Epidemiology and Global Health

Measuring changes in *Plasmodium falciparum* census population size in response to sequential malaria control interventions

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Abstract

Here we introduce a new endpoint “census population size” to evaluate the epidemiology and control of *Plasmodium falciparum* infections, where the parasite, rather than the infected human host, is the unit of measurement. To calculate census population size, we rely on a definition of parasite variation known as multiplicity of infection (MOI_{var}), based on the hyper-diversity of the *var* multigene family. We present a Bayesian approach to estimate MOI_{var} from sequencing and counting the number of unique DBLα tags (or DBLα types) of *var* genes, and derive from it census population size by summation of MOI_{var} in the human population. We track changes in this parasite population size and structure through sequential malaria interventions by indoor residual spraying (IRS) and seasonal malaria chemoprevention (SMC) from 2012 to 2017 in an area of high-seasonal malaria transmission in northern Ghana. Following IRS, which reduced transmission intensity by > 90% and decreased parasite prevalence by ~40-50%, significant reductions in *var* diversity, MOI_{var}, and population size were observed in ~2,000 humans across all ages. These changes, consistent with the loss of diverse parasite genomes, were short lived and 32-months after IRS was discontinued and SMC was introduced, *var* diversity and population size rebounded in all age groups except for the younger children (1-5 years) targeted by SMC. Despite major perturbations from IRS and SMC interventions, the parasite population remained very large and retained the *var* population genetic characteristics of a high-transmission system (high *var* diversity; low *var* repertoire similarity) demonstrating the resilience of *P. falciparum* to short-term interventions in high-burden countries of sub-Saharan Africa.

eLife assessment

This **valuable** study highlights how the diversity of the malaria parasite population diminishes following the initiation of effective control interventions but quickly rebounds as control wanes. The data presented is **solid** and the work shows how genetic studies could be used to monitor changes in disease transmission.

Introduction

Malaria in high-transmission endemic areas of sub-Saharan Africa (SSA) is characterised by vast diversity of the *Plasmodium falciparum* parasites from the perspective of antigenic variation (Chen et al., 2011; Day et al., 2017; Otto et al., 2019; Ruybal-Pesántez et al., 2022, 2017). As with other hosts of hyper-variable pathogens (Futse et al., 2008), children experiencing clinical episodes of malaria, eventually become immune to disease but not to infection. This results in a large reservoir of chronic asymptomatic infections, in hosts of all ages, sustaining transmission to mosquitos. Given the goal of malaria eradication by 2050, it is therefore of interest to examine how the parasite population changes following perturbation by major intervention efforts, both in terms of its size and underlying population genetics.

So, what do we mean by the parasite population size in the case of *P. falciparum* and how do we measure it? Parasite prevalence, detected by microscopy or more sensitive molecular diagnostics (e.g., PCR), describes the proportion of infected human hosts. Studies of *P. falciparum* genetic diversity have shown that the majority of people in high-transmission endemic areas harbour diverse multiclonal infections measured as the complexity or multiplicity of infection (MOI) (e.g., (Anderson et al., 2000; Paul et al., 1995; Smith et al., 1999; Sumner et al., 2021)) with complex population dynamics (Bruce et al., 2000; Farnert et al., 1997). These genetic data indicate much larger parasite population sizes than observed by prevalence of infection alone. Thus, from an ecological perspective, we can consider a human host as a patch carrying a number of "antigenically-distinct infections" of *P. falciparum*. The sum of these antigenically-distinct infections over all hosts provides us with a census of the parasite count of relevance to monitoring and evaluating malaria interventions. We refer to this census population size hereafter simply as population size but make clear that this measure is distinct from effective population size (N_e) as measured by neutral variation.

Diversity of *P. falciparum* single copy surface antigen genes such as circumsporozoite protein (*csp*), merozoite surface protein 1 (*msp1*) or 2 (*msp2*), and apical membrane antigen 1 (*ama1*) have each been widely used to measure MOI (e.g., (Falk et al., 2006; Lerch et al., 2017; Nelson et al., 2019)). They have become part of newer genetic panels (e.g., Paragon v1 (Tessema et al., 2020) and AMPLseq v1 (LaVerriere et al., 2022)) specifically for MOI determination. Typically, MOI is reported as the maximum number of alleles or single locus haplotypes present at the most diverse of these antigen-encoding loci rather than the number of unique multilocus haplotypes of these genes combined, as it is challenging to accurately reconstruct or phase these haplotypes in hosts with an MOI > 3 (Lerch et al., 2019). Each of these genes is under balancing selection with a few geographically-common haplotypes and many very rare haplotypes in moderate-to high-transmission settings (Markwalter et al., 2022; Sumner et al., 2021). Where there is a high probability of co-occurrence of two or more common single locus haplotypes in a host, genotyping each of these single copy antigen genes alone will underestimate MOI. Single nucleotide polymorphism (SNP) panels have been used to define the presence of multiclonal infections with limited reliability to estimate MOI for highly complex infections, typical in high transmission, even with the use of computational methods (Labbé et al., 2023).

As an alternative to genotyping single copy antigen genes and biallelic SNP panels to estimate MOI, we have proposed the use of a fingerprinting methodology known as *var*coding to genotype the hyper-diverse *var* multigene family (~50-60 *var* genes per haploid genome). This method employs a ~450bp region of a *var* gene, known as a DBLα tag encoding the immunogenic Duffy-binding-like alpha (DBLα) domain of PfEMP1 (*Plasmodium falciparum* erythrocyte membrane protein 1), the major surface antigen of the blood stages (Zhang and Deitsch, 2022). Bioinformatic analyses of a large database of exon 1 sequences of *var* genes showed a predominantly 1-to-1 DBLα-*var* relationship, such that each DBLα tag typically represents a unique *var* gene, especially in high transmission (Tan et al., 2023). The extensive diversity of DBLα tags together with the very low percentage of *var* genes shared between parasites (Chen et al., 2011; Day et al., 2017; Ruybal-Pesántez et al., 2022, 2017) facilitate measuring MOI by amplifying, pooling, sequencing, and counting the number of unique DBLα tags (or DBLα types) in a host (Ruybal-Pesántez et al., 2022; Tiedje et al., 2022). From a single PCR with degenerate primers and amplicon sequencing, the method specifically counts the most diverse DBLα types, designated non-upsA, per infection to arrive at a metric we call MOI_{var}. It is not based on assigning haplotypes but exploits the fact that *var* repertoires are non-overlapping, especially in high transmission. Instead, it assumes a set number of non-upsA types per genome based on repeated sampling of 3D7 control isolates accounting for PCR sampling errors to calculate MOI_{var} (Ghansah et al., 2023; Ruybal-Pesántez et al., 2022; Tiedje et al., 2022). Consequently, rather than looking at the diversity of a single copy antigen-encoding gene like *csp*, *msp2*, or *ama1* to calculate MOI, by *var*coding we are looking at sets of 45 non-upsA DBLα types per genome. Prior work has shown that *var*coding is more sensitive to measure MOI in high transmission where there is an extremely high prevalence of multiclonal infections that cannot be accurately phased with either biallelic SNP panels (Ghansah et al., 2023; Labbé et al., 2023; Tessema et al., 2020; Watson et al., 2021) or combinations of single copy antigen genes (Sumner et al., 2021).

Here we report an investigation of changes in parasite population size and structure through two sequential malaria control interventions between 2012 and 2017 in Bongo District located in the Upper East Region of Ghana, one of the 12 highest burden countries in Africa (World Health Organization, 2022). We present a novel Bayesian modification to the published *var*coding approach (Ruybal-Pesántez et al., 2022; Tiedje et al., 2022) that takes into account under-sampling of non-upsA DBLα types in an isolate to estimate MOI_{var} (Ruybal-Pesántez et al., 2022; Tiedje et al., 2022) and therefore population size. We document *P. falciparum* prevalence as well as *var* diversity and population structure from baseline in 2012 through a major perturbation by a short-term indoor residual spraying (IRS) campaign managed under operational conditions, which reduced transmission intensity by > 90% as measured by the entomological inoculation rate (EIR) and decreased parasite prevalence by ~40-50% (Tiedje et al., 2022). Next, we followed what happened to parasite population size more than two years after the IRS intervention was discontinued and seasonal malaria chemoprevention (SMC) was introduced for children between the ages of 3-59 months (i.e., < 5 years) (Wagman et al., 2018). Detectable changes in parasite population size were seen as a consequence of the IRS intervention but this quantity rapidly rebounded 32-months after the intervention ceased. Overall, throughout the IRS, SMC, and subsequent rebound, the parasite population in humans remained large in size and retained the *var* population genetic characteristics of high transmission (i.e., high *var* diversity, low *var* repertoire overlap), demonstrating the overall resilience of the species to survive significant short-term perturbations.

Materials and methods

Human subject ethical approval

The study was reviewed/approved by the ethics committees at the Navrongo Health Research Centre (Ghana), Noguchi Memorial Institute for Medical Research (Ghana), The University of Melbourne (Australia), and The University of Chicago (United States). Details on the study area, study population, inclusion/exclusion criteria, and data collection procedures have been previously described (Tiedje et al., 2022, 2017).

Study design and sample collection

Using an interrupted time-series study design, four age-stratified cross-sectional surveys of ~2,000 participants per survey were undertaken to investigate the impacts of IRS and SMC in combination with long-lasting insecticide-treated nets (LLINs) impregnated with pyrethroids under operational conditions on the asymptomatic *P. falciparum* reservoir from two proximal catchment areas (i.e., Vea/Gowrie and Soe, with a sampling area of ~60 km^2) in Bongo District, Ghana (Tiedje et al., 2022, 2017) (Table supplement 1). Bongo District, located in the Upper East Region, is categorized as high-seasonal malaria transmission based on the World Health Organization's (WHO) "A Framework for Malaria Elimination" (WHO/GMP, 2017) where *P. falciparum* prevalence was ≥ 35% at baseline in 2012 (Tiedje et al., 2022, 2017). These ~2,000 participants of all ages (1-97 years) represent ~15% of the total population that reside in these two catchment areas in Bongo (Tiedje et al., 2017). The four cross-sectional surveys were completed at the end of the wet season (i.e., high-transmission season) and the study can be separated into four distinct study time points: (1) October 2012 (Survey 1) prior to the IRS and SMC (i.e., baseline), (2) October 2014 (Survey 2) two months after the second round of IRS (Actellic 50EC), (3) October 2015 (Survey 3) seven months after the third round of IRS using a long-acting non-pyrethroid insecticide (Actellic 300CS) (Gogue et al., 2020; US President's Malaria Initiative Africa IRS (AIRS) Project, 2016), and finally (4) October 2017 (Survey 4) 32-months after the discontinuation of IRS, but during the deployment of SMC to all children 3-59 months (i.e., < 5 years) (**Figure 1**). Details on the study area, study population, and data collection procedures have been previously described (Tiedje et al., 2022, 2017).

