Number and proportion of *P. falciparum* gametocytes vary from acute infection to chronic parasite carriage despite unaltered sexual commitment rate

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Abstract

In areas of seasonal malaria, resuming transmission every wet season relies on persistent asexual parasites during dry season that maintain the ability to produce gametocytes until the mosquito population resurges, following the start of annual rains. Although human asymptomatic *P. falciparum* reservoirs in the dry season are widely recognized, the longitudinal dynamics of parasite sexual commitment and gametocytogenesis are unclear. Here, we compared the density and proportion of *P. falciparum* gametocytes in blood of asymptomatic subjects during the dry season versus subjects with symptomatic malaria in the wet season. Blood concentrations of phospholipids potentially involved in gametocytogenesis and parasite transcriptomes were also compared. While blood densities of gametocytes and asexual parasites were lower during the dry season, we found that gametocytes were proportionally higher during the dry versus wet season. Levels of parasite transcripts involved in sexual commitment were unaltered throughout the year, and phospholipid content in the plasma throughout the year did not affect sexual commitment in vitro. Our experimental data and mathematical modelling support that gametocyte density and proportion diverge as infections progress from recently transmitted to chronic carriage, without significant alterations in the rate of sexual commitment over time.

Introduction

It has long been known that the malaria-causing parasite *Plasmodium falciparum* requires a mosquito host to complete its lifecycle and to be transmitted between humans (1). However, it remains unclear if in the human host the parasite can dynamically allocate resources to the differentiation of sexual-stage gametocytes that are transmitted to mosquitos, particularly in areas of seasonal malaria where the size of the mosquito population fluctuates dramatically during the dry and wet seasons. During blood feedings, infected Anopheles mosquitoes deposit *P. falciparum* sporozoites in the human dermis that find their way into circulation, reaching the liver where they infect hepatocytes. After 7-10 days of parasite replication, each infected hepatocyte releases thousands of merozoites into the blood stream (2), where they start continuous asexual intra-erythrocytic replication cycles of ~48 h, from merozoite invasion of erythrocytes, through the ring- and trophozoite-stages, until the multinucleated schizont releases new merozoites (3). A small proportion of intra-erythrocytic asexual parasites differentiate into sexual-stage gametocytes (4). Instead of another ~48 h asexual schizogony leading to the 8-30 new merozoites ready to continue asexual cycles (5), the parasite develops into sexually committed schizonts leading to 8-30 sexually committed merozoites that upon infection of new red blood cells (RBCs) each produce one stage I gametocyte (6, 7) that through a period of 8-12 days, will go through five developmental stages (8). Stages II to IV of gametocytaemia take place in the bone marrow or spleen (9, 10), while stage V gametocytes are again present in peripheral blood, each lasting up to ~20 days (11, 12) waiting for another mosquito bite. Following a blood meal gametocytes encounter the right environment for sexual reproduction in the mosquito midgut (13). They transform into female and male gametes that fuse into a zygote originating oocysts that invade the midgut wall to generate sporozoites that can, after reaching the mosquito salivary glands, restart the parasite lifecycle in the next blood meal (14). The continued presence of mosquitoes and humans in a geographical region allows for perennial *P. falciparum* transmission; however, in areas where the dry season is several

months long, the mosquito population can plummet to near zero (15), thereby interrupting transmission. *P. falciparum* faces the added challenge of surviving in its human host for months while waiting for the return of mosquitoes with the ensuing wet season (16-18). During mosquito-free dry periods, gametocyte production is largely wasted, as gametocytes cannot survive to bridge two wet seasons (11, 12). Although curtailing gametocyte production rates during the dry season would seem beneficial to the parasite, such a strategy would require a mechanism to reactivate gametocytogenesis with the return of rain and mosquitos. Host factors such as age (19), haematocrit (20), haemoglobin type (21) and duration of malaria symptoms (22) have been linked to gametocytaemia in individuals with clinical malaria; and higher frequencies of gametocyte positive individuals have been observed in chronic asymptomatic infections compared to acute infections (23-25). Additionally, various in vitro conditions have been shown to modulate sexual commitment and thus gametocytogenesis (6, 26-28). The specific effect of lysophosphatidylcholine (Lyso-PC) in repressing the pro-sexual commitment transcription factor AP2G has been recently identified, with low Lyso-PC concentrations increasing the proportion of sexual forms in vitro (28). Furthermore, gametocyte development 1 (GDV1) has been identified as the de-repressing effector which counters the epigenetic silencing and promotes AP2G expression leading to the induction of gametocytogenesis (29), and homeodomain protein 1 (HDP1) was recently identified as a positive transcriptional regulator essential for gametocyte development (30). Still, it is not fully understood how sexual investment and gametocytaemia is regulated in vivo, and if or how transmission to mosquitoes can be optimized by the parasite to conserve resources. Recently, we have shown that *P. falciparum* parasites isolated at the end of the dry season are transcriptionally distinct from those of subjects with febrile malaria in the transmission season (16, 31), but alteration of sexual commitment and gametocytogenesis were not investigated. The observed transcriptional differences reflected longer parasite circulation within each ~48 h intra-erythrocytic cycle in the dry season, likely increasing splenic clearance of infected cells

and thus maintaining the low parasitaemia observed during the dry season (16). Here, we aimed to examine sexual commitment and gametocytogenesis of parasites persisting through the dry season and explored possible parasite adaptations during mosquito-free periods. We quantified sexual commitment and female and male gametocyte densities in blood samples collected from children at different times of the year and with different clinical presentations, and we related them to total parasite density and infection length. Furthermore, we determined longitudinally the Lyso-PC concentration in plasma of children, and assessed its ability to induce *P. falciparum* sexual commitment and gametocytogenesis in vitro. Finally, we adapted a previously described within-host mathematical model that considers asexual parasites, sexually committed parasites, and gametocytes (32) to infer the dynamics of gametocyte number and proportion as *P. falciparum* infections progress from recently transmitted to chronic asymptomatic carriage over several months.

Results

Sexual stages differ between asymptomatic *P. falciparum* at the end of the dry season and *P. falciparum* causing clinical malaria in the wet season. We first investigated the gametocyte-related transcriptome (33) within a whole genome RNA sequencing dataset (16) comparing persistent *P. falciparum* detected by rapid diagnostic test (RDT) in 12 asymptomatic Malian children at the end of the dry season (May) to *P. falciparum* of 12 age- and gender- matched Malians presenting clinical malaria with higher parasitaemias in the wet season (MAL). Principal component analysis of 333 gametocyte-specific genes with detectable number of reads (out of the 786 defined as gametocyte-related by ((33), S1 Table) showed segregation of transcription profiles based on seasonality (Fig 1A). Differentially expressed genes (DEGs) determined at a false discovery rate threshold of 5% resulted in 205 DEGs, with 146 transcripts up-regulated, and 59 transcripts down-regulated in the dry season samples compared to clinical malaria samples (Fig 1B and S1 Table). These transcriptional differences

prompted us to quantify female and male gametocytes in the blood of the two groups of children collected in the same year and the same cohort study. First, we determined total parasite (PFGLY, PF3D7_1420400) density by qRT-PCR, and observed that it was higher in clinical malaria cases in the transmission season (MAL) than in asymptomatic cases at the end of the dry season (May) (Fig 1C). Then, we determined the density of female (P25, PF3D7_1031000) and male (PFMGET, Pf3D7_1469900) gametocytes, based on a standard curve obtained from sorted gender-specific fluorescent gametocytes (34) (S1 Fig). This analysis revealed higher numbers of both female and male gametocytes per mL of blood in clinical cases of malaria during the wet season (MAL) compared to asymptomatic carriers at the end of the dry season (May) (Fig 1D). However, when we quantified either gametocyte transcript relative to the total *P. falciparum* level present in each subject's sample, normalized to a housekeeping gene transcribed by all parasite stages, we observed an inverse trend. There were proportionally fewer female and male gametocytes in malaria cases in the wet season (MAL) than at the end of the dry season (May), although the difference was significant for the female comparison only (Fig 1E). We also quantified phospholipids known to influence *P. falciparum* sexual commitment in vitro (28) in the plasma of the same children through shotgun lipidomic analysis. Malaria cases in the wet season (MAL) showed lower levels of several species of Lyso-PC (Fig 1F) and lysophosphatidylethanolamine (Lyso-PE) (Fig 1G) families compared to asymptomatic carriers at the end of the dry season (May), which could potentially increase *P. falciparum* sexual commitment in malaria cases (MAL). Nevertheless, transcription of the sexual commitment regulators (AP2G, PF3D7_1222600; and GDV1, PF3D7_0935400), sexually committed schizonts (MSRP1, PF3D7_1335000), or early gametocyte marker (GEXP5, PF3D7_0936600) were not significantly different by qRT-PCR (Fig 1H) nor were they differentially expressed in the abovementioned RNAseq analysis (S1 Table).

