

A cohort study on the duration of *Plasmodium falciparum* infections during the dry season in The Gambia

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Brief summary:

In The Gambia, a cohort of individuals asymptomatically infected with *P. falciparum* was sampled monthly over the dry season. Higher density multi-clonal infections were more likely to persist during the dry season.

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Abstract

Background: In areas where *Plasmodium falciparum* malaria is seasonal, a dry season reservoir of blood-stage infection is essential for initiating transmission during the following wet season.

Methods: In The Gambia, a cohort of 42 individuals with qPCR positive *P. falciparum* infections at the end of the transmission season (December) were followed monthly until the end of the dry season (May) to evaluate infection persistence. The influence of human host and parasitological factors was investigated.

Results: A large proportion of individuals infected at the end of the wet season had detectable infections until the end of the dry season (40.0%; 16/40). At the start of the dry season, the majority of these persistent infections (82%) had parasite densities >10 p/μL compared to only 5.9% of short-lived infections. Persistent infections (59%) were also more likely to be multi-clonal than short-lived infections (5.9%) and were associated with individuals having higher levels of *P. falciparum*-specific antibodies ($p = 0.02$).

Conclusion: Asymptomatic persistent infections were multi-clonal with higher parasite densities at the beginning of the dry season. Screening and treating asymptomatic infections during the dry season may reduce the human reservoir of malaria responsible for initiating transmission in the wet season.

Keywords: *Plasmodium falciparum*, Malaria, transmission, dry season, multiplicity of infection,

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Introduction

In many endemic areas, malaria transmission is seasonal, with most clinical cases occurring during the wet season. *Plasmodium falciparum* infections acquired during this period can persist in asymptomatic individuals throughout the dry season, and if they produce gametocytes, can potentially contribute to transmission at the start of the next wet season [1-3]. These clinically silent infections can persist for many months without treatment [2], and pose a significant hurdle for malaria elimination efforts [4]. Understanding how long infections persist in the dry season in The Gambia and identifying who carries them may inform targeted approaches to reduce the dry season reservoir of malaria and reduce re-introduction of malaria in the subsequent wet season.

Few studies have described the asymptomatic dry season reservoir of malaria and the related parasite and host factors. Asymptomatic, genetically complex infections are common in high transmission areas [5]; also in low transmission settings infections can persist for many months [6, 7]. The host specific response to malaria infection may play an important role by allowing the establishment of chronic infections. Protection against severe malaria is acquired relatively quickly [8, 9], but the ability to control parasitemia at a sub-clinical levels develops more slowly following repeated exposure [10]. In Mali, individuals infected during the dry season had higher *P. falciparum*-specific antibodies than those who were uninfected [11, 12], and older children were more likely to be infected at the end of the dry season compared to younger children [13]. Both findings support the hypothesis that a certain degree of exposure and immunity is required to be asymptotically infected. Interestingly, in Uganda, older children carried infections for a longer time than children under 5 and also adults [14], suggesting that the type of immune response associated with persistent asymptomatic infection may differ from responses that can clear infections [15]. Moreover, it is unclear when parasite specific antibody responses contribute to protection [16, 17] or are simply markers of previous exposure [18]. In a recent study, responses to a panel of *P. falciparum* antigens indicated a higher level of immunity that allowed individuals to control blood-stage infections following controlled exposure to *P. falciparum* sporozoites [19]. Other host factors may also play an important role in the establishment of chronic asymptomatic infections. In Uganda, infections persisted longer in males than females [14] and host factors such as haemoglobinopathies [20] and anaemia [21], known to be associated with the risk and severity of malaria, may also influence the duration of asymptomatic carriage [22].

Besides host factors related to chronic infections, parasites themselves may either be pre-programmed or adapted to persist throughout the dry, non-transmission season. Being able to establish a clinically silent infection that does not activate the immune system may result in a slower clearance rate, a plausible evolutionary strategy for surviving the dry season and ensuring onward transmission. In Mali, where malaria transmission is highly seasonal, parasite populations collected during the wet and dry seasons were similar. Nevertheless, parasites sampled in the dry season appeared to adapt their phenotype, resulting in a longer time in peripheral circulation with increased splenic clearance, potentially a strategy to maintain a clinically and immunologically silent low parasitemia [11]. In this study, we identified individuals infected at the end of the transmission season in The Gambia, determined how long these infections remained detectable during the dry season, and explored the related host and parasite factors.

