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

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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 **convincing** and the work shows how genetic studies could be used to monitor changes in disease transmission.

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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.

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 up to 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 census 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 (Ghansah et al., 2023; 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 insecticidal 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**). LLINs (i.e., PermaNet 2.0, Olyset, or DawaPlus 2.0) were mass distributed in Bongo District by the National Malaria Elimination Programme (NMEP)/Ghana Health Service (GHS) between 2010-2012 and again in 2016 following the discontinuation of IRS (Gogue et al., 2020; Tiedje et al., 2022; US Agency for International Development (USAID) Global Health Supply Chain Program, 2020). In addition, to maintain high coverage of LLINs between these campaigns continuous distribution was undertaken using routine services (i.e., antenatal clinics, school distributions, immunization visits, etc.) (Tiedje et al., 2022). Over the course of this study self-reported LLIN usage the previous night remained high across all age groups, from 89.1% in 2012 (pre-IRS), 83.5% in 2014 (during IRS), 90.6% in 2015 (post-IRS), to 96.8% in 2017 (SMC). 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.,

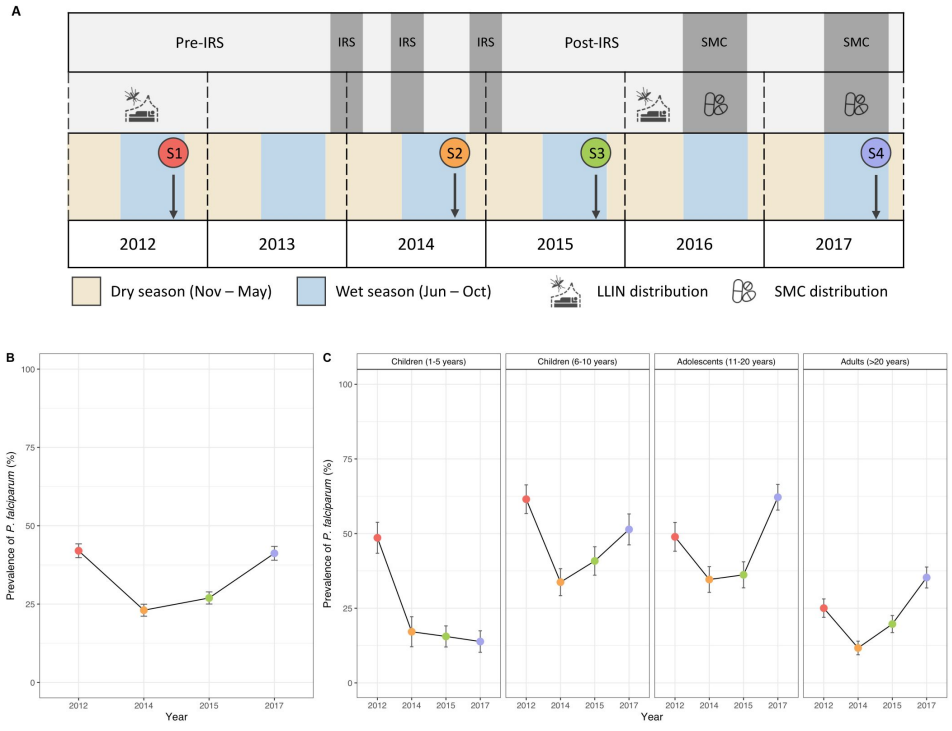

Figure 1.

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 (Tiedje et al., 2022). 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) calculated using the Wald Interval.

2022). Following the IRS, SMC was rolled out in the Upper East Region by the NMEP)/(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 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 sequence region within the *var* genes encoding the DBLα domains of PfEMP1 were amplified using a single-step PCR, pooled, and sequenced on an Illumina platform using the MiSeq 2×300bp paired-end protocol (Supplemental Methods, Figure supplement 1). The raw sequence data was then processed using our previously published customized bioinformatic pipelines (Supplemental Methods, Figure supplement 2). For additional information on the use of these bioinformatic pipelines a detailed tutorial is available (*https://github.com/UniMelb-Day-Lab/tutorialDBLalpha*).

