Biology of Malaria Transmission

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Understanding transmission biology at an individual level is a key component of intervention strategies that target the spread of malaria parasites from human to mosquito. Gametocytes are specialized sexual stages of the malaria parasite life cycle developed during evolution to achieve crucial steps in transmission. As sexual differentiation and transmission are tightly linked, a deeper understanding of molecular and cellular events defining this relationship is essential to combat malaria. Recent advances in the field are gradually revealing mechanisms underlying sexual commitment, gametocyte sequestration, and dynamics of transmissible stages; however, key questions on fundamental gametocyte biology still remain. Moreover, species-specific variation between *Plasmodium falciparum* and *Plasmodium vivax* transmission dynamics pose another significant challenge for worldwide malaria elimination efforts. Here, we review the biology of transmission stages, highlighting numerous factors influencing development and dynamics of gametocytes within the host and determinants of human infectiousness.

Current measures for malaria elimination have been inspired by the Global Malaria Eradication Program (GMEP), which operated under the World Health Organization (WHO) between 1955 and 1969. The strategy of GMEP was largely based on dichlorodiphenyltrichloroethane (DDT)-based indoor residual spraying (DDT-IRS) complemented with mass drug administration (Pampana 1969). Although GMEP was able to eliminate malaria from many regions of the world, it was eventually abandoned because of technical challenges, increasing spread of both insecticide resistance and drug-resistant parasite strains, and lack of continuous political support (Najera et al. 2011). In 2007, the Bill and Melinda Gates Foundation, supported by WHO, called for a campaign to prioritize malaria eradication strategies with renewed focus on blocking transmission (Alonso et al. 2011; malERA Consultative Group on Drugs 2011). Indeed, malaria elimination can only be achieved by interrupting and reducing transmission in a defined area until no parasites remain (Cohen et al. 2010; Alonso et al. 2011). Tools currently used to reduce transmission focus on vector control efforts such as insecticide-treated nets (ITNs) and antimalarial combination therapy including a transmission-blocking drug. Artemisinin-based combination therapies (ACTs) are currently used as a first-line treatment worldwide (Global Partnership to Roll Back Malaria 2001). These efficiently clear asex-

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ual parasites and early transmission stages, whereas mature infectious transmission stages are unaffected. To block transmission, ACT is often combined with the only transmission-blocking drug on the market, Primaquine. However, recent emergence of artemisinin resistance in Southeast Asia asks for urgent evaluation of alternative treatment strategies (Noedl et al. 2008; Dondorp et al. 2009; Mbengue et al. 2015; Straimer et al. 2015).

Of the five *Plasmodium* species known to cause malaria in humans, *Plasmodium falciparum* is lethal and responsible for severe disease pathology and the majority of deaths due to malaria, especially in sub-Saharan Africa. *Plasmodium vivax* typically causes milder infections than *P. falciparum* but has a much greater geographical distribution (Gething et al. 2012). The clinical symptoms of malaria are largely a result of the replication of asexual stages in human blood, but transmission to mosquitoes is only achieved through the development of sexual stages, termed gametocytes. To abrogate transmission of *P. falciparum*, we must be able to clear asexual and sexual stages from the human host, eventually rendering an individual noninfectious to mosquitoes. However, in the case of *P. vivax*, elimination is highly challenging because of the relapse of dormant liver-stage hypnozoites that can persist as a transmission reservoir for several months after the initial infection (White 2011; Dembélé et al. 2014). Lack of sufficient knowledge on the infectivity of asymptomatic and symptomatic individuals harboring transmission parasite stages remains a major gap in understanding malaria transmission. Because of the complex and nonlinear relationship between parasite density in the human host and infectiousness to mosquitoes (Schneider et al. 2007; Bousema et al. 2012; Churcher et al. 2013), our knowledge of strategies used by the parasite for efficient transmission is still far from complete. Recent studies highlighting the bone marrow as the primary site of gametocyte development and sequestration (Farfour et al. 2012; Aguilar et al. 2014; Joice et al. 2014) raises questions on the timing of events from sequestration leading to reentry of mature gametocytes into the bloodstream and potential migration to subdermal capillaries under the skin during an infection. As gametocytes represent a potential bottleneck in transmission, a deeper understanding of transmission biology is required to develop novel tools and strategies toward elimination and eradication of malaria. In this review, we discuss the current knowledge, recent advances, and open questions regarding key aspects of transmission biology, including mechanisms underlying gametocyte development, gametocyte sequestration, and assessment of the infectious reservoir in an individual. Targeted research on the highlighted knowledge gaps will be crucial to identify potential targets for intervention strategies. Although studies on the transmission biology of *P. vivax* are limited compared with *P. falciparum*, we have compared key features of sexual biology between both species.