Methods

Study design

Climate in The Gambia is characterised by a short rainy season and a long dry season. Malaria transmission follows the same pattern, starting in July or August and lasting until December, with little or no clinical cases during the dry season. In December 2016, all residents of four villages (Madina Samako, Sendebu, Njayel, Karanbada) located within 5Km of each other in the Upper River Region, eastern Gambia, were invited to have a finger prick blood sample collected and analysed by VarATS quantitative PCR (qPCR) for *P. falciparum* infection and confirmed that they had not received antimalarial treatment within the 2 weeks before sampling. qPCR-positive individuals were invited for monthly samplings (venous blood draw of 5 to 8mL), with qPCR and qRT-PCR performed from the end of December until the end of the dry season (May), or until they either, (i) they became symptomatic and received treatment immediately, (ii) cleared their infection (negative by qRT-PCR and/or VarATS qPCR two months in a row), or (iii) withdrew from the study (Figure 1a). Subjects with an axillary temperature greater than 37.5°C were screened with a rapid diagnostic test (CareStart) and positives were treated with artemether-lumefantrine, the first line treatment in The Gambia. Written informed consent was obtained from all participants; parents or legal guardians provided a written informed consent for minors (<18 years old). The study was approved by the Gambia Government/Medical Research Council Joint Ethics Committee (SCC 1476) and the London School of Hygiene & Tropical Medicine ethics committee (Ref 10982). In the 2 months preceding this study, the same villages were enrolled in an independent study (Claessens *et al.* manuscript in preparation, SCC 1318, L2015.50) to monitor asymptomatic malaria infection via monthly VarATS qPCR surveys and symptomatic malaria infections by passive case detection and treatment by community-based nurses. Baseline infection status (confirmed qPCR negativity or history of treatment) of individuals with persistent or short-lived infections in October and November 2016 was similar between groups (persistent infections = 32% (7/22), short-lived infections (47% 8/17) Fishers exact test $p = 0.49$) (Table S1 and S2).

Molecular detection of parasites

During the study, presence of *P. falciparum* was assessed using VarATS qPCR as described in [23] from fingerprick (start December screening) or venous blood samples (monthly, end December to May). The parasite stages were quantified by qRT-PCR assays specific for ring stage parasites (*sbp-1*), male gametocytes (*PfMGET*), or female gametocytes (*CCp4*) as described before [24]. In these assays, parasite densities were determined using standard curves of purified parasite stages. Purified male and female gametocytes were generated using the PfDynGFP/P47mCherry reported line to enable sorting of the different sex gametocyte populations, as described before [25] Ring stage parasite standard curves were generated from NF54 parasite cultures following synchronisation as detailed in [26]. Total parasite density was calculated as the sum of the male gametocytes, female gametocytes and ring stage parasites quantified using the qRT-PCR assays. Samples were genotyped using *P. falciparum* merozoite surface protein 2 (*mSP2*) [27]. Briefly, nucleic acid was extracted from 100 μ l whole blood and 5 μ l was used in a nested PCR specific for *mSP2* 3D7 and FC27 allelic families followed by capillary electrophoresis to determine allelic size polymorphisms. Secondary PCR products were run on a 2% w/v agarose gel to check for bands within the correct size range (193 to 506 bp). Secondary PCR products were diluted and 2.5 μ l per sample processed on an ABI 7500 fast

(Applied Biosystem, UK) and resulting chromatograms analyzed using PeakScanner® software version 2 (Applied Biosystems). To distinguish between true peaks and background signal or stutter peaks, a cutoff of 200 relative fluorescent units (RFUs) was applied and fragment sizes must differ by more than 3 bp.

Haemoglobin quantification and genotyping

Haemoglobin density was measured in December using a haemoglobin analyser (HemoCue; AB Leo Diagnostics, Helsingborg, Sweden). Human haemoglobin S (HbS) and C (HbC) were genotyped using previously published methods [28].

