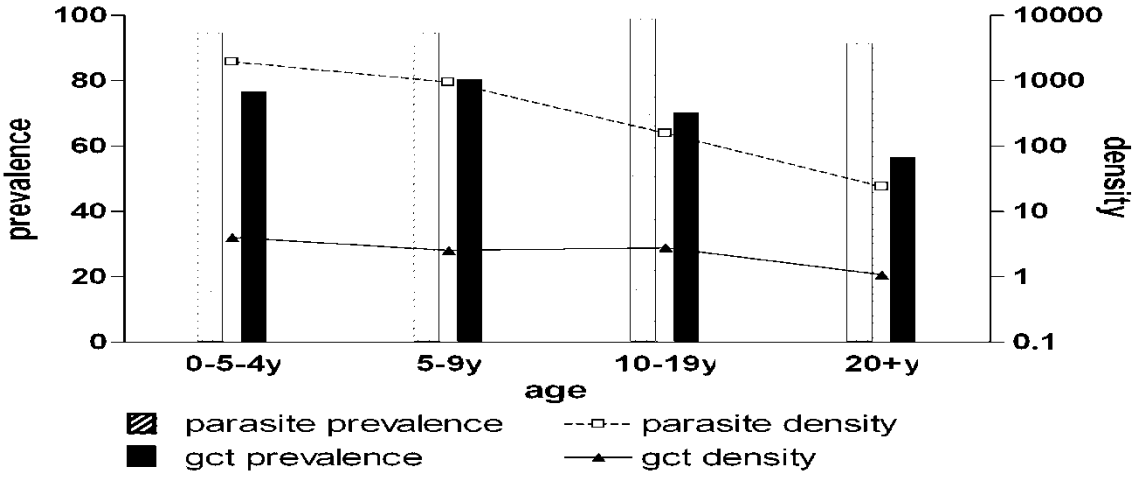

Figure 4. Parasite carriage by quantitative nucleic acid based amplification (QT-NASBA) in different age groups. Total parasite prevalence and density were determined by 18S QT-NASBA; gametocyte prevalence and density by *Pfs*25 QT-NASBA. The number of parasite carriers (with concurrent gametocytaemia) for the different age groups was 0.5-4y: 74(60); 5-9y: 93(79); 10-19y: 117(83); 20+y 108(67).

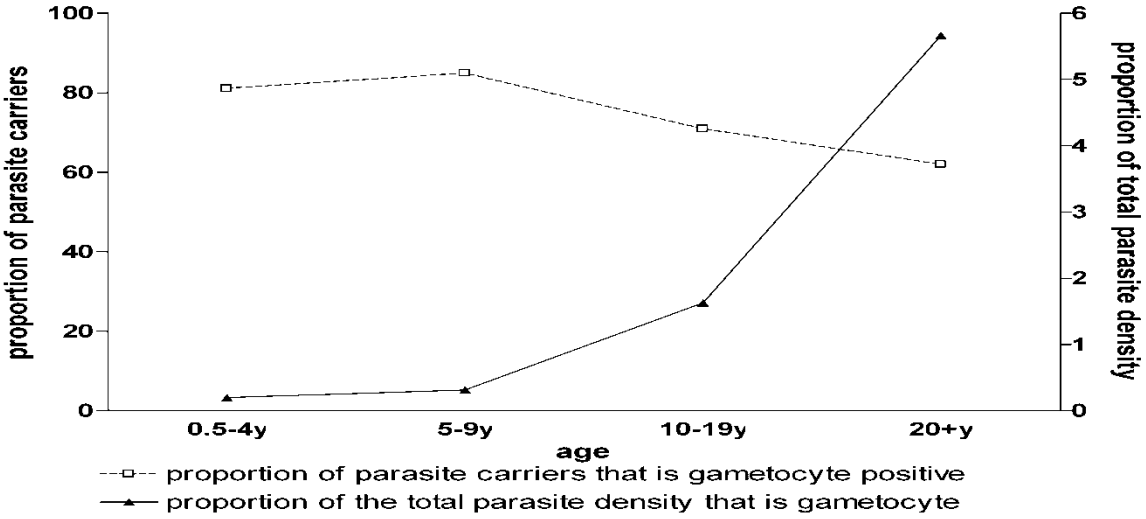

Figure 5. The age-dependent prevalence and density of gametocytes relative to total parasite carriage by QT-NASBA. The number of parasite carriers (with concurrent gametocytaemia) for the different age groups was 0.5-4y: 74(60); 5-9y: 93(79); 10-19y: 117(83); 20+y 108(67).

Discussion

This study indicates that while the global prevalence of asexual parasites and gametocytes is highest in children, the density of gametocytes relative to the total parasite concentration increases with age. This age-dependent increase is apparent by microscopy and by a more sensitive molecular gametocyte detection technique. These findings suggest that the commitment of asexual malaria parasites to the sexual pathway may increase with age.

P. falciparum sexual stage commitment is crucial for the transmission of parasites from man to mosquito and the subsequent spread of malaria in the human population. A better understanding of the factors that influence the switch from asexual to sexual stage development in the human host would therefore provide new opportunities for malaria control. In the study presented here, a large data set collected during cross sectional surveys in two consecutive years was analyzed to explore factors associated with gametocyte production. Numerous studies have shown that the prevalence of gametocytes decreases with age [4,5,22-28]. The present study attempted to determine whether this is the simple consequence of an age-dependent decline in the prevalence and density of asexual parasites [4-6] or if there may be an age-dependent production of gametocytes. The proportion of gametocytes relative to total parasite density increased with age in the current study [2]. Whilst gametocytes only comprised 1.8% of the total parasite population in the youngest children, they comprised 18.2% of the total parasite population in the oldest age-group. These calculations may have been affected by the limited sensitivity of microscopy for detecting low gametocyte densities [29] but the association between age and gametocyte production was supported by two additional lines of evidence.

In the analyses on the proportion of asexual parasite carriers with concurrent gametocytaemia and the proportion of gametocytes among the total parasite population, the group of individuals with microscopically detectable gametocytes but no asexual parasites was excluded. This group was expected to form a relatively small subset of individuals whose asexual parasites were recently cleared by immune responses or antimalarial drugs. Contrary to this expectation, the presence of gametocytes in the absence of asexual parasites detectable by microscopy was not rare: one in six gametocyte carriers had no microscopical evidence of asexual parasitaemia and this proportion increased with age. One theoretical explanation for this observation could be that a higher proportion of

infections were recently cleared by anti-malarial drugs in adults. After the clearance of asexual parasites by chloroquine, the most commonly used antimalarial drug in the study area, gametocytes may have persisted since these are largely unaffected by this drug [30]. No information on recent drug use was recorded in this study but it is unlikely that a difference in drug use between children and adults would explain the observations. Disease symptoms and anti-malarial drug use are more common in children, not in adults [31]. A more likely explanation is that that low density (i.e. microscopically undetectable) malaria infections were more frequently accompanied by microscopically detectable gametocyte densities in older age-groups. This would support an age-dependent increase in commitment to gametocyte production.

A last line of evidence comes from the molecular gametocyte detection tool that is at least 100-fold more sensitive for detection of gametocytes [32]. QT-NASBA detects gametocytes at densities as low as 0.02 gametocytes/µL [2,20]. Similar to microscopical findings, the proportion of gametocytes relative to total parasite density detectable by QT-NASBA increased with age: from 0.2% in the youngest age group to 5.7% in the oldest individuals. These three lines of evidence suggest that the relative density of gametocytes increases with age in an area where transmission is intense and adults have developed an efficient anti-disease and anti-parasite immune response [33,34].

The biological mechanism behind an age-dependent increase in sexual stage commitment is impossible to deduce from epidemiological data. It could plausibly result from epidemiological differences in infections between age-groups or from a response of the parasite to age-acquired immune responses and the resulting lower density of asexual parasites. The latter would suggest a strategic advantage for parasites that may increase gametocytaemia in response to conditions that negatively affect asexual stage parasite multiplication [35]. The developmental decision to enter gametocytogenesis for *P. falciparum* occurs during the formation of the asexual schizont, which can commit its entire progeny of merozoites either to develop once more asexually, or to enter sexual differentiation [36]. This decision may depend on the immune stress experienced by the parasites. Gametocytogenesis in *Plasmodium chabaudi* is increased in immunized compared to naïve mice [37]. Similar findings for *P. falciparum* have been reported when parasites were exposed to immune stimuli *in vitro* [38]. The findings from the current study suggest a similar

mechanism in natural infections where gametocytaemia increases in response to age-acquired immune responses, decreasing asexual parasite densities or other age-dependent factors. Anaemia and drug treatment, likely triggers of gametocyte production [6,12,13] were not directly measured but are likely to be more common in children than adults and can therefore not explain the findings of a higher relative gametocyte density in adults. It is also possible that the association is explained by a longer duration of malaria infections in adults. Children are more likely to develop symptoms and seek treatment or benefit from a prophylactic effect of presumptive treatment [39]. Infections may have a longer average duration in immune adults, giving parasites more time to develop gametocytes. The half-life of gametocytes is 3-6 days [40,41] and the density of gametocytes could therefore increase cumulatively when gametocytes have been produced for a longer period of time while asexual parasite densities are decreasing prior to sampling. Such an effect cannot be ruled out in this study although the asymptomatic nature of the vast majority of infections makes it plausible that most individuals harboured parasites for a sufficiently long time to develop gametocytes [41]. It is also possible that gametocyte mortality is increased in children due to the high asexual parasite density-mediated release of cytokines [42,43], resulting in a lower relative gametocyte density. Gametocyte-clearing immune responses [44] may also have contributed to these observations but there is currently insufficient evidence for the functional relevance of such responses in malaria endemic countries or its age-dependency.

In conclusion, the findings reported in this study suggest that once asexual population growth has been controlled by the host, the transmission benefits of increased gametocyte densities become apparent. These findings require confirmation in longitudinal studies that should ideally use molecular parasite detection tools. Although the patterns observed by QT-NASBA were similar to those by microscopy, the former is preferable since it detects gametocytes over a much wider range of densities [20]. An additional advantage of molecular detection tools is that they would also allow the detection of sexual stage committed asexual parasites [45], which could provide an additional level of detail in studying the dynamics of gametocyte production. These findings have an important implication for malaria control: although the prevalence of asexual parasite and gametocyte carriage decreases with increasing age, adults can be important contributors to the human infectious reservoir. Adults constitute the majority of populations in malaria endemic areas and many

adults harbour (low densities of gametocytes. The current study suggests that the commitment to gametocytaemia may increase in adults, increasing their relative importance for the human infectious reservoir. Adults should therefore be taken into consideration when implementing interventions that aim at reducing malaria transmission.

Acknowledgements

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References

1. Taylor LH, Read AF. Why so few transmission stages? Reproductive restraint by malaria parasites. *Parasitol Today* 1997;**13**(4)**:**135-40.
2. Drakeley C, Sutherland C, Bousema JT, Sauerwein RW, Targett GA. The epidemiology of *Plasmodium falciparum* gametocytes: weapons of mass dispersion. *Trends Parasitol* 2006;**22**(9)**:**424-30.
3. Babiker HA, Schneider P, Reece SE. Gametocytes: insights gained during a decade of molecular monitoring. *Trends Parasitol* 2008;**24**(11)**:**525-30.
4. Akim NIJ, Drakely C, Kingo T, Simon B, Senkoro K, Sauerwein RW. Dynamics of *P. falciparum* gametocytemia in symptomatic patients in an area of intense perennial transmission in Tanzania. *American Journal of Tropical Medicine and Hygiene* 2000;**63**(3-4)**:**199-203.
5. Bousema JT, Gouagna LC, Drakeley CJ, et al. *Plasmodium falciparum* gametocyte carriage in asymptomatic children in western Kenya. *Malar J* 2004;**3**(1)**:**18.
6. Price R, Nosten F, Simpson JA, et al. Risk factors for gametocyte carriage in uncomplicated *falciparum* malaria. *Am J Trop Med Hyg* 1999;**60**(6)**:**1019-23.
7. Sowunmi A, Fateye BA, Adedeji AA, Fehintola FA, Happi TC. Risk factors for gametocyte carriage in uncomplicated *falciparum* malaria in children. *Parasitology* 2004;**129**(Pt 3)**:**255-62.
8. von Seidlein L, Drakeley C, Greenwood B, Walraven G, Targett G. Risk factors for gametocyte carriage in Gambian children. *Am J Trop Med Hyg* 2001;**65**(5)**:**523-7.
9. Abdel-Wahab A, Abdel-Muhsin AA, Ali E, et al. Dynamics of gametocytes among *Plasmodium falciparum* clones in natural infections in an area of highly seasonal transmission. *Journal of Infectious Diseases* 2002;**185**(12)**:**1838-1842.

10. Sowunmi A, Fateye BA. *Plasmodium falciparum* gametocytaemia in Nigerian children: before, during and after treatment with antimalarial drugs. *Trop Med Int Health* 2003;**8**(9)**:**783-92.

11. Barnes KI, Little F, Mabuza A, et al. Increased gametocytemia after treatment: an early parasitological indicator of emerging sulfadoxine-pyrimethamine resistance in *falciparum* malaria. *J Infect Dis* 2008;**197**(11)**:**1605-13.

12. Drakeley CJ, Secka I, Correa S, Greenwood BM, Targett GA. Host haematological factors influencing the transmission of *Plasmodium falciparum* gametocytes to Anopheles gambiae s.s. mosquitoes. *Trop Med Int Health* 1999;**4**(2)**:**131-8.

13. Stepniewska K, Price RN, Sutherland CJ, et al. *Plasmodium falciparum* gametocyte dynamics in areas of different malaria endemicity. *Malar J* 2008;**7:**249.

14. Nassir E, Abdel-Muhsin AM, Suliaman S, et al. Impact of genetic complexity on longevity and gametocytogenesis of *Plasmodium falciparum* during the dry and transmission-free season of eastern Sudan. *Int J Parasitol* 2005;**35**(1)**:**49-55.

15. Taylor LH, Walliker D, Read AF. Mixed-genotype infections of the rodent malaria *Plasmodium* chabaudi are more infectious to mosquitoes than single-genotype infections. *Parasitology* 1997;**115 (Pt 2):**121-32.

16. Bousema JT, Drakeley CJ, Mens PF, et al. Increased *Plasmodium falciparum* gametocyte production in mixed infections with *P. malariae*. *Am J Trop Med Hyg* 2008;**78**(3)**:**442-8.

17. Alano P. *Plasmodium falciparum* gametocytes: still many secrets of a hidden life. *Mol Microbiol* 2007;**66**(2)**:**291-302.

18. Lobo CA, Kumar N. Sexual differentiation and development in the malaria parasite. *Parasitol Today* 1998;**14**(4)**:**146-50.

19. Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-van-Dillen PM, Noordaa Jvd. Rapid and simple method for purification of nucleic acids. *Journal of Clinical Microbiology* 1990;**28**(3)**:**495-503.

20. Schneider P, Bousema T, Omar S, et al. (Sub)microscopic *Plasmodium falciparum* gametocytaemia in Kenyan children after treatment with sulphadoxine-pyrimethamine monotherapy or in combination with artesunate. *Int J Parasitol* 2006;**36**(4)**:**403-8.

21. Ouedraogo AL, de Vlas SJ, Nebie I, et al. Seasonal patterns of *Plasmodium falciparum* gametocyte prevalence and density in a rural population of Burkina Faso. *Acta Trop* 2008;**105**(1)**:**28-34.

22. Boudin C, Lyannaz J, Bosseno MF, Carnevale P, Ambroise-Thomas P. Epidemiology of *Plasmodium falciparum* in a rice field and a savanna area in Burkina Faso: seasonal fluctuations of gametocytaemia and malarial infectivity. *Ann Trop Med Parasitol* 1991;**85**(4)**:**377-85.

23. Boudin C, Olivier M, Molez JF, Chiron JP, Ambroise-Thomas P. High human malarial infectivity to laboratory-bred Anopheles gambiae in a village in Burkina Faso. *Am J Trop Med Hyg* 1993;**48**(5)**:**700-6.

24. Cattani JA, Tulloch JL, Vrbova H, et al. The epidemiology of malaria in a population surrounding Madang, Papua New Guinea. *American Journal of Tropical Medicine and Hygiene* 1986;**35**(1)**:**3-15.

25. Drakeley CJ, Akim NI, Sauerwein RW, Greenwood BM, Targett GA. Estimates of the infectious reservoir of *Plasmodium falciparum* malaria in The Gambia and in Tanzania. *Trans R Soc Trop Med Hyg* 2000;**94**(5)**:**472-6.

26. Molineaux L, Storey J, Cohen JE, Thomas A. A longitudinal study of human malaria in the West African Savanna in the absence of control measures: relationships between different *Plasmodium* species, in particular *P. falciparum* and *P. malariae*. *Am J Trop Med Hyg* 1980;**29**(5)**:**725-37.

27. Ouedraogo AL, Schneider P, de Kruijf M, et al. Age-dependent distribution of *Plasmodium falciparum* gametocytes quantified by Pfs25 real-time QT-NASBA in a cross-sectional study in Burkina Faso. *Am J Trop Med Hyg* 2007;**76**(4)**:**626-30.

28. van der Kolk M, Tebo AE, Nimpaye H, Ndombol DN, Sauerwein RW, Eling WM. Transmission of *Plasmodium falciparum* in urban Yaounde, Cameroon, is seasonal and age-dependent. *Trans R Soc Trop Med Hyg* 2003;**97**(4)**:**375-9.

29. Mulder B, van der Ligt W, Sauerwein R, Verhave JP. Detection of *Plasmodium falciparum* gametocytes with the OBC test and Giemsa-stained thick blood films for malaria transmission studies in Cameroon. *Trans R Soc Trop Med Hyg* 1998;**92**(4)**:**395-6.

30. Butcher GA. Antimalarial drugs and the mosquito transmission of *Plasmodium*. *Int J Parasitol* 1997;**27**(9)**:**975-87.

31. von Seidlein L, Clarke S, Alexander N, et al. Treatment uptake by individuals infected with *Plasmodium falciparum* in rural Gambia, West Africa. *Bull World Health Organ* 2002;**80**(10)**:**790-6.

32. Babiker HA, Schneider P. Application of molecular methods for monitoring transmission stages of malaria parasites. *Biomed Mater* 2008;**3**(3)**:**034007.

33. Modiano D, Petrarca V, Sirima BS, et al. Baseline immunity of the population and impact of insecticide-treated curtains on malaria infection. *Am J Trop Med Hyg* 1998;**59**(2)**:**336-40.

34. Modiano D, Sirima BS, Sawadogo A, et al. Severe malaria in Burkina Faso: influence of age and transmission level on clinical presentation. *American Journal of Tropical Medicine and Hygiene* 1998;**59**(4)**:**539-542.

35. Talman AM, Domarle O, McKenzie FE, Ariey F, Robert V. Gametocytogenesis: the puberty of *Plasmodium* falciparum. *Malar J* 2004;**3:**24.

36. Bruce MC, Alano P, Duthie S, Carter R. Commitment of the malaria parasite *Plasmodium falciparum* to sexual and asexual development. *Parasitology* 1990;**100:**191-200.

37. Buckling A, Read AF. The effect of partial host immunity on the transmission of malaria parasites. *Proc R Soc Lond B Biol Sci* 2001;**268**(1483)**:**2325-30.

38. Smalley ME, Brown J. *Plasmodium falciparum* gametocytogenesis stimulated by lymphocytes and serum from infected Gambian children. *Trans R Soc Trop Med Hyg* 1981;**75**(2)**:**316-7.

39. Gosling RD, Drakeley CJ, Mwita A, Chandramohan D. Presumptive treatment of fever cases as malaria: help or hindrance for malaria control? *Malar J* 2008;**7:**132.

40. Eichner M, Diebner HH, Molineaux L, Collins WE, Jeffery GM, Dietz K. Genesis, sequestration and survival of *Plasmodium falciparum* gametocytes: parameter estimates from fitting a model to malariatherapy data. *Trans R Soc Trop Med Hyg* 2001;**95**(5)**:**497-501.

41. Sinden RE, Smalley ME. Gametocytogenesis of *Plasmodium falciparum* in vitro: the cell-cycle. *Parasitology* 1979;**79**(2)**:**277-96.

42. Karunaweera ND, Carter R, Grau GE, Kwiatkowski D, Del Giudice G, Mendis KN. Tumour necrosis factor-dependent parasite-killing effects during paroxysms in non-immune *Plasmodium vivax* malaria patients. *Clin Exp Immunol* 1992;**88**(3)**:**499-505.

43. Naotunne TS, Karunaweera ND, Del Giudice G, et al. Cytokines kill malaria parasites during infection crisis: extracellular complementary factors are essential. *J Exp Med* 1991;**173**(3)**:**523-9.

44. Saeed M, Roeffen W, Alexander N, Drakeley CJ, Targett GA, Sutherland CJ. *Plasmodium falciparum* antigens on the surface of the gametocyte-infected erythrocyte. *PLoS One* 2008;**3**(5)**:**e2280.

45. Schneider P, Schoone G, Schallig H, et al. Quantification of *Plasmodium falciparum* gametocytes in differential stages of development by quantitative nucleic acid sequence-based amplification. *Mol Biochem Parasitol* 2004;**137**(1)**:**35-41.

Chapter 5

Substantial Contribution of Submicroscopical *Plasmodium falciparum* Gametocyte Carriage to the Infectious Reservoir in an area of Seasonal Transmission

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Summary

Man to mosquito transmission of malaria depends on the presence of the sexual stage parasites, gametocytes, that often circulate at low densities. Gametocyte densities below the microscopical threshold of detection may be sufficient to infect mosquitoes but the importance of submicroscopical gametocyte carriage in different transmission settings is unknown.