DEVELOPMENT OF TRANSMISSION STAGES IN HUMAN MALARIA

The pathology of malaria infection and associated clinical manifestations are predominantly attributed to the asexual erythrocytic stages. During asexual blood stage development, the ring stages develop into replicative schizont forms that release multiple invasive daughter merozoites (Fig. 1A). Within each replication cycle, a small proportion (~0.1%–5%) of asexual parasites develop into male and female sexual stages called gametocytes (Sinden 1983), which are the only stages transmitted to the mosquito vector, albeit not directly contributing to disease pathology. The time required for gametocyte maturation differs strikingly between the different *Plasmodium* species. A *P. falciparum* gametocyte takes ~8–10 days for maturation into five morphologically distinct phases (stages I–V) (Fig. 1B) (Hawking et al. 1971; Sinden et al. 1978). In the other *Plasmodium* species, asexual and sexual cycle are of similar length. *P. vivax* gametocytes require ~48 h for development and disappear from circulation within 3 days of sexual maturation (Sinden and Gilles 2002). In the rodent malaria parasites, *Plasmodium berghei* (Mons et al. 1985) and *Plasmodium yoelii* (Gautret

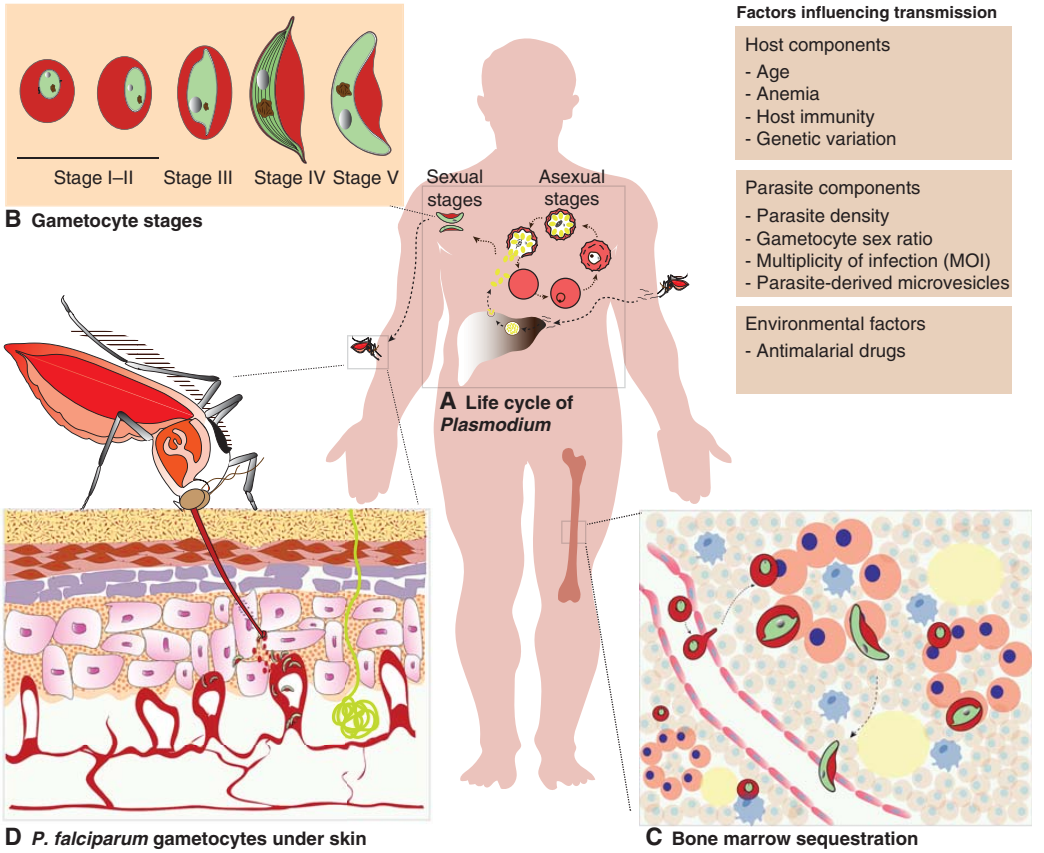

Figure 1. The *Plasmodium falciparum* life cycle in the human host. (*A*) Life cycle of *P. falciparum*. Human malaria infection is initiated when a female anopheline mosquito injects *Plasmodium* sporozoites into the skin during a blood meal. Sporozoites actively reach peripheral circulation and migrate to the liver in which they replicate within hepatocytes forming merozoites that are released into the bloodstream. Merozoites invade red blood cells (RBCs) and develop through ring, trophozoite, and schizont stages before forming new merozoites that are released at schizont egress and reinvade new RBCs. A small proportion of blood stage parasites develop into sexual stages called gametocytes that reach the dermis where they are taken up by another mosquito. After fertilization and sporogonic development in the mosquito midgut, infectious sporozoites are formed that reach the salivary glands for transmission into another host. (*B*) Schematic representation of *P. falciparum* gametocyte developmental stages. Gametocytes undergo five distinct morphological stages during development. Stage I and early stage II are morphologically similar to early stage asexual parasites, and late stage II is the first stage that can be distinguished from asexual trophozoites. Late stage III and stage IV are further elongated and characterized by their spindle shape, whereas in stage V gametocytes, the ends are more rounded forming a crescent shape with minimal visible host cell surface. (*C*) Model of bone marrow sequestration of *P. falciparum* gametocytes. Sexually committed parasitized RBCs home to the bone marrow by binding to endothelial wall of sinusoids followed by transmigration into the extravascular space and undergo development. Alternatively, early asexual parasite stages transmigrate into the extravascular space to produce sexually committed schizonts that release merozoites, which, on reinvasion, begins sexual developmental stages (models also reviewed in Nilsson et al. 2015). Increased rigidity of early gametocytes (Aingaran et al. 2012; Peatey et al. 2013) and the observed binding of immature gametocytes to erythroblastic islands (Joice et al. 2014) favor their maturation in the hematopoietic system. Mature gametocytes exit the microenvironment potentially because of restoration in their deformability (Tiburcio et al. 2012) and intravasate into circulation to be taken up by mosquitoes. (*D*) Model of *P. falciparum* gametocyte localization to the skin. Mature gametocytes preferentially sequester in the subdermal micro capillaries of skin where they are easily accessible to mosquito during a blood meal. (*Inset*) Factors influencing malaria transmission including host, parasite, and environmental conditions are listed.

et al. 1996a), gametocyte maturation requires only 24–27 h. The first recognizable gametocyte stages in *P. falciparum* are round compact forms containing hemozoin. These stages (stage I) and subsequent developmental forms (stage II–IV) are largely absent from blood circulation, but sequester in deep tissue in which they develop into mature sausage-shaped stage V gametocytes and reappear in the blood infective for mosquitoes (Thomson and Robertson 1935; Smalley et al. 1981). The density of mature *P. falciparum* gametocytes in peripheral circulation is typically <100 gametocytes/μL of blood (Drakeley et al. 2006), and in most cases they are present at submicroscopic levels. In contrast to *P. falciparum*, mature *P. vivax* gametocytes are large and round, filling up nearly the entire stippled red blood cell (RBC) with a prominent nucleus (Sinden and Gilles 2002). Because of their faster maturation period compared with *P. falciparum*, *P. vivax* gametocytes are present in blood circulation within a week after mosquito inoculation and before parasite detection by microscopy (Boyd and Stratman-Thomas 1934; Boyd et al. 1936; McKenzie et al. 2007). This poses a significant challenge to *P. vivax* elimination strategies, as infected people may be infectious before parasites are detectable by microscopy (see also below). On ingestion in the mosquito midgut, *P. falciparum* mature gametocytes egress from their host cell, differentiate into male and female gametes triggered by a drop in temperature, increase in pH and xanthurenic acid concentration, and, subsequently, undergo fertilization to form a diploid zygote (Billker et al. 1998, 2000). The zygote develops into motile ookinetes, which penetrate the mosquito midgut and develop into oocysts. *P. falciparum* oocysts mature over a period of 11–16 days (Meis et al. 1992) before releasing infectious sporozoites that migrate to the salivary glands for onward transmission. The likelihood of a mosquito acquiring an infection during a blood meal depends on a wide array of human, parasite, and mosquito factors. The development of gametocytes in humans is vital to the maintenance of malaria transmission and represents a potential bottleneck in the parasite's life cycle. Understanding the biology of gametocyte development and the human infectious reservoir at both the individual and population level is therefore crucial to ablate disease transmission.