Serology

IgG antibodies against 17 *P. falciparum* antigens were quantified at baseline (late December 2016) for each participant using a Luminex MAGPIX® suspension bead array [29]: Circumsporozoite protein (CSP), Erythrocyte binding antigen (EBA140, EBA175 and EBA181); Glutamate rich protein 2 (GLURP-R2); Merozoite surface protein 1-19 (MSP1-19), Merozoite surface protein 2 (MSP2-ch150/9 [3D7 family allele] and MSP2-DD2 [FC27 family allele]); Apical membrane antigen 1 (AMA1), Reticulocyte binding protein homologue (RH2.2, RH4.2, RH5.1), Schizont egress antigen-1 (SEA1), Heat shock protein 40 (HSP40), Skeleton-binding protein 1 (SBP1), and Early transcribed membrane proteins 4 (ETRAMP4 Ag2) and 5 (ETRAMP5 Ag1). Full details of all antigens are in supplementary table 3. These antigens are either expressed during the asexual blood-stage (Etramp, PfSEA, HSP40, SBP1), involved in merozoite invasion (Rh, AMA1, EBA, MSP, GLURP) or localised on the surface of sporozoites (CSP). Serological analysis was conducted on serum samples collected in December, assayed at a dilution of 1:200. Secondary antibody was an R-phycoerythrin conjugated goat anti-human IgG (Jackson Immuno Research, PA, USA; 109-116-098) diluted 1:200. Data are presented as median fluorescence intensity (MFI) (median of individual bead fluorescence values for a given region/antigen specificity) adjusted for inter-plate variation in background reactivity as previously described [29]. A sample was considered positive if the response was above background. The background response was calculated as the mean response of 4 negative controls plus 2 standard deviations. Negative controls were European malaria naïve donors.

Statistical analysis

Infections were classified as 'short-lived' if they were cleared within 2 months, 'persistent' if they remained detectable for 3-6 months and 'treated' if they became symptomatic and were treated. Data were analysed with Graphpad Prism version 9. A normality test (D'Agostino-Pearson) was used to determine if data were normally distributed. Unpaired T test was used to compare 2 groups of parametric data. Kruskal-Wallis with Dunns multiple comparison test was used to compare 3 or more groups of non-parametric data. Fisher's Exact test was used for contingency tables with small expected frequencies. **Spearman's correlation was used to analyse associations with non-parametric data and** Pearson's correlation was used for parametric data. Significance was indicated when the value of $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Results

Malaria prevalence at the end of the transmission season (beginning of December 2016) was 8.0% (74/924), with children under 5 having lower prevalence (2.5%; 5/199) than children 5-15 years old (7.9%; 27/341) and individuals >15 years old (10.9%; 42/384). Complete follow-up data were obtained from 40 individuals, 2 did not complete follow up until negative by PCR, but were parasite positive for at least 3 months so were included in the categorised analysis only (Figure 1a and b). 41% (n=17) of the subjects had *short lived* infections (undetectable by January), 52% (n=22) had *persistent* infections (detectable by PCR for 3-6 months), and 3 infected subjects developed symptoms and were *treated* (7%, all children <15 years of age) (Figure 1b). 40% (16/40) of individuals infected in early December remained PCR positive until the end of the dry season, with most of these individuals (81.3%; 13/16) also carrying gametocytes at the end of the dry season (Figure 2).

No association between the sex of the individual and the duration of infection was found (Table S2). Mean haemoglobin concentration at the start of the dry season was 11.31 g/dL (n=37). HbAA was the most frequent haemoglobin type (75.7%; 28/37), followed by HbAS (21.6%, 8/37), and HbSS (2.7%, 1/37). There was no association between haemoglobin concentration (short-lived= 11.35, persistent = 11.18, treated = 11.30) or type and duration of carriage (Table S4). Persistent infections were slightly more common in the 5-15 year olds (63%; 12/19) than in the other age groups (<5 years - 0.0% [0/4], >15 years - 53% [10/19]) but differences between groups were not statistically significant (Fisher's exact $p = 0.074$) (Figure 2b), and there was no overall difference in the age of individuals with persistent or short-lived infections (Figure S1) ($p=0.93$). Importantly, all children <5 years were offered four rounds of seasonal malaria chemoprevention during the transmission season with the last round being given in November. In persistent infections, asexual parasitemia and gametocytemia declined slowly over the season, with the median asexual parasitemia declining from 214,417 to 10,446 parasites/mL (Figure 2c) and gametocytemia declining from 1,237 to 289 gametocytes/mL (Figure 2d). The ratio of male:female gametocytes was female biased (0.20; CI 95%:0.16-0.23). This sex ratio did not change over time (Figure S2) and is similar to what was observed in other studies with transmissible infections [4].