Membrane feeding experiments were carried out on 80 children below 14 years of age at the end of the wet season in an area of seasonal malaria transmission in Burkina Faso. Gametocytes were quantified by microscopy and by *Pfs25*-based quantitative nucleic acid sequence-based amplification assay (QT-NASBA). The childrens' infectiousness was determined by membrane feeding experiments in which a venous blood sample was offered to locally reared *Anopheles* mosquitoes. Gametocytes were detected in 30.0% (24/80) of the children by microscopy compared to 91.6% (65/71) by QT-NASBA ($p<0.001$). We observed a strong association between QT-NASBA gametocyte density and infection rates ($p=0.007$). Children with microscopically detectable gametocytes were more likely to be infectious (68.2% compared to 31.7% of carriers of submicroscopical gametocytes, $p=0.001$), and on average infected more mosquitoes (13.2% compared to 2.3%, $p<0.001$). However, because of the high prevalence of submicroscopical gametocyte carriage in the study population, carriers of sub-microscopical gametocytes were responsible for 24.2% of the malaria transmission in this population. Submicroscopical gametocyte carriage is common in an area of seasonal transmission in Burkina Faso and contributes substantially to the human infectious reservoir. Submicroscopical gametocyte carriage should therefore be considered when implementing interventions that aim to reduce malaria transmission.

Introduction

The transmission of malaria depends on the presence of mature sexual stage parasites, gametocytes, in the human peripheral blood. Once ingested by a mosquito taking a blood meal, gametocytes develop through different mosquito-specific stages and ultimately result in infection of the mosquito salivary glands with sporozoites. This renders the mosquito infectious to humans. There is growing awareness that targeting gametocytes, either alone or as part of integrated control programmes, is essential for malaria control and elimination efforts [1-4]. The identification of the human infectious reservoir is therefore important for successful malaria control. Gametocytes frequently occur at low densities, making microscopical detection complicated[5]. In the last decade, molecular tools have become available to detect and quantify gametocytes at densities well below the microscopical threshold, in the order of 0.02-10 gametocytes/µL of blood[4]. Using these techniques, it has become evident that the proportion of gametocyte carriers in the population has been grossly underestimated and that the gametocyte reservoir may be 2-5 fold larger than assumed based on microscopy [6,7]. Carriers of gametocytes at submicroscopical levels are capable of infecting mosquitoes [7-19] although at a lower degree than those with gametocytes detectable microscopically in whom gametocytes are present at higher densities [12,18].

The importance of submicroscopical gametocyte carriage for malaria epidemiology and malaria control is the subject of some debate. While carriers of gametocytes at submicroscopical densities were concluded to be as important for the human infectious reservoir as carriers of microscopically-detectable gametocytes in areas of perennial transmission in Kenya [18] and Thailand[16], data from the Gambia suggest that submicroscopical gametocyte carriers only form a very small fraction of the infectious reservoir in this area of seasonal transmission [20]. This suggests that the relevance of submicroscopical gametocyte carriage may depend on transmission settings.

Here, we determine the contribution of submicroscopical densities of gametocytes to the human infectious reservoir in an area of seasonal malaria transmission in Burkina Faso.

Methods

This study was conducted in September-November 2005 in the village of Laye, 30km northwest of Ouagadougou, Burkina Faso. The area is characterised by Sudanese savannah with a marked wet season from June to October and an estimated entomological inoculation rate of 300-500 infective bites per person per year[21]. Asexual parasite carriage in the population shows seasonal fluctuations and was recently estimated at 60-90% in children below 15 years of age and 20-50% in adults[22]. Clearance was received by the Ministry of Health of Burkina Faso. Children below 14 years of age were randomly selected from village census lists and written informed consent was obtained from parents/guardians after the purpose of the study was explained. Children were accompanied to the Centre National de Recherche et de Formation sur le Paludisme (CNRFP) 1 to 2 days after the consenting procedure. At CNRFP, children underwent a clinical examination and their axillary temperature was measured. Children were enrolled in membrane feeding experiments regardless of symptoms or the presence of asexual malaria parasites or gametocytes. Venous blood samples (3mL) were drawn into heparin-containing tubes for membrane feeding and for gametocyte detection both by microscopy and by real-time *Pfs25* quantitative nucleic acid sequence based amplification (QT-NASBA). For all membrane-feeding assays, 3mL venous blood samples were obtained and fed to ~50 locally colony-reared 4-5-day-old female *A. gambiae* sensu stricto mosquitoes. The mosquito colony was established three years prior to the current experiments. Blood was offered via an artificial membrane attached to a water-jacketed glass feeder maintained at 37°C. After 10–15 min, fully fed mosquitoes were selected and kept on glucose at 29°C[7]. Unfed and partially fed mosquitoes were removed by aspiration and discarded. Mosquito midguts were examined after 7 days for the presence of oocysts following dissection in 2% mercurochrome. A second microscopist confirmed the presence of oocysts in midguts that were scored positive.

Only the total number of oocysts per batch of fed mosquitoes was recorded; not the number of oocysts per individual mosquito.Experiments in which a minimum of 10 mosquitoes were examined on day 7 after feeding were included in the analyses. After membrane feedings, children with fever (axillary temperature ≥ 37.5°C) and malaria parasites were treated with artemisinin-based combination therapy according to the national guidelines. Individuals for whom infections other than malaria were suspected

were accompanied to the nearest health facility for appropriate clinical care. The study protocol was viewed and approved by the Ministry of Health of Burkina Faso on August 8th 2000(Research's Authorization number 2000/3174/MS/SG/DEP).

Microscopical detection of *P. falciparum* parasites.

Samples were considered negative if no parasites were detected in 100 fields. Both asexual stage and gametocyte densities were simultaneously assessed by counting against 1000 leucocytes in the thick smear. The lower limit of microscopy for gametocyte quantification was therefore estimated at 8 gametocytes/ µl of blood. Parasite counts were converted to numbers of parasites per µl by assuming a standard count of 8000 leucocytes/µl of blood. Each sample was read independently by two microscopists and the mean density was used. A third reader was involved when the first two readers disagreed about the prevalence of gametocytes or their estimated densities differed ≥30%. In these cases the mean density of the two closest readings was used.

Gametocyte detection by real time Pfs25 QT-NASBA

Gametocyte detection by *Pfs25* QT-NASBA was performed as described elsewhere using a NucliSens EasyQ analyser (Bio-Mérieux) [23,24]. Nucleic acid was extracted from 50-µL blood samples as described by Boom *et al.*[25]. The first part of the RNA extraction was done in the field following the original guanidinium isothiocyanate (GuSCN) RNA extraction method[25] until the nucleic acids were bound to silica dioxide particles. At this point, samples were stored at -20°C and transferred to the laboratory for completion of the extraction and QT-NASBA analysis. The number of gametocytes was calculated in relation to a standard gametocyte stage V dilution series [26], using the time point of amplification at which the fluorescence detecting target amplicons exceeded the mean fluorescence of three negative controls + 20 standard deviations. The *Pfs*25 QT-NASBA technique is gametocyte specific and has a detection limit of 20–100 gametocytes/mL[24]. Samples with *Pfs*25 QT-NASBA gametocyte concentrations <20 gametocytes/mL were considered gametocyte negative.

Sample size considerations

Based on a previous study in the area, we expected a gametocyte prevalence of 10-20% by microscopy and 70-80% by *Pfs*25 QT-NASBA [23]. Including 80 individuals in the membrane

feed experiments would allow us to detect a threefold lower infectiousness of submicroscopical carriers compared to microscopical gametocyte carriers[12,20] when we assumed that 60% of the microscopical gametocyte carriers infected at least one mosquito[18] (Z_α=1.645; Z_β=0.84).

Data analysis

Data analyses were performed using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) and Stata 10 (Statacorp, Texas US). Densities of gametocytes were analysed on a log10-scale. The prevalence of mosquito infection (i.e. whether an individual infected at least one mosquito) and the proportion of infected mosquitoes were used as outcomes of the membrane feeding experiments and were related to *Pfs*25 QT-NASBA gametocyte density, age and fever in logistic and linear regression models. Individual oocyst densities in mosquitoes were not recorded.

Results

We enrolled 80 children in our study who were aged 2.9 – 13.6 years. In line with previous studies from the study area, microscopy indicated an asexual parasite prevalence of 82.5% (66/80) and a gametocyte prevalence of 30.0% (24/80; Table 1)[22,23].

Table 1. Baseline characteristics

Age, median (IQR)	6.2 (4.8 – 9.5)
Sex, % male (n/N)	55.0 (44/80)
Fever, % (n/N)*	22.5 (18/80)
Asexual parasite prevalence, % (n/N)	82.5 (66/80)
Asexual parasite density/µL, median (IQR)¥	1028.5 (585 – 3662)
Symptomatic malaria¶	15.0 (12/80)
Microscopic gametocyte prevalence, % (n/N)	30.0 (24/80)
Microscopic gametocyte density/µL, median (IQR)¥	40.0 (16 – 45)
QT-NASBA gametocyte prevalence, % (n/N)†	91.6 (65/71)
QT-NASBA gametocyte density/µL, median (IQR)	7.9 (1.4 – 48.9)

**Fever=temperature ≥37.5°C; ¥for carriers only; ¶defined as fever with a parasite density ≥500 parasites/µL; †only gametocyte densities ≥20 gametocytes/mL were considered Pfs25 QT-NASBA positive*

When the *Pfs25* QT-NASBA was used for gametocyte detection, 91.6% (65/71) individuals were shown to be carrying gametocytes. There was a strong correlation between gametocyte densities detected by QT-NASBA and microscopy for microscopically gametocyte positive samples (Spearman correlation coefficient = 0.60; p=0.004). For nine individuals RNA collection failed, i.e. no sample was collected, and therefore no QT-NASBA data were available. Membrane feeds were successful for all 80 individuals but, due to mosquito mortality between the day of feeding and the day of dissection, only a total of 74 membrane feeds had at least 10 mosquitoes dissected and were therefore included in the analyses. The proportion of infected mosquitoes was positively associated with *Pfs*25 QT-NASBA gametocyte density (Spearman correlation coefficient=0.34, p=0.007; Figure 1) and was not influenced by a clinical malaria episode (i.e. fever with a parasite density ≥500 parasites/µL) (p=0.18) or the presence of fever (p=0.63).The relation between the proportion of infected mosquitoes and *Pfs*25 QT-NASBA gametocyte density was best described by the equation $Y = 0.0176\text{Ln}(X) + 0.0187$ ($R^2 = 0.153$).

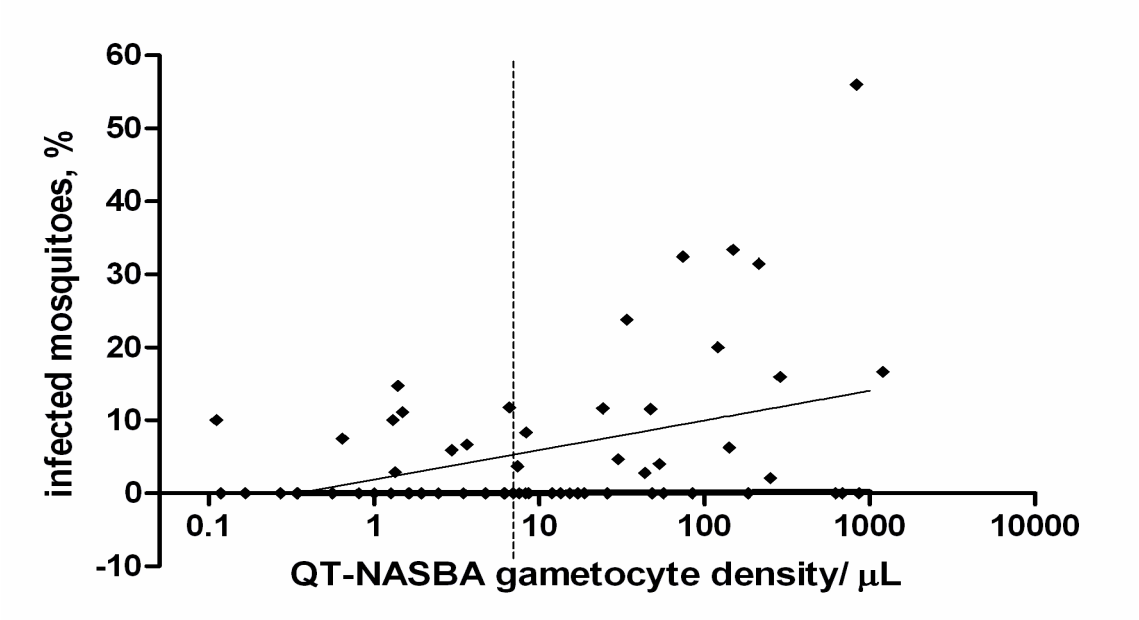

Figure 1. The relationship between *Pfs*25 QT-NASBA gametocyte density and the proportion of infected mosquitoes in membrane feeding experiments.

The solid line indicates the best fitted line (Y = 0.0176Ln(X) + 0.0187; R^2 = 0.153). The dashed line indicates the estimated microscopic threshold for gametocyte detection, 8 gametocytes/μL, when screening 100 high power fields (i.e. ~1000 white blood cells).

Of those children with microscopically detectable gametocytes, 68.2% (15/22) infected at least one mosquito compared to 31.7% (13/41) of children with submicroscopical gametocyte densities (p=0.001; Table 2).

Table 2. Membrane feeding results

	Gametocyte carriage				
	Microscopy –	**Microscopy –**	**Microscopy +**	**Total**	**p-value**
	QT-NASBA –	**QT-NASBA +**	**QT-NASBA +**		
Prevalence in the population, % (n)	6.9 (5)	60.3 (44)	32.9 (24)	100 (73)	
Density at feeding, median (IQR)*	N.D.	7.5 (1.5 – 27.9)	33.8 (2.9 – 120.2)	8.2 (1.6 – 49.7)	0.13
Proportion of infectious individuals, % (n/N)	0.0 (0/5)	31.7 (13/41)	68.2 (15/22)	41.1 (28/68)	0.001¶
Proportion of infected mosquitoes, % (n/N)	0.0 (0/151)	2.3 (28/1202)	13.2 (90/683)	5.8 (118/2036)	<0.001¶
Total number of oocysts/infected mosquitoes¥	0/0	36/28	250/90	286/118	
Relative contribution to transmission	0%	24.2%	75.8%	100.0%	

** by Pfs25 QT-NASBA; ¶p-value for a test for trend; ¥only the total number of oocysts per batch of fed mosquitoes was recorded, not the number of oocysts of individual mosquitoes. Therefore only a summary measure can be presented and no analyses could be done on individual oocyst densities.*
The total number of samples is lower than 80 because QT-NASBA results were not available for 9 individuals. Two individuals without QT-NASBA results that were gametocyte positive by microscopy were included. The relative contribution to transmission was based on the product of the proportion of infected mosquitoes (4th row) and the prevalence of this subgroup in the population (1st row).

The proportion of infected mosquitoes was also higher in carriers of microscopically- compared to submicroscopically-detectable gametocytes (p<0.001). Oocyst densities of individual mosquitoes were not recorded, only the total number of oocysts observed in each experiment. Formal statistical testing on oocyst burden could therefore not be done although the total number of oocysts relative to the number of infected mosquitoes was higher for individuals with microscopically-detectable densities of gametocytes. The relative contribution to transmission of microscopical and submicroscopical gametocyte carriage was determined based on their prevalence in the studied population and the proportion of mosquitoes infected by each group. This resulted in a relative contribution to malaria

transmission of 24.2% for submicroscopical gametocyte carriage, compared to 75.8% for microscopical gametocyte carriage.

Discussion

In this study, we observed that submicroscopical gametocyte carriage is common in children in an area of seasonal malaria in Burkina Faso. Although, on average, carriers of gametocytes at submicroscopical densities infected significantly fewer mosquitoes that themselves developed lower oocyst burdens, the contribution to the infectious reservoir of this age group was considerable and estimated at 24%.

There are numerous reports that suggest that some individuals can infect mosquitoes despite the absence of microscopically detectable gametocytes[7,8,10,12,16,18,19]. However, it is unclear (i) how important this phenomenon is in areas of seasonal malaria[20] and (ii) how important submicroscopical gametocyte densities are for malaria transmission in the general, typically asymptomatic, population. Several detailed studies on the infectiousness of carriers of submicroscopical gametocytes have been carried out, but only after chemotherapeutic treatment of symptomatic malaria cases[7,18,20]. Because children in those studies all had high densities of asexual parasites in the weeks prior to the mosquito feeding experiments, they were more likely to have gametocytes at the time of the membrane feedings[5]. Although it was previously shown that gametocyte carriage may be common in asymptomatic individuals as well[27], findings from clinical trials cannot be extrapolated to the general population. To the best of our knowledge, the infectious reservoir has never been determined in the general population by means of the combination of molecular gametocyte detection tools with membrane feeding experiments used in the study described here. Although our experiments were restricted to children, our findings provide valuable information about the infectiousness of a cross-section of the population living in an area of seasonal malaria transmission. At the end of the wet season, 90% of the children in our study area carried gametocytes[23], two-thirds of them at densities below the microscopical threshold of detection. The infectiousness of individuals with submicroscopical gametocyte densities was lower than that of children with microscopical gametocyte densities in terms of (i) prevalence of infection, (ii) proportion of infected mosquitoes and (iii) oocyst burden in mosquitoes. These observations are largely in

agreement with previous studies[12,18] although a study in symptomatic children in Kenya reported that submicroscopical gametocyte carriers were as likely as microscopical gametocyte carriers to be infectious and only the average number of infected mosquitoes and the oocyst burden were lower for submicroscopical gametocyte carriers[18]. This difference may be due to the different populations: we enrolled asymptomatic children compared to the Kenyan study where children were sampled 14 days after a clinical malaria episode[18]. We nevertheless consider the current findings biologically more plausible since the chance of a submicroscopical gametocyte carrier being classified as 'infectious' (i.e. infecting at least one mosquito) is likely to be lower if submicroscopical gametocyte carriers on average infect a lower proportion of mosquitoes[12,18]. Contrary to a recently published hypothesis that infection of mosquitoes by submicroscopical gametocytaemia may be rare in areas of seasonal malaria transmission[20], our findings suggest that carriers of submicroscopical gametocyte densities may be common in these circumstances. The relative contribution to transmission per gametocyte carrier may be lower for submicroscopical gametocyte carriers but their relative abundance in a population appears to counterbalance this and makes them important contributors to malaria transmission. To reliably determine the influence of transmission intensity and seasonality on the occurrence and infectiousness of submicroscopical gametocyte densities, a direct comparison is needed where the infectiousness of different populations is assessed at several time-points during the season.

Our study has two limitations: we determined the infectious reservoir at the end of the wet season only and restricted our experiments to children. Seasonal patterns in gametocyte carriage[22] make it impossible to draw conclusions about the importance of submicroscopical gametocyte carriage for malaria transmission at other time-points in the season. For this, a series of membrane feeding experiments are needed throughout the year. We have previously reported that submicroscopical gametocyte carriage is less prevalent in adults in our study area[23]. Our data can therefore not be extrapolated to the whole population. Although we observed a significant correlation between *Pfs*25 QT-NASBA gametocyte density and mosquito infection rates, it is not possible to reliably estimate the infectiousness of individuals based on gametocyte density data only. Some children with a gametocyte density below 1 gametocyte/µL were able to infect mosquitoes in our study.

This is surprising since a blood meal, that is on average 2-3µL, should contain at least one male and one female gametocyte to result in infection. However, the phenomenon has been observed before [18] and may be influenced by the aggregation of gametocytes that favours the encounter of males and females [28]. Alternatively, we cannot rule out that artefacts resulting from RNA degradation have resulted in unrealistically low estimates of gametocyte densities in occasional samples. We also observed that some carriers of gametocytes at high density were unable to infect mosquitoes[8,12,18]. This could be partly due to transmission reducing immune responses[8,29]. These immune responses may be inversely related to age[18,29]. Further studies should therefore be conducted at different time-points, include all age groups and preferably incorporate transmission reducing immune responses and sexing of gametocytes to further elucidate the detailed processes that determine the composition of the human infectious reservoir of malaria in a given transmission setting.

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References

1. Carter R, Mendis KN, Miller LH, Molineaux L, Saul A (2000) Malaria transmission-blocking vaccines--how can their development be supported? NatMed 6: 241-244.
2. Sauerwein RW (2007) Malaria transmission-blocking vaccines: the bonus of effective malaria control. Microbes Infect 9: 792-795.
3. White NJ (2008) The role of anti-malarial drugs in eliminating malaria. Malar J 7 Suppl 1: S8.
4. Babiker HA, Schneider P, Reece SE (2008) Gametocytes: insights gained during a decade of molecular monitoring. Trends Parasitol 24: 525-530.
5. Drakeley C, Sutherland C, Bousema JT, Sauerwein RW, Targett GA (2006) The epidemiology of *Plasmodium falciparum* gametocytes: weapons of mass dispersion. Trends Parasitol 22: 424-430.
6. Shekalaghe SA, Bousema JT, Kunei KK, Lushino P, Masokoto A, et al. (2007) Submicroscopic *Plasmodium falciparum* gametocyte carriage is common in an area of low and seasonal transmission in Tanzania. TropMedIntHealth 12: 547-553.