Details of the IRS and SMC interventions

Starting in 2013, the AngloGold Ashanti Malaria Control Programme (AGAMal) in a public-private partnership with the Global Fund, scaled up IRS across all of the Upper East Region of northern Ghana (Gogue et al., 2020). As part of this initiative, three rounds of IRS with organophosphate formulations (i.e., non-pyrethroid) were rolled out prior to the start of the wet season between 2013 and 2015 (**Figure 1A**) in Bongo District (Tiedje et al., 2022). Based on AGAMal's operational reports, IRS coverage in Bongo District was 91.8% in Round 1, 95.6% in Round 2, and finally 96.6% in Round 3 (AGAMal, personal communication). To monitor the impact of the IRS on the local vector population, monthly entomological collections were undertaken between February 2013 and September 2015 (Tiedje et al., 2022). Using these surveys we observed that the monthly entomological inoculation rate (EIR) (infective bites/person/month (ib/p/m)), a measure of local transmission intensity, declined by > 90% at the peak of the wet season between August 2013 (pre-IRS) (EIR = 5.3 ib/p/m) and August 2015 (post-IRS) (EIR = 0.4 ib/p/m) (Tiedje et al., 2022). Following the IRS, SMC was rolled out in the Upper East Region by the National Malaria Elimination Programme (NMEP)/ Ghana Health Service (GHS) starting in 2016 (Gogue et al., 2020) (**Figure 1A**). SMC is the intermittent administration of full treatment courses of an antimalarial to children between the ages of 3-59 months (i.e., < 5 years) (WHO, 2012). Like other countries, the SMC drug combination of choice in Ghana is amodiaquine plus sulfadoxine-pyrimethamine, which is administered at monthly intervals during the high-transmission season (i.e., wet season) (WHO, 2012). The goal of this age-targeted intervention is to both clear current

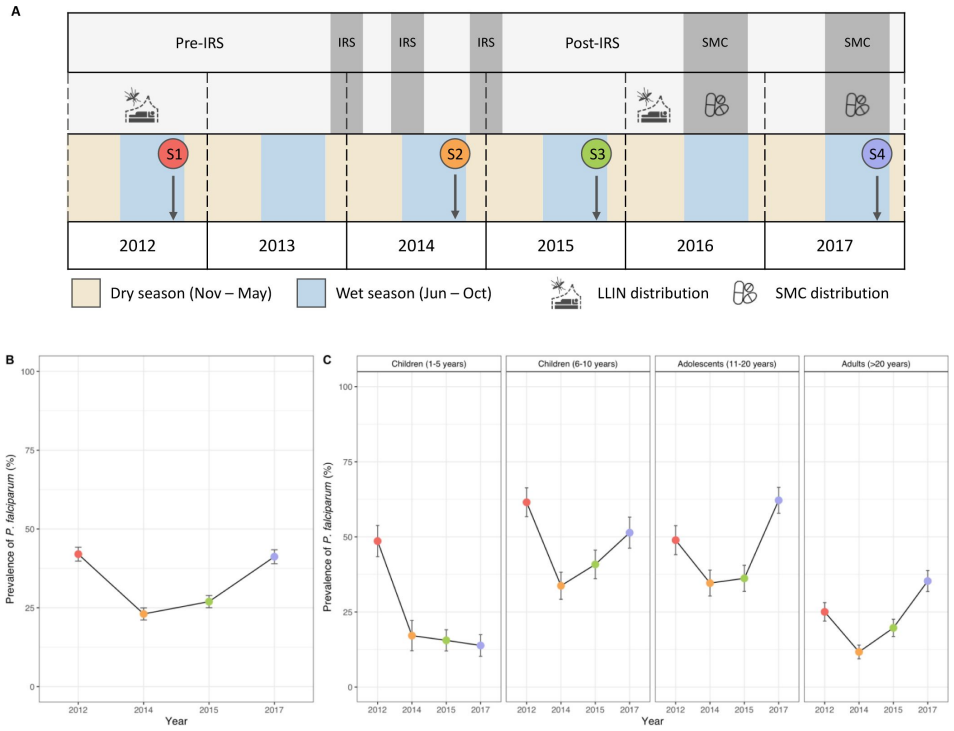

Figure 1.

Study design and changes in the prevalence of microscopic *P. falciparum* infection following the IRS and SMC interventions in Bongo, Ghana.

(A) Four age-stratified cross-sectional surveys of ~2,000 participants per survey were conducted in Bongo, Ghana at the end of the wet seasons in October 2012 (Survey 1, baseline pre-IRS, red), October 2014 (Survey 2, during IRS, orange), October 2015 (Survey 3, post-IRS, green), and October 2017 (Survey 4, SMC, purple) (Table supplement 1) (see Materials and Methods). The three rounds of IRS (grey areas) were implemented between 2013 and 2015 (Tiedje et al., 2022). SMC was distributed to all children < 5 years of age during the wet seasons in 2016 (two rounds between August – September 2016) and 2017 (four rounds between September – December 2017) (Gogue et al., 2020). Both IRS and SMC were implemented against a background of widespread LLIN usage which were distributed across Bongo District by the NMEP/GHS between 2010 – 2012 and again in 2016 (Gogue et al., 2020; Tiedje et al., 2022; US Agency for International Development (USAID) Global Health Supply Chain Program, 2020). Prevalence of microscopic *P. falciparum* infections in the **(B)** study population and **(C)** for all age groups (years) in each survey. Error bars represent the upper and lower limits of the 95% confidence interval (CI).

malaria infections and prevent malarial illness by maintaining a therapeutic concentration of an antimalarial in blood over the period of greatest risk (i.e., high-transmission season). Reported SMC coverage in Bongo District was 92.6% in 2016 (two rounds between August – September 2016) and 94.6% in 2017 (four rounds between September – December 2017) (Gogue et al., 2020).

Var genotyping and sequence analysis

Genomic DNA was extracted from the dried blood spots for all participants with a confirmed microscopic asymptomatic *P. falciparum* infection (i.e., isolate) (2,572 isolates, Table supplement 2) using the QIAamp DNA mini kit (QIAGEN, USA) as previously described (Tiedje et al., 2017). For *var* genotyping or *var*coding, the sequences encoding the DBLα domains of the antigen-encoding *P. falciparum var* genes were amplified by PCR, pooled, and sequenced on a MiSeq 2×300 paired-end cycle protocol as previously published (New York University Genome Technology Center, New York, NY, USA; Australian Genome Research Facility, Melbourne, Australia) (Figure supplement 1) (Day et al., 2017; He et al., 2018; Ruybal-Pesántez et al., 2017). The raw sequence data was then cleaned and processed using our previously published customized bioinformatic pipeline (*https://github.com/UniMelb-Day-Lab/DBLaCleaner*) (He et al., 2018). This pipeline was used to de-multiplex and merge the paired-end reads as well as remove low-quality sequences and chimeras using several filtering parameters (see Figure supplement 2 for additional details). These steps resulted in a total of 291,498 cleaned DBLα sequences for all surveys (Figure supplement 2). To identify the unique DBLα types, we then clustered these cleaned DBLα sequences with 237,910 DBLα sequences previously published from Bongo (He et al., 2018; Pilosof et al., 2019; Rorick et al., 2018) at the standard 96% sequence identity (*https://github.com/UniMelb-Day-Lab/clusterDBLalpha*) (Barry et al., 2007; Day et al., 2017; Ruybal-Pesántez et al., 2017). Our dataset was then further curated by translating the DBLα types (N = 68,507) into amino acid sequences and removing any DBLα types that could not be translated (i.e., contained a stop codon) (N = 137; 0.2%). The remaining DBLα types were then assigned to their most likely DBLα domain class (i.e., DBLα0, DBLα1, or DBLα2) using a hidden Markov model, and further classified based on the association of specific domain classes with semi-conserved upstream promoter sequences (ups) as either upsA or non-upsA DBLα types (*https://github.com/UniMelb-Day-Lab/classifyDBLalpha*) (Ruybal-Pesántez et al., 2017). For additional information on the use of these bioinformatic pipelines a detailed tutorial is available (*https://github.com/UniMelb-Day-Lab/tutorialDBLalpha*). Using a cut off of ≥ 20 DBLα types, DBLα sequencing data was obtained from 2,099 *P. falciparum* isolates (81.6%) with a total of 289,064 DBLα sequences and 53,241 unique DBLα types being identified in the study population (Table supplement 2). This genotyping success was acceptable given that we were working with low-density asymptomatic infections (Table supplement 1). The median *P. falciparum* density was ~4 times higher for isolates with ≥ 20 DBLα types compared to those that gave no or < 20 DBLα types (520 [IQR: 200-1880] parasites/μL vs. 120 [IQR: 40-200] parasites/μL, respectively).

DBLα type diversity

We monitored the impacts of the sequential interventions (i.e., IRS and SMC) on diversity by measuring changes in the population genetics of DBLα types at the population level (i.e., *P. falciparum* reservoir). Diversity was monitored using two measures, DBLα type richness and DBLα type frequency. Richness was defined as the number of unique DBLα types observed (i.e., DBLα type pool size) in each survey or study time point (i.e., 2012, 2014, 2015, and 2017). DBLα type richness, however, does not provide any information about the relative frequencies of the different DBLα types in the population, as they are all weighted equally whether they are observed once or more frequently (e.g., observed in > 20 isolates per survey). To further examine the impacts of the interventions on DBLα type diversity we also assessed the frequency of each unique DBLα type in 2012, 2014, 2015, and 2017. Here we defined DBLα type frequency as the number of times (i.e., number of isolates) a DBLα type was observed in each survey. Both upsA and non-upsA DBLα type diversities were measured due to their different biological features, chromosomal positions (i.e., subtelomeric regions vs. internal or central regions), as well as population genetics

(Falk et al., 2009; Gardner et al., 2002; Jensen et al., 2004; Kaestli et al., 2006; Kalmbach et al., 2010; Kraemer et al., 2007; Kraemer and Smith, 2006; Kyriacou et al., 2006; Lavstsen et al., 2003; Normark et al., 2007; Rottmann et al., 2006; Warimwe et al., 2012, 2009; Zhang and Deitsch, 2022). The proportions of upsA and non-upsA *var* genes in a repertoire or single genome have been defined as ~15-20% and ~80-85%, respectively, based on whole genome sequencing (Rask et al., 2010). The upsA and non-upsA DBLα type proportions were partitioned as expected in our analyses, with the median proportions at the repertoire level being comparable in 2012 (19% upsA and 81% non-upsA), 2014 (22% upsA and 78% non-upsA), 2015 (21% upsA and 79% non-upsA), and 2017 (20% upsA and 80% non-upsA).

Repertoire similarity as defined by pairwise type sharing

To estimate genetic similarity between the DBLα repertoires (i.e., unique DBLα types identified in each isolate) identified from two isolates, pairwise type sharing (PTS) was calculated between all pairs of isolates in each survey as previously described (Barry et al., 2007). PTS, analogous to the Sørensen Index, is a similarity statistic to evaluate the proportion of DBLα types shared between two isolate repertoires (i.e., DBLα repertoire overlap) and ranges from 0 (i.e., no DBLα repertoire overlap) to 1 (i.e., identical DBLα isolate repertoires), where < 0.50 = unrelated, 0.5 = recent recombinants/siblings, > 0.5 = related, and 1 = clones. PTS is a measure of identity-by-state (IBS) used to assess repertoire similarity (or relatedness) between isolates and is not used to infer inheritance from a recent common ancestor (i.e., identity-by-decent (IBD)) (Speed and Balding, 2015).

DBLα isolate repertoire size and number of *P. falciparum var* repertories

For this study we have exploited the unique population structure of non-overlapping DBLα isolate repertoires to estimate isolate MOI_{var}. To calculate MOI_{var}, the non-upsA DBLα types were chosen since not only are they more diverse and less conserved between isolate repertoires (i.e., low median $PTS_{non\text{-}upsA} \leq 0.020$) compared to the upsA DBLα types, but they have also been shown to have a more specific 1-to-1 relationship with a single *var* gene than upsA (Tan et al., 2023). The low to non-existent overlap of repertoires enables an estimation of MOI that relies on the number of non-upsA DBLα types sequenced from an individual's isolate (Ruybal-Pesántez et al., 2022; Tiedje et al., 2022). A constant repertoire size or number of DBLα types in a parasite genome can be used to convert the number of types sequenced in an isolate to its estimated MOI (Ruybal-Pesántez et al., 2022; Tiedje et al., 2022). As such, the approach neglects the measurement error in this size introduced by targeted PCR and amplicon sequencing of *var* genes in an infection.