At the start of the dry season, parasite density in persistent infections was significantly higher than in short-lived infections ($p < 0.0001$ Kruskal-Wallis with Dunns multiple comparison test) (Figure 3a), and duration of infection in months was significantly related to starting parasite density (Spearman's $r = 0.86$, $p < 0.0001$) (Figure 3b). Most persistent infections (82%; 18/22) and only 1 out of 17 short-lived infections had initial parasite densities above the theoretical limit of expert microscopy detection (10p/uL) [30] (Figure 3a). In the end of December samples (first monthly visit after screening), over half of the short-lived infections were undetectable by both the asexual parasite and gametocyte qRT-PCR assays (53%; 9/17), and 76% (13/17) did not have any asexual parasites detected, indicating these individuals had already cleared their infections in the first month. Whereas at the same time point, almost all persistent infections had asexual parasites (20/22) and/or gametocytes (20/22) at densities that could be quantified by the qRT-PCR assays.

Complexity of infection (the number of genetically distinct genotypes within a blood sample) was measured throughout the dry season using *m*sp2 genotyping (Figure 4a). In 21 out of 115 samples,

msp2 genotype could not be determined, likely due to parasite densities being below the assay limit of detection. At the start of the dry season, most infections had less than 2 genotypes (80.5%, 33/41) and the median per subject was 1 (range 1-8). Complexity of infection was significantly higher in the persistent than in the short-lived infections ($p = 0.015$, Kruskal-Wallis with Dunns multiple comparison test). Higher complexity of infection (>2 genotypes) was only observed in persistent infections; all short-lived infections were monoclonal, except for one with 2 genotypes (Figure 4b). The number of genotypes at the start of the dry season also correlated with parasite density (Figure 4c). In every persistent infection, there was at least one *msp2* genotype detected repeatedly throughout the dry season, suggesting chronic infection with the same *P. falciparum* strain as opposed to reinfection (Figure S3).

To assess the role of host immunity in the persistence of *P. falciparum* infection, antibodies were measured at the start of the dry season using a multiplex bead-based assay with a panel of 17 *P. falciparum* antigens. There was a trend for higher *P.f.*-specific antibody responses against 13 out of the 17 antigens in subjects who developed persistent infections compared to those with short-lived infections, $p = 0.02$ (Figure 5a and b). The three Rh family antigens (Rh2, Rh4.2, Rh5.1) were among the four most discriminatory antigens, with higher antibody levels associated with persistent infections, although only Rh5.1 reached statistical significance ($p = 0.03$). Of the other individual antigens, only PfSEA and GLURP ($p = 0.01$, $p = 0.04$, respectively) were significantly associated with subsequent duration of infection (persistent versus short-lived). Individuals with persistent infections tended to respond to more antigens (above background response) than those with short-lived infections, $p = 0.07$ (Figure 5c). The magnitude of total *P.f.*-specific antibody response to all antigens was significantly correlated with age, Spearman's $r = 0.39$ $p = 0.03$. (Figure 5d) and there was also a trend for increased breadth of response with age (Figure 5e).

Discussion

In this study, 40% of individuals infected at the end of the wet season remained asymptotically infected until the end of the dry season, demonstrating the considerable silent reservoir of malaria that exists to re-start the next transmission season in The Gambia. Individuals with persistent infections had higher starting parasite densities, often with multiclonal infections, and higher *P.f.*-specific antibody responses, suggesting that prior malaria exposure is important to maintain the human reservoir of malaria. Targeting these infections may significantly reduce such a reservoir and thus the capacity of starting the next transmission season.