MECHANISMS OF SEXUAL COMMITMENT AND GAMETOCYTE SEQUESTRATION

Factors stimulating gametocytogenesis have been debated over the past decades. Early observational studies of infected individuals suggested that gametocyte production may be associated with clinical symptoms (Miller 1958), but the molecular mechanisms underlying this phenomenon remained unknown. The initiation of gametocytogenesis and modulation of gametocyte production in a natural infection is influenced by host environmental factors, including stress induced by host immunity (Bousema et al. 2006), antimalarial drug treatment (Dunyo et al. 2006), or anemia (Nacher et al. 2002), as well as host genetic factors such as human hemoglobin variants (Fig. 1, see inset, top) (Trager and Gill 1992; Gouagna et al. 2010). Similarly, under in vitro conditions, increased gametocyte production was seen at higher parasite densities (Bruce et al. 1990), in the presence of parasite-conditioned medium (Williams 1999; Dyer and Day 2003), and, on addition of human serum (Smalley and Brown 1981), erythroid progenitor cells (Peatey et al. 2013) or antimalarial drugs (Buckling et al. 1999). Total parasite density in an individual can influence gametocytogenesis as a relatively higher concentration of gametocytes was observed in individuals with low-density infections when compared with those with high-density infections (Drakeley et al. 2006). Evidence from clinical observations during human or experimental infections suggests an increased gametocyte production following drug treatment (Buckling et al. 1997; Price et al. 1999; Bousema et al. 2003; Sowunmi et al. 2011), indicating that inefficient treatment and/or parasite recrudescence are associated with higher gametocyte numbers (Price et al. 1999; Barnes et al. 2008). These studies suggest that selection of drug-resistant parasite clones may be associated with increased chances of transmission;

however, this hypothesis has yet to be systematically tested given the complex relationship between drug resistance and malaria transmission, which involves factors such as multiplicity of resistant clones, transmission intensity, and the genetic nature of resistance traits (Talisuna et al. 2003). Several other conditions have been associated with increased gametocyte production, including the time during transmission season (Ouédraogo et al. 2008), response to mosquito probing or bites from uninfected mosquitoes (Paul et al. 2004), and presence of vector-borne factors in the blood (Fischer et al. 2000).

Host Factors Associated with Gametocytogenesis

Naturally acquired immunity during a malaria infection limits the asexual parasite density, thereby affecting gametocyte production from the asexual precursors. However, there is also evidence for a direct influence of *Plasmodium*-induced host-immune response on gametocytogenesis. Increased gametocyte production in *P. falciparum* cultures was observed on addition of lymphocytes and sera from malaria-infected Gambian children (Smalley and Brown 1981) and after addition of anti–*P. falciparum* antibodies produced by hybridoma cell lines (Ono et al. 1986). Data from epidemiological studies suggest a role for anemia in triggering gametocytogenesis. A high proportion of gametocyte carriers were observed among anemic individuals in studies from Thailand and The Gambia (Price et al. 1999; von Seidlein et al. 2001; Nacher et al. 2002; Stepniewska et al. 2008), but the association may be a result of a longer duration of infection resulting in late gametocyte development in these individuals. More convincing data are from in vitro studies in which *P. falciparum* gametocytogenesis is promoted in the presence of young RBCs or reticulocytes (Trager et al. 1999; Trager 2005). On erythropoietin (EPO) treatment, which induces reticulocytosis, a marked increase in *Plasmodium chabaudi* (Gautret et al. 1996b) and *P. berghei* (Mons 1986) gametocyte production was observed in vivo. However, it is not clear which signal(s) associated with reticulocytosis stimulates gametocytogenesis.

Molecular Mechanism of Sexual Conversion

The rate of gametocyte production has historically been thought to be linked to the parasite's response to hostile growth conditions. Recent studies have started to unravel the molecular and cellular basis for the sexual developmental switch. Evidence from *P. falciparum* in vitro cultures indicates that sexual differentiation can be induced by depletion of nutrients in the parasite environment (culture media) (Williams 1999; Dyer and Day 2003). More recently, two studies have shown that extracellular vesicles (EVs) secreted by *P. falciparum* infected RBCs into the environment (or culture media) act as intercellular communicators to induce gametocytogenesis (Mantel et al. 2013; Regev-Rudzki et al. 2013). Purified EVs from the conditioned media of in vitro *P. falciparum* cultures can be internalized by infected RBCs and stimulate sexual stage development in a dose-dependent manner (Mantel et al. 2013). In addition, Regev-Rudzki et al. (2013) showed that drug treatment induces the release of EVs that can transfer nucleic acids to neighboring parasites promoting sexual conversion in the recipient cells as well as conferring drug resistance. The specific components of EVs that induce gametocytogenesis and the subsequent downstream mechanism in the sexual conversion pathway remain to be elucidated, which may open up new targets to ablate gametocyte development.