Bayesian estimation of MOI_{var} and associated population size

Here, we extend the method to a Bayesian formulation and estimate the posterior distribution for each sampled individual for the probability of different MOI values. From individual posterior distributions, we can then obtain the estimated MOI frequency distribution for the population as a whole. The two pieces of information required for our approach are the measurement error and the prior distribution of MOI. The measurement error is simply the repertoire size distribution, that is, the distribution of the number of non-upsA DBLα types sequenced given MOI = 1, which is empirically available (Figure supplement 3). We refer to it as $P(s \mid MOI=1)$ where s denotes repertoire size. In the following, s denotes the number of non-upsA DBLα types sequenced when MOI > 1. The prior distribution of MOI refers to the belief we have for what the actual MOI distribution might look like at the population level before empirical evidence is taken into consideration. For example, the prior distribution of MOI is likely to centre around a higher value in high-transmission endemic areas than in low-transmission ones.

We can obtain P(s | MOI = m) from the serial convolution of the repertoire size distribution P(s | MOI = 1) and P(s | MOI = m - 1). Starting with the repertoire size distribution given a single infection, we can derive P(s | MOI = m) for *m* equal to 2,3,..., up until a maximum value of 20 (empirically determined), as follows:

$$P(s|MOI=m)=\sum_{x=L}^{U}P(x|MOI=1)\times P(s-x|MOI=m-1) \quad 1.1$$

where *L* and *U* are the lower and upper limit for the repertoire size, 10 and 45 respectively from the empirical repertoire size distribution.

For simplicity, we begin with a uniform prior. We use Bayes' rule to derive a posterior distribution of MOI given a certain number of non-upsA DBLα types sequenced from an individual:

$$P(MOI=j|s)=\frac{P(s|MOI=j)\times P(MOI=j)}{\sum_{i=1}^{k}P(s|MOI=i)\times P(MOI=i)} \quad 1.2$$

where *k* is the maximum value of MOI, here 20, as empirically determined.

To obtain the MOI distribution at the population level, we could either simply pool the maximum *a posteriori* MOI estimate for each sampled individual, or use a technique called mixture distribution. For the latter, we weighed each posterior MOI distribution for each sampled individual equally and sum over all posterior distributions at the individual level to derive the MOI distribution at the population level:

$$f(MOI=m)=\sum_{i=1}^{n}\frac{1}{n}P(MOI=m|s_i) \quad 1.3$$

where *n* is the number of sampled individuals. These two approaches gave similar results for our empirical survey data as determined by the Kolmogorov-Smirnov Test. The obtained distance statistic is close to 0 and the corresponding *p-value* is non-significant across all surveys, indicating that the two estimates were drawn from the same distribution (Table supplement 3). Given this similarity, we present hereafter only the result of pooling the maximum a posteriori MOI estimates. Note that we focused on individuals who had a confirmed microscopic asymptomatic P. *falciparum* infection for our MOI estimation.

To examine alternative priors, we considered empirical MOI distributions described in the literature including the Poisson, hyper-Poisson, and negative binomial distributions (Dietz, 1988; Henry, 2020). The hyper-Poisson and negative binomial distributions can capture the overdispersion seen in the empirical distribution of MOI for certain areas and caused by factors such as heterogeneous biting. We investigated changing the parameters of a negative binomial distribution to generate priors with different means spanning a wide range of MOI values (mean MOI within [~3, ~9]), including those seen in high-transmission endemic areas. We found that the final MOI distribution at the population level is fairly robust against the specification of those parameters. In other words, the estimated MOI distributions at the population level for different parameters do not significantly diverge from each other. The pairwise distance between these distributions measured by KL-divergence centres around 0, and the difference between their mean MOI values across different priors is close to 0 (Figure supplement 3 and 4). Additionally, we performed Pearson correlation tests. We found that the correlation coefficients between the estimated MOI distributions at the population level across different parameters were close to 1 with a highly significant *p-value* (Figure supplement 5 and 6). Thus, we provide in our analyses the estimated population MOI distribution using a uniform prior.

Statistical analysis

We used the R v4.0.2 for all data analyses with the collection of R packages in *tidyverse* being used for data curation along with *base*, *stats*, *gtsummary,* and *epiR* for the statistical analyses (R Core Team, 2018; Sjoberg, D et al., 2021; Stevenson, 2020; Wickham et al., 2019). Continuous variables are presented as medians with interquartile ranges (IQRs) and discrete variables are presented using the observed proportions or calculated values with 95% confidence intervals (CIs). Kaplan-Meier survival curves were generated for the time (i.e., number of surveys) to first event (i.e., when the DBLα type was no longer observed/detected) comparing the upsA and non-upsA DBLα types; *p*-values were determined using the log-rank test using the R packages *survival* and *survminer* (Kassambara et al., 2021; Therneau, 2023). The time interval to first event considered for all survival curves was the number of surveys or year (i.e., 2012, 2014, 2015, and 2017) that each DBLα type was observed and only includes those upsA (N = 2,215) and non-upsA (N = 33,151) DBLα types that were seen at baseline in 2012 (i.e., those DBLα types observed prior to the IRS intervention).

Data availability

The sequences utilized in this study are publicly available in GenBank under BioProject Number: PRJNA 396962. All data associated with this study are available in the main text or the supplemental information.

Results

Between 2013 and 2015, three-rounds of IRS with non-pyrethroid insecticides were implemented across all of Bongo District. Coincident with the > 90% decrease in transmission following IRS (Tiedje et al., 2022), the prevalence of microscopic *P. falciparum* infections compared to the 2012 baseline survey (pre-IRS) declined by 45.2% and 35.7% following the second (2012 to 2014) and the third (2012 to 2015) round of IRS (**Figure 1B**, Table supplement 1). These declines in parasite prevalence were observed across all ages, with the greatest impacts being observed on the younger children (i.e., 1-5 years) who were ~3 times less likely to have an infection in 2015 (post-IRS) compared to 2012 (pre-IRS) (**Figure 1C**, Table supplement 1). These reductions were however short-lived and in 2017, 32-months after the discontinuation of IRS, but during SMC, overall *P. falciparum* prevalence rebounded to 41.2%, or near pre-IRS levels (**Figure 1B**, Table supplement 1). Importantly, this increase in the prevalence of infection in 2017 was only observed among the older age groups (i.e., ≥ 6 years) (**Figure 1C**, Table supplement 1). This difference by age group in 2017 can be attributed to SMC, which only targets children between 3-59 months (i.e., < 5 years). A notable increase in parasite prevalence for adolescents (11-20 years) and adults (>20 years) was found in 2017 relative to 2012 (**Figure 1C**, Table supplement 1).

Next, we wanted to explore changes in population size measured by MOI_{var}. As this metric is based on non-overlap of *var* repertoire diversity of individual isolates, specifically non-upsA DBLα types, we investigated whether DBLα isolate repertoire similarity (or overlap), as measured by PTS, increased following the sequential interventions (i.e., IRS and SMC). **Figure 2** shows that median PTS values for both upsA and non-upsA DBLα types remained low in all surveys although the PTS distributions for both groups changed significantly at each of the study time points relative to the 2012 baseline survey (pre-IRS) (*p-values* < 0.001, Kruskal-Wallis test) (**Figure 2**). However, the change was in the direction of reduced similarity with lower median PTS scores and a larger number of isolates sharing no DBLα types (i.e., PTS = 0) in 2014, 2015, and 2017 compared to 2012. Relevant to measurement of MOI_{var}, the median PTS scores for non-upsA DBLα types were lower following the IRS intervention ($PTS_{non\text{-}upsA}$: 2014 = 0.013 and 2015 = 0.013 vs. $PTS_{non\text{-}upsA}$:

2012 = 0.020). In 2017, the non-upsA PTS distributions shifted back towards higher median PTS scores ($PTS_{non\text{-}upsA}$ = 0.016) and fewer isolates shared no DBLα types relative to 2014 and 2015 (**Figure 2**). To verify this pattern was not influenced by multiclonal infections (MOI_{var} > 1) we also examined isolates with monoclonal infections (MOI_{var} = 1) and found that this non-overlapping structure persisted regardless of infection complexity, particularly for the non-upsA DBLα types (Figure supplement 7). These PTS data make clear that we were dealing with a large, highly diverse parasite population where 99.9% of the isolate comparisons in all surveys had $PTS_{non\text{-}upsA}$ scores ≤ 0.1 (i.e., shared ≤ 10% of their non-upsA DBLα types), indicating that DBLα isolate repertoires were highly unrelated (**Figure 2**). In fact, throughout the IRS, SMC, and subsequent rebound, very few DBLα isolate repertoires were observed to be related, with < 0.003% isolate comparisons in each survey having a $PTS_{non\text{-}upsA}$ ≥ 0.5 (i.e., siblings or recent recombinants) (**Figure 2**)

The raw data of non-upsA DBLα isolate repertoire sizes (Figure supplement 8) were used to estimate MOI_{var} as adjusted using the Bayesian approach (**Figure 3**). We observed that at baseline in 2012, the majority (89.2%) of the population across all ages carried multiclonal infections (median MOI_{var}= 4 [IQR: 2 – 6]) (**Figure 3A**). Following the IRS intervention, the estimated MOI_{var}distributions became more positively skewed, indicating that a lower proportion of participants harboured multiclonal infections with a lower median MOI_{var} in 2014 (64.5%; median MOI_{var} = 2 [IQR: 1 – 3]) and 2015 (71.4%; median MOI_{var} = 2 [IQR: 1 – 3]) compared to 2012 (**Figure 3A**). These reductions in median $MOI_{var}$and the proportion of multiclonal infections, which were observed across all age groups (**Figure 3B**), are consistent with the > 90% decrease in transmission intensity following the IRS in turn reducing exposure to new parasite genomes. However, in 2017, both median MOI_{var} (3 [IQR: 2 – 4]) and the proportion of multiclonal infections (78.9%) rebounded in all age groups, even among the younger children (1-5 years) targeted by SMC (**Figure 3**). While the prevalence of infection in 2017 remained low for the younger children (1-5 years) targeted by SMC, those infected still carried multiclonal infections (84.1% of those infected) (**Figure 3B**). Although the MOI_{var} distributions across all age groups started to rebound in 2017 (i.e., less positively skewed compared to 2014 and 2015) they had not fully recovered to the 2012 baseline patterns (**Figure 3**). This was most apparent among the younger children (1-5 years), as a larger proportion of isolates in 2017, compared to 2012, had MOI_{var} values equal to one or two, while a smaller proportion had MOI_{var} values ≥ 5 (**Figure 3B**).

Population size measured as the number of *P. falciparum var* repertoires circulating in the population during each survey was estimated by summation of isolate MOI_{var}. In 2014 during IRS, this number decreased by 72.4% relative to the 2012 baseline survey (pre-IRS) (**Figure 4A**), whereas prevalence decreased by 54.5% (**Figure 4B**). Although population size increased slightly in 2015 relative to 2014, there were still 64.5% fewer *var* repertoires in the population compared to 2012 (**Figure 4A**) in comparison to a 42.6% decrease in prevalence (**Figure 4B**). Importantly this loss of *var* repertoires in 2014 and 2015 following the IRS intervention was seen for all age groups, with the greatest overall reductions (> 85%) being observed for the younger children (1-5 years) (**Figure 4CD**). However, in 2017, the number of diverse *var* repertoires in the population rebounded, more than doubling between 2015 and 2017 (**Figure 4AB**). This increase in the number of *var* repertoires was seen for all age groups in 2017, except for the younger children (1-5 years) who were directly targeted by SMC (**Figure 4CD**). In fact, the greatest overall increase was observed for the adolescents (11-20 years), where the number of *var* repertoires in 2017 was ~1.4 times higher compared to 2012 (**Figure 4CD**). Similar trends in the number *var* repertoires were also observed for the older children (6-10 years) and adults (>20 years) in 2017, although the rebound was not as striking as that detected for the adolescents.