DBLα sequencing data was obtained from 2,397 *P. falciparum* isolates (93.2%) (Table supplement 2). This genotyping success was acceptable given that we were working with low-density asymptomatic infections (Table supplement 1). 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 289,049 DBLα sequences and 53,238 unique DBLα types being identified in the study population (Table supplement 2). 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 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

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 estimate MOI (Ruybal-Pesántez et al., 2022; Tiedje et al., 2022). This approach, however, neglects the measurement error in this size introduced by targeted PCR and amplicon sequencing of *var* genes in an isolate.

Bayesian estimation of MOI_{var} and associated census 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 (Labbé et al., 2023)). We refer to it as P(s | MOI = 1) where *s* here denotes repertoire size. More generally when MOI ≥ 1, *s* denotes the number of non-upsA DBLα types sequenced or isolate size. 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:

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

where *L* and *U* are the lower and upper limit for the repertoire size, 10 and 45 respectively from the empirical repertoire size distribution (Figure supplement 3 (Labbé et al., 2023)).

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:

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

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:

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

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 supplements 3 and 4). Additionally, the difference between the mean MOI values at the population level obtained from the two approaches is small (Table supplements 3 and 4). Given this similarity, we present the results based on pooling the maximum *a posteriori* MOI estimates for each sampled individual in the main text and include the results based on mixture distribution in the Supplemental Information. Note that we focused on individuals who had confirmed microscopic asymptomatic P. *falciparum* infections 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 therefore focused on a negative binomial distribution and investigated changing its parameters to generate priors with different means spanning a wide range of MOI values (mean MOI within [~1.5, ~6.7]), including those seen in high-transmission endemic areas. A uniform prior and a zero-truncated negative binomial distribution with parameters within the range typical of high-transmission endemic regions (higher mean MOI, for example, ~4.3 versus ~6.7, with tails for higher MOI values in the range of 10-20) produce similar MOI estimates at the population level (Table supplements 5 and 6). However, when setting the parameter range of the zero-truncated negative binomial to be representative of those in low-transmission endemic regions where the empirical MOI distribution centres around monoclonal infections with the majority of MOIs = 1 or 2 (mean MOI ≈ 1.5, no tail at higher MOI values), the final population-level MOI distribution does deviate more from that based on the aforementioned prior and parameter choices (Table supplement 6). The final individual- and population-level MOI estimates are not sensitive to the specifics of the prior MOI distribution as long as this distribution captures the tail for higher MOI values with above-zero probability. The obtained Kolmogorov-Smirnov Test distance statistics and their corresponding *p-values*, the Pearson correlation tests and their corresponding *p-values*, as well as the difference in mean MOI values, for the comparison of the MOI estimates obtained with the different priors are included in Table supplements 5 and 6. Given these comparisons, we provide in our analyses the estimated population MOI distribution using a uniform prior.

Statistical analysis

We used the R v4.3.1 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 or calculated values with the 95% confidence interval (95% CIs) or ± 2 standard deviations (± 2SD). 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 = 2,218) and non-upsA (N = 33,159) DBLα types that were seen at baseline in 2012 (i.e., those DBLα types observed prior to the IRS intervention) (Table supplement 2).

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, respectively (**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 (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 the 2012 (pre-IRS) (**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**). Somewhat unexpectedly, the change was in the direction of reduced similarity (i.e., less overlap) 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 4). 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 $PTS_{non\text{-}upsA}$ ζ 0.5 (i.e., siblings or recent recombinants) (**Figure 2**).

The raw data of non-upsA DBLα isolate repertoire sizes (Figure supplement 5) were used to estimate MOI_{var} as adjusted using the Bayesian approach based on pooling the maximum *a posteriori* MOI estimates (**Figure 3**) and the mixture distribution (Figure supplement 6). 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) predominantly targeted by SMC (**Figure 3**). While the prevalence of infection in 2017 remained low for the younger children (1-5 years), 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**).