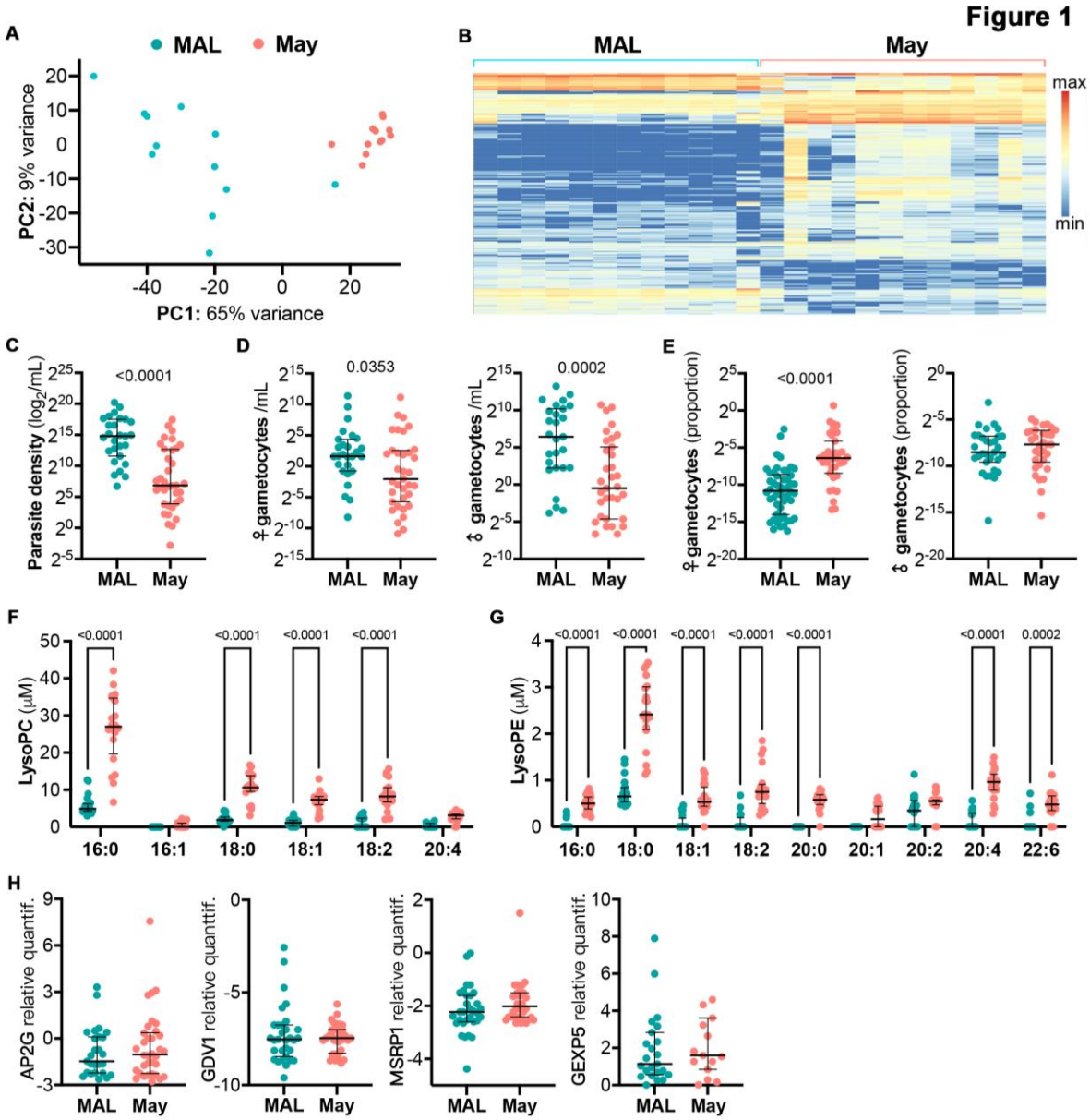


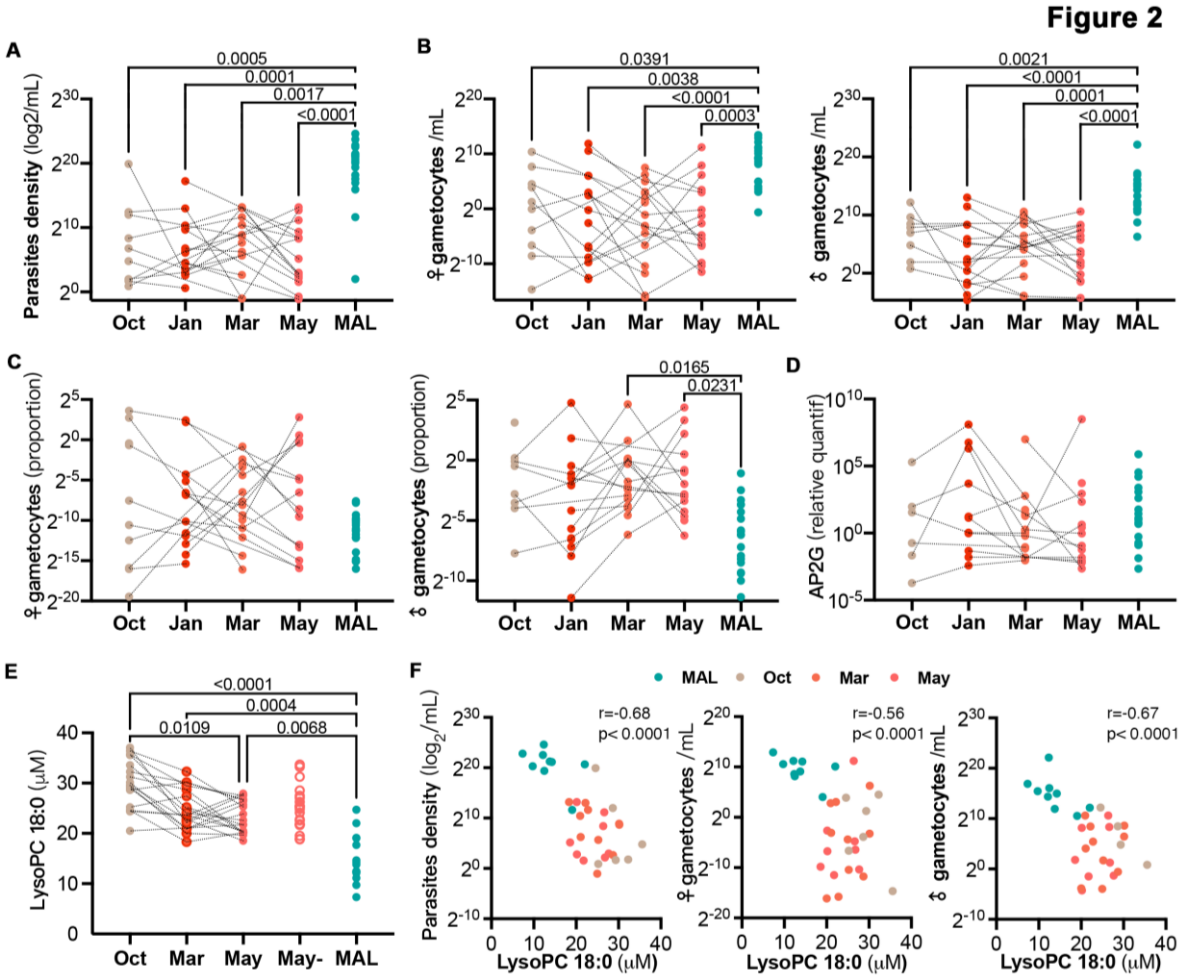
Fig 1. Sexual stages differ between subclinical P. falciparum at the end of the dry season and *P. falciparum* causing clinical malaria in the transmission season. A: Principal components analysis of RNA-Seq data of 333 gametocyte specific *P. falciparum* genes from parasites collected at the end of the dry season and from clinical malaria cases (n= 12 May, 12 MAL). **B**: Heatmap showing normalized reads of 205 gametocyte specific *P. falciparum* of differentially expressed genes (DEGs) (rows) for each subject (columns) from *P. falciparum* collected at the end of the dry season (May) and at the first clinical malaria case (MAL) in the ensuing transmission season. **C:** Asexual parasite density detected by qRT-PCR of RDT^+ subclinical children at the end of the dry season (May = 35) and of children with their first clinical malaria episode (MAL = 27) in the wet season. **D:** Female (left) and male (right) gametocyte densities detected by qRT-PCR of RDT^+ subclinical children at the end of the dry season (May = 35) and of children with their first clinical malaria episode (MAL= 27) in the wet season. **E:** Proportion of female (left) and male (right) gametocytes detected by qRT-PCR of RDT^+ subclinical children at the end of the dry season (May) and of children with their first clinical malaria episode (MAL) in the wet season. **F:** Plasma concentration of Lyso-PC species

of RDT$^+$ subclinical children at the end of the dry season (May) and of children with their first clinical malaria episode (MAL) in the wet season. **G:** Plasma concentration of Lyso-PE species of RDT$^+$ subclinical children at the end of the dry season (May) and of children with their first clinical malaria episode (MAL) in the wet season. **H:** Relative expression of AP2G, GDV1, MSRP1, and GEXP5, detected by qRT-PCR of RDT$^+$ subclinical children at the end of the dry season (May n > 15) and of children with their first clinical malaria episode (MAL n > 23) in the wet season. Lipid data represented as median ± IQR; two-way ANOVA with Tukey multiple comparisons correction. Parasitaemia and gametocytaemia and AP2G, GDV1, MSRP1, and GEXP5 expression data represented as median ± IQR; Mann-Whitney test.