In areas of seasonal malaria transmission, understanding who maintains the parasite reservoir during the dry season may inform control interventions to reduce this reservoir and thereby the resurgence of malaria in the subsequent transmission season [1]. In our study setting, only 8% of village residents had detectable parasites at the end of the transmission season and towards the end of the dry season the prevalence fell to 3%. In our modestly sized study population, we observed no clear relationship between subject age and duration of parasite carriage, although all persistent infections were found in those above 5 years of age. In general, the number of children under 5 infected at the end of the wet season was low compared to other age groups, possibly reflecting the levels of protective immunity, with younger children being more likely to develop symptoms when

infected [7], or the impact of seasonal malaria chemoprevention [31] that is implemented in the study area.

Responses to our panel of 17 *P. falciparum* antigens tested were generally low [18], highlighting the generally low level of malaria exposure in the area. Individuals with persistent infections tended to have higher and broader *P.f.*-specific antibody responses than those with short-lived infections, a finding consistent with previous studies in seasonal settings [11, 12]. In Mali, asymptotically infected children had higher levels of *P.f.*-specific antibodies than children with clinical malaria [13] while higher age (and thus cumulative exposure) was associated with longer duration of asymptomatic infection in Malawi [32], Uganda and Ghana [14, 33]. In our study, we also observed a relationship between age and increasing magnitude and breadth of the immune responses, but this correlation was relatively weak. This supports earlier reports of marked heterogeneity in malaria exposure in The Gambia [3, 34]. In the context of our current findings, we hypothesize that this heterogeneity results in some individuals being more likely to experience repeated infections, resulting in higher levels of immunity and thus increased ability to sustain chronic asymptomatic parasite carriage while age per se is not a strong determinant of immunity.

Each persistent infection had at least one unique genotype that was repeatedly observed, indicating infection persistence. High complexity of infection at the beginning of the dry season was associated with longer duration of asymptomatic carriage, consistent with other studies [35, 36]. This could either be due to multiple clones being received from a single mosquito bite [37], or by the accumulation of clones over time in those who harbour infections for a prolonged period. Complexity of infection and parasite density were also strongly correlated in this study. This could be partly explained by the difficulty of detecting all parasite clones when density is low and falling below the detection limit of the assay [38]. Our estimates of clonal complexity were based on MSP-2 genotyping. Whilst alternative genotyping methods are available, these do not always improve sensitivity [39-41], even at low parasite densities. It is therefore, unlikely that an alternative method would have resulted in different conclusions. Higher parasite density, multi-clonal infections were more likely to persist throughout the dry season. This could indicate infection persistence simply reflects a longer period to clear a larger parasite biomass, although we did not observe clear declines in parasite densities during the dry season among persisting infections. The fact that short-lived infections had lower parasite densities could suggest these infections may have been carried longer and were already on the path to clearance [14]. Although a plausible factor for explaining our findings, available data prior to our cohort inception indicate that a similar proportion of individuals with both persisting and short-lived infections were either confirmed PCR negative or received curative treatment 1-2 months before the current study (Table S1 and S2). We thus conclude that similar proportions of persistent and short-lived infections were acquired very recently. In addition to the explanations of higher parasite biomass or shorter infection age, parasites in persistent infections may be (epi)genetically programmed to survive longer, and/or genetically different from those in short-lived infections, potentially with a less virulent phenotype or slower parasite multiplication rate. This hypothesis is consistent with a recent study from Mali, where similar parasite populations were present in the wet and dry seasons. However, parasites in the dry season were transcriptionally different from those present in the wet season and they were able to adapt their phenotype to secure longer circulation and reduced sequestration, resulting in increased splenic clearance and lower but persistent parasitaemia [11].

Our study was limited by the small sample size, largely governed by the decreasing malaria prevalence in the area [3]. However, we were able to demonstrate that the dry season reservoir of malaria is largely comprised of school aged children and adults who have likely experienced repeated infections. Importantly, persistent infections could potentially be identified at the start of the dry season, given that they were associated with higher initial parasite densities. Our findings confirm ongoing gametocyte production in chronic infections. Future studies may address the infectivity of these infections to mosquitoes and, more importantly for malaria control, determine whether clearing infections that are present at the start of the dry season results in a meaningful reduction in malaria burden in the next transmission season.