Census 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} (**Figure 4**, Table supplement 7). In 2014 during IRS, this number decreased by 72.5% relative to the 2012 baseline survey (pre-IRS) (**Figure 4CE**), whereas prevalence decreased by 54.5% (**Figure 4CE**). Although census population size increased slightly in 2015 relative to 2014 (**Figure 4A**), there were still 64.6% fewer *var* repertoires in the population compared to 2012 (**Figure 4CE**) in comparison to a 42.6% decrease in prevalence (**Figure 4CE**). Importantly this loss of *var* repertoires in 2014 and 2015 following the IRS intervention was seen for all age groups (**Figure 4B**), with the greatest overall reductions (> 85%) being observed for the younger children (1-5 years) (**Figure 4DF**). However, in 2017, the number of diverse *var* repertoires in the population rebounded, more than doubling between 2015 and 2017 (**Figure 4CE**). This increase in the number of *var* repertoires was seen for all age groups in 2017, except for the younger children (1-5 years) where those up to 59 months were targeted by SMC (**Figure 4BD**). 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 4F**). 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 census population size changed considerably during the sequential IRS and SMC interventions, we investigated how the removal or loss of *P. falciparum var* repertoires and subsequent rebound in 2017 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 number of unique DBLα types (upsA = 2,218; non-upsA = 33,159) (**Figure 5**, Table supplement 2) and limited overlap of *var* repertoires (i.e., median $PTS_{non\text{-}upsA}$ ≤ 0.020) seen in a relatively small study population of 685 microscopically positive individuals (**Figure 2**). In 2014, as *P. falciparum* prevalence and census population size declined (**Figure 4**) so too did the

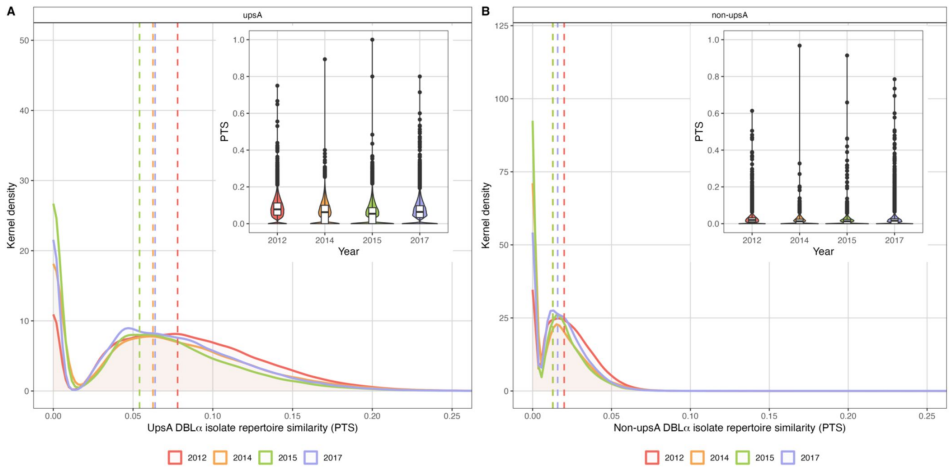

Figure 2.

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 for those isolates 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).