The genetic factors underlying sexual differentiation in *Plasmodium* parasites have remained elusive until recently. Early studies suggested that merozoites released from a schizont commit to either the asexual or sexual pathway (Inselburg 1983; Bruce et al. 1990) and that sexually committed parasites form exclusively male or female gametocytes (Silvestrini et al. 2000). These studies suggested a defined gene expression pathway responsible for commitment in malaria parasites. More recent work revealed that sexual commitment is regulated by a highly conserved apicomplexan-specific transcription factor, ApiAP2-G, both in *P. falciparum* (Kaf-

sack et al. 2014) and in *P. berghei* (Sinha et al. 2014). In *P. berghei*, the disruption of another transcription factor from the AP2 family, AP-2G2, appears to inhibit male gametocyte development and may, therefore, be involved in maintenance of gametocyte sex ratio (Sinha et al. 2014). *P. falciparum* ApiAP2-G was found to be epigenetically regulated by at least two proteins, histone deacetylase 2 (PfHda2) and heterochromatin protein 1 (PfHP1), which causes repression of gametocytogenesis under nonpermissive conditions (Brancucci et al. 2014; Coleman et al. 2014). Conditional knockdown of PfHda1 or PfHP1 in asexual stage parasites in vitro leads to a cascade of gene activation, including *AP2-G*, and induction of gametocyte production (and decreased asexual replication) (Brancucci et al. 2014; Coleman et al. 2014). Together, these findings show epigenetic control of stage conversion; however, the upstream factors regulating these epigenetic control mechanisms remain unknown. In addition to ApiAP2-G-mediated epigenetic regulation, it is likely that additional factors are involved in the onset of gametocytogenesis. Moving forward, it will be important to understand how external stress factors are linked to the molecular mechanism(s) of sexual commitment and gametocyte development.

Sequestration of Transmission Stages

Tissue-specific sequestration of asexual parasite stages is associated with severe malaria pathology such as cerebral malaria and pregnancy-associated malaria (Miller et al. 2002). RBCs infected with the asexual stages of *P. falciparum* (mature trophozoites and schizonts) are sequestered away from peripheral circulation by adhering to endothelial receptors, such as CD36, ICAM-1, and CSA in the microvasculature (for reviews, see Miller et al. 2002; Sherman et al. 2003). Adherence to host receptors is mediated by expression of parasite-derived *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1) on knob-like structures at the surface of infected RBCs (Kilejian 1979; Baruch et al. 1995). Although the transmission stages do not generally contribute to disease pathology, immature stages of *P. falciparum* gametocytes sequester in tissues presumably to avoid clearance by the spleen. In contrast, all developmental stages of *P. vivax* gametocytes can be seen in the blood and no sequestration of *P. vivax* transmission stages has been reported so far. Because *P. vivax* develops exclusively in reticulocytes, RBCs infected with *P. vivax* have an increased surface area and are highly flexible (Suwanarusk et al. 2004; Handayani et al. 2009), which presumably helps them to pass through small capillaries or sinusoidal vessels and avoid clearance by the spleen.

Postmortem case studies from the early 1900s (Marchiafava and Bignami 1894; Thomson and Robertson 1935) and more recent field and clinical reports in the last two decades (Smalley et al. 1981; Farfour et al. 2012; Aguilar et al. 2014) have confirmed the presence of *P. falciparum* immature gametocytes in the spleen and bone marrow of malaria infected patients. Notably, a recent systematic histological and transcriptional analysis of autopsy tissues from children who died from cerebral malaria revealed gametocyte enrichment in the bone marrow parenchyma in which they are predominantly localized to the erythroblastic islands (Joice et al. 2014), suggesting that sexual stage development can occur in erythroid progenitor cells during infection. The underlying mechanism of gametocyte sequestration, including the parasite stages that home to bone marrow, is not yet well defined. However, the prevailing model suggests that sexually committed parasites or immature gametocytes traverse the endothelial barrier and home to the bone marrow parenchyma, in which they undergo development to produce mature gametocytes that eventually intravasate into the peripheral circulation (Fig. 1C) (Nilsson et al. 2015; Pelle et al. 2015). In contrast to asexual stages, early gametocytes do not significantly modify the RBC, as minimal levels of PfEMP-1 are expressed on the infected RBC surface and no significant knob structures were observed (Silvestrini et al. 2012; Tiburcio et al. 2013). This difference in surface protein expression is consistent with in vitro studies in which immature gametocytes showed significantly less, if any, binding to purified host ligands (CD36,