7. Bousema JT, Schneider P, Gouagna LC, Drakeley CJ, Tostmann A, et al. (2006) Moderate Effect of Artemisinin-Based Combination Therapy on Transmission of *Plasmodium* falciparum. JInfectDis 193: 1151-1159.
8. Bonnet S, Gouagna LC, Paul RE, Safeukui I, Meunier JY, et al. (2003) Estimation of malaria transmission from humans to mosquitoes in two neighbouring villages in south Cameroon: evaluation and comparison of several indices. TransRSocTropMedHyg 97: 53-59.
9. Gamage-Mendis AC, Rajakaruna J, Carter R, Mendis KN (1991) Infectious reservoir of *Plasmodium vivax* and *Plasmodium falciparum* malaria in an endemic region of Sri Lanka. American Journal of Tropical Medicine and Hygiene 45: 479-487.
10. Boudin C, Olivier M, Molez JF, Chiron JP, Ambroise-Thomas P (1993) High human malarial infectivity to laboratory-bred Anopheles gambiae in a village in Burkina Faso. AmJTropMedHyg 48: 700-706.
11. Young MD, Hardman NF, et al. (1948) The infectivity of native malarias in South Carolina to Anopheles quadrimaculatus. Am J Trop Med Hyg 28: 303-311.
12. Pethleart A, Prajakwong S, Suwonkerd W, Corthong B, Webber R, et al. (2004) Infectious reservoir of *Plasmodium* infection in Mae Hong Son Province, north-west Thailand. Malar J 3: 34.
13. Jeffery GM, Eyles DE (1955) Infectivity to mosquitoes of *Plasmodium falciparum* as related to gametocyte density and duration of infection. AmJTropMedHyg 4: 781-789.
14. Bonnet S, Gouagna C, Safeukui I, Meunier JY, Boudin C (2000) Comparison of artificial membrane feeding with direct skin feeding to estimate infectiousness of *Plasmodium falciparum* gametocyte carriers to mosquitoes. TransRSocTropMedHyg 94: 103-106.
15. Graves PM (1980) Studies on the use of a membrane feeding technique for infecting Anopheles gambiae with *Plasmodium* falciparum. TransRSocTropMedHyg 74: 738-742.
16. Coleman RE, Kumpitak C, Ponlawat A, Maneechai N, Phunkitchar V, et al. (2004) Infectivity of asymptomatic *Plasmodium*-infected human populations to Anopheles dirus mosquitoes in western Thailand. JMedEntomol 41: 201-208.
17. Githeko AK, Brandling-Bennett AD, Beier M, Atieli F, Owaga M, et al. (1992) The reservoir of *Plasmodium falciparum* malaria in a holoendemic area of western Kenya. TransRSocTropMedHyg 86: 355-358.
18. Schneider P, Bousema JT, Gouagna LC, Otieno S, van dV, et al. (2007) Submicroscopic *Plasmodium falciparum* gametocyte densities frequently result in mosquito infection. AmJTropMedHyg 76: 470-474.
19. Muirhead-Thomson RC (1954) Factors determining the true reservoir of infection of *Plasmodium falciparum* and Wuchereria bacnrofti in a West African village. Transactions of the Royal Society of Tropical Medicine and Hygiene 48: 208-225.
20. Okell LC, Drakeley CJ, Ghani AC, Bousema T, Sutherland CJ (2008) Reduction of transmission from malaria patients by artemisinin combination therapies: a pooled analysis of six randomized trials. MalarJ 7: 125.
21. Cuzin-Ouattara N, Van den Broek AH, Habluetzel A, Diabate A, Sanogo-Ilboudo E, et al. (1999) Wide-scale installation of insecticide-treated curtains confers high levels of protection against malaria transmission in a hyperendemic area of Burkina Faso. Trans R Soc Trop Med Hyg 93: 473-479.
22. Ouedraogo AL, De Vlas SJ, Nebie I, Ilboudo-Sanogo E, Bousema JT, et al. (2008) Seasonal patterns of *Plasmodium falciparum* gametocyte prevalence and density in a rural population of Burkina Faso. Acta Trop 105: 28-34.
23. Ouedraogo AL, Schneider P, de Kruijf M, Nebie I, Verhave JP, et al. (2007) Age-dependent distribution of *Plasmodium falciparum* gametocytes quantified by Pfs25 real-time QT-NASBA in a cross-sectional study in Burkina Faso. AmJTropMedHyg 76: 626-630.
24. Schneider P, Bousema T, Omar S, Gouagna L, Sawa P, et al. (2006) (Sub)microscopic *Plasmodium falciparum* gametocytaemia in Kenyan children after treatment with

sulphadoxine-pyrimethamine monotherapy or in combination with artesunate. IntJParasitol 36: 403-408.

25. Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-van Dillen PM, et al. (1990) Rapid and simple method for purification of nucleic acids. JClinMicrobiol 28: 495-503.
26. Ponnudurai T, Lensen AH, Meis JF, Meuwissen JH (1986) Synchronization of *Plasmodium falciparum* gametocytes using an automated suspension culture system. Parasitology 93 (Pt 2): 263-274.
27. Bousema JT, Gouagna LC, Drakeley CJ, Meutstege AM, Okech BA, et al. (2004) *Plasmodium falciparum* gametocyte carriage in asymptomatic children in western Kenya. Malaria Journal 3: 18.
28. Pichon G, Awono-Ambene HP, Robert V (2000) High heterogeneity in the number of *Plasmodium falciparum* gametocytes in the bloodmeal of mosquitoes fed on the same host. Parasitology 121 (Pt 2): 115-120.
29. Bousema JT, Drakeley CJ, Sauerwein RW (2006) Sexual-Stage Antibody Responses to *P. falciparum* in Endemic Populations. CurrMolMed 6: 223-229.

Chapter 6

Naturally acquired immune responses to *Plasmodium falciparum* sexual stage antigens Pfs48/45 and Pfs230 in an area of seasonal transmission

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Summary

Acquisition of immunity to *P. falciparum* sexual stages is a key determinant for reducing man-mosquito transmission by preventing the fertilisation and the development of the parasite in the mosquito midgut. Naturally acquired immunity against sexual stages may therefore form the basis for the development of transmission blocking vaccines, but studies conducted to date offer little in the way of consistent findings. Here, we describe the acquisition of anti-gametocyte immune responses in malaria exposed individuals in Burkina Faso. A total of 719 blood samples were collected in a series of three cross-sectional surveys at the start, peak and the end of the wet season. The seroprevalence of antibodies with specificity for the sexual stage antigens Pfs48/45 and Pfs230 was twofold lower (22-28%) than that for an asexual blood stage antigen GLURP (65%) or for the pre-erythrocytic stage antigen CSP (54%). The youngest children responded at similar frequencies to all four antigens but, in contrast with the immune responses to GLURP and CSP that increased with age independently of season and area of residence, there was no evidence for a clear age-dependence of responses to Pfs48/45 and Pfs230. Anti-Pfs230 antibodies were most prevalent at the peak of the wet season ($p<0.001$). Our findings suggest that naturally acquired immunity against Pfs48/45 and Pfs230 is a function of recent rather than cumulative exposure to gametocytes.

Introduction

Malaria transmission depends on the presence of infectious sexual stage parasites in human peripheral blood, and naturally acquired immune responses to these or other stages can affect malaria transmission in several ways. An important indirect manner in which they influence malaria transmission is by reducing the number of asexual parasites that are the source of gametocytes. Immune responses can also influence malaria transmission more directly. Antibodies with specificity for sexual stages have been associated with a reduction of *P. falciparum* gametocyte prevalence in semi-immune individuals living in a hyper-endemic area of Irian Jaya, Indonesia[1]. Early stage (stage I and IIa) gametocytes express the parasite protein PfEMP1 on the erythrocyte surface[38] while recently identified proteins may be expressed in later developmental stages[38,46]. Immune responses against these gametocyte-derived surface antigens may be related to direct clearance of gametocytes[47], and may explain why the duration of gametocyte carriage appears to decrease with age[8]. A third way in which antibody responses can affect transmission is by reducing the infectiousness of gametocytes once ingested by mosquitoes.

Gametocytes in infected erythrocytes and gametes that emerge from erythrocytes inside the mosquito midgut express stage-specific antigens on their surfaces[27,50]. These antigens have a role in the fertilization or sporogonic development of malaria parasites in mosquitoes[43,44]. A proportion of gametocytes die in the human host without being passed on to a mosquito, thereby exposing sexual stage antigens to the human immune system. Sexual stage-specific antibodies may be elicited against these antigens[31] and may play a role in transmission-blocking immunity by preventing fertilization or the development of sporogonic stage parasites in the mosquito[4,7,18,20,21,42,44,49]. These antibody responses may reduce the spread of malaria in human populations. A better understanding of naturally acquired sexual stage immunity is thus relevant to malaria control as it may form the basis for the development of malaria transmission blocking vaccines.

Pfs230 and Pfs48/45 are major gametocyte and gamete surface antigens that induce antibody responses in naturally exposed individuals[7,18,21,42,44] that are associated with functional transmission reducing immunity[21,25,42,44].

Here, we describe the profiles of naturally acquired sexual stage immune responses to Pfs230 and Pfs48/45 in individuals from an area of intense seasonal malaria transmission in Burkina Faso.

Methods

Study site and population

The study was conducted in a region close to Ouagadougou, the capital of Burkina Faso (West Africa), where malaria is endemic. Two areas of different endemicity[16] were included in the study to test the effect of transmission intensity on sexual stage immunity. *Plasmodium falciparum* is the predominant malaria species in the region, accounting for 90% of the infections; the remaining 10% being attributed to *P. malariae* and *P. ovale*[24].

The study's participants are members of subsistence farming communities and all are permanent residents in the area. Participants were explained the procedures, risks and benefits involved in the study and their consent was obtained. The study protocol was viewed and received a written approval of the ministry of health of Burkina Faso (Research authorization number 2000/3174/MS/SG/DEP).

Measurement of transmission intensity

Repeated CDC light trap captures were carried out in both areas to estimate transmission intensity according to both area and season. Each area consisted of 3 grouped villages. Indoor mosquito captures extended from June to November 2002. The CDC light trap was placed close to the bed of the sleeper in randomly selected houses and mosquito capture was done from 7:00 pm to 6:00 am. Mosquito species were identified morphologically, counted and stored in tubes with silica gel. A representative sample of Anopheles gambiae mosquito thoraces and heads were examined for *P. falciparum* circumsporozoite protein (CSP) positivity index using routine CSP ELISA[2]. The monthly sporozoite rate was estimated, testing representative samples (approximately 50% of all caught mosquitoes from each trap) of randomly selected specimens of mosquitoes from each village. The entomological inoculation rate (EIR) was calculated as the product of the sporozoite rate by the biting rate.

Blood sample collection

The seasonally-spaced cross-sectional surveys for parasitological and serological data collection coincided with entomological data collection in 2002. At each survey, approximately 300 individuals (~150 from each area of residence) were randomly selected from village census lists aiming to include 60 individuals (10 per village) from each of five pre-defined age-groups: 1-4, 5-9, 10-14, 15-29 and ≥30 years of age. Individually selected individuals were invited to a sampling point and were systematically included until the required sample sizes were reached.

For parasites counts, a blood slide film was made from finger prick blood of each individual. For specific anti-plasmodial antibody measurements, 500 µL finger prick blood samples were drawn. Plasma was separated by centrifugation and stored at –20°C before use. Subjects with fever (body temperature ≥37.5°C) were treated with an anti-malarial drug (CQ) following the national policy for malaria treatment in the year of 2002.

Microscopical detection of *P. falciparum* parasites.

Slides were read independently by two microscopists, each examining 100 microscopic fields, and the mean density was used. A third reader was involved when the first two readers disagreed about the prevalence or estimated densities differed ≥30%. In these cases the mean density of the two closest readings was used. Asexual stage and gametocyte densities were simultaneously assessed by counting against 1000 leucocytes in the thick smear. The lower limit of microscopy for gametocyte quantification was estimated at 5 parasites/µL of blood. Parasite counts were converted to numbers of parasites per µL by assuming a standard count of 8000 leucocytes/µl of blood.

Antigens

Plasma IgG with specificity for Pfs48/45 and Pfs230 antigens derived from an extract of mature *P. falciparum* NF54 strain gametocytes were measured by ELISA (see below). For this purpose, mature gametocytes were produced in an automated static culture system in red blood cells of blood group O+ and non immune AB serum[39] and harvested after 13-14 days. Gametocyte purification was previously described[50]. Briefly, mature gametocytes were isolated at 37°C to prevent their activation. Parasite culture was loaded on a cushion of 63% Percoll (GE-Healthcare:17-0891-01) and centrifuged for 30 min at 1,500g. The purified

gametocytes were then aliquoted and stored at –70°C before use. For extraction of Pfs48/45 and Pfs230 enriched proteins, gametocytes were re-suspended in 1% sodium desoxycholate / TrisNaCl and 1mM phenylmethylsulfonyl fluoride (PMSF), incubated for 10 min at room temperature and spin 13,000 rpm for 10 min. The supernatant was collected as Pfs48/45 and Pfs230-enriched antigens extract and diluted in 0.25% PBSTM (2.5% milk and 0.05% tween20 in PBS) for use. The synthetic peptide $NANP_6$ corresponding to the repeat region of the circumsporozoïte protein and the synthetic peptide GLURP GMP85-213 LR67[48] were used in standardized ELISA for sporozoite- and asexual blood stage-specific antibody detection respectively (see below).

Pfs48/45 and Pfs230 IgG Enzyme Linked Immunosorbent Assays (ELISA)

Samples selection

Although samples for serology were available from almost all participants, it was not possible to screen all samples for the presence of sexual stage antibodies due to the labour intensiveness and costs involved in the necessary antigen preparation. ELISA was therefore performed on a representative sub-sample of randomly selected samples. For this purpose, samples were randomly selected from the list of available samples for each age-group and season separately. Initially, a number of 150 samples per season i.e. 25 in age groups 1-4 and 5-9; 50 samples in age groups 10-19 and ≥20 years of age were randomly selected for testing both Pfs48/45 and Pfs230 antigens. Samples sizes were approximately similar for both antigens tests at the start of the wet season (137 for Pfs48/45 and 136 for Pfs230), peak of the wet season (149 and 148 respectively) and end of the wet season (125 and 129 respectively). To exclude possible variation in gametocyte antigen preparations, only samples that were concurrently tested for both Pfs48/45 and Pfs230 antigens extracted from the same batch of gametocytes were used for data analysis. This resulted in an overall 130 samples at the start and end of the wet season and 150 at the peak of the wet season explaining the slight inconsistencies in the sample sizes between groups of seasons, age groups and areas of different endemicity.

ELISA experiment

The presence of anti-Pfs48/45 and anti-Pfs230 IgG antibodies in plasma samples was determined by coating 10µg/mL of anti-Pfs48/45 rat monoclonal antibody (mAb) 85RF45.3[45]

or anti-Pfs230 rat mAb 63F6D7-F(ab)2[41], diluted in PBS, in the wells of 96-well polystyrene U-bottom ELISA-hard plates (Sterilin®, International Medical Products B.V., Zutphen, the Netherlands). Free sites were blocked with 5% milk (Marvel, Premier International Foods Ltd, Spalding, Lincs, United Kingdom) in PBS and Pfs48/45 and Pfs230 antigens contained in 50 µL gametocyte extract (250,000 gametocytes equivalents/well) were captured by overnight incubation at 4°C. The buffer (0.25% PBSTM) used for dilution of gametocyte extract was added to control wells (i.e. no extract added) for background measurement. A dilution (1:100) in 0.25% PBS/TweenMilk of the test plasma was added to the wells prepared with and without antigen (control wells); and incubated for 2 hours at room temperature. The plate was washed and bound IgG antibodies were detected by addition of 100µL of 1:30,000 diluted Goat-anti Human IgG-PO (H+L; Pierce) for 1h 30 min at room temperature. Wells were washed with PBS and subsequently incubated with tetramethyl benzidine (TMB) substrate solution for 20 min. The colour reaction was stopped with 4N H2SO4, and the optical density (OD) was read at 450 nm in an Anthos 2001 Microplate Reader (Labtec BV). All plasma samples were tested in duplicate. Three non-immune plasmas from Dutch blood bank donors as negative controls and one positive control plasma of a Dutch man that have been exposed to malaria for almost 30 years in sub-Saharan Africa were included per plate. The value for IgG titer (OD) of a sample was expressed as the difference in OD between the antigen and control wells. The cutoff was calculated as the mean OD of negative controls plus two standard deviations. A sample was considered positive if its background-adjusted OD was above the cutoff.

GLURP and $NANP_6$ IgG ELISA

To evaluate anti-asexual blood stage or anti-sporozoite antibody responses, 0.2µg/mL of GLURP85-213 in 0.05M carbonate buffer (50µL/well) or 1µg/mL of $NANP_6$ in PBS (50 µL/well) were coated in flat bottom high binding microtiter 96 well plates (NUNCTM Maxisorp, Nalge Nunc International Corp, Life Techn, The Netherlands) at 4°C. Coated plates were incubated overnight at 4°C and washed with PBST. Free sites were blocked with 150 µl/well of 2.5% milk/PBS (Marvel). Subsequently, the blocker was washed off and plates incubated 1h at room temperature with 50 µl of 1:200 plasma diluted in PBSTM for GLURP ELISA or with 50 µl of 1:100 diluted plasma for anti-sprorozoitic ELISA. Plates were washed and incubated for 1 hour with rabbit anti-human IgG-Peroxidase (Dako, P-214) diluted

1:10,000 in PMSTM before reaction with the substrate (TMB). The staining was stopped after 15 min of reaction by 4N H2SO4 and the plate was read as described in Pfs48/45-Pfs230 ELISA. Plasma was considered positive if the OD value was greater than two standard deviations above the mean of the negative control plasmas from Dutch blood bank donors.

Statistical analysis

Study participants were categorized into groups by age (1-4 years, 5-9 years, 10-19 years and ≥20 years). The statistical analysis was performed using SPSS version 14.0 (SPSS Inc., Chicago, IL, USA). The influence of age on antibody prevalence was tested using logistic regression analyses using age in categories. Multivariate regression models allowed for confounding effects of age, season and area of residence. The Pearson χ2 test was used for comparing proportions and trends in dichotomous variables. Spearman's rank correlation test was used to assess association between antibody levels of tested antigens. The level of significance was set at a two-tailed $P < 0.05$.

The association between Pfs230 and Pfs48/45 antibody responses and functional transmission reducing activity (TRA) in the Standard Membrane Feeding Assay in previous studies (9, 11, 18) was presented after categorization of TRA as >50% and >90% reduction (9, 11, 18) and calculating odds ratios (OR) with 95% confidence intervals (95% CI).

Results

Entomology

A representative sample of 4525 mosquitoes was tested for transmission intensity estimates. EIR details per season and area of residence are summarized in Table 1. Overall, the mean EIR was estimated to be 28.5 infective bites/person/month (ib/p/m) in the whole study area and this varied by season (1.47; 69.57; and 14.67 ib/p/m at start, peak and end-wet season, respectively) and area of residence (52.4 ib/p/m versus 3.05 ib/p/m).

Parasitology

A total of 719 blood slide samples were collected over the three cross-sectional surveys. At the site with the highest endemicity, 147, 101 and 130 samples were collected at the start, peak and end of the wet season, respectively. At the site of lower endemicity, these figures were 149, 50 and 142, respectively. The overall proportion of individuals

harbouring asexual parasites was 63.4% (456/709); while 18.9% (136/709) carried gametocytes. There was a significant age-dependent decrease in both prevalence of asexual parasites (OR = 0.54, p < 0.001, 95% CI 0.46-0.63) and gametocytes (OR = 0.61, p < 0.001, 95% CI 0.51-0.74) and in the density of asexual parasites (β = -0.027, se(β) = 0.003, p < 0.001) and gametocytes (β = -0.009, se(β) = 0.022, p = 0.004) after adjustment for season and area of residence.

Parasite prevalence and density showed seasonal fluctuations. The prevalence of asexual parasites was significantly higher at the peak compared to the start (p < 0.001) and the end of the wet season (p = 0.004). Similarly we observed a higher asexual parasite density at the peak compared to the start (p < 0.001) and the end of the wet season (p = 0.01). Details on gametocyte prevalence are presented in Table 1. Similar to asexual parasites, gametocytes were more prevalent at the peak (28.5%, 43/151) compared to the start of the wet season (22%, 65/296) (p = 0.1) and were least prevalent at the end of the wet season (10.3%, 28/272) (p < 0.001). The median density of gametocytes/□L blood was higher at the peak (24, IQR 24-72) compared to the start (24, IQR 16-40) (p=0.03) and, at a borderline level of significance, lower compared to the end of the wet season (40, IQR 40-57) (p = 0.09). Despite a substantial difference in EIR between them, there was no significant variation in asexual parasite and gametocyte prevalence between the study areas. The prevalence of asexual parasites was 61% (208/341) in the low transmission area and 65.6% (248/378) in the high transmission area (p = 0.2). The distribution of gametocytes in both areas is shown in Table 1. The proportion of gametocytes carriers was 17% (60/341) in the low transmission area and 20.1% (76/378) in the high transmission area (p = 0.4). This equality in parasite prevalence could reflect the high endemicity of malaria across the entire area (16).

Table 1. Entomological inoculation rates and gametocyte indices in the study population.

Season			Start wet	Peak wet	End wet
Number of traps		Low endemic	36	51	54
		High endemic	36	50	53
EIR, (95% CI)		Low endemic	0.0	9.16	0.0
		High endemic	2.9	129.9	29.3
Gametocyte prevalence	1-4 years, % (n/N)	Low endemic	20.0 (6/30)	40.0 (4/10)	24.0 (6/25)
		High endemic	43.3 (13/30)	42.9 (9/21)	14.8 (4/27)
	5-9 years, % (n/N)	Low endemic	30.0 (9/30)	40.0 (4/10)	9.7 (3/31)
		High endemic	34.5 (10/29)	36.8 (7/19)	6.9 (2/29)
	10-19 years, % (n/N)	Low endemic	17.3 (9/52)	42.1 (8/19)	13.7 (7/51)
		High endemic	26.5 (13/49)	22.9 (8/35)	5.9 (2/34)
	20+ years, % (n/N)	Low endemic	2.7 (1/37)	9.1 (1/11)	5.7 (2/35)
		High endemic	10.3 (4/39)	7.7 (2/26)	5.0 (2/40)
Total			22.0 (65/296)	28.5 (43/151)	10.3 (28/272)

Low endemic = area with lower endemicity; high endemic = area with higher endemicity

Table 2. Factors associated with antibody prevalence to *P. falciparum* pre-erythrocytic and asexual blood stage antigens (2A) and sexual stage antigens (2B).