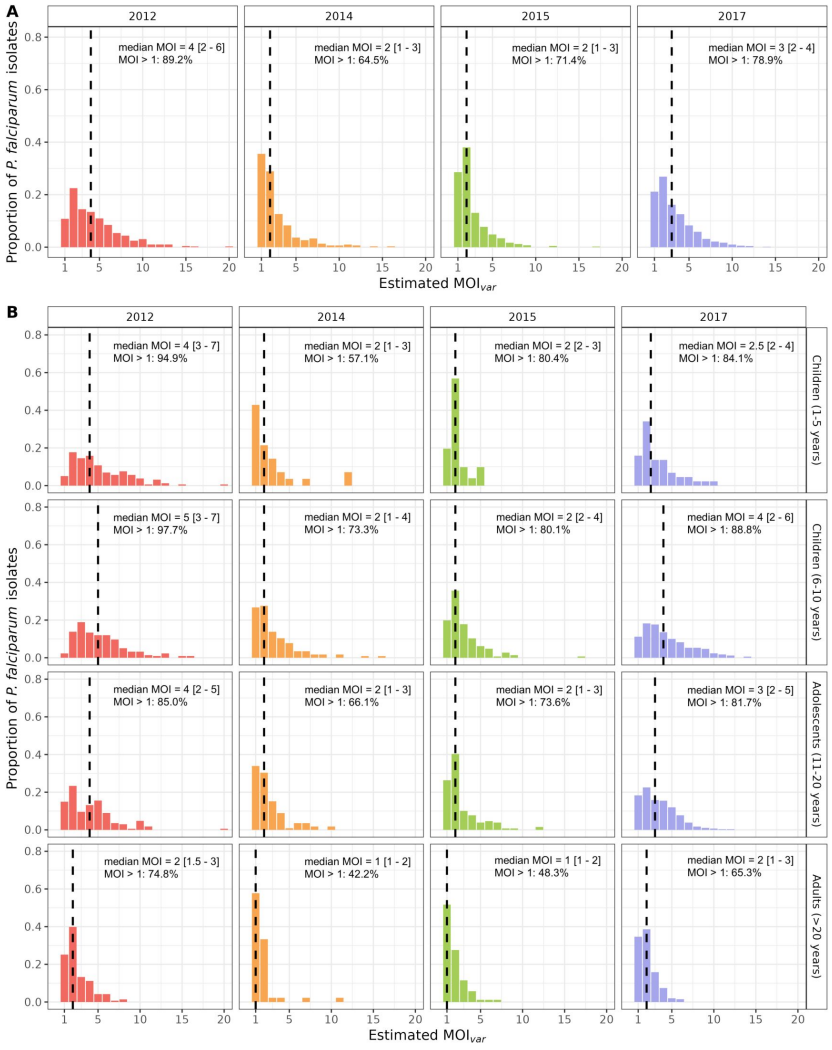

Figure 3.

MOI_{var} distributions in 2012 (pre-IRS, red), 2014 (during IRS, orange), 2015 (post-IRS, green), and 2017 (SMC, purple) based on pooling the maximum *a posteriori* MOI estimates.

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 supplements 2 and 7). 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).

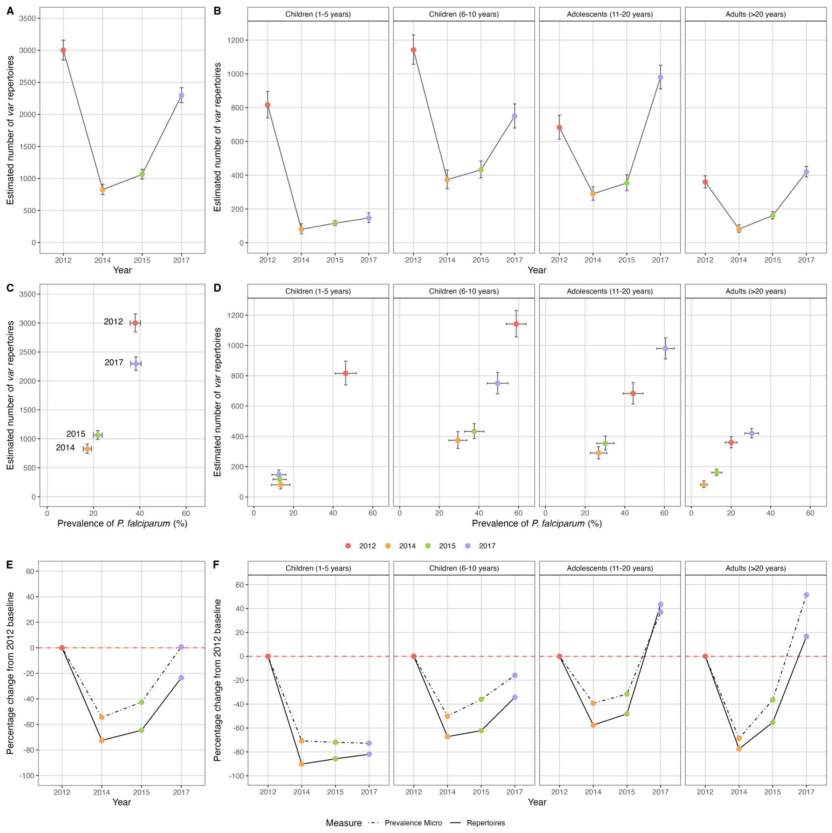

Figure 4.