Sexual commitment and gametocytogenesis dynamic in persisting infections. We then determined the dynamics of *P. falciparum* sexual commitment and gametocytogenesis in persistently infected individuals, and examined associations between plasma Lyso-PC and Lyso-PE, monitoring longitudinally ~20 children who were continuously infected from the wet season until the end of the dry season. We quantified the number of total parasites, and fully formed female and male gametocytes by qRT-PCR at four different time points in asymptomatic children persistently infected since the wet season (Oct) through the dry season until its end (Jan, Mar and May); and in a parallel group of age-matched individuals presenting with clinical malaria during the ensuing wet season (MAL). We observed that total parasite density was higher in malaria cases (MAL), than at any time-point of the asymptomatic infections, whether in the wet (Oct) or the dry season (Jan, Mar and May), while no differences were found between any of the time points of asymptomatic infections (Oct, Jan, Mar and May) (Fig 2A). Similarly, we observed higher densities of both female and male gametocytes in malaria cases (MAL) than at any time point of the asymptomatic infections (Oct, Jan, Mar and May), and again no differences within the persisting period (Fig 2B). Conversely, the proportion of male gametocytes was higher at later time points of persisting infections (Mar or May) than in malaria cases during the transmission season (MAL) (Fig 2C). Moreover, we found no significant difference in the sexual commitment regulator AP2G between incident (MAL) or persistent infections at any point analyzed (Oct, Mar and May) (Fig 2D). Quantification of Lyso-PC 18:0 in malaria cases compared to asymptomatic infections progressing from the wet into the dry season again resulted in decreased Lyso-PC values in

clinical malaria cases (MAL) compared to asymptomatic infections (Oct, Mar and May) (Fig 2E). We further detected a decrease of Lyso-PC from the wet season (Oct) to the end of the dry season (May), but no difference was seen between the two available time points of the dry season (Mar and May) (Fig 2E). Furthermore, Lyso-PC 18:0 levels in persistent infections (Oct, Mar and May) were comparable to non-infected individuals at the end of the dry season (May^{-}), indicating that asymptomatic infections have no impact on Lyso-PC 18:0 (Fig 2E and S2 Fig). Accordingly, a relation between Lyso-PC levels and parasitaemia or gametocytaemia was only significant when considering clinical malaria plus all asymptomatic samples (Oct, Mar, May and MAL) ($r=-0.68$ $p< 0.0001$; $r=-0.56$ $p< 0.008$ and $r=-0.67$ $p< 0.0001$ for parasitaemia, female and male gametocytaemia respectively), but disappeared when each group was analyzed independently, or in all persisting asymptomatic infections analyzed together (Fig 2F).

Fig 2. Sexual commitment and gametocytogenesis dynamic in persisting infections. A: Parasite, **B:** Female (left) and male (right) gametocyte, and **C:** Proportion of female (left) and male (right) gametocytes densities detected by qRT-PCR of RDT^{+} subclinical children at different times during the wet (Oct n = 10) and the dry season (Jan, Mar and May n > 15), and during clinical malaria episodes (MAL n = 19). **D:** Relative expression of AP2G detected by qRT-PCR of RDT^{+} subclinical children at different times during the wet (Oct n = 6) and the dry season (Jan, Mar and May n > 11), and during clinical malaria episodes (MAL n = 19). **E:** Plasma concentration of Lyso-PC 18:0 of RDT^{+} subclinical children at different times during the wet (Oct n = 17) and the dry season (Mar and May n = 19), and during clinical malaria episodes (MAL n = 15). Parasite gene expression and lipid data were compared through Kruskal-Wallis test with multiple comparisons. **F**: Correlation between Lyso-PC 18:0 and parasite (left), female gametocyte (canter) or male gametocyte (right) densities detected by qRT-PCR detected by qRT-PCR of RDT^{+} subclinical children at different times during the wet (Oct n = 10) and the dry season (Mar and May n > 15), and during clinical malaria episodes (MAL n = 19). *P* and r determined by Pearson correlation.



Decreased Lyso-PC during clinical malaria does not induce higher sexual commitment or gametocytaemia. We then questioned if the low levels of Lyso-PC and Lyso-PE species in plasma of malaria cases compared to persistent infections at the end of the dry season (Fig 1F and 2E) could induce *P. falciparum* sexual conversion or gametocytaemia differently in vitro. We exposed parasites of the *P. falciparum* 2004 line (Pf2004) to plasma of children collected during clinical malaria cases (MAL) versus those of children with asymptomatic infections at the end of the dry season (May) alongside the conditions described by Brancucci et al. to induce or inhibit sexual convertion and gametocyteamia. As reported earlier (28), we observed that 24 h after culturing Pf2004 without plasma or serum (noPl), and thus no Lyso-PC, over minimal fatty acid medium (MFA), AP2G expression increased compared to the same parasites cultured in the presence of German control plasma (PlDE) (Fig 3A). However, MFA

medium supplementation with 25% plasma from children presenting with clinical malaria during the wet season (PlMAL), versus plasma from children with asymptomatic infections at the end of the dry season (PlMay) resulted in similar APG2 induction 48 h after beginning of treatment (Fig 3A), and similar conversion rate of parasites into gametocytes on day 5 after treatment (defined by = d5gametocytes/ d2parasites) (Fig 3B). Absence of plasma (noPl) resulted in higher gametocytaemia than observed on the German pooled plasma condition (PlDE) (Fig 3B), plasmas from malaria cases (PlMAL) or asymptomatic infections at the end of the dry season (PlMay) revealed similar conversion rates (Fig 3B). Although the 2-4 fold Lyso-PC differences between non-infected or asymptomatic children and malaria cases were significant (Fig 1F-G and Fig 2E), these were much lower than the several orders of magnitude included in the standard curve used to establish that gametocyte conversion responds to low Lyso-PC in the culture medium (28), and were thus likely insufficient to impose alterations in sexual commitment or gametocytaemia, as seen by similar AP2G induction and gametocyte conversion in vitro (Fig 3).

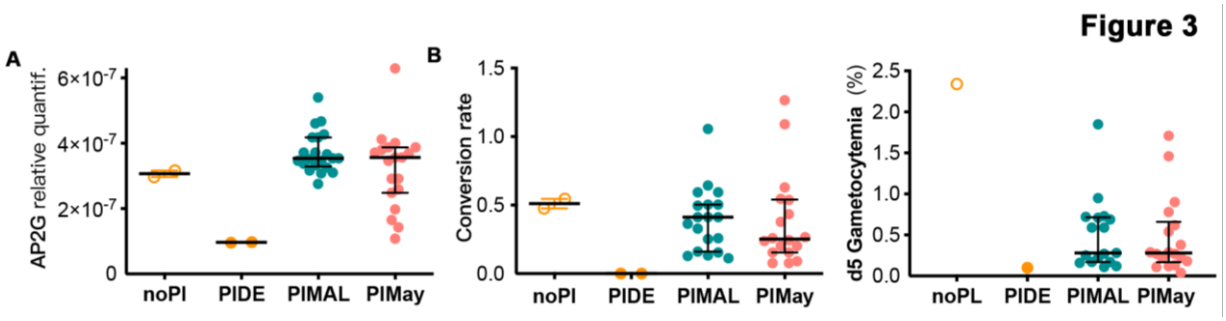


Fig 3. Decreased Lyso-PC of clinical malaria cases does not induce higher sexual commitment or gametocytaemia in vitro. A: Relative expression of AP2G detected by qRT-PCR of Pf2004 24 h after 24 h culture with 25% plasma collected during clinical malaria episodes (MAL n = 21), or at the end of the dry season (May n = 19). **B**: Pf2004 conversion rate (gametocytes d5/parasites d2) (left) and gametocyte density (right) detected by microscopy, after 24 h culture with 25% plasma collected during clinical malaria episodes in the wet season (MAL n = 19) or at the end of the dry season (May n = 18), alongside no plasma (noPl) and German (PlDE) conditions. Data represented as median ± IQR; Mann-Whitney test.