2A

		NANP-6		GLURP	
		Univariate Odd ratio (95% CI)	Multivariate Odd ratio (95% CI)	Univariate Odd ratio (95% CI)	Multivariate Odd ratio (95% CI)
Age	1-4 yr	1 (ref)	1 (ref)	1 (ref)	1 (ref)
	N	127		142	
	5-9 yr	2.51 (1.48-4.25)	2.57 (1.51-34.38)	4.88 (2.96-8.04)	4.88 (2.96-8.04)
	N	144		147	
	10-19 yr	4.05 (2.49-6.59)	4.29 (2.62-7.03)	5.60 (3.56-8.82)	5.59 (3.55-8.80)
	N	230		236	
	20+ yr	16.27 (9.19-28.82)	17.16 (9.61-30.62)	6.44 (3.96-10.48)	6.46 (3.97-10.51)
	N	175		184	
Season	Start wet	1 (ref)	1 (ref)	1 (ref)	
	N	269		293	
	Peak wet	6.57 (3.96-10.91)	10.59 (5.91-18.98)	0.75 (0.50-1.14)	
	N	143		148	
	End wet	1.18 (0.84-1.66)	1.33 (0.90-1.96)	0.96 (0.67-1.36)	
	N	264		268	
Area	Low tran.	1 (ref)	1 (ref)	1 (ref)	
	N	326		337	
	High tran.	1.73 (1.27-2.35)	1.93 (1.38-2.71)	0.92 (0.67-1.25)	
	N	350		372	
Asexual	Absent	1 (ref)		1 (ref)	
	N	244		259	
	Present	0.72 (0.53-0.99)		1.08 (0.79-1.49)	
	N	432		450	
Gametocyte	Absent	1 (ref)	1 (ref)	1 (ref)	1 (ref)
	N	544		575	
	Present	0.57 (0.39-0.84)	0.62 (0.38-1.00)	0.52 (0.35-0.76)	0.58 (0.38-0.90)
	N	132		134	

ref=reference; tran.=transmission intensity; sero-reactivity related to a given variable was adjusted for all others variables that primarily play a significant role in the univariate model

2B

		Pfs48/45		Pfs230	
		Univariate Odd ratio (95% CI)	Multivariate Odd ratio (95% CI)	Univariate Odd ratio (95% CI)	Multivariate Odd ratio (95% CI)
Age	1-4 yr N	1 (ref) 85	1 (ref)	1 (ref) 84	1 (ref)
	5-9 yr N	0.17 (006-0.47) 84	0.16 (0.06-0.47)	0.27 (0.12-0.58) 83	0.27 (0.12-0.58)
	10-19 yr N	0.53 (0.28-1.03) 138	0.52 (0.26-1.00)	0.74 (0.42-1.31) 137	0.74 (0.41-1.31)
	20+ yr N	1.65 (0.89-3.05) 108	1.67 (0.89-3.11)	0.65 (0.35-1.19) 105	0.64 (034-1.18)
Season	Start wet N	1 (ref) 137		1 (ref) 136	1 (ref)
	Peak wet N	1.35 (0.78-2.32) 149		4.92 (2.85-8.50) 148	4.93 (2.85-8.52)
	End wet N	0.69 (0.37-1.28) 129		0.73 (0.37-1.44) 125	0.73 (0.37-1.45)
Area	Low tran. N	1 (ref)140		1 (ref) 138	
	High tran. N	0.69 (0.43-1.12) 275		1.20 (0.76-1.91) 271	
Asexual	Absent N	1 (ref) 131		1 (ref) 128	
	Present N	0.65 (0.40-1.05) 284		0.98 (0.62-1.55) 281	
Gametocyte	Absent N	1 (ref) 326		1 (ref) 321	
	Present N	0.72 (0.40-1.31) 89		1.06 (0.63-1.78) 88	

ref=reference; tran.=transmission intensity; sero-reactivity related to a given variable was adjusted for all others variables that primarily play a significant role in the univariate model

Serology

Plasma samples were screened for antibody response profiles in the population and related to age (Figure 1), season (Figure 2) and area of residence (Figure 3). The average

prevalence of IgG antibodies was 54.3% (367/676) for the sporozoite antigen $NANP_6$ and 64.9% (460/709) for the asexual blood stage antigen GLURP. In contrast to sporozoite and asexual blood stage antigens, sexual stage-specific antibody responses were only detected in a minority of the samples: 22.2% (92/415) contained anti-Pfs48/45 IgG and 28.6% (117/409) anti-Pfs230 IgG.

Effect of age on antibody responses

Among children below 5 years, the prevalence of antibodies with specificity for $NANP_6$ (23.6%) and GLURP (32.4%) was broadly similar to those for Pfs48/45 (27.1%) and Pfs230 (38.1%). As expected, the prevalence of asexual stage antibodies to $NANP_6$ and GLURP increased significantly with age (Adjusted OR = 2.35, $p < 0.001$, 95% CI 1.98-2.78; Adjusted OR = 1.79, $p < 0.001$, 95% CI 1.53-2.08 respectively) reflecting cumulative exposure to infection, while no evidence of an age-dependent increase in sexual stage-specific antibody responses was observed (Table 2).

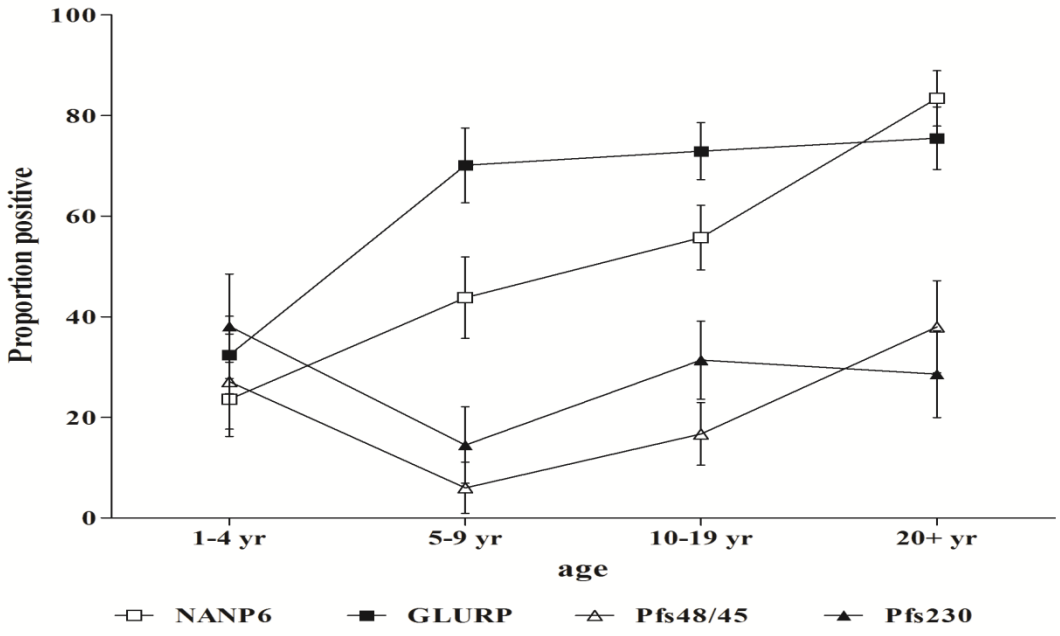

Figure 1. Prevalence of antibodies against pre-erythrocytic stage ($NANP_6$), asexual blood stage (GLURP) and sexual stage (Pfs48/45 and Pfs230) in relation to age.

The *error bars show the ± limits of the 95% confidence intervals. The number of plasma samples tested per age group (1-4, 5-9, 10-19 and ≥20 years of age) are 127, 144, 230 and 175 for $NANP_6$; 142, 147, 236, 184 and 184 for GLURP; 85, 84, 138 and 108 for Pfs48/45 and 84, 83, 137 and 105 for Pfs230 respectively.*

The seroprevalences for Pfs48/45 and Pfs230 in the youngest children (27.1% and 38.1% respectively) were comparable to those in adult (38% and 28.6% respectively). However, there was a significant decline of Pfsf48/45- and Pfs230-specific antibody prevalence from 1-4 year-olds to 5-9 year-olds (χ^2 = 13.61, p = 0.001 and χ^2 = 12.02, p < 0.001 respectively) followed subsequently by a significant increase with increasing age (OR = 3.09, p < 0.001, 95% CI 2.02-4.71 and OR = 1.41, p = 04, 95% CI 1.01-1.97 respectively) (Figure 1).

Differences between seasons and areas

GLURP antibody responses showed no variation according to season or area whilst, conversely, the prevalence of $NANP_6$ antibodies differed by both season (Figure 2) and area of residence (Figure 3). Antibodies with specificity for $NANP_6$ were detected in 83.9% of individuals at the peak wet season while this proportion was significantly lower at the start (44.2%) and at the end (48.5%) of the wet season. Thus the prevalence of $NANP_6$ antibodies at the peak wet season was significantly higher than either at the start (χ^2= 60.34, p < 0.001) or the end of the wet season (χ^2 = 48.91, p < 0.001). There was also a difference in $NANP_6$ antibody prevalence between the area of high transmission (60.9%) and the area of lower transmission (47.2%; χ^2 = 12.61, p < 0.001).

The prevalence of sexual stage-specific antibodies in relation to season is presented in Figure 2.

Pfs48/45 antibody prevalence increased at the peak of the wet season (27.5%) but did not significantly differ from the prevalence at either the start (21.9%) or the end of the wet season (16.3%). The variation in Pfs230-specific antibody prevalence was significant, reaching 51.4% at the peak compared to 17.6% at the start (χ^2 = 35.29, p < 0.001) and 13.6% at the end of the wet season (χ^2= 42.99, p < 0.001). This increased Pfs230 antibody prevalence at the peak of the wet season remained significant after adjustment for age (Adjusted OR = 4.93, p < 0.001, 95% CI 2.85-8.52, Table 2). Both antigens are on the surface of the gametocyte and were expected to have shown similar patterns of immune response. The difference observed in their immune responses may be dependent on the difference in their immunogenicity, Pfs230 being more immunogenic than Pfs48/45 (22).

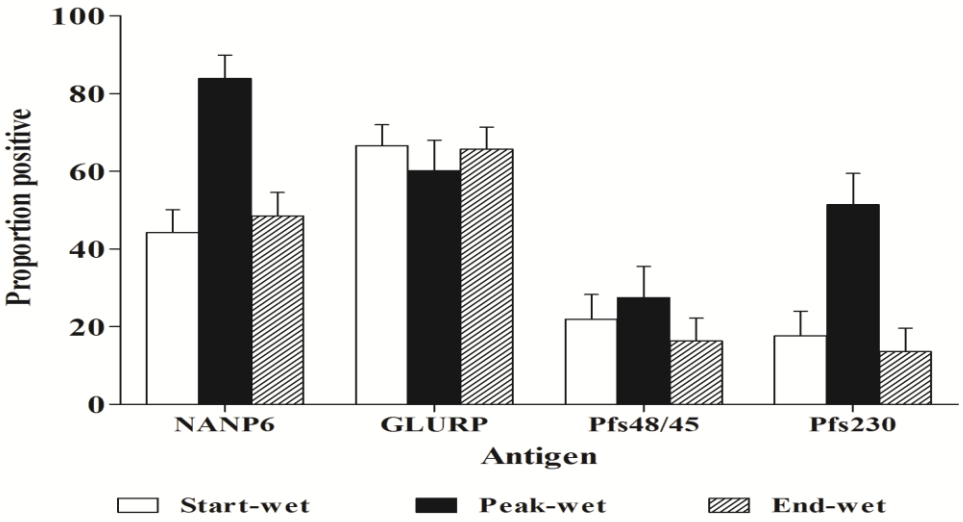

Figure 2. Prevalence of antibodies against $NANP_6$, GLURP, Pfs48/45 and Pfs230 in relation to season. *Bars indicate the proportion of positive individuals in each area of residence. Error bars indicate the upper limit of the 95% confidence interval around the proportion. The number of individuals (with antibodies) during the different seasons was: start-wet season: 119 (269) for $NANP_6$, 195 (293) for GLURP, 30 (137) for Pfs48/45, 24 (136) for Pfs230; peak-wet season: 120 (143) for $NANP_6$, 89 (148) for GLURP, 41 (149) for Pfs48/45 and 76 (148) for Pfs230; end-wet season: 128 (264) for $NANP_6$, 92 (176) for GLURP, 17 (125) for Pfs48/45 and 21 (129) for Pfs230.*

In terms of area of residence, there was no difference in the prevalence of antibodies with specificity for either Pfs48/45 or Pfs230 (Table 2).

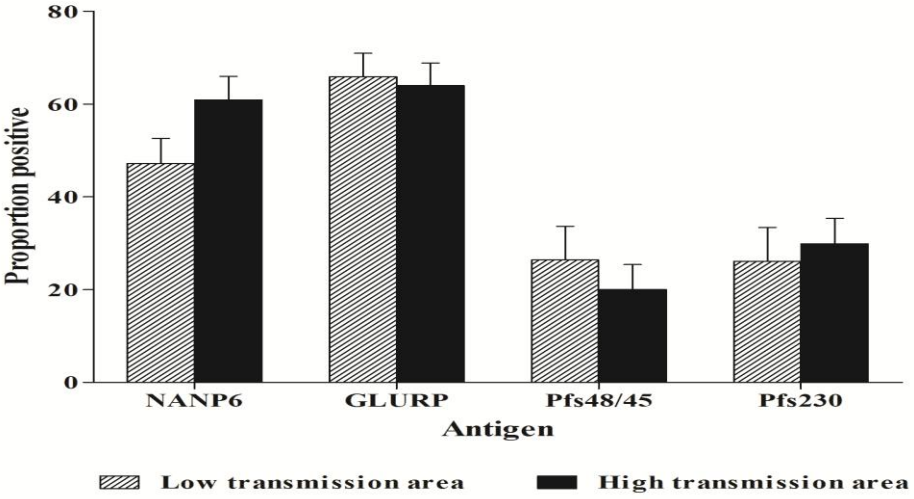

Figure 3. Prevalence of antibodies against $NANP_6$, GLURP, Pfs48/45 and Pfs230 in relation to the area of residence. *Bars indicate the proportion of positive individuals in each area of*

residence. Error bars indicate the upper limit of the 95% confidence interval around the proportion. The number of positive individuals in both area of residences was: high transmission area: 213 (350) for $NANP_6$, 238 (372) for GLURP, 55 (275) for Pfs48/45 and 81 (271) for Pfs230; low transmission area: 154 (326) for $NANP_6$, 222 (337) for GLURP, 37 (140) for Pfs48/45 and 36 (138) for Pfs230.

The relationship between antibody responses and parasite carriage is shown in Table 2. Both anti-$NANP_6$ and anti-GLURP immune responses were significantly higher in individuals negative to gametocytes (Adjusted OR = 0.62, 95% CI 0.38-1.00, p = 0.04 for $NANP_6$ and Adjusted OR = 0.58, p = 0.01, 95% CI 0.38-0.90 for GLURP).The seroprevalences for Pfs48/45 and Pfs230 antibodies were not influenced by the concurrent presence of asexual parasites or gametocytes.

Correlation of antibody responses between the different antigens

We also examined the correlation between antibody responses at the individual level. As presented in Table 3, the levels of antibody directed to $NANP_6$ and Pfs230 and those to Pfs230 and Pfs48/45 were strongly correlated ($p \leq 0.001$.) Antibody levels to GLURP were associated with those to $NANP_6$.

Table 3. Spearman rank correlation of level of anti-sporozoite and anti-asexual blood stage antibody with level of anti-sexual stage antibody.

	$NANP_6$ *r* (*P*-value) N	GLURP *r* (*P*-value) N	Pfs48/45 *r* (*P*-value) N	Pfs230 *r* (*P*-value) N
$NANP_6$		0.209 (<0.001) 671	0.53 (0.29) 390	0.170 (0.001) 384
GLURP			-0.067 (0.17) 413	-0.039 (0.43) 407
Pfs48/45				0.414 (<0.001) 409
Pfs230				

r = spearman correlation coefficient; N = number of individuals. Paired $NANP_6$-GLURP, $NANP_6$-Pfs230 and Pfs48/45-Pfs230 data showed strong correlations ($p \leq 0.001$).

Table 4. The association between sexual stage antibody prevalence and functional transmission reducing activity in three studies using the same ELISA methodology.

		Sexual stage antibody responses			
		Pfs48/45 antibody prevalence (n=350)		Pfs230 antibody prevalence (n=344)	
Functional transmission reducing activity		OR (95% CI)	p-value	OR (95% CI)	p-value
	>50% reduction	3.76 (2.27-6.23)	<0.001	1.87 (1.18-2.96)	0.007
	>90% reduction	6.08 (3.21-11.49)	<0.001	2.84 (1.47-5.50)	0.002

This summary table combines data from Tanzania and Indonesia (9, 11, 18) that used identical ELISA protocols and related antibody prevalence to functional transmission reducing activity (i.e. the percentage reduction in mosquito oocyst numbers in test samples compared with controls) in standard membrane feeding assays. OR=odds ratio; 95% CI=95% confidence interval

Discussion

As in previous studies[6,9,18,23,26,43,44], we found evidence for naturally acquired immunity against *P. falciparum* sexual stages. Overall, 22 to 28% of our samples were positive for sexual stage-specific antibodies while 54 to 65% were positive for pre-erythrocytic- and asexual blood stage-specific antibodies. Unlike immune responses to $NANP_6$ and GLURP, we found no evidence for age as a predictor for anti-Pfs48/45 and anti-Pfs230 antibody responses. Our data do reveal that sexual stage-specific antibody responses increase markedly during the transmission season. This would indicate a role for recent exposure to parasite antigens in the short-term boosting of sexual stage-specific immunity, although this requires confirmation in longitudinal studies.

The lower prevalence of sexual stage-specific antibodies compared with either pre-erythrocytic- or asexual stage-specific antibodies in the same study population confirms previous findings[6,13,18]. In contrast to antibodies directed to sexual stage antigens, those directed to an asexual blood stage antigen (GLURP) increased with age, as described before[14,15,17,19,32], with no apparent season-dependent changes[17,19]. This would suggest that anti-GLURP antibody responses are both long-lived and stable even when antigen exposure decreases in the low transmission season and/or with increasing age. If sexual stage-specific antibody responses persisted in the absence of boosting, we would expect to see a similar pattern of increasing antibody prevalence with increasing age, but, despite a significant increase in the prevalence of sexual stage-specific antibodies from 5-9 years of age onward in our population, the close similarity of the prevalences in the youngest children and in adults above 20 years old of age effectively obscured any such association.

A single recent study reported an age-dependent increase of sexual stage-specific antibody responses, although it should be noted that that study concerned pre- and post-treatment determinations in residents of an area with a very low rate of transmission and is therefore not directly comparable with the study we report here[11]. The results of other studies of the age-dependence of such responses are inconsistent[6,18,30]. In Cameroon, the level of transmission blocking immunity, as measured in membrane feeding experiments, was not related to age[3,5]. The lack of age-dependence of sexual stage-specific antibody responses has often been attributed to their short-lived nature and hence a requirement for frequent boosting[30,40]. Cumulative immune memory, likely the result of repeated exposure to

infections, is thought to form the basis of the age-dependent increase of asexual blood stage-specific immune responses[51.] Immature and/or short-term memory for responses directed to sexual stage antigens is plausible[30] and possibly explained by a predominantly T-cell independent induction of the immune response[29].

Interestingly, the prevalence of sexual stage-specific IgG responses in the youngest children in our study group was similar to that for the asexual (GLURP) and pre-erythrocytic ($NANP_6$) antigens, suggesting that sexual stage-specific immune responses may readily develop in response to antigen exposure early in life. Since age-related cumulative exposure seems to have little effect on sexual stage immune responses, we speculate that the greater prevalence in young children may reflect the initial immune response to gametocytes as observed in individuals after a single or limited number of exposures to infection with either P. falciparum[6,22,33] or *P. vivax*[31]. The remarkable nadir in both Pfs48/45 and Pfs230 seroprevalences in children aged 5-9 years old was previously observed in populations exposed to endemic malaria. The studies found that sexual stage immune responses decreased with age in the younger age groups but there was no further decline in older age groups[6,18]. Sexual stage antibody responses in young children are likely to be the result of high gametocyte exposure in this age group. In older children, gametocyte exposure decreases, possibly explaining the reduction in antibody prevalence. In adults gametocyte exposure may be lowest but sexual stage commitment during infections may be relatively increased[34] and antibody responses to sexual stage antigens may become more long-lived, reflecting a maturation of the immune response.