Estimated number and relative change in the 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 (i.e., census population size) for those isolates with DBLα sequencing data (Table supplements 2 and 7) in the **(A)** study population and **(B)** for all age groups (years). The estimated number of *var* repertoires vs. *P. falciparum* prevalence for **(C)** study population and **(D)** for all age groups (years) (Table supplement 7). The percentage change in *P. falciparum* prevalence (black dotted 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% change) for the **(E)** study population and **(F)** for all age groups (years). Error bars in **(A-D)** represent the upper and lower limits of the 95% confidence intervals (95% CIs). The 95% CIs for the number of *var* repertoires (i.e., census population size) were calculated based on a bootstrap approach. We resampled 10,000 replicates from the original population-level distribution with replacement. Each resampled replicate has the same size as the original sample. We then derive the 95% CI based on the distribution of the resampled replicates. The 95% CIs for *P. falciparum* prevalence (%) were calculated using the Wald Interval.

number of DBLα types, resulting in a 32.2% 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 46.0% 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 census 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 7). 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 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 in 2012 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 were observed at multiple study time points (i.e., 2012, 2014, 2015, and 2017), albeit in different isolate repertoires, 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 (**Figure 4**) 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 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,

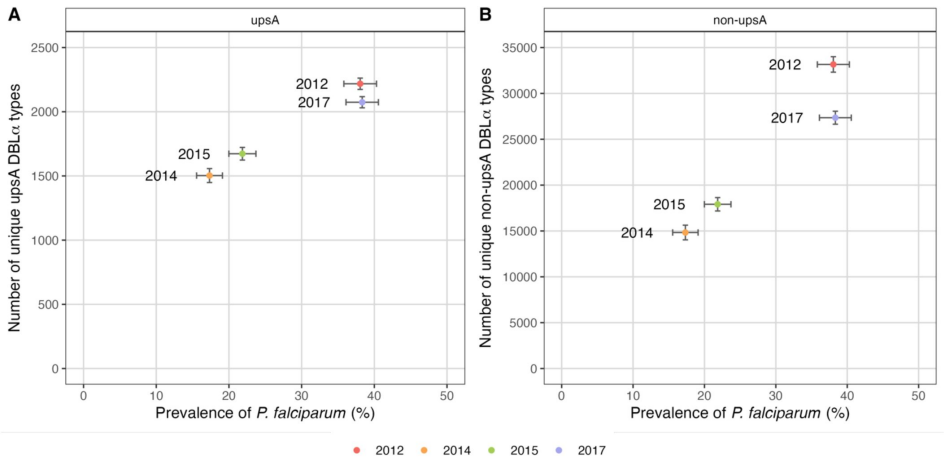

Figure 5.

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). Error bars represent the upper and lower limits of the 95% confidence intervals (95% CIs) for the *P. falciparum* prevalence (%; x-axis) and ± 2 standard deviations (± 2SD) for the number of unique upsA and non-upsA DBLα types (y-axis). The 95% CIs for *P. falciparum* prevalence (%) were calculated using the Wald Interval. The ± 2SD for the number of unique upsA and non-upsA DBLα types were calculated based on a bootstrap approach. We resampled 10,000 replicates from the original population-level distribution with replacement. Each resampled replicate has the same size as the original sample. We then derive the standard deviation (SD) based on the distribution of the resampled replicates.