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1 **Figures**

2

3 **Figure 1: Study design and flow**

4 (A) Illustration of study design (B) Study flow diagram. Subjects were screened in early December 2016 and
 5 asymptomatic parasite positive individuals (by VarATS qPCR) were selected at the end of the wet season
 6 (December 2016) and were recruited into a longitudinal study with monthly samples to determine duration
 7 of infection from end of December until the end of the dry season (May) or until they (i) cleared their
 8 infection (negative by PCR), or (ii) became symptomatic and received treatment.

9 **Figure 2. Duration of detectable parasite carriage in the blood during the dry season**

10 Subjects (n=42) who were parasite positive by VarATS qPCR in fingerprick blood samples in December were
 11 followed monthly until PCR negative or treated. Two subjects who did not complete follow up were
 12 parasite positive for at least 3 months (and thus classified as persistent infections) are excluded from the
 13 Kaplan Meier graphs. (A) Number of subjects parasite positive each month (n=40). (B) Number of subjects
 14 positive each month by age category. (C) Asexual parasite densities over time in the persistent infections
 15 group, measured in venous blood samples by qRT-PCR for ring stage parasite (*SBP-1*). (D) Total gametocyte
 16 densities (males + females) over time in the persistent infections group, measured in venous blood samples
 17 by qRT-PCR for male (*PfMGET*) and female (*CCp4*) gametocytes. Light grey lines show individual responses
 18 and the dark grey line shows the median response.

19 **Figure 3. Level of parasitemia at the beginning of the dry season and subsequent duration of detectable 20 parasitemia**

21 **Total parasite density (sum of asexual parasites and male and female gametocytes) for each subject
 22 (n=42) by qRT-PCR at the start of the dry season. (A) Total parasite density for each individual, by group.**
 23 Groups compared by Kruskal-Wallis with Dunns multiple comparison test. **(B) Spearmans correlation
 24 between parasite density and duration of infection for the persistent and short-lived infection groups.**

25

26 **Figure 4. Genotypic complexity of parasite infection and persistent detectability through the dry season**

27 The number of clones present in each individual was determined by *msp2* genotyping. (A) Number of
 28 clones for each individual over time, displayed by group. The black cross indicates where a sample was
 29 missing and a '0' indicates the number of genotypes could not be determined. (B) Number of clones at the
 30 start of the dry season in the December or January sample, by group. For 7 subjects there is no genotyping
 31 data available from December or January. Groups compared by Kruskal-Wallis with Dunns multiple
 32 comparison test. (C) Spearman's correlation between number of clones and total parasite density at the
 33 start of the dry season (sum of asexual parasites and gametocytes determined by qRT-PCR). Persistent
 34 infections (red circles), short-lived infections (black circles), treated (filled circles). Vertical dashed line
 35 indicates the theoretical limit of expert microscopy detection

36 **Figure 5. Antibody levels to a panel of *P. falciparum* antigens in relation to duration of detectable**
37 **infection in the dry season**

38 Antibody responses were measured to a panel of 17 *P. falciparum* antigens using a multiplex bead-based
39 assay. (A) Panels show the individual subject responses to each antigen, by short-lived or persistent
40 infection group, ordered by decreasing differences between the two groups. Dashed line indicates average
41 background response. (B) Sum of the ranked response to all 13 upregulated antigens by group. (C) Breadth
42 of the response to all 17 antigens, defined as the number of antigens each individual responded to above
43 background by group. (D) Spearmans correlation between age and sum of the ranked response to all 17
44 antigens (treated - grey filled circles). (E) Number of antigens each individual responded to above
45 background by age group. Box plots show the median with the whiskers indicating the minimum and
46 maximum. Groups in (B) and (C) compared by unpaired T test.

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49 **Funding**

50 This work was supported by grants from the Netherlands Organization for Scientific Research (Vidi
51 fellowship NWO 016.158.306) and the Bill & Melinda Gates Foundation (INDIE OPP1173572), the joint
52 MRC/LSHTM fellowship, the French National Research Agency (18-CE15-0009-01).