ICAM-1) (Day et al. 1998) and to bone marrow endothelium or other endothelial cell lines (Silvestrini et al. 2012). Altogether, these data suggest that gametocyte sequestration is PfEMP-1 independent, but may involve other exported parasite molecule(s) required for bone marrow homing and association with erythroblast islands in parenchyma. Based on *Plasmodium*-specific serum responses from malaria patients, the surface antigens of gametocytes seem to be distinct from those on the surface of asexual infected RBCs (Saeed et al. 2008), indicating that host–parasite interactions in tissues are likely gametocyte-specific. Products of multigene families involved in host cell modifications, such as STEVOR and RIFIN, are expressed during gametocyte development (McRobert et al. 2004; Petter et al. 2008), but their functional role in gametocytogenesis or cytoadherence has not been shown. Nevertheless, a switch in deformability of mature gametocytes (Aingaran et al. 2012; Dearnley et al. 2012; Tiburcio et al. 2012) is accompanied by reorganization of STEVOR in the RBC membrane of mature gametocytes (Tiburcio et al. 2012). Taken together, the sequestration and subsequent development of immature gametocytes in the bone marrow and spleen is likely to be maintained through mechanical retention and as-yet-uncharacterized binding properties, whereas the switch in deformability at the mature stage V gametocyte stage may facilitate their release from sequestration sites into the periphery.

Extravascular sequestration in the bone marrow may not only help young gametocytes to evade host immune responses and/or undergo development, but also provide a nutrient-rich and aerobic environment with abundant young RBCs for ideal gametocyte development. In agreement with this hypothesis, in vitro data indicates an enhanced invasion of erythroid progenitor cells with a concomitant increase in gametocyte formation within young RBCs (Tamez et al. 2009; Peatey et al. 2013). More focused studies are required to decipher the mechanism of gametocyte sequestration, including identification of parasite stages that home to the bone marrow, the receptor–ligand interactions involved, if any, and importantly, whether sexual commitment occurs in the periphery or in the bone marrow microenvironment. Answers to these outstanding questions on gametocyte sequestration are crucial to understand transmission dynamics and to design novel transmission intervention strategies.

DETERMINANTS OF INFECTIOUSNESS

In the human host, a small subset of total parasite population differentiates into mature gametocytes, some of which may be ingested by a mosquito during a blood meal. Subsequent fertilization of gametes and development into sporozoites within the mosquito makes it infectious to another human. The human infectious reservoir is defined as the proportion of a population capable of successfully infecting mosquitoes (Drakeley et al. 2000). Gametocyte carriage and successful transmission from human to mosquito is influenced by several factors including age (Ouédraogo et al. 2010), gametocyte density (Robert et al. 2000; Schneider et al. 2007; Ouédraogo et al. 2009), gametocyte sex ratio (Robert et al. 1996b; Mitri et al. 2009), antimalarial drug treatment (Buckling et al. 1999; Robert et al. 2000; Sowunmi et al. 2004), and host immunity (Fig. 1, see inset, top) (Saeed et al. 2008; Sutherland 2009). Although mature gametocyte presence in the blood has long been thought to be crucial for an efficient transmission, these stages were rarely detected by microscopy (Dowling and Shute 1966; Bejon et al. 2006) and, therefore, it was previously assumed that only a small proportion of malaria-infected individuals carried gametocytes. With the use of sensitive molecular assays, it is now clear that gametocytes are present in most malaria infections and at highly variable densities (Schneider et al. 2007; Shekalaghe et al. 2007) that can successfully infect mosquitoes (Schneider et al. 2007; Bousema et al. 2012; Churcher et al. 2013). Given the highly variable and nonlinear relationship between gametocyte density and mosquito infection rate (Bousema et al. 2012; Churcher et al. 2013), quantifying the human component of the infectious reservoir is a challenging task. Mosquito-feeding assays have been routinely

used to quantify the infectiousness of an individual and to evaluate the effects of transmission-blocking vaccines or gametocytocidal drugs. In direct skin-feeding assays, *Anopheles* mosquitoes are allowed to take a blood meal by direct contact with the skin of an individual recapitulating a natural infection (Bousema et al. 2012). In membrane-feeding assays, there are two types: (1) Direct membrane-feeding assay (DMFA) involves feeding uninfected *Anopheles* mosquitoes with a blood sample drawn from a naturally infected individual through an artificial membrane (parafilm) in the presence of autologous sera, and (2) standard membrane feeding assay (SMFA) involves feeding mosquitoes with in vitro cultured gametocytes mixed with RBCs and human serum through the membrane in a device (Bousema et al. 2012). Mosquito infectivity is measured in these assays by quantifying the number of oocysts present in the mosquito midgut (either as mean oocyst density or oocyst prevalence across mosquitoes) following a maturation period of 7 to 8 days. Although direct skin feeding assays may yield a more accurate estimate of human transmission potential, membrane-feeding assays are appropriate to compare infectiousness between individuals and to assess transmission-reducing interventions (Bousema et al. 2012; Miura et al. 2013). The SMFA, compared with DMFA, can be performed under standardized laboratory conditions and is considered the gold standard for measuring transmission-reducing activity. A more detailed review of the human infectious reservoir at the epidemiological level and tools to measure transmission is covered in Bousema and Drakely (2016).