Progressing infection leads to apparent differences in gametocytogenesis. We observed decreased densities (Fig 1D and Fig 2B) but higher proportions (Fig 1E and Fig 2C) of female

and male gametocytes in the dry versus wet season, or in asymptomatic versus clinical malaria in the wet season (Fig 2C), without concomitant differences in the expression of regulators of sexual commitment, or transcriptional markers of sexually committed schizonts or early gametocytes (Fig 1H and Fig 2E and S1 Table). To further investigate this apparent conflict, we first ascertained if there were indeed indications of altered gametocytogenesis between parasites persisting through the dry season and parasites causing malaria in the wet season. Specifically, we investigated in more detail the transcriptional profiles of early and late-stage gametocytes. Using markers of early or late gametocyte development gathered from different sources ((35, 36) and Jeninga et al., unpublished Feb 2022) and intersecting them with the RNAseq data of clinical malaria cases and dry season samples shown in Fig 1, we identified 159 gametocyte-specific transcripts with quantifiable reads in samples from individuals with circulating parasites persisting through the dry season and parasites causing malaria in the wet season. Of those, 105 transcripts define early gametocytes; and 54 transcripts are characteristic of late-stage gametocytes (highlighted in S1 Table). Expression of these transcripts in asymptomatic carriers at the end of the dry season (May) and malaria cases (MAL) in the wet season showed an enrichment of upregulated transcripts of late-stage gametocytes in the dry season (81,48%) (44/54 genes), which clearly contrasted with the proportion of early-gametocyte transcripts or total *P. falciparum* transcripts upregulated at that time, which showed (39,05% (41/105 genes) and 33,4% (1131/3381 genes) upregulated in the dry season respectively (Fig 4A).

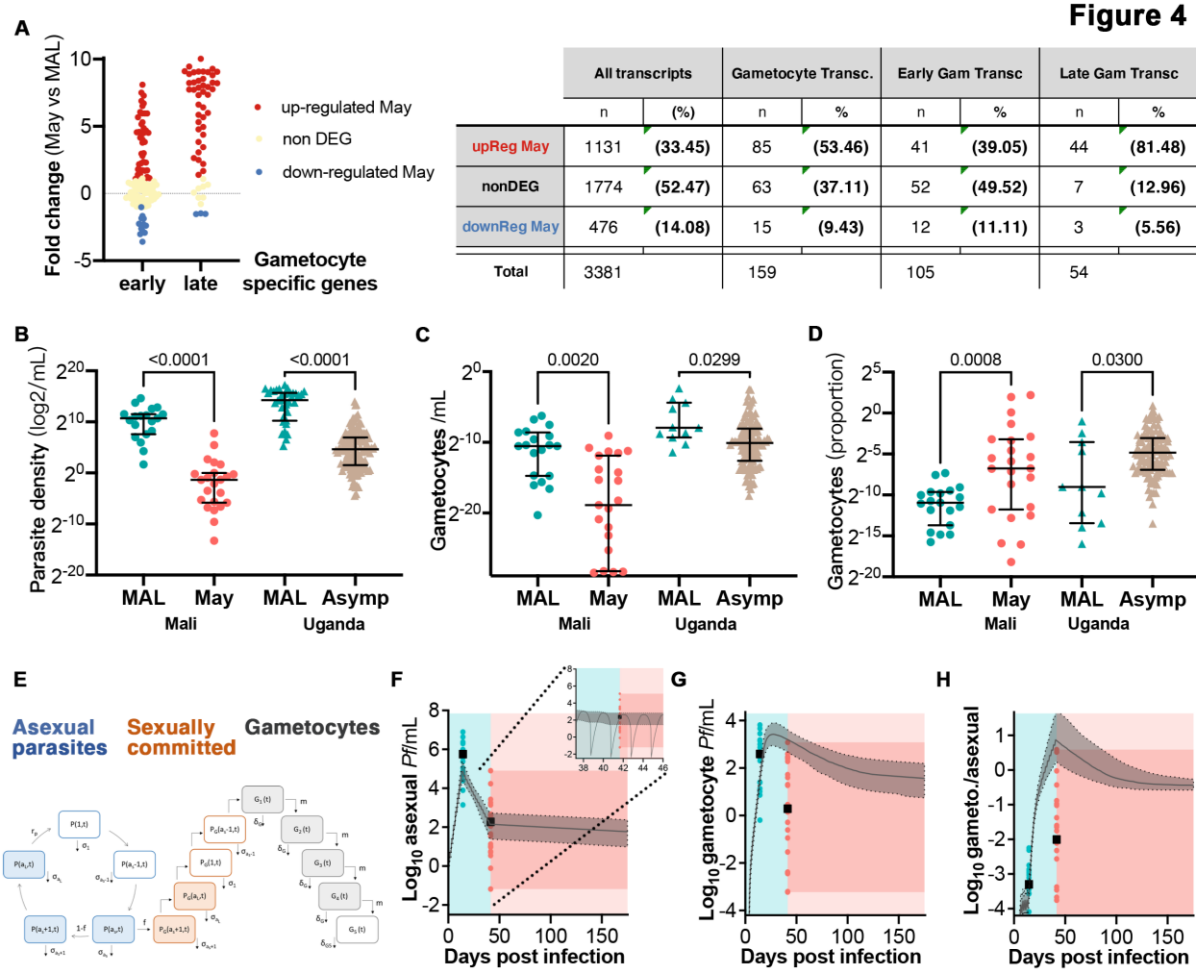
The lack of difference in sexual commitment or early-stage gametocyte transcripts, along with the strong difference in late-stage gametocyte transcript between the end of the dry season (May) malaria cases (MAL) parasitaemias hints not at a difference in gametocyte production, but rather an effect during the 8-12 days of gametocyte development, in both/either the sexual and/or the asexual parasite compartments, suggesting that length of infection may play a role in the results observed. Hence, we investigated if the observed patterns of gametocyte

densities and proportions were generalizable to non-seasonal settings where infections can also persist. While Mali shows a very contrasting seasonal pattern with 5-6 months interruption of transmission every year (15), Uganda has uninterrupted year-round transmission with peaks in times of stronger rain (37). RT-qPCR data quantifying transcripts of total parasite (varATS) and gametocyte (P25, PF3D7_1031000) densities from samples of 150 Ugandan individuals with asymptomatic parasitaemias persisting for over one month (n=122) and age-matched individuals presenting with clinical malaria (n=38) had been previously published (37) and were retrieved from ClinEpiDB (38). The data, determined by the area under the curve over the course of asymptomatic infection divided by the time of infection, indicated that parasite and gametocyte densities were increased in clinical malaria cases (MAL) compared to asymptomatic infections (Asymp) (Fig 4B and C); while the proportion of gametocyte transcripts relative to total *P. falciparum* transcripts showed again an inverse trend (Fig 4D). As seen with the Malian samples, the proportion of gametocytes was higher in persisting parasitaemias (Asymp) compared to malaria cases (MAL) (Fig 4D). Malian and Ugandan samples vary in seasonality of transmission, but share the fact that clinical malaria cases are likely recently transmitted (39), while asymptomatic parasitaemias have persisted longer than one month in the Uganda cohort, and at least 5 months in the Malian setting. The parallel in infection length between samples of symptomatic clinical malaria and asymptomatic infections in the two contexts led us to hypothesize that gametocyte numbers and proportions change dynamically in the course of a *P. falciparum* infection, leading to the observed patterns in Mali and Uganda. To address this, we devised a mathematical model including three different parasite populations_ asexual parasites (P), sexually committed parasites (P_G) and gametocytes (G), all of which can circulate or be adherent as previously developed (32) (Fig 4E). As the original model by Cao et al. was not able to sufficiently explain the observed dynamics when fitted to the data, we extended the model in different ways. First, we incorporated our recent observation of increased clearance of longer-circulating infected