53 **Conflict of interest**

54 The authors declare no conflict of interest.

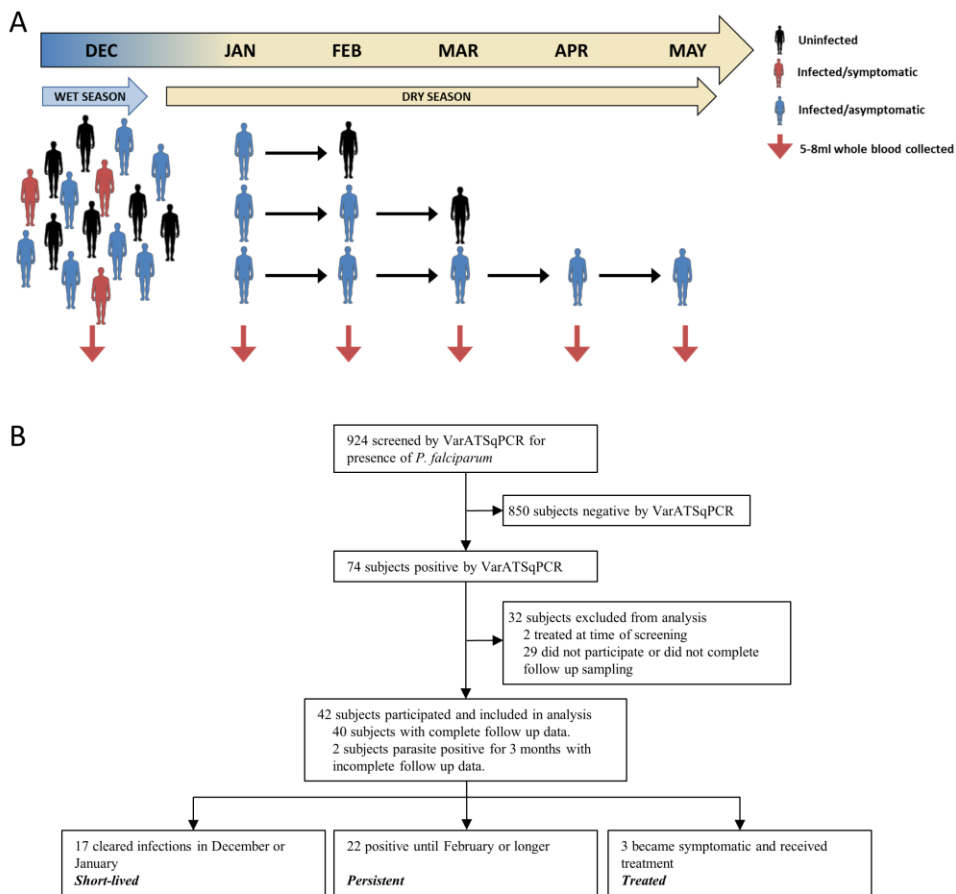
55 **Acknowledgments**

56 We would like to thank Julia Mwesigwa and all fieldworkers for sample collection.

57

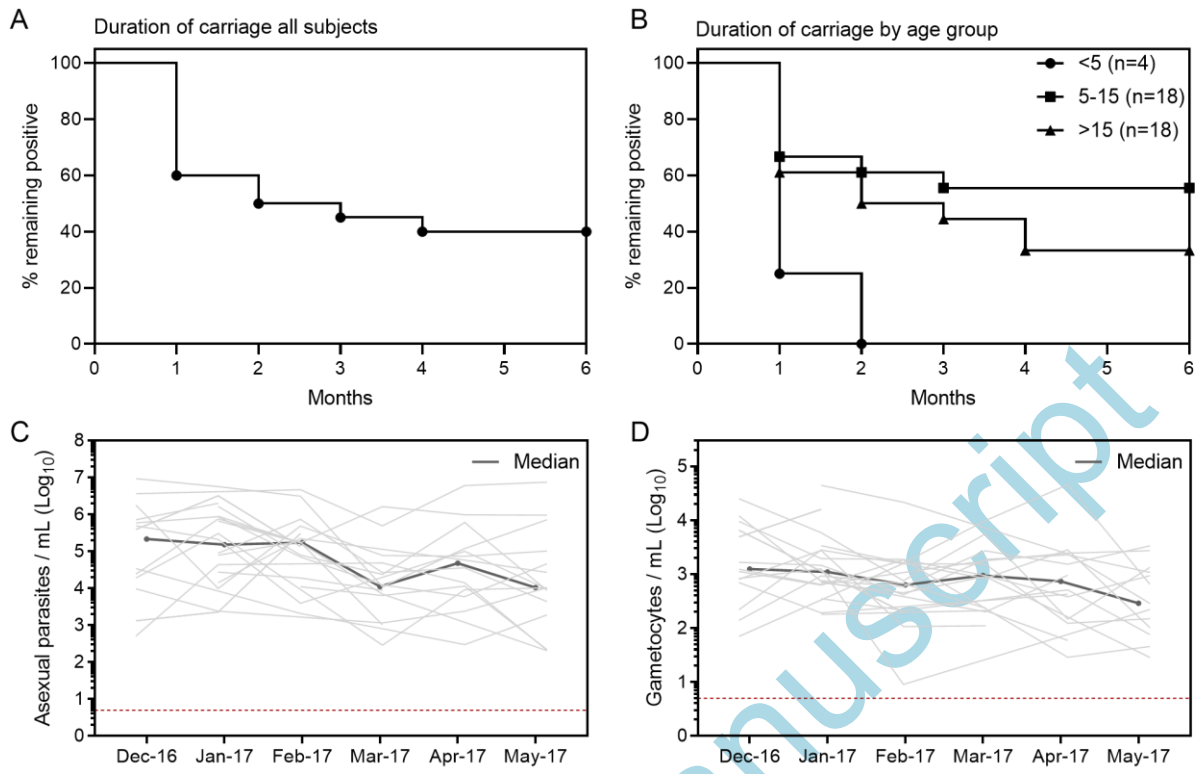