An important finding from the study reported here is that sexual stage-specific antibody responses may vary with season. Pfs230-specific antibody prevalence, in particular, increased during the peak of the transmission season, suggesting that the level of sexual stage-specific antibodies may reflect recent exposure. A possible association between sexual stage immunity and recent antigen exposure is also suggested by the correlation between $NANP_6$ and anti-sexual stage antibodies. In contrast to those directed to GLURP, $NANP_6$-specific antibody responses seemed to be short-lived. Thus the pattern of variation in $NANP_6$ antibody responses closely reflected the seasonality of transmission in our study, indicating that seroreactivity to $NANP_6$ is dependent on recent exposure and therefore highly susceptible to changes in sporozoite exposure. The close association we observed

between $NANP_6$-and Pfs230-specific antibodies may be an indication that the immune responses to sexual stage antigens are therefore also related to recent exposure to infection, explaining the seasonality of the anti-Pfs230 antibody response. Our results showed that many infections with asexual parasites are accompanied by gametocytes[35,37] and this proportion will be further increased if sub-microscopic gametocyte densities are considered[10]. Since asexual parasite carriage increases during the transmission season[36], exposure to gametocytes increases in parallel, possibly explaining the seasonality of anti-sexual stage IgG responses. However, the seasonality of sexual stage-specific immune responses[28] has been rarely studied, making any comparison difficult. The importance of recent exposure to infection for the acquisition of sexual stage-specific immunity has been reported from previous studies in which the prevalence of anti-Pfs48/45 and anti-Pfs230 antibodies increased in migrants from a non-endemic to an endemic area[9] and where functional transmission reducing immunity was shown to increase during the transmission season[12]. We did not determine functional transmission reducing immune responses in the current dataset. Three studies that used the same methodology for determining total IgG antibody responses to Pfs48/45 and Pfs230 did relate these responses to functional transmission reducing activity (TRA) in the standard membrane feeding assay[9,11,18]. When combined, these data show a strong association between Pfs48/45 and Pfs230 antibody prevalence and different levels of TRA (Table 4). These data support the current findings on sexual stage antibody responses that plausibly reflect associations between functional TRA and age, season and recent exposure to malaria antigens.

We further tried to explore the relevance of recent exposure for the acquisition of parasite stage-specific immunity by relating antibody responses to concurrent asexual parasite and gametocyte carriage. Our results showed that $NANP_6$ and GLURP –specific antibodies appear to negatively associate with gametocytes suggesting that immune responders to these two antigens are more likely to have a shorter period of asexual parasite carriage that will translate in a lower likelihood of gametocyte carriage. However, we found no effect of anti-Pfs48/45 or anti-Pfs230 antibodies on gametocyte carriage. Concurrent gametocytes may be positively related to development of sexual stage-specific immune responses[11] although a straightforward relationship is not always evident[6,18]. Immune responses to gametocyte antigens induced prior to sampling may persist for several weeks after gametocyte

clearance[11] or following reduction of gametocytaemia to sub-microscopic levels, whilst acquisition/boosting of responses to gametocytes circulating at the time of sampling may take longer e.g. after their death/destruction and subsequent clearance. Intuitively, then, an antibody response that is boosted by recent gametocyte antigen exposure can better be determined in studies that determine gametocyte carriage at both microscopic and submicroscopic level repeatedly and relate sexual stage-specific antibody responses to previous as well as current gametocyte exposure.

In summary, plasma samples from malaria exposed individuals were analyzed to provide indications of the development of naturally-acquired immunity to *P. falciparum* sexual stages. Our findings indicate that sexual stage-specific immune responses are naturally acquired in the study population and that they are a function of recent rather than cumulative exposure to gametocytes. This occurs after limited exposures to gametocytes in the youngest age-groups, but boosting is also evident during the peak of the transmission season. A next step would be to determine the functional importance of sexual stage-specific antibody responses in this population that, despite developing both asexual and sexual stage-specific antibody responses, is repeatedly exposed to intense transmission[35,36].

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References

1. Baird, J. K., T. R. Jones, Purnomo, S. Masbar, S. Ratiwayanto, and B. Leksana. 1991. Evidence for specific suppression of gametocytemia by *Plasmodium falciparum* in residents of hyperendemic Irian Jaya. Am J Trop Med Hyg 44:183-190.
2. Beier, J. C., P. V. Perkins, R. A. Wirtz, R. E. Whitmire, M. Mugambi, and W. T. Hockmeyer. 1987. Field evaluation of an enzyme-linked immunosorbent assay (ELISA) for *Plasmodium falciparum* sporozoite detection in anopheline mosquitoes from Kenya. Am J Trop Med Hyg 36:459-468.

3. Boudin, C., A. Diop, A. Gaye, L. Gadiaga, C. Gouagna, I. Safeukui, and S. Bonnet. 2005. *Plasmodium falciparum* transmission blocking immunity in three areas with perennial or seasonal endemicity and different levels of transmission. Am J Trop Med Hyg 73:1090-1095.
4. Boudin, C., M. Olivier, J. F. Molez, J. P. Chiron, and P. Ambroise-Thomas. 1993. High human malarial infectivity to laboratory-bred Anopheles gambiae in a village in Burkina Faso. Am J Trop Med Hyg 48:700-706.
5. Boudin, C., M. Van Der Kolk, T. Tchuinkam, C. Gouagna, S. Bonnet, I. Safeukui, B. Mulder, J. Y. Meunier, and J. P. Verhave. 2004. *Plasmodium falciparum* transmission blocking immunity under conditions of low and high endemicity in Cameroon. Parasite Immunol 26:105-110.
6. Bousema, J. T., C. J. Drakeley, J. Kihonda, J. C. Hendriks, N. I. Akim, W. Roeffen, and R. W. Sauerwein. 2007. A longitudinal study of immune responses to *Plasmodium falciparum* sexual stage antigens in Tanzanian adults. Parasite Immunol 29:309-317.
7. Bousema, J. T., C. J. Drakeley, and R. W. Sauerwein. 2006. Sexual-stage antibody responses to *P. falciparum* in endemic populations. Curr Mol Med 6:223-229.
8. Bousema, J. T., L. C. Gouagna, C. J. Drakeley, A. M. Meutstege, B. A. Okech, I. N. Akim, J. C. Beier, J. I. Githure, and R. W. Sauerwein. 2004. *Plasmodium falciparum* gametocyte carriage in asymptomatic children in western Kenya. Malar J 3:18.
9. Bousema, J. T., W. Roeffen, M. van der Kolk, S. J. de Vlas, M. van de Vegte-Bolmer, M. J. Bangs, K. Teelen, L. Kurniawan, J. D. Maguire, J. K. Baird, and R. W. Sauerwein. 2006. Rapid onset of transmission-reducing antibodies in javanese migrants exposed to malaria in papua, indonesia. Am J Trop Med Hyg 74:425-431.
10. Bousema, T., and C. Drakeley. 2011. Epidemiology and infectivity of *Plasmodium falciparum* and *Plasmodium vivax* gametocytes in relation to malaria control and elimination. Clin Microbiol Rev 24:377-410.
11. Bousema, T., W. Roeffen, H. Meijerink, H. Mwerinde, S. Mwakalinga, G. J. van Gemert, M. van de Vegte-Bolmer, F. Mosha, G. Targett, E. M. Riley, R. Sauerwein, and C. Drakeley. 2010. The dynamics of naturally acquired immune responses to *Plasmodium falciparum* sexual stage antigens Pfs230 & Pfs48/45 in a low endemic area in Tanzania. PLoS One 5:e14114.
12. Bousema, T., C. J. Sutherland, T. S. Churcher, B. Mulder, L. C. Gouagna, E. M. Riley, G. A. Targett, and C. J. Drakeley. 2010. Human immune responses that reduce the transmission of *Plasmodium falciparum* in African populations. Int J Parasitol.
13. Carter, R., P. M. Graves, I. A. Quakyi, and M. F. Good. 1989. Restricted or absent immune responses in human populations to *Plasmodium falciparum* gamete antigens that are targets of malaria transmission-blocking antibodies. J Exp Med 169:135-147.
14. Cohen, S., and G. A. Butcher. 1971. Serum antibody in acquired malarial immunity. Trans R Soc Trop Med Hyg 65:125-135.
15. Cohen, S., G. A. Butcher, G. H. Mitchell, J. A. Deans, and J. Langhorne. 1977. Acquired immunity and vaccination in malaria. Am J Trop Med Hyg 26:223-232.
16. Cuzin-Ouattara, N., A. H. Van den Broek, A. Habluetzel, A. Diabate, E. Sanogo-Ilboudo, D. A. Diallo, S. N. Cousens, and F. Esposito. 1999. Wide-scale installation of insecticide-treated curtains confers high levels of protection against malaria transmission in a hyperendemic area of Burkina Faso. Trans R Soc Trop Med Hyg 93:473-479.
17. Dodoo, D., M. Theisen, J. A. Kurtzhals, B. D. Akanmori, K. A. Koram, S. Jepsen, F. K. Nkrumah, T. G. Theander, and L. Hviid. 2000. Naturally acquired antibodies to the glutamate-rich protein are associated with protection against *Plasmodium falciparum* malaria. J Infect Dis 181:1202-1205.
18. Drakeley, C. J., J. T. Bousema, N. I. Akim, K. Teelen, W. Roeffen, A. H. Lensen, M. Bolmer, W. Eling, and R. W. Sauerwein. 2006. Transmission-reducing immunity is inversely related to age in *Plasmodium falciparum* gametocyte carriers. Parasite Immunol 28:185-190.
19. Dziegiel, M., P. Rowe, S. Bennett, S. J. Allen, O. Olerup, A. Gottschau, M. Borre, and E. M. Riley. 1993. Immunoglobulin M and G antibody responses to *Plasmodium falciparum*

glutamate-rich protein: correlation with clinical immunity in Gambian children. Infect Immun 61:103-108.

20. Graves, P. M., T. R. Burkot, R. Carter, J. A. Cattani, M. Lagog, J. Parker, B. J. Brabin, F. D. Gibson, D. J. Bradley, and M. P. Alpers. 1988. Measurement of malarial infectivity of human populations to mosquitoes in the Madang area, Papua, New Guinea. Parasitology 96 (Pt 2):251-263.
21. Graves, P. M., R. Carter, T. R. Burkot, I. A. Quakyi, and N. Kumar. 1988. Antibodies to *Plasmodium falciparum* gamete surface antigens in Papua New Guinea sera. Parasite Immunol 10:209-218.
22. Graves, P. M., A. Doubrovsky, R. Carter, S. Eida, and P. Beckers. 1990. High frequency of antibody response to *Plasmodium falciparum* gametocyte antigens during acute malaria infections in Papua New Guinea highlanders. Am J Trop Med Hyg 42:515-520.
23. Graves, P. M., R. A. Wirtz, R. Carter, T. R. Burkot, M. Looker, and G. A. Targett. 1988. Naturally occurring antibodies to an epitope on *Plasmodium falciparum* gametes detected by monoclonal antibody-based competitive enzyme-linked immunosorbent assay. Infect Immun 56:2818-2821.
24. Habluetzel, A., N. Cuzin, D. A. Diallo, I. Nebie, S. Belem, S. N. Cousens, and F. Esposito. 1999. Insecticide-treated curtains reduce the prevalence and intensity of malaria infection in Burkina Faso. Trop Med Int Health 4:557-564.
25. Healer, J., D. McGuinness, R. Carter, and E. Riley. 1999. Transmission-blocking immunity to *Plasmodium falciparum* in malaria-immune individuals is associated with antibodies to the gamete surface protein Pfs230. Parasitology 119 (Pt 5):425-433.
26. Healer, J., D. McGuinness, P. Hopcroft, S. Haley, R. Carter, and E. Riley. 1997. Complement-mediated lysis of *Plasmodium falciparum* gametes by malaria-immune human sera is associated with antibodies to the gamete surface antigen Pfs230. Infect Immun 65:3017-3023.
27. Kumar, N. 1987. Target antigens of malaria transmission blocking immunity exist as a stable membrane bound complex. Parasite Immunol 9:321-335.
28. Marrelli, M. T., R. S. Malafronte, and J. K. Kloetzel. 1997. Seasonal variation of anti-*Plasmodium falciparum* antibodies directed against a repetitive peptide of gametocyte antigen pfs2400 in inhabitants in the State of Amapa, Brazil. Acta Trop 63:167-177.
29. Mendis, K. N., and R. Carter. 1991. Transmission blocking immunity may provide clues that antimalarial immunity is largely T-independent. Res Immunol 142:687-690.
30. Mendis, K. N., P. H. David, and R. Carter. 1990. Human immune responses against sexual stages of malaria parasites: considerations for malaria vaccines. Int J Parasitol 20:497-502.
31. Mendis, K. N., Y. D. Munesinghe, Y. N. de Silva, I. Keragalla, and R. Carter. 1987. Malaria transmission-blocking immunity induced by natural infections of *Plasmodium vivax* in humans. Infect Immun 55:369-372.
32. Molineaux, L., and G. Gramiccia. 1980. Parasitology, chapter five in "THE GARKI PROJECT. Research on the Epidemiology and Control of Malaria in the Sudan Savanna of West Africa". WHO report:109-172.
33. Ong, C. S., K. Y. Zhang, S. J. Eida, P. M. Graves, C. Dow, M. Looker, N. C. Rogers, P. L. Chiodini, and G. A. Targett. 1990. The primary antibody response of malaria patients to *Plasmodium falciparum* sexual stage antigens which are potential transmission blocking vaccine candidates. Parasite Immunol 12:447-456.
34. Ouedraogo, A. L., T. Bousema, S. J. de Vlas, N. Cuzin-Ouattara, J. P. Verhave, C. Drakeley, A. J. Luty, and R. Sauerwein. 2010. The plasticity of *Plasmodium falciparum* gametocytaemia in relation to age in Burkina Faso. Malar J 9:281.
35. Ouedraogo, A. L., T. Bousema, P. Schneider, S. J. de Vlas, E. Ilboudo-Sanogo, N. Cuzin-Ouattara, I. Nebie, W. Roeffen, J. P. Verhave, A. J. Luty, and R. Sauerwein. 2009. Substantial

contribution of submicroscopical *Plasmodium falciparum* gametocyte carriage to the infectious reservoir in an area of seasonal transmission. PLoS One 4:e8410.

36. Ouedraogo, A. L., S. J. de Vlas, I. Nebie, E. Ilboudo-Sanogo, J. T. Bousema, A. S. Ouattara, J. P. Verhave, N. Cuzin-Ouattara, and R. W. Sauerwein. 2008. Seasonal patterns of *Plasmodium falciparum* gametocyte prevalence and density in a rural population of Burkina Faso. Acta Trop 105:28-34.
37. Ouedraogo, A. L., P. Schneider, M. de Kruijf, I. Nebie, J. P. Verhave, N. Cuzin-Ouattara, and R. W. Sauerwein. 2007. Age-dependent distribution of *Plasmodium falciparum* gametocytes quantified by Pfs25 real-time QT-NASBA in a cross-sectional study in Burkina Faso. Am J Trop Med Hyg 76:626-630.
38. Piper, K. P., R. E. Hayward, M. J. Cox, and K. P. Day. 1999. Malaria transmission and Naturally acquired immunity to PfEMP-1. Infection and Immunity 67:6369-6374.
39. Ponnudurai, T., A. H. Lensen, A. D. Leeuwenberg, and J. H. Meuwissen. 1982. Cultivation of fertile *Plasmodium falciparum* gametocytes in semi-automated systems. 1. Static cultures. Transactions of the Royal Society of Tropical Medicine and Hygiene 76:812-818.
40. Ranawaka, M. B., Y. D. Munesinghe, D. M. de Silva, R. Carter, and K. N. Mendis. 1988. Boosting of transmission-blocking immunity during natural *Plasmodium vivax* infections in humans depends upon frequent reinfection. Infect Immun 56:1820-1824.
41. Roeffen, W., P. J. Beckers, K. Teelen, T. Lensen, R. W. Sauerwein, J. H. Meuwissen, and W. Eling. 1995. *Plasmodium* falciparum: a comparison of the activity of Pfs230-specific antibodies in an assay of transmission-blocking immunity and specific competition ELISAs. Exp Parasitol 80:15-26.
42. Roeffen, W., F. Geeraedts, W. Eling, P. Beckers, B. Wizel, N. Kumar, T. Lensen, and R. Sauerwein. 1995. Transmission blockade of *Plasmodium falciparum* malaria by anti-Pfs230-specific antibodies is isotype dependent. Infect Immun 63:467-471.
43. Roeffen, W., T. Lensen, B. Mulder, K. Teelen, R. Sauerwein, W. Eling, J. H. Meuwissen, and P. Beckers. 1994. Transmission blocking immunity as observed in a feeder system and serological reactivity to Pfs 48/45 and Pfs230 in field sera. Mem Inst Oswaldo Cruz 89 Suppl 2:13-15.
44. Roeffen, W., B. Mulder, K. Teelen, M. Bolmer, W. Eling, G. A. Targett, P. J. Beckers, and R. Sauerwein. 1996. Association between anti-Pfs48/45 reactivity and *P. falciparum* transmission-blocking activity in sera from Cameroon. Parasite Immunol 18:103-109.
45. Roeffen, W., K. Teelen, J. van As, M. vd Vegte-Bolmer, W. Eling, and R. Sauerwein. 2001. *Plasmodium* falciparum: production and characterization of rat monoclonal antibodies specific for the sexual-stage Pfs48/45 antigen. Exp Parasitol 97:45-49.
46. Saeed, M., W. Roeffen, N. Alexander, C. J. Drakeley, G. A. Targett, and C. J. Sutherland. 2008. *Plasmodium falciparum* antigens on the surface of the gametocyte-infected erythrocyte. PLoS One 3:e2280.
47. Sutherland, C. J. 2009. Surface antigens of *Plasmodium falciparum* gametocytes--a new class of transmission-blocking vaccine targets? Mol Biochem Parasitol 166:93-98.
48. Theisen, M., D. Dodoo, A. Toure-Balde, S. Soe, G. Corradin, K. K. Koram, J. A. Kurtzhals, L. Hviid, T. Theander, B. Akanmori, M. Ndiaye, and P. Druilhe. 2001. Selection of glutamate-rich protein long synthetic peptides for vaccine development: antigenicity and relationship with clinical protection and immunogenicity. Infect Immun 69:5223-5229.
49. van der Kolk, M., S. J. de Vlas, and R. W. Sauerwein. 2006. Reduction and enhancement of *Plasmodium falciparum* transmission by endemic human sera. Int J Parasitol 36:1091-1095.
50. Vermeulen, A. N., T. Ponnudurai, P. J. Beckers, J. P. Verhave, M. A. Smits, and J. H. Meuwissen. 1985. Sequential expression of antigens on sexual stages of *Plasmodium falciparum* accessible to transmission-blocking antibodies in the mosquito. J Exp Med 162:1460-1476.

51. Weiss, G. E., B. Traore, K. Kayentao, A. Ongoiba, S. Doumbo, D. Doumtabe, Y. Kone, S. Dia, A. Guindo, A. Traore, C. Y. Huang, K. Miura, M. Mircetic, S. Li, A. Baughman, D. L. Narum, L. H. Miller, O. K. Doumbo, S. K. Pierce, and P. D. Crompton. 2010. The *Plasmodium* falciparum-specific human memory B cell compartment expands gradually with repeated malaria infections. PLoS Pathog 6:e1000912.

Chapter 7

General discussion

The work reported in this thesis describes the prevalence of gametocytes in human populations in Burkina Faso and factors related to their transmission from man to mosquito. Gametocyte detection in human blood samples is primarily based on microscopy and has included the more sensitive molecular technique Pfs25 QT-NASBA to quantify sub-microscopical gametocytaemia. The infectiousness of gametocytes in the field is determined by direct membrane feeding assays and the contribution of both microscopical and sub-microscopical gametocytaemia to malaria transmission. Antibody responses against transmission blocking vaccine candidate antigens Pfs48/45 and Pfs230, important modulators of gametocyte infectivity, are detected in field sera and their implication in transmission reducing activity (TRA) discussed. The overall results are integrated in a final discussion on their relevance for malaria transmission reducing interventions, malaria control and elimination strategies.

7.1. The epidemiology of *Plasmodium falciparum* gametocytes

Gametocytes and *Anopheles* mosquito vectors are the two components necessary for malaria transmission in humans. Successful malaria transmission from man to mosquito depends on the presence of infectious gametocytes in the peripheral blood of the human host. It is well acknowledged that targeting gametocytes either alone or following the introduction of control interventions will contribute to reducing malaria transmission and thereby assist in lessening malaria associated morbidity and mortality[1-3].

In Chapter 2 of this thesis, we determined whether season plays a role in the prevalence of gametocytes in an area of high malaria endemicity in Burkina Faso. Knowledge of seasonal gametocyte distribution can contribute to the appropriate design of interventions; correctly timed transmission reducing interventions may allow a more efficient reduction of malaria transmission. In our study, approximately one fifth of the study population harbored gametocytes as detected by microscopy. There was a clear increase in the prevalence and density of gametocytes during the peak transmission season (peak of the rainy season) compared to the period when transmission was practically absent (dry season). At the peak transmission season, we observed a positive association between gametocyte prevalence and density with asexual parasite density. This positive association between gametocytes

and high densities of asexual parasites is not novel[4-7]. In a low endemic area of Sudan, a 4-fold increase from the dry season to the wet season has been observed in the prevalence of gametocytes[10]. In areas with different malaria endemicity in Tanzania, the proportion of gametocyte carriers increased significantly from the dry to the wet season with a difference of 4 to 21%[11,12]. In Cameroon, similar seasonal trends in gametocyte prevalence have also been observed[10,13,14]. Most adults carrying gametocytes concurrently carried low densities of asexual parasites[8,9] suggesting that gametocyte carriage is not always associated on high asexual parasite densities[2]. Asexual parasite density is possibly not the only signal that drives gametocyte prevalence at the peak transmission season, since human immune responses that target asexual blood stage replication, induced by super-infections (a new infection establishing in parallel with an existing one) during the wet season[10,15] may also increase gametocyte production. Although not monitored during our study, it is also possible that hematological disruptions like anemia, commonly observed in children exposed to intense transmission, associate with high gametocyte prevalence[7,16,17] during the wet transmission season.

The two-fold increased gametocyte prevalence at the start of the wet season compared to that during the dry season is intriguing. This rise appeared to be independent of an increased exposure to sporozoites (which occurred after the start of the transmission season), age or asexual parasite density. We hypothesized that uninfected mosquitoes bites, particularly more common during this period of the year, may induce gametocyte production. This association was previously suggested[18] and would indicate an adaptation of parasites to ensure that an up-regulation of gametocytogenesis strategically coincides with an increase in the presence of mosquitoe bites to maximize transmission efficiency. However, recent studies by Billingsley and colleagues[19] and Shutler and colleagues[20] reported equivocal results on gametocyte production rates after mosquito probing indicating that the association between sexual stage commitment and mosquito bites needs confirmation in future studies, as well as the elucidation of the possible stimulus for gametocytogenesis.