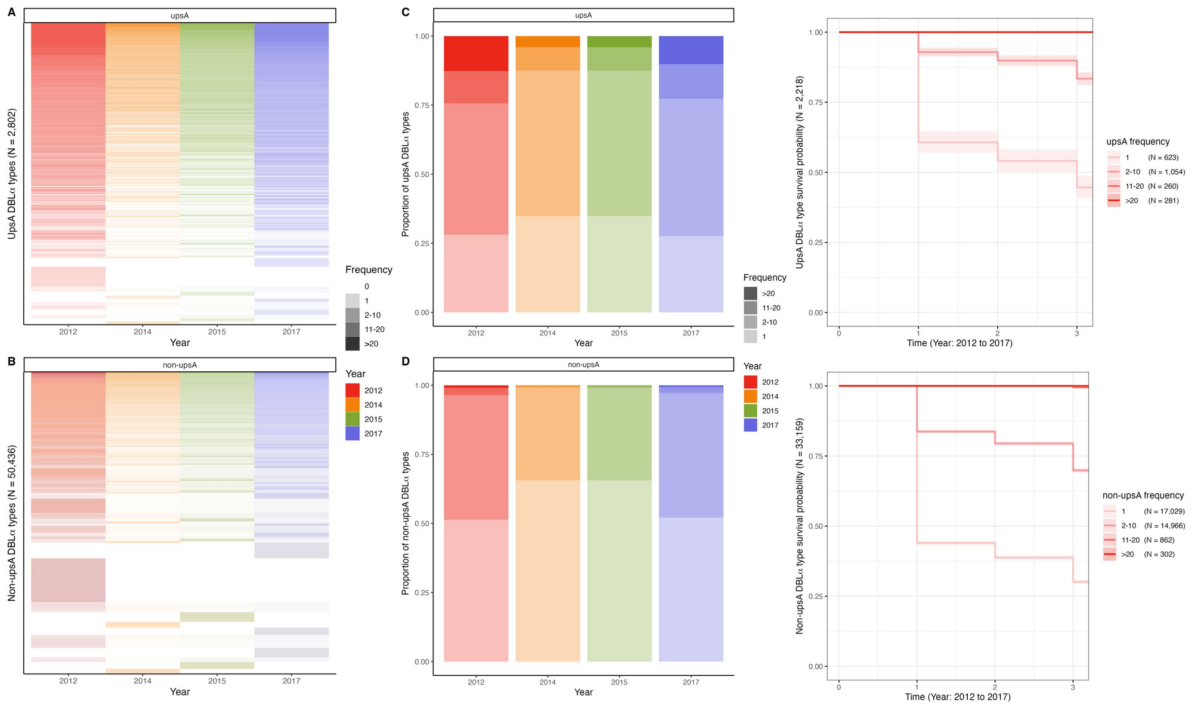

Figure 6.

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 2,802 upsA DBLα types and the 50,436 non-upsA DBLα types (Table supplement 2). 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 7). 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 upper and lower limits of the 95% confidence intervals (95% CIs) with 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 = 2,218) and non-upsA (N = 33,159) DBLα types that were seen at baseline in 2012 (pre-IRS) as indicated in red (Table supplement 2). 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 **(E)** upsA and **(F)** non-upsA DBLα types overlap in the figure.

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*.

Census population size is a total enumeration or count of infections in a given population sample and over a given time period in an ecological sense, distinct from the formal effective population size (N_e) used in population genetics (Charlesworth, 2009). Given the low overlap between *var* repertoires of parasites observed in monoclonal infections (MOI = 1), the census population size calculated in Bongo, Ghana translates to a diversity of strains or repertoires. The distinction of census population size in terms of infection counts and effective population size from population genetics has been made before for pathogens, including the seasonal influenza virus and the measles virus (Bedford et al., 2011); it is also a distinction made in the ecological literature for non-pathogen populations (Palstra and Fraser, 2012). The census population size of a given population sample depends of course on sample size and was used here for comparisons across time of samples of the same depth (i.e., ~2,000 individuals). There is, however, a simple map between census population size and mean MOI, as one can simply divide or multiply by the sample size, respectively, to convert between the two quantities (Table supplement 7). Therefore, one can extrapolate from the census population size of a given population sample to that of the whole population of local hosts to compare across studies that differ in sampling depth. What is needed for this extrapolation is a stable mean MOI relative to the sample size or sampling depth, which is indeed the case in this study (Figure supplement 8) and can be easily checked in other studies. Given the typical duration of infection, we expect our population size to be representative of a per-generation measure.