erythrocytes in persisting parasitaemias compared to recently transmitted parasites (16), which promoted different removal rates of asexual and sexually-committed parasites during distinct phases of acute and chronic *P. falciparum* infection in our model (see Methods and Source code 1). In addition, we distinguish between three phases *during P. falciparum* infection in which the parasite multiplication factor varies due to immune response or adhesive properties of the parasite. Using an iterative Bayesian fitting-approach to fit the model to the symptomatic and asymptomatic parasitaemia and gametocytaemia levels of the Malian cohort, we quantitatively assessed several biological parameters including sexual commitment rate, gametocyte circulation time, parasite multiplication rate and gametocyte sequestration time, thereby confirming previously determined ranges (32, 40) (S3 Fig). Our adapted model was able to provide a dynamic depiction of parasitaemia levels, and gametocyte densities and proportions, as we observed within the Malian data (Fig 4F-H). We defined that the death rate of asexual and sexually committed parasites increased in later stages of the 48 h cycle, shifting toward the removal of older parasite stages and leading to a constant but oscillating number of asexual parasite in circulation (highlighted in the box-inlay of Fig 4F). This can, to some extent, explain the large variation seen in parasitaemias, especially during the chronic phase, which additionally may arise from different collection time points after start of infection for the individual samples (i.e., pooling of the individual measurements from Oct-May and calculation of average for each individual by the area under the curve over the course of blood collections during the asymptomatic phase and fixing it to one time point for fitting (see Methods)). Keeping a constant sexual commitment rate, f, throughout acute and chronic infection phases, our model predicts a higher number and lower proportions of gametocytes in the acute phase_ a trend that is inverted during the transition phase of infection due to effective removal of asexual parasites by an active immune response (Fig 4G and H). During the chronic phase of infection, when parasitaemias remain constant or are slowly decreasing, gametocyte proportions are decreasing as well until reaching an equilibrium roughly 4-5

months after the start of blood stage infection. Overall, our model provides a dynamic depiction of gametocyte densities and proportions similar to the data we observed in Mali and in Uganda, without altering the sexual commitment rate (Fig 4C and D).

Figure 4



	All transcripts		Gametocyte Transc.		Early Gam Transc		Late Gam Transc	
	n	(%)	n	%	n	%	n	%
upReg May	1131	(33.45)	85	(53.46)	41	(39.05)	44	(81.48)
nonDEG	1774	(52.47)	63	(37.11)	52	(49.52)	7	(12.96)
downReg May	476	(14.08)	15	(9.43)	12	(11.11)	3	(5.56)
Total	3381		159		105		54	

Fig 4 | Progressing infection leads to apparent differences in gametocytogenesis. A, Fold change between parasites collected at the end of the dry season and from clinical malaria cases (May n= 12, MAL n= 12) of early and late gametocyte transcripts according to López-Barragán et al., 2011; Poran et al., 2017 and Petter unpublished data. Each dot represents a gene and color identifies range in fold change, red: upregulated in May, yellow: not DEG, and blue: downregulated in May). **B**, Parasitaemia, **C**, gametocytaemia and **D**, proportion of gametocyte in symptomatic (MAL n = 19 and 38 in Mali and Uganda respectively) and asymptomatic infections (May n = 23 and Asymp n = 122) in Mali and Uganda quantified by RT-qPCR. Data represented as median ± IQR; multiple non-parametric test with Tukey-correction for multiple comparisons. **E**, Diagram of the mathematical model to describe parasite dynamics including individual compartments, transitions and death rates; Asexual parasites (P) replicate in a 48 h cycle, being cleared at an age dependent death rate (σ_a). A fraction of asexual parasites (f) transitions to the sexually committed parasite compartment (P_G), goes through a round of replication and develops into gametocytes (G). Gametocytes mature over 5 distinct stages in independent model compartments. Asexual parasites (blue),

sexually committed parasites (orange) and gametocytes (grey) are indicated shaded if not circulating due to sequestration. **F**, Model prediction and clinical data of asexual parasites. The small inlay indicates a zoom-in showing the oscillating parasite dynamics within 48 h-intervals. **G**, gametocytes and **h**, the proportion of gametocytes per mL blood. Individual data points and median (black square) of wet (aqua circles) and dry (orange circles) season data throughout acute (aqua shaded area) and chronic (salmon shaded area) phase of infection are shown. Median fit (grey line) and 95% prediction interval of 100 model predictions of the 6th PyABC generation are shown as grey shaded area.

Discussion

The relative absence of mosquitoes during the months-long dry season and the short lifespan of *P. falciparum* sexual-stage gametocytes (11, 12) renders the production of gametocytes ineffective during most of the dry season; however, gametocytes must be available to resume malaria transmission when mosquitos return with the wet season. Whether *P. falciparum* maintains a constant rate of production of sexual forms or adjusts gametocytogenesis to maximize transmission has been unclear. In this study, with samples from Malians exposed to alternating 6-month dry and wet seasons, and samples from clinical malaria and asymptomatic *P. falciparum* infection from year-round transmission region of Uganda, we showed that symptomatic cases of malaria consistently have higher parasite and stage V gametocyte densities than asymptomatic infections; however, the proportion of gametocytes increases in the asymptomatic persisting infections (Fig 1, 2 and 3). Additionally, our data from Mali indicated that transcription of genes characterizing sexually committed parasites and stage I gametocytes (41, 42) quantified by RT-qPCR did not appear affected by the season or clinical symptoms (Fig 1 and 2), nor were reads of over 100 genes characteristic of early gametocytes and detected by RNAseq of circulating parasites particularly enriched for DEGs. This, clearly contrast with representative expressed genes of late gametocytes that were very much enriched of transcripts upregulated in persisting asymptomatic infections at the end of the dry season (Fig 4 and S1 Table).

Our work suggests that gametocytogenesis remains constant as infections progress from recent transmission to chronic carriage, and it is the difference in asexual survival/replication rates that promotes different gametocyte densities, independently of environmental factors. Although it has been suggested that *P. falciparum* investment in gametocyte production adjusts to host nutrient availability (43), drug pressure (22), transmission intensity (44), and even seasonality (45, 46), results are often inconsistent and potentially affected by the dynamics of asexual parasite growth, without necessarily indicating altered sexual commitment. In Sudan, following the long dry season, gametocyte density estimates have been reported to increase prior to the highest transmission peak (47), suggesting a higher gametocyte investment. However, in Burkina Faso, gametocyte densities were higher at the peak and end of the transmission season rather than during the dry season (48), while the efficiency of membrane feeding to infect mosquitoes was increased early in the transmission season compared to peak of the malaria season or the dry season (49). Nevertheless, these studies did not monitor longitudinally the same parasite population during the dry and early wet season. In a recent study also in Burkina Faso, transmission was shown to be more effective in persistent than in incident infections, but no data was collected after day 35, and collections were all done during the wet season (25) when a mix of more and less recently transmitted parasite clones is likely in individuals without symptoms.

In vitro, depletion of Lyso-PC or choline have been shown to induce AP2G expression, leading to increased gametocyte production (28, 50), but associations in humans are inconsistent. In Rwanda, low Lyso-PC associated with higher parasite densities (51); and in Ghana, although correlations with parasitaemia were not reported, Lyso-PC levels in human blood samples were inconsistently related with gametocyte-committed ring stages converting into gametocytes in vitro (42). Our data show, like Orikiiriza et al., an association of lower Lyso-PC levels with increased parasitaemia and severity of malaria (Fig 1F-G and 2E), but the

association was lost at low parasitaemias, and persistent infection at the end of the dry season did not affect Lyso-PC compared to non-infected individuals (Fig 2F and S2 Fig).

The effect of drugs on sexual conversion in vitro has been previously reported. Specifically, sub-curative doses of dihydroartemisinin (DHA) stimulate AP2G expression and sexual conversion when drug exposure occurs at the trophozoite stage, but not at the early ring stage. The observed increase could not synergize nor add to increases promoted by depletion of choline, suggesting, as DHA affects lipids, that the two effects might be the same (50). Our RNAseq (S1 Table) and RT-qPCR (Fig 1H and 2D) data show constant low-level expression of AP2G, the master regulator of gametocytogenesis, and equally unaltered expression of transcripts characteristic of committed schizont and early gametocytes (S1 Table and Fig 1H), suggesting that reductions in circulating Lyso-PC induced by uncomplicated clinical malaria are not capable of affecting *P. falciparum* sexual commitment. Accordingly, we observed in vitro that supplementation with plasma from clinical malaria cases (low Lyso-PC) or end of the dry season persistently infected individuals (normal Lyso-PC) did not differentially induce AP2G expression or the rate of sexual conversion (Fig 3), suggesting that Lyso-PC differences introduced by *P. falciparum* infection are not sufficient to trigger the mechanisms demonstrated by Brancucci et al. and Portugaliza et al.. Nevertheless, it is possible that Lyso-PC levels in particular bone marrow niches are sufficiently low to trigger parasites' sexual commitment, although this would require that infected erythrocytes be at that niche to respond to the stimulus.