Our findings also reveal that age drives gametocyte distribution. We further looked at this age effect in Chapter 3 and 4 using the molecular QT-NASBA technique for gametocyte detection. In Chapter 3, gametocyte prevalence and density were found to vary with age both by microscopy and QT-NASBA[9,21]. Our finding that gametocyte carriage is

negatively related to age, being more common in younger individuals, is supported by several studies[4,5,11,13-15,22,23]. Our data suggest that asexual parasite densities may primarily be responsible for the age-related decrease of gametocyte prevalence being an indirect consequence of the age related-decrease of asexual parasite densities[9]. We hypothesize that asexual stage immunity that gradually increases with age is accompanied by asexual parasite clearance that ultimately result in a lower prevalence of gametocytes in adults. However this seems somehow to be compensated by an increase gametocyte production with age as observed in Chapter 4.

In Chapter 4, we try to verify the above mentioned hypothesis by determining whether the age-related decrease of gametocyte carriage is a consequence of an age-dependent clearance of asexual parasites[2] or if there may be an age-dependent production of gametocytes. We observed that the proportion of gametocyte carriers relative to the total parasite population in increased in adults compared with the youngest children both by microscopy (from 1.8% to 18.2%) and by QT-NASBA (0.5% to 5.7%). Individuals that carry only gametocytes also increased in proportion from children (13.4%) to adults (45.6%). In subjects carrying only gametocytes, all asexual parasites may have converted to gametocytes or may have been cleared either by treatment or by partial immunity. Because antimalarial treatment is a rare event in adults and naturally acquired immunity is not sterilizing, this may suggest that asexual parasites declined to submicroscopical level while induced gametocytaemia persisted for several months[24-26]. This actually demonstrates that gametocyte production increases with increasing age independent of asexual parasite carriage that itself decreases with increasing age. From our epidemiological study, it is difficult to directly identify the cause for this increase in gametocyte production with age. Although we overall observed an age-related increase of gametocyte production, it is clear that gametocyte carriage stabilizes at a lower level in adults compared to children likely because of the lower carriage of their generators, i.e. the asexual parasites. A plausible explanation for this increase in gametocyte production in adults would be that fitness advantages for the parasite may increase the shift from asexual parasite to gametocyte in response to stressful conditions commonly observed in adults and possibly related to age-acquired immunitythat negatively affect asexual stage parasite multiplication. Together with findings from chapter 3, this indicates that asexual parasites may be the main drivers of gametocyte carriage but an age-dependent increase in gametocytogenesis could be an

(incomplete) compensatory mechanism for the lower asexual parasite densities in older individuals.

Due to their overall higher representation in the total population, our findings suggest that the contribution of adults to the human infectious reservoir is probably substantially greater than appreciated to date under the assumption that these low gametocytaemias remain infectious.

7.2. The contribution of submicroscopic gametocyte carriage to malaria transmission

When gametocyte carriers are identified in field studies, gametocyte densities in the peripheral blood are at least an order of magnitude lower than those of asexual parasites[4,5,9,14,17,21,27,28]. Little is known about the reasons for this paucity of gametocytes as compared to asexual parasites in endemic areas[29]. There is some evidence that gametocyte-specific immune responses can down-regulate gametocytaemia independent of asexual stage-specific[30] immunity but the nature and targets of such functional immunity have not been identified. At least part of the low gametocyte prevalence can be explained by the relative insensitivity of microscopy for detecting gametocytes. In our study, thick smears were read by two independent microscopists and gametocytes counted against 500 leucocytes in 100 microscopic fields with a limit of detection of 16 gametocytes/μL. Consequently, individuals with less than 16 gametocytes/μL were unlikely to be detected by microscopy. In this context, false negative gametocytaemia is not a rare event[26]. We determined the relevance of this phenomenon by asking several readers to read the same slide for gametocytes (figure 1).

The likelihood of gametocyte detection increased substantially with increasing the number of observers. Our results demonstrate our routine standard protocol (2 miscroscopists once read the same slide) detected gametocytes in 18% of the slides while this increased to 33% when 5 microscopists read the same slide twice. Although this finding indicates that gametocyte detection by microscopy can be improved, this will substantially increase the workload for an already laborious technique and the reliability of the final result is still dependent on the experience of the slide readers.

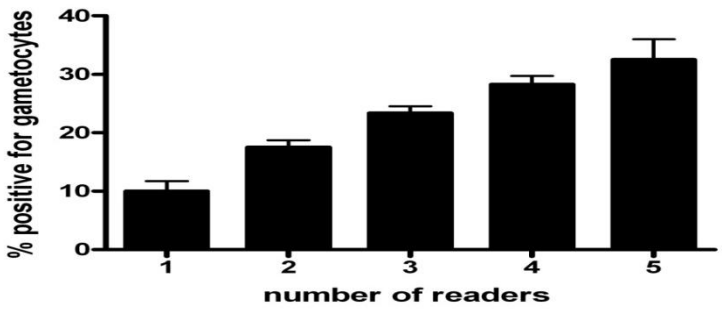

Figure 1: The rate of gametocyte positivity as determined by microscopy increases with increasing the number of slide readers. Each slide has been read twice by all the 5 readers (i.e. the maximum number of times a slide was read was 10 times); there was variation in the microscopic fields that were examined.

These limitations demonstrate the low sensitivity of microscopy and its inaccuracy in gametocyte quantification at very low concentrations. The prevalence of gametocytes in malaria endemic areas is grossly underestimated when using standard microscopy.

In Chapter 3, Pfs25 Real-Time QT-NASBA, which has a lower detection limit of 20-100 gametocytes/mL[31] and 10 asexual parasites/mL[32], is used to detect and quantify gametocyte density. Pfs25 QT-NASBA in our study confirmed the age-related pattern in the prevalence of gametocytes that was previously reported by microscopy[4,5,14,15,22]. The overall prevalence of gametocytes as detected by microscopy (~20%) increased 3.3 fold by QT-NASBA to 70%, indicating that a considerable proportion of gametocyte carriers is missed by microscopy (Chapter 3)[9]. In a highly endemic area in Kenya, gametocyte prevalence in clinical malaria cases was reported to be 86% by QT-NASBA compared to 22% by microscopy. In a low transmission setting of Tanzania, the prevalence of sub-microscopical gametocytaemia reached 15% in the general population while an estimate by microscopy indicated only ~1% gametocyte carriers[33]. These findings provide evidence that sub-microscopical gametocyte carriers are common in endemic areas and may be relevant for malaria transmission. We next investigated this issue by directly determining the infectivity of microscopic and submicroscopic gametocyte carriers by membrane feeding assays. This was the first study to use molecular gametocyte detection tools in combination with direct assessment of gametocyte infectivity in a general population.

In Chapter 5, we used direct membrane feeding experiments to determine the infectiousness of malaria exposed children and subsequently the contribution of microscopical and sub-microscopical gametocytaemia to transmission. Blood was offered to mosquitoes via a thin stretched artificial membrane attached to a water-jacketed glass feeder maintained at 37°C. During the entire process, mosquitoes were handled gently to reduce stress which may increase mosquitoes' mortality and affect the final results. Mosquitoes were allowed to feed during 10-15 min and dissected 7-8 days later for oocyst detection. The findings from our asymptomatic population of children in the absence of anti-malarial treatment are clear. The proportion of carriers of sub-microscopical gametocytes was approximately 60% contributing around 25% to the overall malaria transmission while carriers of gametocytes detectable by microscopy representing 33% and contributed 75% to overall transmission. In Kenya, children with sub-microscopical densities of gametocytes equally contributed to transmission compared to those with microscopically detectable gametocytes[34]. In our recent study including individuals of all ages (Ouedraogo and others, unpublished data) the proportion of carriers of submicroscopical and microscopical gametocytes were 49% and 16% respectively and contributed 51% and 47% to malaria transmission, respectively. In the study described in this thesis, the infectiousness of very low densities of gametocytes was evident although the proportion of infected mosquitoes was lower compared to higher gametocyte densities. Assuming that in a membrane feeding experiment with gametocyte carriers, fully fed mosquitoes engorged up to 2 μL of blood (and all partially fed mosquitoes are discarded), the theoretic minimum density would be 1 gametocyte/μL: one male and one female gametocyte. In our experiments, mosquito infection occurred at densities estimated below 1 gametocyte/μL. Under laboratory conditions, the lowest gametocyte density from culture resulting in mosquito infections was in the range 250-300 gametocytes/mL[34]. In contrast, results from the field show infectious gametocyte densities below 100 gametocytes/mL[34] and a few samples considered to be gametocyte negative by both microscopy and QT-NASBA (Chapter 4) were even found to be infectious in Burkina Faso[25] and Kenya[34,35]. In these cases, RNA degradation during sample collection or processing may explain why in mosquito infections from apparently gametocyte negative samples[36] may still infect mosquitoes. From these combined data, it is evident that mosquito infections can occur at extremely low gametocyte densities. This phenomenon is very important, possibly explained by the

clustering of gametocytes in blood meal[37] that may be influenced by adhesive 'nanotubes[38], thus requires further study in field and laboratory settings. RNA extraction controls show the potential disturbing influence of RNA degradation/failure to extract RNA.

Importantly, we also observed that high gametocyte densities fail to infect mosquito. Although higher gametocyte densities favor mosquito infection[28,34], a positive association with infectiousness is generally found to be weak[22,39-42]. There may be several explanations. Theoretically, only two gametocytes (male and female) in 2μL of blood meal are sufficiently to infect a single mosquito but the reality might be different under natural conditions. In individuals living in malaria endemic areas, the gametocyte sex ratio is female biased[43-47] with one male gametocyte for 3 to 4 female gametocytes[26,48] but this ratio can vary significantly during the course of individual infections and may affect intra-individual transmission dynamics[48,49]. The non-infectiousness of high gametocyte densities may be related to this and/or reflect the presence of transmission reducing immunity that affects the success of transmission from man to mosquito[50-56].

7.3. The relevance of sexual stage immunity for the reduction of malaria transmission

Immunity may act against gametocyte-derived antigens in the human circulation by reducing their density and / or infectiousness. In semi-immune individuals, a 3-fold reduction of *P. falciparum* gametocyte prevalence has been associated with non-specific anti-gametocyte antibodies[30]. Other studies further showed that naturally induced antibodies were capable of targeting younger stage I and IIa gametocytes against PfEMP1 expressed on asexual and infected red blood cells[57,58] but the functional relevance of this response in the field[30] and in the lab remains elusive. Antibodies against gametocyte and gamete surface antigens[59-61] can arrest the development of the sporogonic stage development in the mosquito midgut[51,54-56,62,63], thereby reducing transmission from man to mosquito. This type of immunity may serve as a model for the development of transmission blocking vaccines and possibly could have an impact on malaria control efforts if it can be understood well. However, it is not sufficiently clear to which extent sexual stage immunity is present in individuals living in malaria endemic areas and affecting malaria transmission.

We studied the acquisition of sexual stage immune responses in humans exposed to seasonal malaria transmission (Chapter 6). In a series of cross sectional surveys conducted at 3 month intervals at different points of the transmission season (start, peak and end). A lower proportion of sera showed detectable sexual stage serum antibodies (~28%) compared to asexual stage antibodies (54-65%) likely as a result of the lower prevalence of gametocyte carriers in the area (i.e. a relative lower exposure to gametocyte antigens compared to asexual parasite antigens). The development of immune responses against sexual stage antigens has previously been shown in longitudinal studies to be a function of recent rather than cumulative exposure to gametocytes[64-66] suggesting that this type of immunity is possibly short lived requiring frequent boosting[65-67] for sustained responses over a long period of time. Our study was not designed to monitor gametocyte exposure prior to blood sampling points, making it difficult to study the dynamics of sexual stage immune responses. In our cross-sectional surveys, we used two approaches to investigate the nature of sexual stage immune responses by: i) studying the age and season dependency, ii) comparing asexual and pre-erythrocytic immune responses to those against sexual stage antigens and iii) studying the relationship between concurrent gametocyte carriage and circulating sexual stage antibodies.

Our data show that contrary to the age-related pattern of asexual and pre-erythrocytic antibody responses, an age-dependence in the development of sexual stage immune responses is not apparent. Despite the age-related increase of the seroprevalence for sexual stage antigens from children above 5 years, we found similar prevalences of sexual stage specific immune responses in youngest children and adults. This lack of age effect on sexual stage immune responses is not novel and has been attributed to their possible short immune memory. Similar to anti- GLURP antibodies generally found age[51,68-72] but not season[70,71]related, sexual stage antibody responses could have increased with increasing age without considerable seasonal fluctuations if their immune memory was long-lived. In long-lived immune responses, antibody responses may reach a steady state after repeated exposure with long-lived plasma cells generating persisting antibody production. We do not observe any evidence for a consistent antibody production in older individuals. Instead, sexual stage immune responses show a seasonal response pattern that resembles that of short-lived pre-erythrocytic antibody responses.

$NANP_6$ and Pfs230 immune responses are correlated in our analyses, possibly because both may depend on recent exposure to malaria antigen. It is also possible that the same individuals recently exposed to gametocytes were also recently exposed to sporozoite antigens (earlier in their recent infection). Recent exposure to gametocytes during the transmission season seems to occur in our study area where asexual parasite carriage is generally elevated at that time point[21], and being associated with gametocytes[9,35].

Our data did not show an apparent relationship between the presence of sexual stage antibodies and concurrent gametocytaemias, in line with some[51,73] but not all studies[65]. This lack of an association may be explained by the timing of sampling. Sexual stage antibodies induced before sampling may persist for several weeks after gametocyte clearance[65] but may also be low when gametocytaemia is reduced to sub-microscopic levels, whilst its acquisition in the presence of gametocytes may take longer. This would indicate that the acquisition of sexual stage immune responses may be susceptible to recent[50,64] rather than concurrent exposure to gametocytes. This is a limitation of the study design. Longitudinal studies will be required to plausibly address the issue of the induction of sexual stage immune responses in relation to antigen exposure. Our findings only provide indirect evidence for a short-lived nature of sexual stage immune responses.

Although transmission reducing activity (TRA) was not measured in the same study, both Pfs230 and Pfs48/45 antigens have been acknowledged as targets of natural antibody responses[51,53] that correlate with functional TRA[49, 51,53,55,56]. These natural antibodies mediating TRA have been found in gametocyte carriers in endemic areas[34,52,54] and in individuals shortly after their first exposure to gametocytes, such as travelers or migrants from non endemic to endemic areas or children experiencing their first malaria infections [55,56]. In a recent study (Ouedraogo and others, unpublished data) conducted in the same area as the present study, 15% of sera were able to block/reduce malaria transmission from man to mosquito by 30-97% as measured in the standard membrane feeding assay.

A positive and significant correlation is found between TRA and specific anti-Pfs48/45 antibodies (Figure 2). In some cases, high antibody titers were not associated with blocking of transmission. A next step would be to determine the longevity of TRA in the area and to which extent transmission reducing immunity can be enhanced by transmission blocking vaccines.

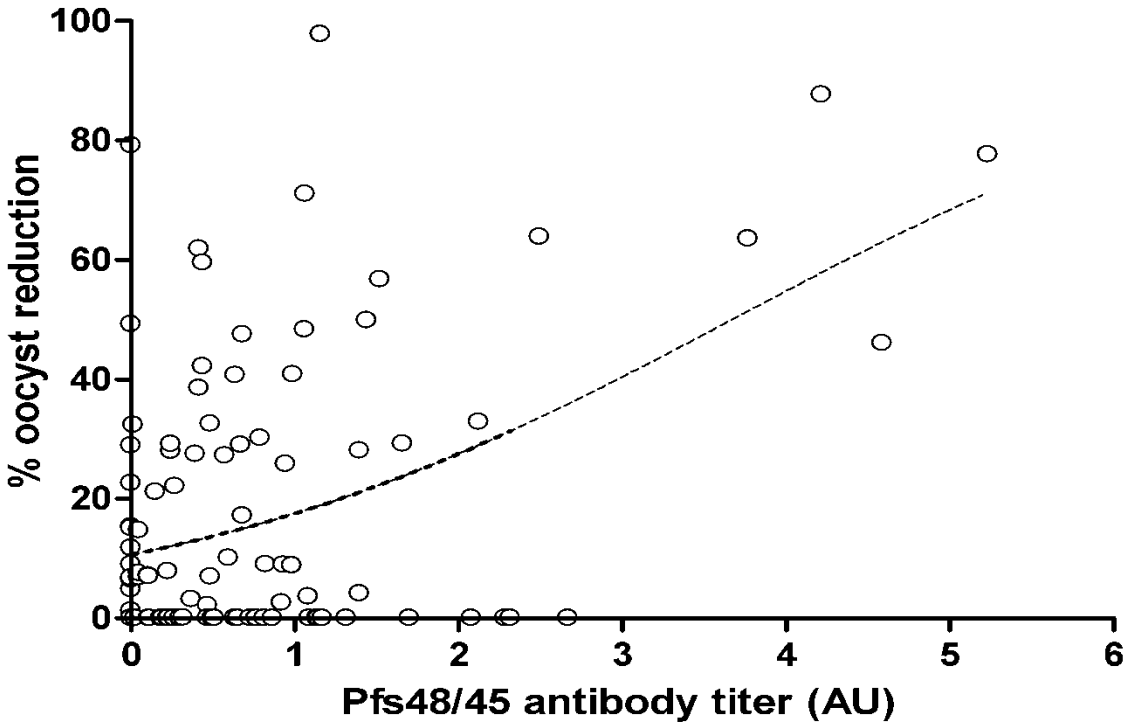

Figure 2: Purified antibodies reduce man-mosquito transmission by 30-97% while TRA correlates (Spearman correlation = 024, p = 0.03) with Pfs48/45 antibody levels

The dashed line represents the best fit for the association between Pfs48/45 antibody concentration and % oocyst reduction; % oocyst reduction = 100/1+10$^{((3.673 - Pfs48/45\ AU)*0.2517)}$

7.4. Malaria control and future directions

There is renewed interest in local malaria elimination leading toward more widespread eradication. This interest is encouraged by the recent declines observed in the incidence of clinical cases and deaths in some of sub-Saharan endemic countries[74-78]. Vector control tools such as LLINs and IRS can reduce transmission[72,79] and the burden of malaria[80,81] and the WHO has justifiably called to scale up the use of these measures. However, neither vector control nor standard approaches to provide efficacious antimalarial drugs to symptomatic malaria cases have resulted in malaria elimination in any of the areas where they were employed, either alone or in combination. Additional tools that target the human side of malaria transmission are likely to be needed to interrupt the transmission cycle. The great challenge is to clear the malaria parasites from populations or to reduce the transmission of

gametocytes from man to mosquito. Interventions that aim to prevent transmission require a thorough understanding of the human infectious reservoir. Most of the studies presented in this thesis can be seen in this light: increasing our understanding of the human infectious reservoir, its dynamics and factors influencing the association between asexual parasites, gametocytes, immune responses and malaria transmission to mosquitoes. Our findings indicate that the infectious reservoir comprises all age groups, symptomatic and asymptomatic parasite carriers harboring microscopic or submicroscopic gametocyte densities. This implies that transmission reducing interventions should target the whole population. The necessity for a high coverage is underlined by the basic reproduction number of malaria, which may be >100 in populations where malaria vectors are present [82]. In other words, a single infectious individuals can cause dozens up to >100 secondary cases in a malaria-naïve population, for instance a population several years after successful malaria elimination[82]. Several interventions are available or will become available for malaria transmission control.

Reducing the human gametocytaemic population

Asymptomatic carriers of the parasite are important contributors to continuous malaria transmission and remain unaffected by conventional treatment approaches where drug administration is restricted to those with symptomatic malaria[83]. The efficacy of drug interventions will therefore depend on the proportion of parasite carriers that seek treatment. In situations where parasites are commonly carried asymptomatically, as was the case in all studies in this thesis, an alternative strategy is needed to include all parasite carriers. This strategy may comprise mass drug administration (MDA) where individuals receive a curative dose of antimalarials regardless of symptoms for parasite clearance[3,84]. Treatment of individuals with artemisinin derivatives should be recommended for this purpose because they effectively reduceor prevent young gametocyte stages, rapidly clear the asexual parasites from which gametocytes are derived and considerably reduce infectiousness after treatment compared to non-ACT treatment[85,86]. These interventions should be implemented at selected optimal periods. In seasonal malaria transmission settings, e.g. Burkina Faso, the optimal time of year for mass therapy to reduce the overall prevalence of asexual parasitaemia as an endpoint should be during the dry season[87]. Community interventions to target asymptomatic carriers can reduce the parasite

prevalence up to 75% as reported recently [88-90]. The predicted efficacy, optimal delivery strategy and drug of choice will depend on the transmission context. There is currently too little experience with MDA, especially in African settings, to judge the potential of this public health tool.

Reducing the infectiousness of the malaria parasite

A reduction in malaria transmission can also be achieved through the reduction of the parasite infectiousness. A malaria transmission blocking vaccine (MTBV) reduces the number of mosquitoes that are infected by a gametocytaemic vaccinated individual. As a consequence, an MTBV reduces the proportion of infected mosquitoes in a population and thereby the chance for individuals to become infected and the burden of disease[91]. Considerable progress has been made in the development of MTBV and several candidates are currently in the pipeline for clinical trials. Because MTBV targets are transmission stages that cause no clinical symptoms, they are generally considered altruistic with no personal benefit as they do not result in clinical protection for the vaccinee. At the public health level, a MTBV may be seen as advantageous since vaccinated individuals would lead to reductions in the reproduction rate of the parasite and in certain circumstances the spread of drug resistant parasite strains.