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Figure 1



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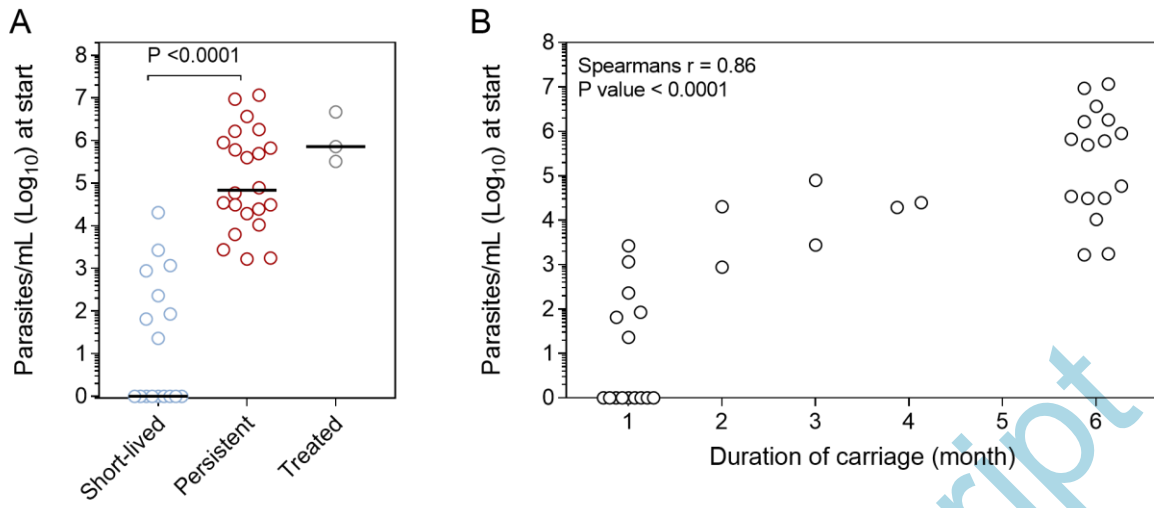
Figure 2



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Figure 3



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Figure 4