As parasite population size changed considerably during the sequential interventions, we investigated how the removal or loss of *P. falciparum var* repertoires and subsequent rebound altered DBLα type richness, measured as the number of unique upsA and non-upsA DBLα types in the parasite population in each survey. Richness at baseline in 2012 (pre-IRS) was high with a large

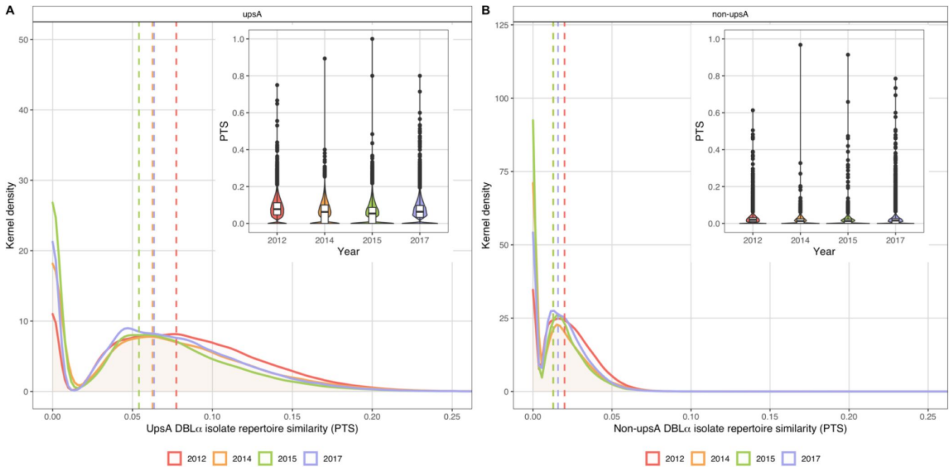

Figure 2.

Sharing of upsA and non-upsA DBLα types among the DBLα isolate repertoires in 2012 (pre-IRS, red), 2014 (during IRS, orange), 2015 (post-IRS, green), and 2017 (SMC, purple).

The overlapping density and violin plots (upper right-hand corners) show the distribution of PTS scores (i.e., DBLα isolate repertoire similarity) between the **(A)** upsA and **(B)** non-upsA DBLα isolate repertoires with DBLα sequencing data (Table supplement 2) in each survey. The PTS scales in the density plots have been zoomed-in to provide better visualization of the upsA and non-upsA DBLα types PTS distributions. The colored dashed lines in the density plots indicate the median PTS scores in each survey for the upsA (2012 (red) = 0.078, 2014 (orange) = 0.063, 2015 (green) = 0.054, and 2017 (purple) = 0.064) and non-upsA (2012 (red) = 0.020, 2014 (orange) = 0.013, 2015 (green) = 0.013, and 2017 (purple) = 0.016) DBLα types. *Note:* The non-upsA median PTS values in 2014 (orange) and 2015 (green) were both 0.013, and overlap in the figure. In the PTS violin plots the central box plots indicate the medians (centre line), interquartile ranges (IQR, upper and lower quartiles), whiskers (1.5x IQR), and outliers (points).

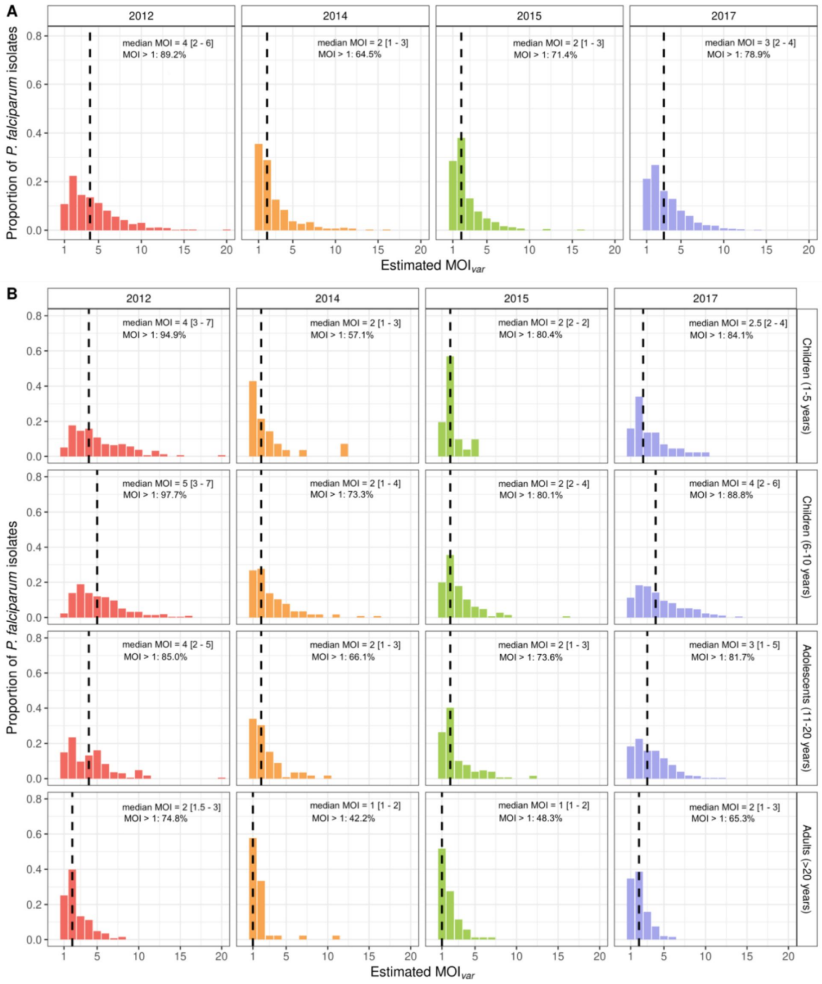

Figure 3.

MOI_{var} distributions in 2012 (pre-IRS, red), 2014 (during IRS, orange), 2015 (post-IRS, green), and 2017 (SMC, purple).

Estimated MOI_{var} distributions for the **(A)** study population and **(B)** for all age groups (years) in each survey for those isolates with DBLα sequencing data (Table supplement 2). The median MOI_{var} values are indicated with the black dashed lines and have been provided in the top right corner (median MOI_{var}value [interquartile range, upper and lower quartiles]) along with the percentage of *P. falciparum* infections that were multiclonal (MOI_{var} > 1) in each survey and age group (years).

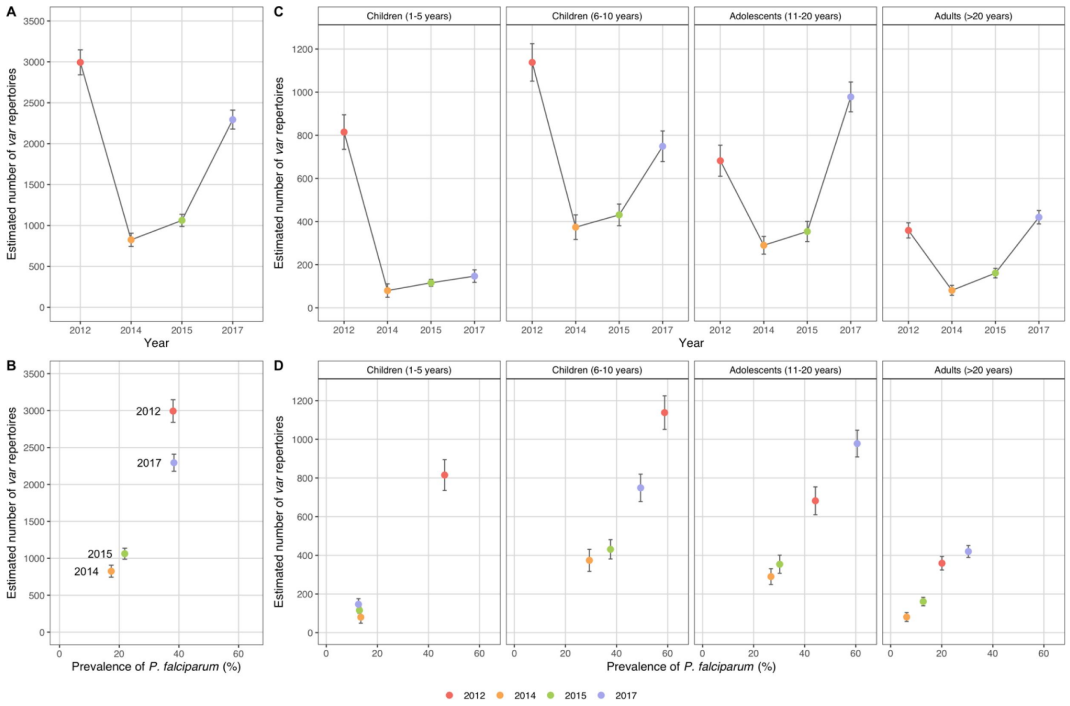

Figure 4.

Estimated number of *P. falciparum var* repertoires in 2012 (pre-IRS, red), 2014 (during IRS, orange), 2015 (post-IRS, green), and 2017 (SMC, purple).

The estimated number of *var* repertoires for the study population in 2012 (N = 2,994), 2014 (N = 825), 2015 (N = 1062), and 2017 (N = 2,294) **(A)** in each survey and **(B)** vs. *P. falciparum* prevalence for those isolates with DBLα sequencing data (Table supplement 2). To explore this further we also estimated the number of *var* repertoires in all age groups (years) **(C)** in each survey and **(D)** vs. *P. falciparum* prevalence for those isolates with DBLα sequencing data. Error bars represent the upper and lower limits of the 95% confidence interval (CI) for the estimated number of *var* repertoires.

number of unique DBLα types (upsA = 2,215; non-upsA = 33,151) (**Figure 5**, Table supplement 2) and limited overlap of *var* repertoires (i.e., median $PTS_{non\text{-}upsA} \leq 0.020$) seen in a relatively small study population of 685 microscopically positive individuals (**Figure 2**). In 2014, as *P. falciparum* prevalence and population size declined (**Figure 4**) so too did the number of DBLα types, resulting in a 32.1% and 55.3% reduction in richness for the upsA and non-upsA DBLα types, respectively compared to 2012 (**Figure 5**, Table supplement 2). Again in 2015, as *P. falciparum* prevalence and population size remained low (**Figure 4**) DBLα type richness was still less than that observed in 2012 (24.6% and 45.9% reduction for upsA and non-upsA DBLα types, respectively) (**Figure 5**, Table supplement 2). Finally, in 2017, we found that upsA and non-upsA DBLα type richness rebounded relative to 2014 and 2015, coincident with the increase in *P. falciparum* prevalence and parasite population size (**Figure 4**, **Figure 5**).

Given this reduction in DBLα type richness following the IRS intervention and subsequent rebound in 2017, we wanted to explore whether the loss of richness was influenced by the frequency of individual DBLα types in the parasite population within and among surveys. To answer this, we defined the relative frequency of individual DBLα types in all isolates in each survey (**Figure 6**, Figure supplement 9). We discovered that individual upsA and non-upsA DBLα types were not all at equal frequencies within a survey and among surveys. They could be classified as frequent (i.e., observed in 11-20 or > 20 isolates), less frequent (i.e., observed in 2-10 isolates) or only seen once, at baseline in 2012 (**Figure 6AB**). In 2014 and 2015, following the IRS, there was a significant increase in the proportion of upsA and non-upsA DBLα types in the lower frequency categories (*p-value* < 0.001, Mann-Whitney U test), with all DBLα types becoming rarer in the population (**Figure 6CD**). This change can be attributed to the removal of *P. falciparum* *var* repertoires (**Figure 4**) with associated loss of upsA and non-upsA DBLα type richness (**Figure 5**), which disproportionally affected those DBLα types seen once. This shift to all DBLα types becoming rarer following IRS changed in 2017, where the proportion of DBLα types in the more frequent categories (i.e., 2-10, 11-20, or > 20 isolates) significantly increased while the proportion seen once decreased (*p-values* < 0.001, Mann-Whitney U tests) (**Figure 6CD**). Data in **Figure 6** (A-D) pointed to a differential effect of the IRS intervention and subsequent rebound on the less frequent upsA and non-upsA DBLα types vs. those that were classified as frequent, where those DBLα types that were most frequent persisted longitudinally.