Previous reports highlighting differences in gametocytogenesis as a product of altered sexual commitment did not account for longevity of infection, nor for dynamic changes within the asexual compartment during a persistent infection. Greischar et al. demonstrated the difficulty of quantifying true transmission investment by comparing existing quantification methods on simulated data where true transmission investment is known (52), and Prajapati and colleagues relied on in vitro culture of sexually committed circulating parasites to define

samples with higher and lower commitment rates (53). Also, our study has limitations; on one hand it is possible that our quantifications of parasite and gametocyte densities are affected by imperfectly comparable primer efficiencies, which also make difficult comparisons of female, male and total gametocyte densities across different transcripts measurements; and on the other, although we do not have true longitudinal data, our model assumes a transitions from a faster to a slower growth rate due to the development of immunity which has been recorded from malaria-therapy used to treat neurosyphilis patients (54).

Despite these limitations, we show empirically and reinforce with a mathematical model, that the proportion of sexual stages is inversely related in recently transmitted versus chronic infections, without alteration in the expression of regulators of sexual commitment and while maintaining a constant rate of sexual commitment. Our recent study in which we found that infections that persist beyond the acute immune response exhibit prolonged circulation and increased splenic clearance (16) allowed us to complement the model by Cao et al. with an increased asexual parasite death rate following the immuno-responsive period, which promoted the progressively increased representation of sexual stages in later phases of the infection.

Gametocyte proportions are low early on in infection because asexual replication efficiently increases asexual parasite numbers while adaptive immunity is absent, and the lag of ~8 days in gametocyte development in the bone marrow has its impact. In our model we propose that later, as immunity prevents fast asexual growth and the parasites persisting further are more efficiently removed in the spleen, the opposite lag effect is then shown, and the proportion of gametocytes finally reflects, despite keeping the same sexual commitment rate, that of parasitaemias ~8 days behind.

In conclusion, our data is consistent with a model in which *P. falciparum* maintains a constant rate of sexual commitment such that the proportion of gametocytes increases over time as

infection persists and asexual parasite numbers decline more rapidly, a strategy that may help overcome the vector-free period and increase transmission efficiency.

Materials and Methods

Gametocyte-specific gene expression. We used RNAseq data of leucocyte-depleted blood pellets from 12 children with asymptomatic malaria at the end of the dry season and an age- and gender-matched cohort presenting with their first malaria case in the ensuing transmission season reported in (16). Originally 808 gametocyte specific genes were reported (33), and we counted 786 after excluding repetitions and converting nomenclature to its most recent version (S1 Table). Out of these 786 genes, we detected normalized reads of 333 gametocyte-specific genes in the RNAseq data set (16) to perform a principal component analysis. Using Deseq2, 205 genes were determined to be differentially expressed between the dry season and malaria cases and were plotted in the heatmap shown in Fig 1B.

Study subjects and ethical approval. Approval by the Ethics Committee of Heidelberg University Hospital, the Faculty of Medicine, Pharmacy and Odontostomatology (FMPOS) at the University of Bamako, and the National Institute of Allergy and Infectious Diseases of the National Institutes of Health Institutional Review Board was obtained to conduct the study registered at ClinicalTrials.gov with the identifier NCT01322581. Clinical data and samples were obtained from 2017 to 2019 in a cohort study previously described (55), conducted in Kalifabougou, Mali, where malaria transmission occurs between June and December, and antimalarial drugs are provided by a single clinic and pharmacy. All participants and the parents/guardians of included children provided written informed consent. Exclusion criteria at enrolment included haemoglobin <7 g/dL, axillary temperature ≥37.5°C, acute systemic illness, or use of antimalarial or immunosuppressive medications in the 30 days preceding enrolment. Clinical malaria cases were prospectively determined by passive surveillance and defined by

axillary temperature ≥37.5°C, ≥2500 asexual parasites/µL of blood, and no other evident cause of fever. Malaria episodes were treated with a 3-day standard course of artemether/lumefantrine according to Malian national guidelines. Cross-sectional clinical visits and blood draws were performed late in the transmission season (October), and at the beginning (January), mid (March) and end (May) of the dry season.

Sample collection. Venous blood (4 mL if donor age was below 4 years, and 8 mL if above) was drawn by venepuncture on wet and dry season cross-sectional visits, and at first malaria episode of the transmission season. Samples are referred as MAL for malaria cases or by time point of collection. Blood was collected in sodium citrate-containing cell preparation tubes (Vacutainer CPT Tubes, BD) and transported to the laboratory where RBC pellet and plasma were separated and stored at −80°C within three hours of blood draw. Plasma used for lipidomic analysis and induction of gametocytogenesis was separated by centrifugation and immediately frozen in liquid N_2.

RNA extraction and quantification of parasite transcripts. RNA was isolated from 200 µl RBC pellet by phenol-chloroform extraction using TRIzol (Ambion), followed by DNAse treatment using the DNAse I Amplification Grade Enzyme (Invitrogen). RNA was quantified by absorption measurements at 230, 260 and 280 nm on a Citation3 Imaging plate reader, followed by cDNA synthesis using the Superscript IV Enzyme (Invitrogen) according to manufacturer instructions. Taqman RT-qPCR multiplex assay (Applied Biosystems) was performed on a qTower (Analytic Jena) with primers to quantify the female gametocyte marker ookinete surface protein P25 (PF3D7_1031000), the male gametocyte transcript PFMGET (Pf3D7_1469900), the master regulator of sexual commitment Ap2G (PF3D7_1222600) and a parasite housekeeping gene (Glycine-tRNA ligase, PF3D7_1420400). Amplification efficiency (E) was estimated on the basis of the equation E=(10^(-1/slope)-1)x100 (S1 Fig). NF54 parasite line expressing

DyneinGFP and carrying an episomal P47mCherry plasmid (DynGFP/P47mCherry) was used for sorting of male and female gametocytes through their respective specific mCherry and/or GFP expression (34). Standard curves of 10-10^6/ml of sorter-purified female or male gametocytes were generated by 10-fold serial dilution in 50% haematocrit (S1 Fig). qPCR expression of female-specific P25 in female (mCherry+) sorted parasites was high and decreased with gametocyte densities, while male gametocytes (GFP+) show ~12-fold lower expression of P25, which may be explained by ~6% impurity originating from the opposite sex during sorting (S1 Fig). Likewise, PfMGET expression was approximately 10-fold higher expressed in male gametocytes (GFP+) compared to females (mCherry+), consitent with 5-7% impurity. Linear regression fit for the independent populations revealed a good fit with R^2-values of 0.93-0.97 by P25 expression and 0.96-0.98 by PfMGET expression (S1 Fig). The asexual parasites standard curve, was generated with Pf2004 asexual culture was counted by FACS and Glycine-tRNA ligase expression was quantified in dilution series of 10-10^7 asexual late rings per ml. The combination of asexual and sexual standard curves was used for qPCR quantification of female and male proportion of total parasitaemias throughout the season. To account for fluorescence leakage between channels a compensation matrix was calculated based on single color fluorescence measurements by an application specialist at Analytic Jena. Gexp5 (PF3D7_0936600) expression levels were quantified in a Taqman RT-qPCR duplex assay together with Glycine-tRNA ligase as a housekeeping gene. Expression levels of GDV1 (PF3D7_0935400), and MRSP1 (PF3D7_1335100) were determined in further Taqman RT-qPCR assays. All primers and probes sequences are listed in the key resources table on S2 Table. Relative quantification was performed by $\Delta\Delta$Ct method using Glycine-tRNA ligase as housekeeping gene and MAL samples a calibrator group.

Parasite culture and preparation of a standard curve for gametocyte quantification.

Pf2004 parasites were cultured at 37° C in 5% O_2, 5% CO_2 and 90% N_2, at 5% haematocrit in

RPMI-1640 medium (with L-glutamine and HEPES (Gibco)). Parasite culture medium was supplemented with 0.24% Sodium Bicarbonate (Gibco), 100µM Hypoxanthine (cc pro), 25 µg/mL gentamycin (Gibco)), and 0.25% Albumax II (Gibco). Medium was changed daily, parasitaemia assessed by Giemsa-stained blood smear and the culture split according to experimental needs. *PfDyn*GFP/*PfP47*mCherry parasite lines (56) were cultured in presence of 15 µg/ml of Blasticidin-S-HCL (Invitrogen) and 2 µM Pyrimethamine (Sigma Aldrich) as described for non-integrating plasmids and P52 integrating plasmid. Parasites were grown to >4% parasitaemia when 50 mM N-Acetylglucosamine (Sigma Aldrich) was added to the culture to eliminate asexual stages. After 4 consecutive days of N-Acetylglucosamine treatment, culture in complete RPMI was resumed. Gametocyte development was monitored daily by smear and on day 5 after start of gametocyte induction magnetic enrichment was performed with MACS columns (Miltenyi Biotec). Gametocytes were sorted on a BD FACSAria III on day 8, keeping cells at 4° C in suspended activation buffer, (gating strategy in S1 Fig). RNA of a defined numbers of sorted male and female gametocytes was extracted and reverse transcribed, a standard curve prepared by serial dilution and used for RT-qPCR as described above.