Relationship between Gametocyte Density and Infectiousness to Mosquitoes

Human infectiousness is linked to asexual parasite density and gametocyte density in the blood. Although there is a positive correlation between gametocyte density and mosquito infection rate (Schneider et al. 2007; Ouédraogo et al. 2009), the association is highly variable and complex at low gametocyte densities (van der Kolk et al. 2005). High gametocyte densities do not necessarily result in mosquito infection (Graves et al. 1988; Gamage-Mendis et al. 1991; Schneider et al. 2007), whereas individuals with low densities that carry no observable gametocytes have been found to be infectious (Jeffery and Eyles 1955; Muirhead-Thomson 1998; Coleman et al. 2004; Schneider et al. 2007). This heterogeneity in mosquito infection may result in part from sampling bias: Samples are generally collected via venipuncture but there may be a specified localization or clustering of gametocytes in the human vasculature during a blood meal (Pichon et al. 2000). Similarly, in *P. vivax* infections, the relationship between gametocyte density and mosquito infection is poorly defined which is attributed to limited sensitivity of microscopy to detect and differentiate gametocyte stages (Gamage-Mendis et al. 1991; Bharti et al. 2006). In one study, *P. vivax* infected patients were infectious to mosquitoes immediately after the appearance of asexual parasites detected by microscopy, but significantly before the emergence of gametocytes (Jeffery 1952). Similarly, several reports of infectivity at undetectable *P. vivax* gametocytemia have been published (Gamage-Mendis et al. 1991; Sattabongkot et al. 1991; McKenzie et al. 2002; Coleman et al. 2004; Pethleart et al. 2004; Bharti et al. 2006). Comparative studies of *P. falciparum* and *P. vivax* indicate that malaria transmission by *P. vivax* parasites is likely to be highly efficient, with lower gametocyte densities typically resulting in mosquito infection (Pukrittayakamee et al. 2008). The use of more sensitive tools, such as QT-NASBA to detect late gametocyte-specific mRNA markers, will help to evaluate the association between *P. vivax* gametocyte density and mosquito infection (Beurskens et al. 2009). Moreover, *P. vivax* transmission is much faster and more persistent than *P. falciparum* because of their ability to form gametocytes early and the relapse of blood stage infections caused by reactivation of hypnozoites (Galinski et al. 2013).

Dynamics of Parasite Genotypes (Complexity of Infection)

The evolutionary success of malaria transmission is attributed to the genetic complexity of

P. falciparum gametocytes in a natural infection and their infectivity to mosquitoes. A high degree of clonal multiplicity persists in malaria-endemic regions that varies with transmission intensity (Robert et al. 1996a), whereas cross-mating in mosquitoes produces new strains in the next generation. Multiple parasite clones in an individual were found to be equally transmissible to mosquitoes with a strong correlation between multiplicity of infection and frequency of cross-mating (Hill and Babiker 1995). However, in some cases, not all clones from the same infection were infectious resulting in less variable genotypes in the infected individual (Paul et al. 1995). In natural infections in The Gambia at the end of the dry season, a higher transmissibility of multiple clones was notably observed even if they existed as a minority parasite population in asymptomatic individuals (Nwakanma et al. 2008). Existing in vitro and population studies suggest that dynamics and degree of *P. falciparum* gametocyte production is associated with the parasite's genetic background (Graves et al. 1984; Teklehaimanot et al. 1987; Abdel-Wahab et al. 2002); however, factors favoring the production of different gametocyte clones within a mixed population are unknown.

Gametocyte Sex Ratio

Gametocyte sex ratio is one of the critical determinants of malaria transmission. Because one male gametocyte produces eight microgametes during final maturation, whereas the female gametocyte develops into one macrogamete, malaria parasites generally produce more female gametocytes than male, and they can also modulate the sex ratio during an infection. An optimal ratio of three or four females to one male gametocyte is commonly observed in *P. falciparum* infections (Robert et al. 1996b, 2003; Kar et al. 2009), but this varies considerably over the course of an infection and between malaria-endemic regions (Paul et al. 2002; Talman et al. 2004; Sowunmi et al. 2008). A higher production of male gametocytes relative to female gametocytes maximizes the success of transmission and this is especially important at lower gametocyte densities (Reece et al. 2008) where there is a need for male bias. The sex ratio is also influenced by stimulation of erythropoiesis (anemic state) and presence of competing parasite strains, which tends to favor a male-biased sex ratio in the infection (Paul et al. 2002; Reece et al. 2008). Differences in gametocyte sex ratio may be associated with the parasite's general response to host immunity during an infection (Paul et al. 2000) and/or stress factors affecting gametocytogenesis including parasite density (Reece et al. 2008).