To explore this observation further we restricted the longitudinal analysis to those DBLα types from the baseline survey in 2012 (pre-IRS). We compared the probability of survival for the DBLα types identified at baseline and found that the upsA DBLα types persisted significantly longer in the population relative to the non-upsA DBLα types ($p < 0.001$, log-rank test), despite the IRS intervention. The simple explanation being that although the upsA DBLα types had lower richness (**Figure 5**), a larger proportion was classified as frequent indicating that multiple copies existed in the population compared to the non-upsA DBLα types (**Figure 6CD**). Furthermore, when we examined survival using the frequency categories, the upsA and non-upsA DBLα types that persisted and were observed at multiple study time points (i.e., 2012, 2014, 2015, and 2017) were those that were most frequent (i.e., observed in 11-20 and >20 isolates) in the population at baseline in 2012 (**Figure 6EF**). As expected, the DBLα types that were only observed once in 2012, were significantly less likely to be seen longitudinally (*p-value* < 0.001, log-rank test) (**Figure 6EF**). These differential changes in DBLα type richness with respect to rare vs frequent DBLα types are a consequence of changes in census population size with interventions where each isolate repertoire is composed of many rare DBLα types as defined by PTS (**Figure 2**).

Discussion

P. falciparum populations in high-transmission endemic areas in SSA, are characterised by extensive diversity, high rates of recombination, as well as frequent multiclonal infections. Here we defined census population size of *P. falciparum* to understand total parasite diversity in a

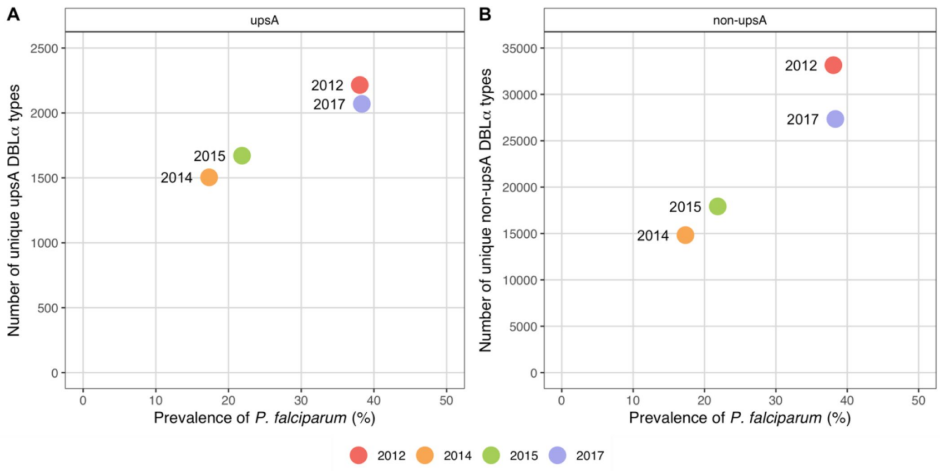

Figure 5.

UpsA and non-upsA DBLα type richness in 2012 (pre-IRS, red), 2014 (during IRS, orange), 2015 (post-IRS, green), and 2017 (SMC, purple).

Number of unique **(A)** upsA and **(B)** non-upsA DBLα types (i.e., richness) observed in each survey vs. *P. falciparum* prevalence based on those isolates with DBLα sequencing data (Table supplement 2).

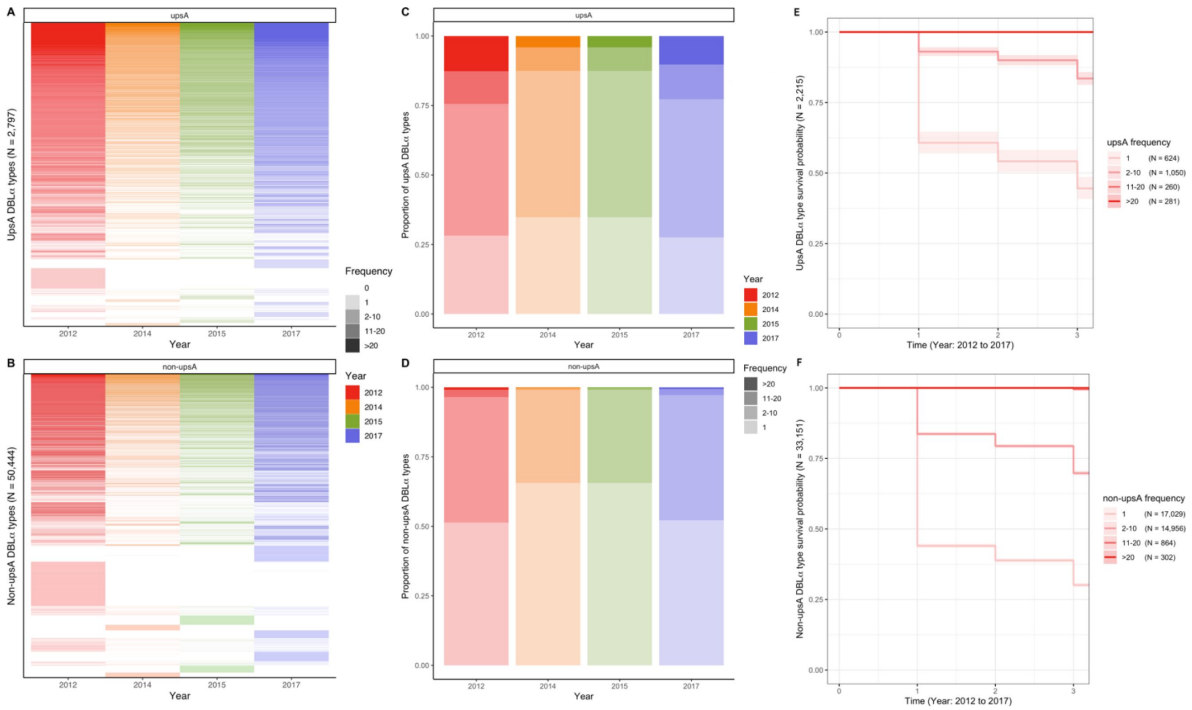

Figure 6.

UpsA and non-upsA DBLα type frequencies and survival in 2012 (pre-IRS, red), 2014 (during IRS, orange), 2015 (post-IRS, green), and 2017 (SMC, purple).

Heatmaps showing the patterns of diversity for the **(A)** upsA and **(B)** non-upsA DBLα types. The columns represent all the upsA and non-upsA DBLα types observed in the four surveys, and the rows represent each of the 2,797 upsA DBLα types and the 50,444 non-upsA DBLα types. White rows are used to denote the absence of a specific DBLα type, while the presence of a DBLα type is indicated in colour and further categorised (colour gradations) based on the frequency or the number of times (i.e., number of isolates) a DBLα type was observed in each survey (Frequency categories: 1, 2-10, 11-20, > 20 isolates; Note the frequency category cut-offs were chosen based on the frequency distributions in Figure supplement 9). The proportions of **(C)** upsA and **(D)** non-upsA DBLα types in each survey based on the number of times (i.e., number of isolates) they were observed in each survey. Kaplan-Meier survival curves for the **(E)** upsA and **(F)** non-upsA DBLα types across time (2012 to 2017) categorised based on their frequency at baseline in 2012 (pre-IRS, red). The colored shaded areas represent the 95% confidence intervals (CIs) and the number **(N)** of upsA and non-upsA DBLα types in each frequency category are provided in parenthesis. These survival curves include only those upsA (N = 2,215) and non-upsA (N = 33,151) DBLα types that were seen at baseline in 2012 (pre-IRS) as indicated in red. The x-axis indicates time where time “0” denotes 2012 (pre-IRS), “1” denotes 2014 (during IRS), “2” denotes 2015 (post-IRS), and finally “3” denotes 2017 (SMC). *Note:* In the survival curves the 11-20 and > 20 frequency categories for both the **(C)** upsA and **(D)** non-upsA DBLα types overlap in the figure.

human population and explore the age-specific efficacy of malaria interventions to reduce this metric in such areas as typified by Bongo, Ghana. Census population size proved more informative than parasite prevalence alone because it captures "within" host parasite population size as MOI, rather than using the infected host per se as a unit of population size. Whilst the concept of census population size is agnostic of how you measure MOI, the extensive DBLα isolate repertoire diversity presented makes a strong case for fingerprinting parasite isolates by *var*coding in high transmission. This is opposed to looking at allelic diversity of a single copy antigen gene such as *csp* that is under balancing selection.

By *var*coding we identified a very large census parasite population size in a relatively small human population of ~2,000 individuals at baseline and captured age-specific changes in this metric in response to sequential malaria control interventions. IRS reduced the MOI_{var} parasite population size substantially with the greatest reductions (85%) seen in the younger children (i.e., 1-5 years). More than two years after cessation of IRS, rebound in 2017 was rapid in all age groups with the exception of younger children targeted by SMC. Population sizes in adolescents and adults showed they carried more infections in 2017 than at baseline in 2012. This is indicative of a loss of immunity during IRS which may relate to the observed loss of *var* richness, especially the many rare types. This warrants further investigation of changes in variant-specific immunity. During and following the IRS and SMC interventions, *var* diversity remained high and *var* repertoire overlap remained low, reflecting characteristic properties of high transmission and demonstrating the overall resilience of the species to survive significant short-term perturbations. Combining interventions and targeting older age groups or the whole community with chemoprevention would no doubt have a much greater impact on reducing the diversity of the reservoir of infection.

What was striking about the Bongo study was the speed with which rebound in MOI_{var} per person and census population size occurred, once the short-term IRS was discontinued. We looked for a potential explanation in our genetic data. PTS and population frequency data showed that many of the DBLα types occurred in multiple repertoires or genomes. This enabled the survival of these more frequent DBLα types through the interventions, facilitating rebound by maintenance of this diversity. The other notable population genetic result of our study was the failure to increase similarity (or relatedness by state) of *var* repertoires by reducing transmission by > 90% via IRS. From a baseline of a very large population size with very low overlap in repertoires you need outcrossing to create relatedness. However, this was less likely to happen due to reduced transmission as a result of IRS. Rebound, with associated increases in transmission, led to a small increase in *var* repertoire similarity. This is the opposite to what has been observed in areas of lower transmission under intense malaria control where the intensity of interventions led to increased genome similarity, as assessed by IBD, from a starting point of much lower genome diversity and greater relatedness (Daniels et al., 2015).

Our molecular approach to measure population size has been to sum MOI_{var} in individual hosts with microscopically-detectable infections. Like any diagnostic method there are limits to sensitivity and specificity, which can be more or less tolerated dependent upon the purpose of the study. Here we have looked at relative changes in population size with sequential interventions using an interrupted time series study design and observed changes by measuring MOI_{var}. We have accounted for missing DBLα type data where complete *var* repertoires may not have been sequenced using a Bayesian method based on empirical knowledge of the measurement error. This approach has conceptual relation to the Bayesian approach by Johnson and Larremore (Johnson and Larremore, 2022) to estimate complete repertoire size of, and overlap between, monoclonal infections from incomplete sampling of DBLα types. Our measurement of population size based on MOI_{var} will be subject to other sampling errors which may in the end be more significant (discussed in detail in Labbé et al. (Labbé et al., 2023)). For example, low parasitemia

typical of asymptomatic infections, small blood volumes, clinical status, and/or within host dynamics, including synchronicity, will all create sampling problems but these are common to all measures of MOI.

Previously, we have drawn attention to underestimation of the number of DBLα types of related parasites from a cross by *var* genotyping (Labbé et al., 2023). Such related parasites must be created frequently in high transmission due to extensive outcrossing (Babiker et al., 1994; Paul et al., 1995). Single clone genomics experiments using biallelic SNPs from whole genome sequencing data have also detected related parasites using IBD in clinical infections from humans in a high-transmission area of Malawi (Nkhoma et al., 2020). Here we have analysed only low density, chronic asymptomatic infections under strong immune selection in semi-immune hosts whom we have shown select against related parasites (He et al., 2018; Ruybal-Pesántez et al., 2022). When considering the importance of possible exclusion of parasites related by descent, the only sure way to detect such parasites in high transmission is by single cell genomics, a methodology of limited application to malaria surveillance due to practicality and cost of scale up. Again, the error from failure to sample related infections must be weighed up against the issues of under-sampling as described above.