Phospholipid analysis. May and MAL plasma samples (shown in Fig 1) were analyzed by shotgun lipidomics at Lipotype as described in (57). Briefly, automated samples extraction was followed by direct sample infusion and high-resolution Orbitrap mass spectrometry including lipid class-specific internal standards to assure absolute quantification of LysoPC and LysoPE, using LipotypeXplorer for identification of lipids in the mass spectra. Oct, Mar, May and MAL samples (shown in Fig 2) were analyzed by Liquid chromatography-mass spectrometry (LC-MS). Samples were extracted, processed, and analyzed at the Lipidomics Unit at the Institute of Physiological Chemistry in Mainz, using the liquid-liquid extraction protocol as described (58). All values have been normalized to analyzed plasma volume.

In vitro induction of *P. falciparum* sexual commitment and gametocytogenesis. In vitro induction of *P. falciparum* sexual commitment was performed as described by (Brancucci et al., 2015). Pf2004 parasites were synchronized to a 4 h window by repeated treatment with 5% sorbitol and MACS purification. Parasites at 26 ± 2 h post invasion (hpi) were plated on 48-well plates in 500 □l culture volume at 0.8-1% parasitaemia and 2.5% haematocrit and cultured in minimum fatty acid medium (mFA, RPMI1640 with 100 µM hypoxanthine, 25 µg/ml gentamycin, 0.39% fatty-acid-free BSA, 30 µM palmitic acid and 30 µM oleic acid) supplemented with 25% plasma from asymptomatic children at the end of the dry season (PlMay), or 25% plasma from malaria cases during the rainy season (PlMAL), in addition to the conditions used by Brancucci et al. (mFA alone, and mFA supplemented with 25% German plasma (PlDE)). After 24 h of treatment, the medium was changed to culture medium and subsequently changed daily. At 48 h after beginning of treatment 30 IU/ml heparin were added to block re-invasion of parasites and eliminate asexual forms. At 0, 48 and 120 h post treatment RBC pellets were collected for RNA quantification of gametocyte markers by RT-qPCR; parasitaemia and gametocytaemia determination by flow cytometry (50 µl of culture stained with 5x SYBR Green II (Invitrogen) and 7,5 µM MitoTracker (Applied Biosystems) for 30 min at 37 °C and acquired with CountBright Absolute Counting Beads (Thermo Ficher) on a FACS Canto II or an LSR II and analyzed using FlowJo software 10.2 or higher versions (Tree Star)). Additionally, Giemsa-stained blood smears were prepared at 48 h and 120 h post-treatment and blindly analyzed by microscopy, counting RBCs, parasites, and gametocytes in 18 fields of view. Multiplication rate of parasites was calculated as $\frac{parasitemia\ 48\ h}{parasitemia\ 0\ h}$, and conversion rate as $\frac{gametocytemia\ 120\ h}{parasitemia\ 48\ h}$.

Analysis of early and late gametocyte markers in RNA-seq data. We compiled a list of early and late gametocyte specific genes based on three RNA-seq studies comparing distinct parasite stages, data from Poran et al. 2017obatined after request from the corresponding author (compared day 2 versus day 9 of gametocytogenesis by single cell RNAseq); well as data from Lopez-Barragan et al. 2011 (compared day 4 versus day 15 of gametocytogenesis by bulk RNAseq) and Jeninga et al., unpublished (compared day 4 of gametocytogenesis versus day 10 of FACS sorted male and female gametocytes by bulk RNAseq). Poran et al. selected early and late gametocyte genes as genes showing a 20-fold higher expression in early or late gametocytes over the respective other group. Late gametocyte genes were also required to show a 20-fold higher expression in late gametocytes compared to ring stage parasites, this was not the case for early gametocyte genes. In Lopez-Barrgagan's data, early gametocyte genes were defined as genes having a 10-fold expression increase in early gametocytes compared to late gametocytes. Genes showing a 20-fold higher expression in late stages were designated as late gametocyte genes. Expression differences to asexual stages were not considered. In Jeninga et al. unpublished genes showing a 3-fold or higher expression difference to both asexual stages and late gametocytes were selected as early gametocyte genes, regardless of gametocyte sex. Intersecting the full list of early and late gametocyte genes with genes transcribed in circulating parasites stages, defined by RNAseq data of Andrade et al., generated a final list of 109 early gametocyte genes and 54 late gametocyte genes to evaluate expression differences of these genes between dry and rainy seasons.

Quantification of parasites in samples of Ugandan asymptomatic carriers by RT-qPCR. We compared the Malian data from dry and wet season with data from 150 Ugandan individuals enrolled in a 2-year longitudinal study including monthly standardized clinical evaluation and blood collection (37) retrieved from ClinEpiDB

(https://clinepidb.org/ce/app/record/dataset/DS_51b40fe2e2122). Asymptomatic *P. falciparum* positive individuals (20.4 years ± 17.2, median: 13.3 years) were included based on the following criteria: i) ≥ two PCR+ time points, ii) ≤ 3 negative time points in between positive, iii) same clone (determined by deep amplicon sequencing of atypical membrane antigen 1 (AMA1) as described in (59) present at all time points of one asymptomatic infection. Additionally, we used samples from an age-matched group of 38 clinical malaria cases (tympanic temperature > 38°C or history of fever in the previous 24 hours and *P. falciparum* positivity by microscopy). *P. falciparum* parasite and gametocyte density in blood samples were measured by RT-qPCR of multi copy conserved *var* gene acidic terminal sequence (60) and P25 (P25, PF3D7_1031000), respectively. Average levels of parasitaemia and gametocytaemia during each asymptomatic infection, were estimated as the area under curve over the course of infection divided by length of infection. For symptomatic infection only the quantification before treatment on day of diagnosis was considered. For comparison between two data sets, a multiple non-parametric comparison between groups of asymptomatic and symptomatic in Mali and Uganda has been performed using Tukey-Correction for multiple comparisons.

Mathematical model. The developed mathematical model extends a model used previously to describe asexual parasite and gametocytes dynamics (32). The model considers 3 different main parasite populations, i.e., distinguishing between asexual parasites (P), sexually committed parasites (P_G) and gametocytes (G) (Fig 4E). Asexual parasites develop and replicate within a red blood cell over a cycle of length a_L=48h after which mature schizonts burst and release a number of r_P = 18-32 merozoites that are able to invade new red blood cells. During this replication cycle, parasites are assumed to be removed through splenic clearance and active immune response. In extension to the previous model, we assume that this removal of asexual and committed parasites follows an age dependent death rate, σ_a that

differs in distinct phases of acute and chronic *P. falciparum* infection. The following iterative equation describes asexual parasite growth at discrete time steps for parasite age, *a*, and time, *t*, increments of one hour.

$$P(a,t) = \begin{cases} P(a-1,t-1)e^{-\sigma_a} \ , a = 2,3,\dots,a_L \\ r_p \ P(a_L,t-1)e^{-\sigma_a} \ , a = 1 \end{cases} \quad (1)$$

$$P(a_s + 1,t) = (1-f)P(a_s\,,t-1)e^{-\sigma_a} \quad (2)$$

The rate of parasite removal, σ_a, is assumed to increase with parasite age within one replicative cycle and described by a sigmoidal curve given by:

$$\sigma_a = \sigma(a) = \frac{\sigma_{max}}{1+e^{p_\sigma(T_\sigma - a)}} \quad (3)$$

The parameter σ_{max} defines the maximal rate of parasite removal, T_σ, the age at which 50% of parasites are removed, and p_σ a scaling parameter.