The wide-spread nature of *Plasmodium falciparum* gametocytes described in this thesis and the contribution of very low gametocyte densities to transmission illustrate the importance of MTBV while the rapid induction of natural TRA in association with naturally acquired immune responses to sexual stage antigens illustrates its relevance to controlling malaria transmission in natural settings. Clearly, the benefits of such a vaccine would be enhanced when implemented in an integrated control strategy, although, as with any vaccine, issues such as the longevity of any immunity induced and the capacity for boosting of that immunity by naturally-acquired infection will affect its longer-term impact.

References

1. Carter R, Mendis KN, Miller LH, Molineaux L, Saul A. Malaria transmission-blocking vaccines--how can their development be supported? *Nat Med* 2000;**6**(3)**:**241-4.
2. Drakeley C, Sutherland C, Bousema JT, Sauerwein RW, Targett GA. The epidemiology of *Plasmodium falciparum* gametocytes: weapons of mass dispersion. *Trends Parasitol* 2006;**22**(9)**:**424-30.

3. von Seidlein L, Walraven G, Milligan PJ, et al. The effect of mass administration of sulfadoxine-pyrimethamine combined with artesunate on malaria incidence: a double-blind, community-randomized, placebo-controlled trial in The Gambia. *Trans R Soc Trop Med Hyg* 2003;**97**(2)**:**217-25.
4. Akim NIJ, Drakely C, Kingo T, Simon B, Senkoro K, Sauerwein RW. Dynamics of *P. falciparum* gametocytemia in symptomatic patients in an area of intense perennial transmission in Tanzania. *American Journal of Tropical Medicine and Hygiene* 2000;**63**(3-4)**:**199-203.
5. Bousema JT, Gouagna LC, Drakeley CJ, et al. *Plasmodium falciparum* gametocyte carriage in asymptomatic children in western Kenya. *Malar J* 2004;**3**(1)**:**18.
6. Bruce MC, Alano P, Duthie S, Carter R. Commitment of the malaria parasite *Plasmodium falciparum* to sexual and asexual development. *Parasitology* 1990;**100:**191-200.
7. Price R, Nosten F, Simpson JA, et al. Risk factors for gametocyte carriage in uncomplicated *falciparum* malaria. *Am J Trop Med Hyg* 1999;**60**(6)**:**1019-23.
8. Ouedraogo AL, Bousema T, de Vlas SJ, et al. The plasticity of *Plasmodium falciparum* gametocytaemia in relation to age in Burkina Faso. *Malar J* 2010;**9:**281.
9. Ouedraogo AL, Schneider P, de Kruijf M, et al. Age-dependent distribution of *Plasmodium falciparum* gametocytes quantified by Pfs25 real-time QT-NASBA in a cross-sectional study in Burkina Faso. *Am J Trop Med Hyg* 2007;**76**(4)**:**626-30.
10. Abdel-Wahab A, Abdel-Muhsin AA, Ali E, et al. Dynamics of gametocytes among *Plasmodium falciparum* clones in natural infections in an area of highly seasonal transmission. *Journal of Infectious Diseases* 2002;**185**(12)**:**1838-1842.
11. Drakeley CJ, Akim NI, Sauerwein RW, Greenwood BM, Targett GA. Estimates of the infectious reservoir of *Plasmodium falciparum* malaria in The Gambia and in Tanzania. *Trans R Soc Trop Med Hyg* 2000;**94**(5)**:**472-6.
12. Drakeley CJ, Carneiro I, Reyburn H, et al. Altitude-dependent and -independent variations in *Plasmodium falciparum* prevalence in northeastern Tanzania. *J Infect Dis* 2005;**191**(10)**:**1589-98.
13. Molineaux L, Gramiccia G. Parasitology, chapter five in "THE GARKI PROJECT. Research on the Epidemiology and Control of Malaria in the Sudan Savanna of West Africa". *WHO report* 1980**:**109-172.
14. van der Kolk M, Tebo AE, Nimpaye H, Ndombol DN, Sauerwein RW, Eling WM. Transmission of *Plasmodium falciparum* in urban Yaounde, Cameroon, is seasonal and age-dependent. *Trans R Soc Trop Med Hyg* 2003;**97**(4)**:**375-9.
15. Boudin C, Lyannaz J, Bosseno MF, Carnevale P, Ambroise-Thomas P. Epidemiology of *Plasmodium falciparum* in a rice field and a savanna area in Burkina Faso: seasonal fluctuations of gametocytaemia and malarial infectivity. *Ann Trop Med Parasitol* 1991;**85**(4)**:**377-85.
16. Drakeley CJ, Secka I, Correa S, Greenwood BM, Targett GA. Host haematological factors influencing the transmission of *Plasmodium falciparum* gametocytes to Anopheles gambiae s.s. mosquitoes. *Trop Med Int Health* 1999;**4**(2)**:**131-8.
17. Stepniewska K, Price RN, Sutherland CJ, et al. *Plasmodium falciparum* gametocyte dynamics in areas of different malaria endemicity. *Malar J* 2008;**7:**249.
18. Paul RE, Diallo M, Brey PT. Mosquitoes and transmission of malaria parasites - not just vectors. *Malar J* 2004;**3:**39.
19. Billingsley PF, Snook LS, Johnston VJ. Malaria parasite growth is stimulated by mosquito probing. *Biol Lett* 2005;**1**(2)**:**185-9.
20. Shutler D, Reece SE, Mullie A, Billingsley PF, Read AF. Rodent malaria parasites *Plasmodium* chabaudi and P. vinckei do not increase their rates of gametocytogenesis in response to mosquito probing. *Proc Biol Sci* 2005;**272**(1579)**:**2397-402.

21. Ouedraogo AL, de Vlas SJ, Nebie I, et al. Seasonal patterns of *Plasmodium falciparum* gametocyte prevalence and density in a rural population of Burkina Faso. *Acta Trop* 2008;**105**(1)**:**28-34.
22. Boudin C, Olivier M, Molez JF, Chiron JP, Ambroise-Thomas P. High human malarial infectivity to laboratory-bred Anopheles gambiae in a village in Burkina Faso. *Am J Trop Med Hyg* 1993;**48**(5)**:**700-6.
23. Cattani JA, Tulloch JL, Vrbova H, et al. The epidemiology of malaria in a population surrounding Madang, Papua New Guinea. *American Journal of Tropical Medicine and Hygiene* 1986;**35**(1)**:**3-15.
24. Bousema T, Okell L, Shekalaghe S, et al. Revisiting the circulation time of *Plasmodium falciparum* gametocytes: molecular detection methods to estimate the duration of gametocyte carriage and the effect of gametocytocidal drugs. *Malar J* 2010;**9:**136.
25. Eichner M, Diebner HH, Molineaux L, Collins WE, Jeffery GM, Dietz K. Genesis, sequestration and survival of *Plasmodium falciparum* gametocytes: parameter estimates from fitting a model to malariatherapy data. *Trans R Soc Trop Med Hyg* 2001;**95**(5)**:**497-501.
26. Sinden RE. Sexual development of malarial parasites. *Adv Parasitol* 1983;**22:**153-216.
27. Drakeley CJ, Flobbe K, Greenwood BM, Targett GA. *Plasmodium falciparum* gametocytes in Gambian adults. *Ann Trop Med Parasitol* 2000;**94**(4)**:**399-401.
28. Tchuinkam T, Mulder B, Dechering K, et al. Experimental infections of Anopheles gambiae with *Plasmodium falciparum* of naturally infected gametocyte carriers in Cameroon: factors influencing the infectivity to mosquitoes. *Trop Med Parasitol* 1993;**44**(4)**:**271-6.
29. Taylor LH, Read AF. Why so few transmission stages? Reproductive restraint by malaria parasites. *Parasitol Today* 1997;**13**(4)**:**135-40.
30. Baird JK, Jones TR, Purnomo, Masbar S, Ratiwayanto S, Leksana B. Evidence for specific suppression of gametocytemia by *Plasmodium falciparum* in residents of hyperendemic Irian Jaya. *Am J Trop Med Hyg* 1991;**44**(2)**:**183-90.
31. Schneider P, Schoone G, Schallig H, et al. Quantification of *Plasmodium falciparum* gametocytes in differential stages of development by quantitative nucleic acid sequence-based amplification. *Mol Biochem Parasitol* 2004;**137**(1)**:**35-41.
32. Schoone GJ, Oskam L, Kroon NCM, Schallig HDFH, Omar SA. Detection and quantification of *Plasmodium falciparum* in blood samples using quantitative nucleic acid sequence-based amplification. *Journal of Clinical Microbiology* 2000;**38**(1)**:**4072-4075.
33. Shekalaghe SA, Bousema JT, Kunei KK, et al. Submicroscopic *Plasmodium falciparum* gametocyte carriage is common in an area of low and seasonal transmission in Tanzania. *Trop Med Int Health* 2007;**12**(4)**:**547-53.
34. Schneider P, Bousema JT, Gouagna LC, et al. Submicroscopic *Plasmodium falciparum* gametocyte densities frequently result in mosquito infection. *Am J Trop Med Hyg* 2007;**76**(3)**:**470-4.
35. Ouedraogo AL, Bousema T, Schneider P, et al. Substantial contribution of submicroscopical *Plasmodium falciparum* gametocyte carriage to the infectious reservoir in an area of seasonal transmission. *PLoS One* 2009;**4**(12)**:**e8410.
36. Bousema JT. Interrupting malaria transmission: the effects of drugs and immunity on the transmissibility of *Plasmodium falciparum*. *Thesis Radboud University Nijmegen:1.* 2007.
37. Pichon G, Awono-Ambene HP, Robert V. High heterogeneity in the number of *Plasmodium falciparum* gametocytes in the bloodmeal of mosquitoes fed on the same host. *Parasitology* 2000;**121 (Pt 2):**115-20.
38. Rupp I, Sologub L, Williamson KC, et al. Malaria parasites form filamentous cell-to-cell connections during reproduction in the mosquito midgut. *Cell Res* 2010.
39. Graves PM. Studies on the use of a membrane feeding technique for infecting Anopheles gambiae with *Plasmodium* falciparum. *Trans R Soc Trop Med Hyg* 1980;**74**(6)**:**738-42.

40. Graves PM, Burkot TR, Carter R, et al. Measurement of malarial infectivity of human populations to mosquitoes in the Madang area, Papua, New Guinea. *Parasitology* 1988;**96 (Pt 2):**251-63.
41. Muirhead-Thomson RC. Low gametocyte thresholds of infection of Anopheles with *Plasmodium* falciparum; a significant factor in malaria epidemiology. *Br Med J* 1954;**4853:**68-70.
42. Mulder B, Tchuinkam T, Dechering K, et al. Malaria transmission-blocking activity in experimental infections of Anopheles gambiae from naturally infected *Plasmodium falciparum* gametocyte carriers. *Trans R Soc Trop Med Hyg* 1994;**88**(1)**:**121-5.
43. Read AF, Narara A, Nee S, Keymer AE, Day KP. Gametocyte sex ratios as indirect measures of outcrossing rates in malaria. *Parasitology* 1991;**104:**387-395.
44. Robert V, Boudin C. [Biology of man-mosquito *Plasmodium* transmission]. *Bull Soc Pathol Exot* 2003;**96**(1)**:**6-20.
45. Robert V, Read AF, Essong J, et al. Effect of gametocyte sex ratio on infectivity of *Plasmodium falciparum* to Anopheles gambiae. *Trans R Soc Trop Med Hyg* 1996;**90**(6)**:**621-4.
46. Sowunmi A, Balogun ST, Gbotosho GO, Happi CT. *Plasmodium falciparum* gametocyte sex ratios in symptomatic children treated with antimalarial drugs. *Acta Trop* 2009;**109**(2)**:**108-17.
47. Sowunmi A, Gbotosho GO, Happi CT, Folarin OA, Balogun ST. Population structure of *Plasmodium falciparum* gametocyte sex ratios in malarious children in an endemic area. *Parasitol Int* 2009;**58**(4)**:**438-43.
48. Paul REL, Coulson TN, Raibaud A, Brey PT. Sex determination in malaria parasites. *Science* 2000;**287:**128-131.
49. Robert V, Sokhna CS, Rogier C, Ariey F, Trape JF. Sex ratio of *Plasmodium falciparum* gametocytes in inhabitants of Dielmo, Senegal. *Parasitology* 2003;**127**(Pt 1)**:**1-8.
50. Bousema T, Sutherland CJ, Churcher TS, et al. Human immune responses that reduce the transmission of *Plasmodium falciparum* in African populations. *Int J Parasitol*.
51. Drakeley CJ, Bousema JT, Akim NI, et al. Transmission-reducing immunity is inversely related to age in *Plasmodium falciparum* gametocyte carriers. *Parasite Immunol* 2006;**28**(5)**:**185-90.
52. Healer J, McGuinness D, Carter R, Riley E. Transmission-blocking immunity to *Plasmodium falciparum* in malaria-immune individuals is associated with antibodies to the gamete surface protein Pfs230. *Parasitology* 1999;**119 (Pt 5):**425-33.
53. Mendis KN, Munesinghe YD, de Silva YN, Keragalla I, Carter R. Malaria transmission-blocking immunity induced by natural infections of *Plasmodium vivax* in humans. *Infect Immun* 1987;**55**(2)**:**369-72.
54. Roeffen W, Geeraedts F, Eling W, et al. Transmission blockade of *Plasmodium falciparum* malaria by anti-Pfs230-specific antibodies is isotype dependent. *Infect Immun* 1995;**63**(2)**:**467-71.
55. Roeffen W, Mulder B, Teelen K, et al. Association between anti-Pfs48/45 reactivity and *P. falciparum* transmission-blocking activity in sera from Cameroon. *Parasite Immunol* 1996;**18**(2)**:**103-9.
56. van der Kolk M, de Vlas SJ, Sauerwein RW. Reduction and enhancement of *Plasmodium falciparum* transmission by endemic human sera. *Int J Parasitol* 2006;**36**(10-11)**:**1091-5.
57. Piper KP, Hayward RE, Cox MJ, Day KP. Malaria transmission and Naturally acquired immunity to PfEMP-1. *Infection and Immunity* 1999;**67**(12)**:**6369-6374.
58. Saeed M, Roeffen W, Alexander N, Drakeley CJ, Targett GA, Sutherland CJ. *Plasmodium falciparum* antigens on the surface of the gametocyte-infected erythrocyte. *PLoS One* 2008;**3**(5)**:**e2280.
59. Kumar N. Target antigens of malaria transmission blocking immunity exist as a stable membrane bound complex. *Parasite Immunol* 1987;**9**(3)**:**321-35.

60. Vermeulen AN, Ponnudurai T, Beckers PJ, Verhave JP, Smits MA, Meuwissen JH. Sequential expression of antigens on sexual stages of *Plasmodium falciparum* accessible to transmission-blocking antibodies in the mosquito. *J Exp Med* 1985;**162**(5)**:**1460-76.
61. Vermeulen AN, Roeffen WF, Henderik JB, Ponnudurai T, Beckers PJ, Meuwissen JH. *Plasmodium falciparum* transmission blocking monoclonal antibodies recognize mon*oval*ently expressed epitopes. *Dev Biol Stand* 1985;**62:**91-7.
62. Bousema JT, Drakeley CJ, Sauerwein RW. Sexual-stage antibody responses to *P. falciparum* in endemic populations. *Curr Mol Med* 2006;**6**(2)**:**223-9.
63. Graves PM, Wirtz RA, Carter R, Burkot TR, Looker M, Targett GA. Naturally occurring antibodies to an epitope on *Plasmodium falciparum* gametes detected by monoclonal antibody-based competitive enzyme-linked immunosorbent assay. *Infect Immun* 1988;**56**(11)**:**2818-21.
64. Bousema JT, Roeffen W, van der Kolk M, et al. Rapid onset of transmission-reducing antibodies in javanese migrants exposed to malaria in papua, indonesia. *Am J Trop Med Hyg* 2006;**74**(3)**:**425-31.
65. Bousema T, Roeffen W, Meijerink H, et al. The dynamics of naturally acquired immune responses to *Plasmodium falciparum* sexual stage antigens Pfs230 & Pfs48/45 in a low endemic area in Tanzania. *PLoS One*;**5**(11)**:**e14114.
66. Ranawaka MB, Munesinghe YD, de Silva DM, Carter R, Mendis KN. Boosting of transmission-blocking immunity during natural *Plasmodium vivax* infections in humans depends upon frequent reinfection. *Infect Immun* 1988;**56**(7)**:**1820-4.
67. Mendis KN, David PH, Carter R. Human immune responses against sexual stages of malaria parasites: considerations for malaria vaccines. *Int J Parasitol* 1990;**20**(4)**:**497-502.
68. Cohen S, Butcher GA. Serum antibody in acquired malarial immunity. *Trans R Soc Trop Med Hyg* 1971;**65**(2)**:**125-35.
69. Cohen S, Butcher GA, Mitchell GH, Deans JA, Langhorne J. Acquired immunity and vaccination in malaria. *Am J Trop Med Hyg* 1977;**26**(6 Pt 2)**:**223-32.
70. Dodoo D, Theisen M, Kurtzhals JA, et al. Naturally acquired antibodies to the glutamate-rich protein are associated with protection against *Plasmodium falciparum* malaria. *J Infect Dis* 2000;**181**(3)**:**1202-5.
71. Dziegiel M, Rowe P, Bennett S, et al. Immunoglobulin M and G antibody responses to *Plasmodium falciparum* glutamate-rich protein: correlation with clinical immunity in Gambian children. *Infect Immun* 1993;**61**(1)**:**103-8.
72. Molineaux L, Gramiccia G. THE GARKI PROJECT. Research on the Epidemiology and Control of Malaria in the Sudan Savanna of West Africa. *WHO report* 1980**:**311.
73. Bousema JT, Drakeley CJ, Kihonda J, et al. A longitudinal study of immune responses to *Plasmodium falciparum* sexual stage antigens in Tanzanian adults. *Parasite Immunol* 2007;**29**(6)**:**309-17.
74. Bouyou-Akotet MK, Mawili-Mboumba DP, Kendjo E, et al. Evidence of decline of malaria in the general hospital of Libreville, Gabon from 2000 to 2008. *Malar J* 2009;**8**(1)**:**300.
75. Ceesay SJ, Casals-Pascual C, Erskine J, et al. Changes in malaria indices between 1999 and 2007 in The Gambia: a retrospective analysis. *Lancet* 2008;**372**(9649)**:**1545-54.
76. Muller O, Ye M, Louis VR, Sie A. Malaria in sub-Saharan Africa. *Lancet* 2009;**373**(9658)**:**122.
77. Okiro EA, Hay SI, Gikandi PW, et al. The decline in paediatric malaria admissions on the coast of Kenya. *Malar J* 2007;**6:**151.
78. O'Meara WP, Mangeni JN, Steketee R, Greenwood B. Changes in the burden of malaria in sub-Saharan Africa. *Lancet Infect Dis* 2010;**10**(8)**:**545-55.
79. Killeen GF, Smith TA, Ferguson HM, et al. Preventing childhood malaria in Africa by protecting adults from mosquitoes with insecticide-treated nets. *PLoS Med* 2007;**4**(7)**:**e229.
80. Lengeler C. Insecticide-treated bed nets and curtains for preventing malaria. *Cochrane Database Syst Rev* 2004(2)**:**CD000363.

81. Sharp BL, Kleinschmidt I, Streat E, et al. Seven years of regional malaria control collaboration--Mozambique, South Africa, and Swaziland. *Am J Trop Med Hyg* 2007;**76**(1)**:**42-7.
82. Smith DL, McKenzie FE, Snow RW, Hay SI. Revisiting the basic reproductive number for malaria and its implications for malaria control. *PLoS Biol* 2007;**5**(3)**:**e42.
83. Okell LC, Drakeley CJ, Bousema T, Whitty CJ, Ghani AC. Modelling the impact of artemisinin combination therapy and long-acting treatments on malaria transmission intensity. *PLoS Med* 2008;**5**(11)**:**e226; discussion e226.
84. Kaneko A, Taleo G, Kalkoa M, Yamar S, Kobayakawa T, Bjorkman A. Malaria eradication on islands. *Lancet* 2000;**356**(9241)**:**1560-4.
85. Bousema JT, Schneider P, Gouagna LC, et al. Moderate effect of artemisinin-based combination therapy on transmission of *Plasmodium* falciparum. *J Infect Dis* 2006;**193**(8)**:**1151-9.
86. Sutherland CJ, Ord R, Dunyo S, et al. Reduction of malaria transmission to Anopheles mosquitoes with a six-dose regimen of co-artemether. *PLoS Med* 2005;**2**(4)**:**e92.
87. Shekalaghe S. Reducing malaria transmission in low endemic areas in tanzania by antimalarial drugs: Epidemiological and Intervention Studies. *Thesis Radboud University Nijmegen:1.* 2010.
88. Dicko A, Diallo AI, Tembine I, et al. Intermittent Preventive Treatment of Malaria Provides Substantial Protection against Malaria in Children Already Protected by an Insecticide-Treated Bednet in Mali: A Randomised, Double-Blind, Placebo-Controlled Trial. *PLoS Med* 2011;**8**(2)**:**e1000407.
89. Konate AT, Yaro JB, Ouedraogo AZ, et al. Intermittent Preventive Treatment of Malaria Provides Substantial Protection against Malaria in Children Already Protected by an Insecticide-Treated Bednet in Burkina Faso: A Randomised, Double-Blind, Placebo-Controlled Trial. *PLoS Med* 2011;**8**(2)**:**e1000408.
90. Schellenberg D, Menendez C, Aponte JJ, et al. Intermittent preventive antimalarial treatment for Tanzanian infants: follow-up to age 2 years of a randomised, placebo-controlled trial. *Lancet* 2005;**365**(9469)**:**1481-3.
91. Sauerwein RW. Malaria transmission-blocking vaccines: the bonus of effective malaria control. *Microbes Infect* 2007;**9**(6)**:**792-5.