In summary, our findings provide parasite population insights into why rebound is the inevitable consequence of such short-term IRS interventions unless you simultaneously target the highly diverse, long-lived parasite population in humans, not just children < 5 years by SMC. Of potential translational significance for malaria molecular surveillance, we identify new metrics, especially MOI_{var} and census population size as well as *var* frequency category, informative to monitor and evaluate interventions in high-transmission areas with multiclonal infections and high rates of outcrossing. Such metrics could be used longitudinally to detect incremental gains of transmission-reducing interventions, including IRS, LLINs, and vaccines to perturb the high-transmission characteristics of the parasite population in humans in high-burden countries in SSA.

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Data availability statement

The sequences utilized in this study are publicly available in GenBank under BioProject Number: PRJNA 396962. All data associated with this study are available in the main text or in the Supporting Information. All custom code is available in an open source repository: (1) DBLαCleaner pipeline is available at*https://github.com/UniMelb-Day-Lab/DBLaCleaner*, (2) clusterDBLalpha pipeline is available at *https://github.com/Unimelb-Day-Lab/clusterDBLalpha*, and the (3) classifyDBLalpha pipeline is available at *https://github.com/Unimelb-Day-Lab/classifyDBLalpha*. A dataset to demo this custom code is available at *https://github.com/UniMelb-Day-Lab/tutorialDBLalpha*. For additional information on the use of the Bayesian approach to estimate MOI_{var} please see *https://github.com/qzhan321/Bayesian-formulation-varcoding-MOI-estimation*.

Benefit sharing statement

A research collaboration was developed with scientists from Ghana based at the Navrongo Health Research Centre and the Noguchi Memorial Institute for Medical Research. All collaborators are included as co-authors and the relevant results from the research has been shared with key stakeholders and the local community (i.e., Paramount Chief of Bongo, divisional Chiefs, Queen Mothers, and community members) as well as the Bongo District Health Directorate and the Regional Health Directorate (Upper East Region). Before this research was undertaken, informed consent was sought and obtained from the key stakeholders and the local community in Bongo District. In addition, members of the local community were trained as field workers and were directly involved in liaising with the local community and in the collection of the study data. The contribution of these individuals to this research is described in the Acknowledgements. This research addresses a priority concern regarding malaria control and the impact of interventions. These concerns are relevant for both the local community as well as for the National Malaria Elimination Programme in Ghana.

Author contributions

K.P.D and M.P conceptualized the study of population size using MOI_{var}. K.A.K and K.P.D conceived and designed the field study with input from A.R.O and K.E.T, who together with O.B coordinated the field studies. K.E.T, S.R-P, and S.L.D processed the samples and performed the genotyping experiments. G.T-H developed and validated the bioinformatic pipelines. K.E.T, S.R-P, and D.C.A processed, cleaned, and curated the datasets for analysis. Q.Z developed and implemented the Bayesian estimations. K.E.T, Q.Z, M.P, and K.P.D analysed the data with contributions from S.R-P, Q.H, and M-H.T. K.E.T and Q.Z visualized the data. K.P.D, M.P, and K.E.T wrote the original draft of the manuscript. Q.Z., S.R-P, G.T-H, Q.H, M-H.T, D.C.A, S.L.D, O. B, A.R.O, and K.AK reviewed and edited manuscript. M.P and K.P.D supervised the research. A.R.O, K.A.K, M.P, and K.P.D acquired the funding.

Competing interests

The authors declare no competing interests.

References

Anderson TJC *et al.* (2000) **Microsatellite markers reveal a spectrum of population structures in the malaria parasite Plasmodium falciparum** *Mol Biol Evol* **17**:1467–1482 https://doi.org/10.1093/oxfordjournals.molbev.a026247

Babiker HA, Ranford-Cartwright LC, Currie D, Charlwood JD, Billingsley P, Teuscher T, Walliker D. (1994) **Random mating in a natural population of the malaria parasite Plasmodium falciparum** *Parasitology* **109**:413–421 https://doi.org/10.1017/S0031182000080665

Barry AE, Leliwa-Sytek A, Tavul L, Imrie H, Migot-Nabias F, Brown SM, McVean GA V, Day KP. (2007) **Population genomics of the immune evasion (var) genes of Plasmodium falciparum** *PLoS Pathog* **3** https://doi.org/10.1371/journal.ppat.0030034

Bruce MC, Galinski MR, Barnwell JW, Donnelly CA, Walmsley M, Alpers MP, Walliker D, Day KP. (2000) **Genetic diversity and dynamics of plasmodium falciparum and P. vivax populations in multiply infected children with asymptomatic malaria infections in Papua New Guinea** *Parasitology* **121**:257–272 https://doi.org/10.1017/S0031182099006356

Chen DS *et al.* (2011) **A molecular epidemiological study of var gene diversity to characterize the reservoir of Plasmodium falciparum in humans in Africa** *PLoS One* **6** https://doi.org/10.1371/journal.pone.0016629

Daniels RF *et al.* (2015) **Modeling malaria genomics reveals transmission decline and rebound in Senegal** *PNAS* **112**:7067–7072 https://doi.org/10.1073/pnas.1505691112

Day KP *et al.* (2017) **Evidence of Strain Structure in Plasmodium falciparum Var Gene Repertoires in Children from Gabon** *West Africa. PNAS* **114**:E4103–E4111 https://doi.org/10.1073/pnas.1613018114

Dietz K. (1988) **Mathematical models for transmission and control of malariaMalaria** *Principles and Practice of Malariology*

Falk N, Kaestli M, Qi W, Ott M, Baea K, Cortés A, Beck H-P. (2009) **Analysis of Plasmodium falciparum var genes expressed in children from Papua New Guinea** *J Infect Dis* **200**:347–356 https://doi.org/10.1086/600071

Falk N, Maire N, Sama W, Owusu-Agyei S, Smith T, Beck HP, Felger I. (2006) **Comparison of PCR-RFLP and genescan-based genotyping for analyzing infection dynamics of Plasmodium falciparum** *Am J Trop Med Hyg* **74**:944–950

Farnert A, Snounou G, Rooth I, Bjorkman A. (1997) **Daily dynamics of Plasmodium falciparum subpopulations in asymptomatic children in a holoendemic area** *Am J Trop Med Hyg* **56**:538–547 https://doi.org/10.4269/ajtmh.1997.56.538

Futse JE, Brayton KA, Dark MJ, Knowles DP, Palmer GH. (2008) **Superinfection as a driver of genomic diversification in antigenically variant pathogens** *Proc Natl Acad Sci U S A* **105**:2123–2127 https://doi.org/10.1073/pnas.0710333105

Gardner MJ *et al.* (2002) **Genome sequence of the human malaria parasite Plasmodium falciparum** *Nature* **419**:498–511 https://doi.org/10.1038/nature01097

Ghansah A, Tiedje KE, Argyropoulos DC, Onwona CO, Deed SL, Labbé F, Oduro AR, Koram KA, Pascual M, Day KP. (2023) **Comparison of molecular surveillance methods to assess changes in the population genetics of Plasmodium falciparum in high transmission** *Front Parasitol* **2**

Gogue C *et al.* (2020) **An observational analysis of the impact of indoor residual spraying in Northern, Upper East, and Upper West Regions of Ghana: 2014 through 2017** *Malar J* **19**:1–13 https://doi.org/10.1186/s12936-020-03318-1

He Q, Pilosof S, Tiedje KE, Ruybal-Pesántez S, Artzy-Randrup Y, Baskerville EB, Day KP, Pascual M. (2018) **Networks of genetic similarity reveal non-neutral processes shape strain structure in Plasmodium falciparum** *Nat Commun* **9** https://doi.org/10.1038/s41467-018-04219-3

Henry JM. (2020) **A hybrid model for the effects of treatment and demography on malaria superinfection** *J Theor Biol* **491**

Jensen ATR *et al.* (2004) **Plasmodium falciparum associated with severe childhood malaria preferentially expresses PfEMP1 encoded by group A var genes** *J Exp Med* **199**:1179–1190 https://doi.org/10.1084/jem.20040274

Johnson EK, Larremore DB. (2022) **Bayesian estimation of community size and overlap from random subsamples** *PLoS Comput Biol* **18**:1–16 https://doi.org/10.1371/journal.pcbi.1010451

Kaestli M, Cockburn I a, Cortés A, Baea K, Rowe JA, Beck H-P. (2006) **Virulence of malaria is associated with differential expression of Plasmodium falciparum var gene subgroups in a case-control study** *J Infect Dis* **193**:1567–1574

Kalmbach Y, Rottmann M, Kombila M, Kremsner PG, Beck H-P, Kun JFJ. (2010) **Differential var gene expression in children with malaria and antidromic effects on host gene expression** *J Infect Dis* **202**:313–317 https://doi.org/10.1086/653586

Kassambara A, Kosinski M, Biecek P, Scheipl F. (2021) **. survminer: Drawing Survival Curves using ggplot2. Kraemer SM, Kyes SA, Aggarwal G, Springer AL, Nelson SO, Christodoulou Z, Smith LM, Wang W** *Levin E*

Newbold CI, Myler PJ, Smith JD. (2007) **Patterns of gene recombination shape var gene repertoires in Plasmodium falciparum: comparisons of geographically diverse isolates** *BMC Genomics* **8** https://doi.org/10.1186/1471-2164-8-45

Kraemer SM, Smith JD. (2006) **A family affair: var genes, PfEMP1 binding, and malaria disease** *Curr Opin Microbiol* https://doi.org/10.1016/j.mib.2006.06.006

Kyriacou HM, Stone GN, Challis RJ, Raza A, Lyke KE, Thera MA, Koné AK, Doumbo OK, Plowe C V., Rowe JA. (2006) **Differential var gene transcription in Plasmodium falciparum isolates from patients with cerebral malaria compared to hyperparasitaemia** *Mol Biochem Parasitol* **150**:211–218 https://doi.org/10.1016/j.molbiopara.2006.08.005

Labbé F, He Q, Zhan Q, Tiedje KE, Argyropoulos DC, Tan MH, Ghansah A, Day KP, Pascual M. (2023) **Neutral vs . non-neutral genetic footprints of Plasmodium falciparum multiclonal infections** *PLoS Comput Biol* **19** https://doi.org/10.1101/2022.06.27.497801

LaVerriere E *et al.* (2022) **Design and implementation of multiplexed amplicon sequencing panels to serve genomic epidemiology of infectious disease: A malaria case study** *Mol Ecol Resour* :2285–2303 https://doi.org/10.1111/1755-0998.13622

Lavstsen T, Salanti A, Jensen ATR, Arnot DE, Theander TG. (2003) **Sub-grouping of Plasmodium falciparum 3D7 var genes based on sequence analysis of coding and non-coding regions** *Malar J* **2** https://doi.org/10.1186/1475-2875-2-27

Lerch A, Koepfli C, Hofmann NE, Kattenberg JH, Rosanas-Urgell A, Betuela I, Mueller I, Felger I. (2019) **Longitudinal tracking and quantification of individual Plasmodium falciparum clones in complex infections** *Sci Rep* **9**:1–8 https://doi.org/10.1038/s41598-019-39656-7

Lerch A, Koepfli C, Hofmann NE, Messerli C, Wilcox S, Kattenberg JH, Betuela I, O'Connor L, Mueller I, Felger I. (2017) **Development of amplicon deep sequencing markers and data analysis pipeline for genotyping multi-clonal malaria infections** *BMC Genomics* **18**:1–13 https://doi.org/10.1186/s12864-017-4260-y

Markwalter CF *et al.* (2022) **Plasmodium falciparum importation does not sustain malaria transmission in a semi-arid region of Kenya** *PLOS Glob Public Heal* **2** https://doi.org/10.1371/journal.pgph.0000807