At parasite trophozoite stage of a_s= 20hpi parasites adhere to the endothelium when at the same time a fraction, f, of parasites enters sexual commitment. Committed parasites continue the rest of the replication cycle producing only committed parasites that enter the first gametocyte stage at the sequestration age, a_s, of the following replication cycle.

$$P_G(a_s+1,t) = f\,P(a_s,t-1)e^{-\sigma_a} \quad (4)$$

$$P_G\,(a,t) = \begin{cases} P(a-1,t-1)e^{-\sigma_a}, a = 2,3,\dots,a_L \ except\ a = a_s\ and\ a = \ a_s+1 \\ r_p \ P(a_L,t-1)e^{-\sigma_a} \ , a = 1 \end{cases} \quad (5)$$

Gametocytes develop over 5 distinct stages of which only mature stage 5 is found in circulation. The development of gametocytes is modelled by:

$$G_1(t) = \ G_1(t-1)e^{-(m-\delta_G)} + \frac{P_G(a_s-1,t-1)e^{-\sigma_a}(1-e^{-(m+\delta_G)})}{m+\delta_G} \quad (6)$$

$$G_2(t) = G_2(t-1)e^{-(m-\delta_G)} + \frac{mG_1\ (t-1)\ (1-e^{-(m+\delta_G)})}{m+\delta_G} \qquad (7)$$

$$G_3(t) = G_3(t-1)e^{-(m-\delta_G)} + \frac{mG_2\ (t-1)\ (1-e^{-(m+\delta_G)})}{m+\delta_G} \qquad (8)$$

$$G_4(t) = G_4(t-1)e^{-(m-\delta_G)} + \frac{mG_3\ (t-1)\ (1-e^{-(m+\delta_G)})}{m+\delta_G} \qquad (9)$$

$$G_5(t) = G_5(t-1)e^{-(\delta_{G5})} + \frac{mG_4\ (t-1)\ (1-e^{-(\delta_{G5})})}{\delta_{G5}} \qquad (10)$$

Immature gametocytes, that are sequestered, mature with a maturation rate, m, and are lost by death rate δ_G , while mature circulating gametocytes are removed by a splenic clearance rate δ_{G5}. As pointed out above the whole model is simulated by discrete time-steps of one-hour increments.

Accounting for different phases of P. falciparum infection: In our model the start of the infection is marked by the beginning of blood stage infection, where we assume an initial parasite load, IPL, with parasite age normally distributed with mean, μ_{IPL} and standard deviation σ_{IPL}. During the acute phase of infection parasitaemias increase rapidly leading to clinical symptoms within the first 2 weeks of blood stage infection (61). The acute phase of infection is accompanied by the generation of an adaptive immune response, including the production of effective antibodies, that takes ~14 days to develop (62). To account for higher parasite removal due to the immune response, we assume an increased maximal parasite removal rate σ_{max2} for age-dependent parasite loss when an adaptive immune response is active. Increased parasite removal is considered to occur 14 days post start of blood stage malaria infection and

indicates the beginning of the transition phase from the acute towards the chronic asymptomatic phase of infection. In addition, as selection for less adhesive and less virulent parasite strains by an active immune response is considered to be crucial for the maintenance of a malaria infection over the course of several dry season months (16), we accounted for this effect in our model by an increased value of T_σ for Eq. (3) during the chronic asymptomatic phase, i.e., replacing T_σ by $T_{\sigma,2}$. This parameter change shifts clearance rates towards affecting older asexual parasite stages, leading to a constant, but oscillating number of asexual parasite numbers in circulation.

Parameter estimation: The model described in Eqs (1-10) was applied to the data assuming a fixed parasite life-cycle period of a_L=48 h, a sequestration age of a_S= 20 hpi, as well as a parameter of T_σ=32 h and P_σ=0.7 characterizing age-dependent parasite removal during the acute infection phase. All other parameters were fitted to the both symptomatic and asymptomatic time points simultaneously using an approximative Bayesian computing approach (63). Prior distributions for the individual parameters spanning biologically reasonable ranges have been selected in accordance with previous studies (Table 1). Due to the high model complexity and parameter dependencies, estimations of multiple pyABC-runs diverge to different local minima for the individual parameters. To obtain robust results for the parameters indicating phase-dependent changes, several independent pyABC fitting runs were compared and the 6th generation was chosen where parameter posterior distributions would still overlap. For the fitting, values of individual patients in the dry season are condensed to one time point by calculating the average levels based on the area under the curve over the course of dry season patient surveillance. Values were log10-transformed to ensure comparability of parasitaemia and gametocytemia. In addition, wet and dry season measurements were assumed to represent the parasite dynamics at 2 and 6 weeks post infection, respectively. Sensitivity of the results with regard to these time points was

investigated. All code to run and analyze the mathematical model are available at https://github.com/GrawLab/MalariaReplication.

Table 1. List of model parameters and respective prior distributions. All prior distributions were uniformly distributed given the indicated ranges.

Parameter	Unit	Description	Prior range	Reference for prior range	89% CI of posterior range
ipl	Parasites /ml	Initial parasite load	1 - 10	(Hodgson et al. 2015) (Schats et al. 2015)	1.47 - 6.54
μ_{ipl}	h	Initial parasite age	4 - 10	(Eichner et al. 2001)	4.41 - 8.22
σ_{ipl}	h	Standard deviation of initial parasite age	4.5 - 6.5	(Cao et al. 2019)	4.89 - 5.81
$\mathbf{r_p}$		Replication factor	18 - 32		2.28 - 3.13 x 10^1
σ_{max}	h^{-1}	Maximal removal rates during acute phase of infection	0.01- 0.15	(Andrade et al. 2020)	0.81 - 1.07 x10^{-1}
σ_{max2}	h^{-1}	Maximal removal rates during transition phase of infection	0.01- 0.4		2.13 - 2.39 x10^{-1}
δ_G	h^{-1}	Death rate immature gametocytes	1e-05 – 1e-04	(Cao et al. 2019)	3.53 - 8.60 x10^{-5}
δ_{G5}	h^{-1}	Death rate mature gametocytes	1.39 - 4.17 x10^{-3}	(Cao et al. 2019)	1.79 - 3.98 x10^{-3}
m	h^{-1}	Gametocyte maturation rate	1.6 - 4.6 x 10^{-2}	(Cao et al. 2019)	2.76 - 4.29 x10^{-2}
f	h^{-1}	Commitment rate	0.5 - 1.0 x 10^{-2}	(Cao et al. 2019), (Eichner et al. 2001)	5.41 - 7.68 x10^{-3}
$\mathbf{a_L}$	h	Life cycle length	48		
$\mathbf{a_s}$	h	Sequestration age	20		
$\mathbf{T_\sigma}$	h	Age at which 50% of parasites are removed during acute phase of infection	32	(Andrade et al. 2020)	
$\mathbf{T_{\sigma 2}}$	h	Age at which 50% of parasites are removed during chronic phase of infection			33.6 - 34.7
$\mathbf{P_\sigma}$		Shape parameter	0.7	(Andrade et al. 2020)	
$\sigma_{max}/\sigma_{max2}$	h	Ratio of maximal removal rate	0.025 - 15		2.06 - 2.89

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Competing Interest Statement

The authors declare that they have no conflict of interest.

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Figure supplement legends

S1 Figure. A: Flow cytometry gating strategy to identify and sort PfDynGFP/ PfP47 gametocytes by mCherry and GFP expression into three populations (female: mCherry-positive, male I: mCherry- GFP-positive, male II: GFP-positive). **B:** Pre-sort gametocyte distribution, and post-sorting purity verification of female and male gametocytes. **C:** Serial dilutions of P25, PfMGET, AP2g, and Glycine-tRNA ligase synthetic DNA to determine curves' slopes and primer efficiency by qPCR. **D:** Standard curves determined with serial dilutions of sorted-purified female and male gametocytes, showing expression of female marker P25 (left) and male marker PfMGET (right).

S2 Figure. Plasma concentration of Lyso-PC 16:0 and 18:0 of RDT+ asymptomatic children at different times during the wet (Oct) and the dry season (Mar and May), and during clinical malaria episodes (MAL).

S3 Figure. A: Posterior distribution of 12 model parameters generated of 100 fits of 6th pyABC generation of one representative pyABC fitting run. **B:** Estimates of biological parameters in distinct phases of P. falciparum infection shown as 89% credibility interval of the posterior distributions. Sexual commitment rate was calculated by fx100%. The parasite multiplication factor is calculated by $\exp(-a_L)(1-f)$ with the term $\exp(-a_L)(1-f)$ describing the fraction of surviving asexual parasite after death and sexual conversion per replication cycle. Gametocyte sequestration time was calculated by 4/m in relation to the 4 sequestered states (stage I to IV) (Cao et al. 2019). While calculations of the model where done in 1 h increments, all estimates shown in S3 Figure. B are converted to per day.

Table supplement legends

S1 Table. Gene Ids and descriptions of transcripts defined as gametocyte-specific by Painter et al., 2017; transcripts detected in RNA sequencing dataset (Andrade et al., 2020) comparing persistent *P. falciparum* in at the end of the dry season (May) to *P. falciparum* of clinical malaria in the wet season (MAL); and transcripts defining early or late gametocyte development gathered from Poran et al., 2017 after request from the corresponding author, López-Barragán et al., 2011, and Jeninga et al., unpublished. (pink and blue shaded rows highlight transcripts up or down regulated in May vs. MAL, respectively.)

S2 Table. Key Resources Table