Gametocyte Localization to the Skin Compartment

The availability of transmission stages ready to be picked up in a mosquito's blood meal is an essential component of human infectiousness and for efficient transmission, as mature gametocytes in circulation must traverse the microvasculature to reach the dermis. There is evidence for preferential parasite localization in the skin compartment. In a diagnostic study from the early 1950s conducted in the endemic regions of the Belgian Congo, skin scarification smears from acute and chronic malaria patients (children and adults) had a higher frequency of mature schizonts and gametocytes compared with thick peripheral blood films (van den and Chardome 1951; Chardome and Janssen 1952). Similarly, in the rodent *P. chabaudi* model, gametocyte numbers were higher in the mosquito's blood meal immediately after engorgement than in the venous tail blood (Gautret et al. 1996b), suggesting an enrichment of parasites in the skin microvasculature. A recent autopsy study of cerebral malaria patients from Malawi showed significant parasite sequestration in the skin microvasculature in a subset of patients by histological analysis (Milner et al. 2015); however, the stage composition of these parasites has not been determined. In another study, infection rates of mosquitoes that were fed directly on the skin were 2.4-fold higher than those observed after feeding on venous blood samples through an artificial membrane (Bousema et al. 2012). These studies suggest that infectious *P. falciparum* mature gametocytes may preferentially localize to subdermal

capillaries beneath the skin (Fig. 1D). In addition, there is a distinct possibility that the parasite modulates gametocyte densities or sequestration according to the peak hours when mosquitoes take a blood meal (Hawking et al. 1971; Garnham 1974). Although conclusive evidence is still lacking, this scenario would likely enhance transmission success and maintain genetic diversity in the population by increasing the possibility of cotransmission of male and female gametocytes and of multiple genotypes in an infection. The increased deformability and subsequent interaction with specific receptors may aid in the localization of mature gametocytes to subdermal capillaries. This hypothesis needs to be rigorously tested using mosquito feeding assays and in vivo models.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Understanding the biology of transmission stages in the human host leading to mosquito infectivity will be essential to successfully overcome current challenges in malaria elimination efforts. Despite their crucial role in transmission, many fundamental questions of gametocyte biology and development remain to be answered. In this review, we have discussed the sexual biology of malaria parasites and determinants of infectiousness emphasizing key factors influencing transmission in a human host. Transmission-blocking intervention strategies targeting sexual stages should take into account molecular targets including the genetic pathways of sexual conversion, sequestration mechanisms, intercellular parasite communication mechanisms, and gametocyte deformability. Increasing evidence in recent years point to the existence of a niche for gametocyte development in the extravascular environment of bone marrow (and presumably in the spleen) (Farfour et al. 2012; Aguilar et al. 2014; Joice et al. 2014) acting as the parasite's hideout. Identification of stage-specific markers and development of novel tools to study sequestration of immature gametocytes are warranted. Another important open question is how the developmental decision between asexual and sexual commitment is induced. The environmental cues influencing this decision are likely connected to precise signaling pathways leading to changes in gene expression. With the identification of genetic master regulators of sexual commitment (AP-2 and DOZI) (Mair et al. 2006; Kafsack et al. 2014; Sinha et al. 2014), the transcriptional and translational mechanisms regulating induction and maturation of gametocytes are becoming clearer. It remains to be answered if this regulation is conserved across *Plasmodium* species and whether a specific environment in the vertebrate host favors sexual differentiation. Finally, a major challenge for future research is to understand and define the infectious potential of malaria-infected individuals. Although highly sensitive molecular detection tools such as quantitative real-time PCR (qRT-PCR), RT-loop-mediated isothermal amplification (RT-LAMP), and quantitative nucleic acid-sequence-based amplification (QT-NASBA) indicate that a high proportion of asymptomatic individuals carry submicroscopic infections (Schneider et al. 2007; Shekalaghe et al. 2007; Bousema and Drakeley 2011), elimination of lingering parasite forms in these individuals is important. In conclusion, combined human, parasite, mosquito, and environmental factors play a key role in influencing transmission and the human infectious reservoir overall. To better understand the infectious reservoir in an individual and to develop transmission-blocking drugs and vaccines, further research on the basic biology of gametocytes and their dynamics within the host is essential. Building on exciting advances in the field discussed here, studies addressing the remaining knowledge gaps in transmission biology will open up new avenues for feasible targets to interrupt malaria transmission.

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