Nelson CS, Sumner KM, Freedman E, Saelens JW, Obala AA, Mangeni JN, Taylor SM, O'Meara WP. (2019) **High-resolution micro-epidemiology of parasite spatial and temporal dynamics in a high malaria transmission setting in Kenya** *Nat Commun* **10** https://doi.org/10.1038/s41467-019-13578-4

Nkhoma SC *et al.* (2020) **Co-transmission of Related Malaria Parasite Lineages Shapes Within-Host Parasite Diversity** *Cell Host Microbe* **27**:93–103 https://doi.org/10.1016/j.chom.2019.12.001

Normark J *et al.* (2007) **PfEMP1-DBL1alpha amino acid motifs in severe disease states of Plasmodium falciparum malaria** *Proc Natl Acad Sci U S A* **104**:15835–40 https://doi.org/10.1073/pnas.0610485104

Otto TD, Assefa SA, Böhme U, Sanders MJ, Kwiatkowski DP, Berriman M, Newbold C. (2019) **Evolutionary analysis of the most polymorphic gene family in falciparum malaria** *Wellcome Open Res* **4**:1–29 https://doi.org/10.12688/wellcomeopenres.15590.1

Paul RE, Packer MJ, Walmsley M, Lagog M, Ranford-Cartwright LC, Paru R, Day KP. (1995) **Mating patterns in malaria parasite populations of Papua New Guinea** *Science (80-)* **269**:1709–1711

Pilosof S, He Q, Tiedje KE, Ruybal-Pesántez S, Day KP, Pascual M. (2019) **Competition for hosts modulates vast antigenic diversity to generate persistent strain structure in Plasmodium falciparum** *PLOS Biol* **17** https://doi.org/10.1371/journal.pbio.3000336

R Core Team. (2018) **R: A Language and Environment for Statistical Computing** *R Found Stat Comput*

Rask TS *et al.* (2010) **Plasmodium falciparum erythrocyte membrane protein 1 diversity in seven genomes - divide and conquer** *PLoS Comput Biol* **6** https://doi.org/10.1371/journal.pcbi.1000933

Rorick MM, Artzy-Randrup Y, Ruybal-Pesántez S, Tiedje KE, Rask TS, Oduro A, Ghansah A, Koram K, Day KP, Pascual M. (2018) **Signatures of competition and strain structure within the major blood-stage antigen of P. falciparum in a local community in Ghana** *Ecol Evol* **8**:3574–3588

Rottmann M, Lavstsen T, Mugasa JP, Kaestli M, Jensen ATR, Müller D, Theander T, Beck H-PP. (2006) **Differential expression of var gene groups is associated with morbidity caused by Plasmodium falciparum infection in Tanzanian children** *Infect Immun* **74**:3904–3911 https://doi.org/10.1128/IAI.02073-05

Ruybal-Pesántez S *et al.* (2022) **Age-specific patterns of DBLa var diversity can explain why residents of high malaria transmission areas remain susceptible to Plasmodium falciparum blood stage infection throughout life** *Int J Parasitol* **20**:721–731

Ruybal-Pesántez S, Tiedje KE, Tonkin-Hill G, Rask TS, Kamya MR, Greenhouse B, Dorsey G, Duffy MF, Day KP. (2017) **Population genomics of virulence genes of Plasmodium falciparum in clinical isolates from Uganda** *Sci Rep* **7** https://doi.org/10.1038/s41598-017-11814-9

Sjoberg D, Whiting K, Curry M, Lavery J, Larmarange J. (2021) **Reproducible Summary Tables with the gtsummary Package** *R Journal* **13**:570–580

Smith T, Felger I, Fraser-Hurt N, Beck H-PP. (1999) **Effect of insecticide-treated falciparum infections bed nets on the dynamics of multiple Plasmodium falciparum infections** *Trans R Soc Trop Med Hyg* **93**:S1/53–S1/57 https://doi.org/10.1016/S0035-9203(99)90328-0

Speed D, Balding DJ. (2015) **Relatedness in the post-genomic era: Is it still useful?** *Nat Rev Genet* **16**:33–44 https://doi.org/10.1038/nrg3821

Stevenson M. (2020) **epiR: Tools for the analysis of epidemiological data**

Sumner KM, Freedman E, Abel L, Obala A, Pence BW, Wesolowski A, Meshnick SR, Prudhomme-O'Meara W, Taylor SM. (2021) **Genotyping cognate Plasmodium falciparum in humans and mosquitoes to estimate onward transmission of asymptomatic infections** *Nat Commun* **12**:1–12 https://doi.org/10.1038/s41467-021-21269-2

Tan MH, Shim H, Chan Y, Day KP. (2023) **Unravelling chaos for malaria surveillance : Relationship between DBLα types and var genes in Plasmodium falciparum** *Front Parasitol* **1** https://doi.org/10.3389/fpara.2022.1006341

Tessema SK *et al.* (2020) **Sensitive, highly multiplexed sequencing of microhaplotypes from the Plasmodium falciparum heterozygome** *J Infect Dis* **225**:1227–1237

Therneau T. (2023) **A Package for Survival Analysis in R**

Tiedje KE, Oduro AR, Agongo G, Anyorigiya T, Azongo D, Awine T, Ghansah A, Pascual M, Koram KA, Day KP. (2017) **Seasonal Variation in the Epidemiology of Asymptomatic Plasmodium falciparum Infections Across Two Catchment Areas in Bongo District, Ghana** *Am J Trop Med Hyg* **97**:199–212 https://doi.org/10.4269/ajtmh.16-0959

Tiedje KE *et al.* (2022) **Indoor residual spraying with a non-pyrethroid insecticide reduces the reservoir of Plasmodium falciparum in a high-transmission area in northern Ghana** *PLOS Glob Public Heal* **2** https://doi.org/10.1371/journal.pgph.0000285

US Agency for International Development (USAID) Global Health Supply Chain Program. (2020) **Technical Breif: Data visability makes all the difference in Ghana's 2018 LLIN mass distribution campaign**

US President's Malaria Initiative Africa IRS (AIRS) Project (2016) **. Entomological monitoring of the PMI AIRS program in northern Ghana: 2016 annual report** *Bethesda*

Wagman J *et al.* (2018) **An observational analysis of the impact of indoor residual spraying with non-pyrethroid insecticides on the incidence of malaria in Ségou Region, Mali: 2012–2015** *Malar J* **17** https://doi.org/10.1186/s12936-017-2168-2

Warimwe GM *et al.* (2012) **Prognostic indicators of life-threatening malaria are associated with distinct parasite variant antigen profiles** *Sci Transl Med* **4** https://doi.org/10.1126/scitranslmed.3003247

Warimwe GM, Keane TM, Fegan G, Musyoki JN, Newton CRJC, Pain A, Berriman M, Marsh K, Bull PC. (2009) **Plasmodium falciparum var gene expression is modified by host immunity** *Proc Natl Acad Sci U S A* **106**:21801–21806

Watson OJ *et al.* (2021) **Evaluating the Performance of Malaria Genetics for Inferring Changes in Transmission Intensity Using Transmission Modeling** *Mol Biol Evol* **38**:274–289 https://doi.org/10.1093/molbev/msaa225

WHO/GMP (2017) **A Framework for Malaria Elimination** *Geneva World Health Organization*

WHO (2012) **WHO Policy Recommendation: Seasonal Malaria Chemoprevention (SMC) for Plasmodium falciparum malaria control in highly seasonal transmission areas of the Sahel sub-region in Africa**

Wickham H *et al.* (2019) **Welcome to the Tidyverse** *J Open Source Softw* **4** https://doi.org/10.21105/joss.01686

World Health Organization (2022) **World Malaria Report 2022** *World Health Organization*

Zhang X, Deitsch KW. (2022) **The mystery of persistent, asymptomatic Plasmodium falciparum infections** *Curr Opin Microbiol* **70** https://doi.org/10.1016/j.mib.2022.102231

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Reviewer #1 (Public Review):

Tiedje et al. investigated the transient impact of indoor residual spraying (IRS) followed by seasonal malaria chemoprevention (SMC) on the plasmodium falciparum parasite population in a high transmission setting. The parasite population was characterized by sequencing the highly variable DBLα tag as a proxy for var genes, a method known as varcoding. Varcoding presents a unique opportunity due to the extraordinary diversity observed as well as the extremely low overlap of repertoires between parasite strains. The authors also present a new Bayesian approach to estimating individual multiplicity of infection (MOI) from the measured DBLα repertoire, addressing some of the potential shortcomings of the approach that have been previously discussed. The authors also present a new epidemiological endpoint, the so-called "census population size", to evaluate the impact of interventions.

This study provides a nice example of how varcoding technology can be leveraged, as well as the importance of using diverse genetic markers for characterizing populations, especially in the context of high transmission. The data are robust and clearly show the transient impact of IRS in a high transmission setting, however, some aspects of the analysis are confusing.

1. Approaching MOI estimation with a Bayesian framework is a well-received addition to the varcoding methodology that helps to address the uncertainty associated with not knowing the true repertoire size. It's unfortunate that while the authors clearly explored the ability to estimate the population MOI distribution, they opted to use only MAP estimates. Embracing the Bayesian methodology fully would have been interesting, as the posterior distribution of population MOI could have been better explored.

2. The "census population size" endpoint has unclear utility. It is defined as the sum of MOI across measured samples, making it sensitive to the total number of samples collected and genotyped. This means that the values are not comparable outside of this study, and are only roughly comparable between strata in the context of prevalence where we understand that approximately the same number of samples were collected. In contrast, mean MOI would be insensitive to differences in sample size, why was this not explored? It's also unclear in what way this is a "census". While the sample size is certainly large, it is nowhere near a complete enumeration of the parasite population in question, as evidenced by the extremely low level of pairwise type sharing in the observed data.

3. The extraordinary diversity of DBLα presents challenges to analyzing the data. The authors explore the variability in repertoire richness and frequency over the course of the study, noting that richness rapidly declined following IRS and later rebounded, while the frequency of rare types increased, and then later declined back to baseline levels. The authors attribute this to fundamental changes in population structure. While there may have been some changes to the population, the observed differences in richness as well as frequency before and after IRS may also be compatible with simply sampling fewer cases, and thus fewer DBLα sequences. The shift back to frequency and richness that is similar to pre-IRS also coincides with a similar total number of samples collected. The authors explore this to some degree with their survival analysis, demonstrating that a substantial number of rare sequences did not persist between timepoints and that rarer sequences had a higher probability of dropping out. This might also be explained by the extreme stochasticity of the highly diverse DBLα, especially for rare sequences that are observed only once, rather than any fundamental shifts in the population structure.

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Reviewer #2 (Public Review):

In this manuscript, Tiedje and colleagues longitudinally track changes in parasite numbers across four time points as a way of assessing the effect of malaria control interventions in Ghana. Some of the study results have been reported previously, and in this publication, the authors focus on age-stratification of the results. Malaria prevalence was lower in all age groups after IRS. Follow-up with SMC, however, maintained lower parasite prevalence in the targeted age group but not the population as a whole. Additionally, they observe that diversity measures rebounds more slowly than prevalence measures. Overall, I found these results clear, convincing, and well-presented. They add to a growing literature that demonstrates the relevance of asymptomatic reservoirs.

There is growing interest in developing an expanded toolkit for genomic epidemiology in malaria, and detecting changes in transmission intensity is one major application. As the authors summarize, there is no one-size-fits-all approach, and the Bayesian MOIvar estimate developed here has the potential to complement currently used methods. I find its extension to a calculation of absolute parasite numbers appealing as this could serve as both a conceptually straightforward and biologically meaningful metric. However, I am not fully convinced the current implementation will be applied meaningfully across additional studies.