Chapter 8

Summary
Résumé
Samenvatting

Summary

Malaria is the most frequent parasitic disease affecting humans around the globe. Amongst the five species of *Plasmodium* responsible for human malaria, *Plasmodium falciparum* is the most pathogenic, causing up to 800,000 deaths per year. The parasite life cycle comprises several developmental stages. Asexual blood stage parasites multiply in red blood cells, causing clinical disease. Sexual stage parasites, called gametocytes, develop within red blood cells from a small proportion of asexual blood stage parasites but they do not cause symptoms and are exclusively responsible for transmitting the infection to female anopheline mosquitoes during blood feeding. Infected mosquitoes can in turn infect other humans during subsequent feeds. Although efforts are being made to target sexual stages as part of an integrated malaria control strategy, the extent of the human reservoir of gametocytes is poorly defined, representing a major obstacle to the future success of such efforts.

This thesis aimed to accurately quantify gametocytes in humans and to investigate their infectiousness under natural conditions, in order to contribute to our understanding of the biology of malaria transmission, as well as to inform control strategists about the best way to target gametocytes in the context of malaria control. For this purpose, we designed our studies to determine: i) age-specific and temporal changes in gametocyte prevalence (Chapter 2, 3&4); ii) the contribution of sub-microscopic gametocyte densities to the human infectious reservoir (Chapter 5) and iii) the dynamics of naturally acquired transmission-reducing immunity (Chapter 6).

Our findings show that microscopically detectable gametocyte concentrations are most prevalent in children and during the wet season. This is primarily due to the higher asexual parasite densities found both in children and in the wet season, although we found indications that human and mosquito immune factors may also play a role (Chapter 2). The use of the molecular QT-NASBA technique, a much more sensitive method than microscopy for detecting low gametocyte concentrations, indicates that gametocyte prevalence is 3.3 fold higher (70%) than estimated based on microscopy (17%), revealing the widespread occurrence of sub-microscopic gametocyte densities not only in children but also in asymptomatic adults (Chapter 3). Since gametocyte occurrence seems to be positively related to high densities of asexual parasites, it is surprising that sub-microscopic

gametocytes are highly prevalent in adults, who commonly harbour low asexual parasite densities. This paradox is explored in more detail in Chapter 4 of this thesis where we describe increased gametocyte production rates in adults. The proportion of gametocytes relative to the total parasite population increases with age, suggesting that gametocyte commitment may be higher in adults. From an evolutionary point of view, this would be an advantageous strategy for parasites: if acquired human immunity reduces asexual parasite growth rates, parasites increase their investment in gametocytes to maximize the chance of transmission to mosquitoes. Although transmission success is positively associated with gametocyte density, we showed that low gametocyte densities are nevertheless highly efficient in transmitting the infection. The contribution of sub-microscopic gametocytes to the infectious reservoir is found to be substantial (Chapter 5). Although low density gametocytaemias result in a lower proportion of infected mosquitoes, their relevance for malaria transmission is compensated for by their high frequency in the whole population. As a consequence, the overall contribution of sub-microscopic gametocyte densities to malaria transmission is approximately 25%. These findings on gametocyte infectiousness are derived from children and cannot therefore be extrapolated to the whole population. However, older age-groups should not be seen as having negligible responsibility for malaria transmission as they represent a substantial proportion of the general population and they harbour a remarkably high prevalence of (low density) gametocytes.

Despite efficient malaria transmission in our study area, gametocytes from our volunteers' blood samples were not always infectious to mosquitoes in experimental feeding assays. Their infectiousness may be affected by factors including naturally acquired transmission-reducing immunity. In Chapter 6, we show that antibody responses against transmission blocking vaccine candidate antigens are naturally acquired in our study population and that their induction is a function of recent rather than cumulative exposure to malaria. These immune responses are not necessarily highest in adults and may reduce man-to-mosquito transmission by preventing the fertilization and development of the parasite in the mosquito midgut.

In conclusion, the work from this thesis reveals an age- and season-structured allocation of gametocytes with a high prevalence and relevance of sub-microscopic gametocyte densities. Whilst immune responses to sexual stage antigens are naturally

acquired in the study populations, transmission is efficiently maintained. This emphasizes the need for malaria transmission control strategies to reduce malaria transmission and ultimately the burden of malaria disease.

Résumé

Le paludisme demeure la parasitose la plus fréquente affectant l'homme. Des cinq espèces responsable du paludisme humain, *Plasmodium falciparum* est la plus pathogénique responsable d'au moins 800 000 décès par an. Le cycle du parasite comprend plusieurs formes parasitaires parmi lesquelles la forme asexuée dont la multiplication au sein des globules rouges provoque les manifestations cliniques de la maladie. Les formes sexuées, encore appelés gamétocytes sont produites chez l'hôte humain à partir d'une petite proportion de formes asexuées. Contrairement aux formes asexuées, les gamétocytes ne causent pas de symptômes et sont exclusivement responsables de la transmission de l'infection à Anophèles femelle lors de la prise de son repas sanguin. Le moustique infecté peut ainsi en retour infecter d'autres êtres humains lors de ses prochains repas sanguin. Quoique à travers de nombreux programmes intégrés de control du paludisme, des efforts sont entrepris pour éliminer les gamétocytes, le manque de clarté sur l'étendu du réservoir humain de gamétocytes constitue un obstacle majeur quand à l'efficacité de tels efforts.

La présente thèse avait pour but de déterminer l'étendu du réservoir de gamétocytes chez l'homme et d'évaluer leur infectivité dans des conditions naturelles afin de collecter et rendre disponible des informations utiles aux stratégies de control du paludisme et éventuellement à son élimination. Pour cela, nous avons planifier nos activités de recherche comme suit afin de : i) déterminer les variations des taux de portage gamétocytaire sous l'effet de l'âge et de la saison (Chapitre 2, 3&4); ii) déterminer la contribution des faibles densités de gamétocytes au réservoir infectieux (Chapitre 5) et enfin iii) étudier la dynamique de l'immunité naturelle acquise réduisant la transmission du paludisme (Chapitre 6).

Nos résultats montrent que les gamétocytes quantifiés par la microscopie sont assez prévalentes chez l'enfant mais aussi pendant la saison de pluie. Cette observation s'expliquerait par la presence de fortes densités de parasites asexués chez l'enfant et pendant la saison de pluie quoique nos résultats laissent croire que des facteurs immunitaires chez l'homme et le vecteur puissent jouer un rôle (Chapitre 2). L'utilisation de la technique QT-NASBA, une technique moléculaire plus sensible que la microscopie, montre que la prévalence des gamétocytes était 3,3 fois plus élevée (70%) que celle basée sur la microscopie (17%) révélant ainsi une forte fréquence de faibles densités

gamétocytaires chez l'enfant mais aussi chez l'adulte (Chapitre 3). Cette observation nous est plutôt apparue surprenant vue que les fortes densités de parasites asexués, favorables à la production de gamétocytes, sont moins fréquentes chez l'adulte. En étudiant le phénomène de plus près dans le Chapitre 4, nous avons observé une augmentation de la production gamétocytaire chez l'adulte à partir de faibles densités de parasites asexués. Effectivement, dans ce Chapitre 4, nous montrons que la proportion de gamétocytes relative à la population totale de parasites augmentait avec l'âge, ce qui suggère que la faculté du parasite asexué à se transformer en parasite sexué augmente avec l'âge de l'individu. D'un point de vu évolutif, il pourrait s'agir d'une stratégie de survie pour le parasite. En effet, le parasite asexué gagnerait pour sa survie en se transformant en parasite sexuée pour être transferré chez le moustique plutôt que de subir une réduction de son taux de multiplication sous une pression stressante chez l'adulte.

Alors qu'une forte densité gamétocytaire apparait être un facteur clé pour la transmission home-moustique, nous montré au Chapitre 5 que les faibles densités de gamétocytes pouvaient aussi être efficaces pour transmettre l'infection au moustique. Même si les individus porteurs de faibles gametocytémies infectent moins de moustiques, ils restent importants pour la transmission du paludisme car leur proportion dans la population générale est assez importante à tel point que leur part de contribution au réservoir infectieux avoisinerait les 25%. Ces résultats ne peuvent être extrapolés à toute la population car provenant de données receuillies seulement au sein d'une population d'enfants. Cependant il est prudent de souligner que les individus plus âgés ne sont pas à négliger en matière de transmission du paludisme vue leur représentativité dans la population et leur forte susceptibilité à porter des gamétocytes quoique de faible densité.

Malgré une transmission intense observée dans notre zone d'étude, nous montrons que des échantillons de sang infectés de gamétocytes n'étaient pas toujours infectieux lors de nos séances d'infection expérimentales de moustiques. En effet, l'infectivité du gamétocyte est sujet à des facteurs dont l'immunité naturelle réduisant la transmission. Au Chapitre 6, nous montrons que des anticorps dirigées contre des antigènes candidats au vaccin bloquant la transmission du paludisme étaient naturellement acquises dans notre population d'étude. Nous montrons que ce type d'immunité se développe à la suite d'une exposition récente aux gamétocytes plutôt qu'à une exposition répétée et prolongée. Cette

réponse immunitaire est plutôt similaire à celle observée chez les individus plus âgés et est susceptible de réduire la transmission homme-moustique en prévenant la fécondation et le développement des formes sexuées du parasite chez le moustique.

Pour conclure, le travail décrit dans cette thèse met en exergue l'existence d'une allocation structurée des gamétocytes en fonction de l'âge et de la saison ainsi qu'une fréquence importante des gamnetocytémies non détectables par la microscopie. Malgré qu'une immunité anti-gamétocytes soit naturellement acquise dans les populations, nous constatons que la transmission du paludisme est remarquablement efficace et continue. Cela démontre quel à point il y a un besoin urgent de stratégies visant à réduire la transmission et conséquemment le fardeau de la maladie.

Samenvatting

Malaria is wereldwijd één van de meest voorkomende parasitaire ziekten bij de mens. Van de vijf *Plasmodium* species die verantwoordelijk zijn voor malaria in de mens, is *Plasmodium falciparum* de meest gevaarlijke, met 800.000 doden per jaar. De levenscyclus van de parasiet bestaat uit verschillende ontwikkelingsstadia. Aseksuele bloedstadium parasieten vermenigvuldigen zich in rode bloedcellen en veroorzaken de symptomen die leiden tot klinische ziekte. Seksueel stadium parasieten, ook wel gametocyten genoemd, ontwikkelen zich in de rode bloedcel uit een klein deel van de aseksuele bloedstadia. Deze gametocyten veroorzaken geen symptomen maar zijn verantwoordelijk voor de transmissie van de parasiet van mens naar de vrouwelijke anopheles mug. Met een nieuw bloedmaal brengen deze geïnfecteerde muggen de parasiet weer over op de mens. Slechts weinig is bekend over het humane reservoir van gametocyten, ondanks vele inspanningen om het seksuele stadium gericht te bestrijden als onderdeel van een geïntegreerde malaria controle strategie. Het gebrek aan kennis beperkt het toekomstig succes van deze dergelijke strategieën.

Het doel van dit proefschrift is het kwantificeren van de gametocyten in de mens en te onderzoeken hoe infectieus deze zijn in natuurlijke omstandigheden. Onze bevindingen dragen bij aan een beter inzicht in de biologie van malaria transmissie en aan betere controle strategieën voor de aanpak van gametocyten in the context van malaria controle of eliminatie. Wij hebben in onze studies daarom het volgende onderzocht: i) leeftijdsspecifieke en tijdelijke veranderingen in gametocyt prevalentie (hoofdstuk 2,3,4); ii) de bijdrage van submicroscopische gametocytemie aan het totale humane infectieuze reservoir (hoofdstuk 5) en iii) de dynamiek van natuurlijk verkregen transmissie reducerende immuniteit (hoofdstuk 6).

Uit onze resultaten blijkt dat het dragen van microscopisch detecteerbare gametocyten vooral voorkomt bij kinderen en tijdens het regenseizoen. Dit is voornamelijk het gevolg van hogere concentraties aseksuele parasieten. Wij vonden echter ook aanwijzingen dat immuun factoren van mens en mug hierin een rol spelen (hoofdstuk 2). Voor onze onderzoeken maakten wij gebruik van de moleculaire QT-NASBA techniek, een

methode die veel sensitiever gametocyten detecteert dan microscopie. Hiermee laten wij zien dat de gametocyt prevalentie 3.3 maal zo hoog is als schattingen gebaseerd op microscopie (70% versus 17%). Dit toont het wijdverspreide voorkomen van submicroscopische gametocyten aan, niet alleen in kinderen maar ook in asymptomatische volwassenen (hoofdstuk 3). Ondanks de positieve relatie tussen aseksuele parasitemie en gametocyten dichtheid, hebben volwassenen, met gewoonlijk lage aantallen aseksuele parasieten, vaak submicroscopische gametocyten. Deze paradox wordt in hoofdstuk 4 in dit proefschrift meer uitgewerkt waarin wij een verhoogde gametocyt productie in volwassenen beschrijven. De verhouding tussen gametocyten en de totale hoeveelheid parasieten verhoogt met oudere leeftijd, wat suggereert dat sexuele differentiatiedruk sterker is in volwassenen. Evolutionair gezien zou dit een overlevingsstrategie van de parasiet zijn: als door verworven immuniteit het aantal aseksuele parasieten in volwassenen verlaagt, verhoogt de parasiet zijn investering in gametocyten, om zijn transmissiekansen te verhogen. Hoewel transmissiesucces positief geassocieerd is met gametocyt concentratie, hebben wij aangetoond dat lage gametocyt concentraties bijzonder infectieus zijn. Hoewel een lage gametocytemie zorgt voor relatief weinig geïnfecteerde muggen, is het voorkomen hiervan relevant voor malaria transmissie vanwege de grote aantallen gametocytendragers. De bijdrage van submicroscopische gametocyten aan het totaal infectieuze reservoir is dus substantieel (hoofdstuk 5) met een totale bijdrage aan malaria transmissie van ongeveer 25%. Bovenstaand onderzoek is enkel verricht in kinderen waardoor de resultaten niet direct geëxtrapoleerd kunnen worden naar de gehele populatie. Echter, oudere leeftijdsgroepen zijn een niet te verwaarlozen factor in malaria transmissie omdat deze een groot deel van de gehele populatie uitmaken en een opvallend vaak submicroscopische gametocytemie hebben.

De gametocyten afkomstig uit bloedmonsters van onze vrijwilligers waren niet altijd infectieus voor muggen in experimentele voedingsassays, ondanks een efficiënte malaria transmissie in ons studiegebied. De besmettelijkheid kan door verschillende factoren zijn verlaagd, zoals door natuurlijk verworven transmissie reducerende immuniteit. In hoofstuk 6 laten we zien dat antilichaam reacties tegen transmissie blokkerende vaccine kandidaat antigenen in onze studiepopulatie natuurlijk verworven worden. De immuun responsen

verlagen de transmissie van mens naar mug door het voorkomen van bevruchting en ontwikkeling van de parasiet in de muggenmaag. Deze responses zijn niet altijd het hoogst in volwassenen en inductie van de responsen is meer een functie van recente blootstelling dan een cumulatieve blootstelling aan malaria.

Concluderend laat dit proefschrift een leeftijds- en seizoens afhankelijke gametocytemie zien met een belangrijke rol voor submicroscopische gametocyt concentraties. Ondanks natuurlijk verworven immuniteit tegen seksuele antigenen bleef de transmissie behouden in de onderzoekspopulaties. Dit benadrukt de noodzaak voor controle strategieën die malaria transmissie en daarmee malaria morbiditeit reduceren.

Bibliography

Ouédraogo AL, Will Roeffen, Adrian J F Luty, Sake J de Vlas, Issa Nebie, Ilboudo-Sanogo Edith, Nadine Cuzin-Ouattara, Karina Teleen, Alfred B Tiono, Sodiomon Bienvenu Sirima, Jan-Peter Verhave, Teun Bousema and Robert Sauerwein. Naturally Acquired Immune Responses to *Plasmodium falciparum* Sexual Stage Antigens Pfs48/45 and Pfs230 in an Area of Seasonal Transmission. Infection and Immunity 79(12): 4957-64 (2011)

Ouédraogo AL, Teun Bousema, Sake J de Vlas, Nadine Cuzin-Ouattara, Jan-Peter Verhave, Chris Drakeley, Adrian JF Luty, Robert Sauerwein. The plasticity of *Plasmodium falciparum* gametocytaemia in relation to age in Burkina Faso. Malaria Journal 9:281 (2010)

Ouédraogo AL, Bousema T, Schneider P, de Vlas SJ, Ilboudo-Sanogo E, Cuzin-Ouattara N, Nébié I, Roeffen W, Verhave JP, Luty AJ, Sauerwein R. Substantial Contribution of Submicroscopical *Plasmodium falciparum* Gametocyte Carriage to the Infectious Reservoir in an Area of Seasonal Transmission. PLoS One 4(12):e8410 (2009)

Ouédraogo AL, de Vlas SJ, Nébié I, Ilboudo-Sanogo E, Bousema JT, Ouattara AS, Verhave JP, Cuzin-Ouattara N, Sauerwein RW. Seasonal patterns of *Plasmodium falciparum* gametocyte prevalence and density in a rural population of Burkina Faso. Acta Trop. 105(1):28-34 (2008)

Ouedraogo AL, Schneider P, De Kruif M, Nebie I, Verhave JP, Cuzin-Ouattara N and Sauerwein RW . Age-dependent distribution of *Plasmodium falciparum* gametocytes quantified by Pfs25 real-time QT-NASBA in a cross-sectional study in Burkina Faso. Am. J. Trop. Med. Hyg. 76(4): 626-630 (2007)

Sanou GS, Tiendrebeogo RW, **Ouedraogo AL**, Diarra A, Ouedraogo A, Yaro JB, Ouedraogo E, Verra F, Behr C, Troye-Blomberg M, Modiano D, Dolo A, Torcia MG, Traore Y, Sirima SB, Nebie I. Haematological parameters, natural regulatory CD4+CD25+FOXP3+ T cells and gammadelta T cells among two sympatric ethnic groups having different susceptibility to malaria in Burkina Faso. BMC Res Notes. 5(1):76 (2012)

Cherif MK, Sanou GS, Maiga B, Elisabeth I, **Ouédraogo AL**, Bougouma EC, Diarra A, Ouédraogo A, Ouattara AS, Troye-Blomberg M, Dolo A, Cavanagh DR, Theisen M, Modiano D, Sirima SB, Nebié I. FcγRIIa polymorphism and anti-malaria specific IgG and IgG subclass responsese in populations differing in susceptibility to malaria in Burkina Faso. Scand J Immunol. doi: 10.1111/j.1365-3083.2012.02690.x. (2012)

Sirima SB, Tiono AB, Ouédraogo A, Diarra A, **Ouédraogo AL**, Yaro JB, Ouédraogo E, Gansané A, Bougouma EC, Konaté AT, Kaboré Y, Traoré A, Roma C, Soulama I, Luty AJ, Cousens S, Nébié I. Safety and immunogenicity of the malaria vaccine candidate MSP3 long synthetic peptide in 12-24 months-old Burkinabe children. PLoS One. 2009 Oct 26;4(10):e7549.

Paganotti GM, Babiker HA, Modiano D, Sirima BS, Verra F, Konate A, **Ouedraogo AL,** Diarra A, Mackinnon MJ, Coluzzi M, Walliker D.Genetic complexity of *Plasmodium falciparum* in two ethnic groups of Burkina Faso with marked differences in susceptibility to malaria. Am J Trop Med Hyg. 2004 Aug; 71(2):173-8.

Okell LC, Bousema T, Griffin JT, **Ouédraogo AL**, Ghani AC, Drakeley CJ. Molecular detection of malaria: what lies beneath the microscopy detection threshold and is it relevant for control? *(Submitted)*

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Curriculum vitae

André Lin Ouédraogo was born in Ouagadougou (Capital city of Burkina Faso, West Africa). He is married and is living in Ouagadougou. His primary and secondary education provided him the bachelor degree in life sciences (1993) from the Lycée Philippe Zinda Kabore de Ouagadougou. He is then awarded a scholarship to study at the university of Sciences and technology Houari Boumedienne in Algers (Algeria). In 1999, he received his engineer diploma in Genie Biologique on a research-based topic carried out at the Microbiology Centre for Development of Nuclear Techniques on biological tissues (Commissariat de l'Energie Atomique d'Alger). In 2002 he received his Master degree in Microbiology and Biochemistry at the University of Ouagadougou from an internship on malaria research at the Centre National de Recherche et de Formation sur le Paludisme (CNRFP). From 2003, he pursued doctoral studies and defended a PhD early in 2008 at the University of Ouagadougou. On a request from Pr. dr. RW Sauerwein, with the aim of gaining more insights in the field of malaria transmission, he was granted in 2007 the NFP fellowship for PhD research at Radboud University Nijmegen Medical Centre (RUNMC). He is / was local Principal Investigator of the following projects: i) "Assessment of the Infectious Reservoir of Malaria - AFIRM - " (Sponsor: LSHTM, 2012-2014)"; ii) "A double blind randomized controlled trial of artemether-lumefantrine alone and in combination with ivermectin to reduce post-treatment malaria transmission – ACTIVE - (Sponsor: LSHTM, 2012-2013)"; iii) "Transmission of Plasmodium falciparum from man to mosquito: the infectious reservoir in sites of different exposure in Burkina Faso, assessed by QT-NASBA and a bio-assay (Sponsor: RUNMC, 2007-2010); iv) "Quantitative survey of human movement relevant to disease transmission in Burkina Faso (Sponsor: Imperial College London, 2011)"; v) "Molecular tool for high throughput detection of infected mosquitoes in membrane feeding assays in Burkina Faso (Sponsor: LSHTM, 2011-2012)".