1. I find the term "census population size" problematic as the groups being analyzed (hosts grouped by age at a single time point) do not delineate distinct parasite populations. Separate parasite lineages are not moving through time within these host bins. Rather, there is a single parasite population that is stochastically divided across hosts at each time point. I find this distinction important for interpreting the results and remaining mindful that the 2,000 samples at each time point comprise a subsample of the true population. Instead of "census population size", I suggest simplifying it to "census count" or "parasite lineage count".

It would be fascinating to use the obtained results to model absolute parasite numbers at the whole population level (taking into account, for instance, the age structure of the population), and I do hope this group takes that on at some point even if it remains outside the scope of this paper. Such work could enable calculations of absolute---rather than relative---fitness and help us further understand parasite distributions across hosts.

2. I'm uncertain how to contextualize the diversity results without taking into account the total number of samples analyzed in each group. Because of this, I would like a further

explanation as to why the authors consider absolute parasite count more relevant than the combined MOI distribution itself (which would have sample count as a denominator). It seems to me that the "per host" component is needed to compare across age groups and time points---let alone different studies.

3. Thinking about the applicability of this approach to other studies, I would be interested in a larger treatment of how overlapping DBLa repertoires would impact MOIvar estimates. Is there a definable upper bound above which the method is unreliable? Alternatively, can repertoire overlap be incorporated into the MOI estimator?

Smaller comments:
- Figure 1 provides confidence intervals for the prevalence estimates, but these aren't carried through on the other plots (and Figure 5 has lost CIs for both metrics). The relationship between prevalence and diversity is one of the interesting points in this paper, and it would be helpful to have CIs for both metrics when they are directly compared.

- https://doi.org/10.7554/eLife.91411.1.sa1

Reviewer #3 (Public Review):

Summary:
The manuscript coins a term "the census population size" which they define from the diversity of malaria parasites observed in the human community. They use it to explore changes in parasite diversity in more than 2000 people in Ghana following different control interventions.

Strengths:
This is a good demonstration of how genetic information can be used to augment routinely recorded epidemiological and entomological data to understand the dynamics of malaria and how it is controlled. The genetic information does add to our understanding, though by how much is currently unclear (in this setting it says the same thing as age-stratified parasite prevalence), and its relevance moving forward will depend on the practicalities and cost of the data collection and analysis. Nevertheless, this is a great dataset with good analysis and a good attempt to understand more about what is going on in the parasite population.

Weaknesses:
Overall the manuscript is well-written and generally comprehensively explained. Some terms could be clarified to help the reader and I had some issues with a section of the methods and some of the more definitive statements given the evidence supporting them.

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Author Response

The following is the authors' response to the original reviews.

> *1. The authors are to be commended for a well written paper on the relevance Census population size as an endpoint to monitor the epidemiology and control of Plasmodium falciparum infections. The work capitalizes on longitudinal cohort of a population ~2,000 individuals in the high, seasonal transmission of P. falciparum in Ghana between 2012 and 2017. This work is of interest for epidemiologists and could impact on malaria control programs.*

We thank the reviewing editors for acknowledging that our paper is well written. We assess over ~8,000 samples from ~2,000 individuals through sequential interventions. The study is

unique in terms of characterisation of all ages and unprecedented in depth of sampling of all ages to define the asymptomatic reservoir of infection in a local community experiencing high, seasonal transmission in the Sahel.

> 1. *Specifically, the authors oversell their partial (i.e., DBL-alpha) var gene sequencing data. Defining "var gene repertoires" based on sequencing of a short, relatively conserved sequence (the DBLalpha domain) of a rather large gene is misleading.*

The reviewing editors' opinion that "Defining "var gene repertoires" based on sequencing of a short, relatively conserved sequence (the DBL-alpha domain) of a rather large gene is misleading" suggests that the reviewers either do not understand the application of the varcoding methodology to measure MOI and/or have not had the opportunity to read Tan et al. (Tan et al., 2023), which directly addresses the validity of counting DBLα tags as a proxy for numbers of var genes in order to calculate MOI. We also published a paper in January 2023 in PLOS Computational Biology by Labbe et al. (Labbé et al., 2023) using simulated genome data, which shows that in high transmission using DBLα is superior to other computational methods to measure MOI.

Here we point to a possible misunderstanding about our method. We do not ''define'' individual P. falciparum var repertoires per se. Instead, we estimate how many var repertoires there are in an isolate in the absence of understanding of what DBLα sequences belong to an individual genome. Importantly, measuring MOIvar is not based on assigning haplotypes but instead, it assumes a set number of non-upsA DBLα types per genome derived from control data accounting for PCR sampling errors to calculate MOI. This adjustment is achieved using the novel Bayesian approach presented in the paper. The method relies on the extensive diversity of the var gene family together with the very low percentage of var genes shared between parasites in high transmission (e.g., Figure 2) to facilitate measuring MOI by counting the number of non-upsA DBLα types.

The analyses in Tan et al. (2023) provide the key data to support our methodology to count DBLα tags as a "proxy" for the number of var genes and hence estimate the number of var repertoires in an infection. Tan et al. (2023) analysed full length exon 1 sequences of var genes from Sanger sequencing and showed a predominantly 1:1 relationship between DBLα encoding sequences and var genes, especially in high transmission (including our study area). This 1:1 relationship was strongest for nonupsA DBLα tags, which are the focus of the MOI estimation.

Based on the published evidence from Tan et al. (2023) and Labbe et al. (2023), we strongly disagree with the comment that we are "Selling this approach as a "new method" for estimating the "census population size" is definitively overselling what DBL-alpha sequencing can offer". What is new is the metric census population size which we state is agnostic of how you measure MOI.

> 1. *The use of census population size quite interesting to evaluate the impact of interventions (IRS, SMC).*

We agree that census population size is interesting as a metric to evaluate the impact of interventions. Figure 3 summarises age-specific MOI data and shows the variability in distributions of MOI by age and intervention. Census population size in Figure 4A provides a summary metric to capture the changes in distribution indicated by the median MOIs. Our recent paper by Labbe et al. (2023) highlights the importance of understanding these MOI distributions rather than the medians, generally reported in malaria epidemiology research.

> *1. The concept of census population size should be agnostic of how the MOI is measured, so maybe one way to address the limitation of using DBL-alpha would be to show that measurement of MOIs using another method leads to similar results.*

The paper is about introducing the metric of census population size and is not a comparison of methods to measure MOI.

We justify our choice of method in the introduction quoting our published control data in Tan et al. (2023) and Labbe et al. (2023), while stating the concept is agnostic of how you measure MOI in the discussion.

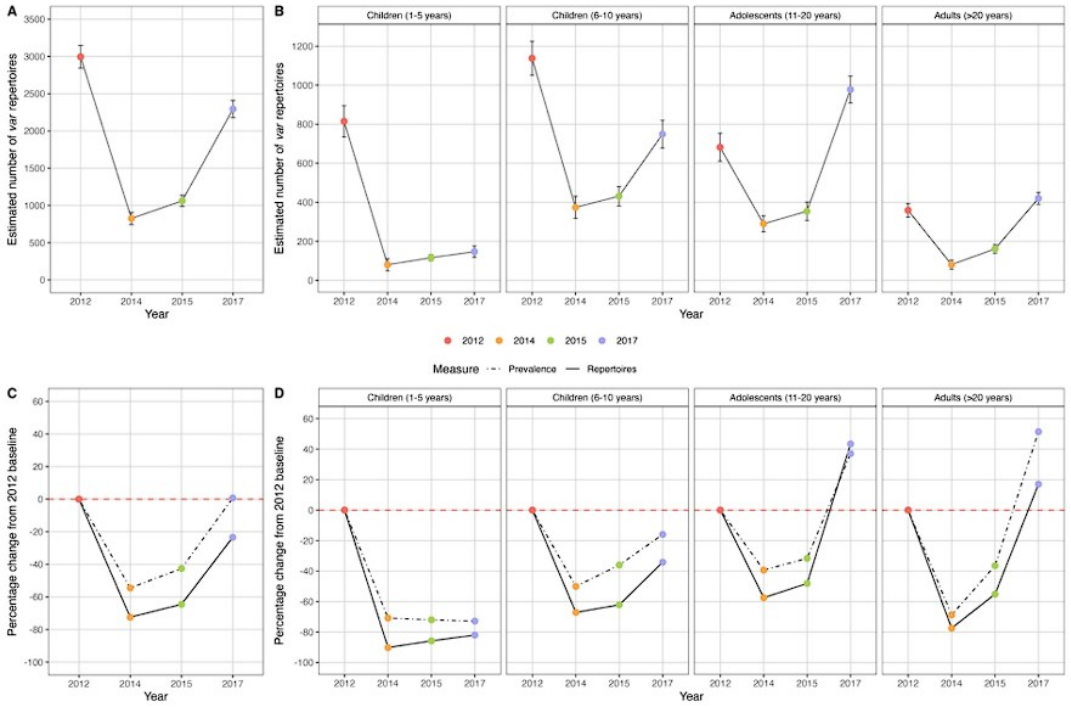
Given published data (Labbé et al., 2023; Tan et al., 2023) to justify measurement of MOI using DBLα sequences in high transmission, there is no need to repeat 2,752 samples with another method to explore census population size in the context of malaria interventions. We also point out the limitations with SNPs and single copy antigen genes to detect MOI, especially in high transmission, in the introduction (Lines 79-93) and Labbe et al. (2023).

> *1. Moreover, it is not fully convincing that the census population size is more informative than parasite prevalence alone, as the graphs in figure 1B (prevalence) and 4A (var repertoires) look rather similar. Census population size is complementary to prevalence where the former gives a measure of the parasite population size, and the latter describes the proportion of infected hosts. Overall, we see greater percentage change in census parasite population size vs. parasite prevalence as a consequence of the significant changes in distribution of MOI by age as related to the interventions (Figure 3). Figure 4B compares census population size (y-axis) to prevalence (x-axis).*

Attached find an updated version of Figure 4 (see below), which may make the comparison clearer by showing the percentage change in each parameter at the four time points (i.e., 2012, 2014, 2015, and 2017).

Author response image 1.

Estimated number and relative change in the number of P. falciparum var repertoires from 2012 (pre-IRS, red) to 2014 (during IRS, orange), 3 2015 (post-IRS, green), and 2017 (SMC, purple). The estimated number of var repertoires for those isolates with DBLα sequencing data for the (A) study population 4 in 2012 (N = 2,994), 2014 (N = 825), 2015 (N = 1062), and 2017 (N = 2,294) and (B) for all age groups (years). The percentage change in prevalence (black dotted 5 line) and the estimated number of var repertoires (black solid line) in 2014, 2015, and 2017 compared to the 2012 baseline survey (red dashed horizontal line at 0% 6 change) for the (C) study population and (D) for all age groups (years) based on those microscopic P. falciparum isolates with DBLα sequencing data (Table 7 supplement 2). Error bars in (A) and (B) represent the upper and lower limits of the 95% confidence interval (CI) for the estimated number of var repertoires.



Upon reflection, we could also explain the relationship of census population size to MOI distributions in more detail in the text.

REFERENCES

Ghansah A, Tiedje KE, Argyropoulos DC, Onwona CO, Deed SL, Labbé F, Oduro AR, Koram KA, Pascual M, Day KP. 2023. Comparison of molecular surveillance methods to assess changes in the population genetics of Plasmodium falciparum in high transmission. Frontiers in Parasitology 2:1067966. doi:doi: 10.3389/fpara.2023.1067966

Labbé F, He Q, Zhan Q, Tiedje KE, Argyropoulos DC, Tan MH, Ghansah A, Day KP, Pascual M. 2023. Neutral vs . non-neutral genetic footprints of Plasmodium falciparum multiclonal infections. PLoS Comput Biol 19:e1010816. doi:doi.org/10.1101/2022.06.27.497801

Tan MH, Shim H, Chan Y, Day KP. 2023. Unravelling chaos for malaria surveillance : Relationship between DBLα types and var genes in Plasmodium falciparum. Frontiers in Parasitology 1. doi:https://doi.org/10.3389/fpara.2022.1006341