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 (1-5 years). More than two years after cessation of IRS, rebound in 2017 was rapid in all age groups, except for the younger children (1-5 years) where those up to 59 months were targeted by SMC. Population sizes in adolescents (11-20 years) and adults (> 20 years) 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 the potential underestimation of the number of DBLα types of related parasites generated by a cross, when using *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 low-to moderate-density, chronic, asymptomatic infections (see Table supplement 1) under strong immune selection in semi-immune hosts whom we have shown select against parasites with high PTS scores consistent with relatedness by decent (Day et al., 2017; 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 infections related by descent must be weighed up against the issues of under-sampling as described above.

The Bayesian approach of the *var*coding method relies on the low or limiting similarity of *var* repertoires infecting individual human hosts. As such, it would appear to break down as the *var* repertoire overlap moves away from extremely low, and therefore, for locations with lower transmission intensity. Interestingly, this is not the case in the numerical simulations of Labbé et al. (Labbé et al., 2023), for a gradient of three transmission intensities, from high to low, with the original *var*coding method performing well across the gradient. This robustness of the method may arise from a nonlinear and fast transition from low to high overlap that is accompanied by MOI changing rapidly from primarily multiclonal (MOI > 1) to monoclonal (MOI = 1) infections. This matter needs to be investigated further in the future, including ways to extend the Bayesian approach to explicitly include the distribution of *var* repertoire overlap.

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.

Acknowledgements

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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, the Supporting Information, or upon reasonable request for research purposes to the corresponding author, Prof. Karen Day (karen.day@unimelb.edu.au). 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), the Bongo District and Upper East Regional Health Directorates, as well as Ghana National Malaria Elimination Programme. Before this research was undertaken, informed consent was sought and obtained from the key stakeholders, traditional leadership, 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 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 in Bongo District, as well as for the National Malaria Elimination Programme in Ghana.

Additional information

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.

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Editors

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 rebound more slowly than prevalence measures. This adds to a growing literature that demonstrates the relevance of asymptomatic reservoirs.

Strengths:

Overall, I found these results clear, convincing, and well-presented. 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, particularly in regions with high diversity/transmission. 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.

Weaknesses:

While I understand the conceptual importance of distinguishing among parasite prevalence, mean MOI, and absolute parasite number, I am not fully convinced by this manuscript's implementation of "census population size". The authors reference the population genetic literature, but within the context of that field, "census population size" refers to the total population size (which, if not formally counted, can be extrapolated) as opposed to "effective population" size, which accounts for a multitude of demographic factors. There is often interesting biology to be gleaned from the magnitude of difference between N and Ne. In this manuscript, however, "census population size" is used to describe the number of distinct

parasites detected within a sample, not a population. As a result, the counts do not have an immediate population genetic interpretation and cannot be directly compared to Ne. This doesn't negate their usefulness but does complicate the use of a standard population genetic term. In contrast, I think that sample parasite count will be most useful in an epidemiological context, where the total number of sampled parasites can be contrasted with other metrics to help us better understand how parasites are divided across hosts, space and time. However, for this use, I find it problematic that the metric does not appear to correct for variations in participant number. For instance, in this study, participant numbers especially varied across time for 1-5 year-olds (N=356, 216, 405, and 354 in 2012, 2014, 2015, and 2017 respectively). This sample size variability is accounted for with other metrics like mean MOI. In sum, while the manuscript opens up an interesting discussion, I'm left with an incomplete understanding of the robustness and interpretability of the new proposed metric.

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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:

None

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Author response:

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

> ***Public Reviews:***
>
> ***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.*

We thank the reviewer for appreciating the extension of _var_coding we present here. We believe the comment on maximum *a posteriori* (MAP) refers to the way we obtained population-level MOI from the individual MOI estimates. We would like to note that reliance on MAP was only one of two approaches we described, although we then presented only MAP. Having calculated both, we did not observe major differences between the two, for this data set. Nonetheless, we revised the manuscript to include the result based on the mixture distribution which considers all the individual MOI distributions in the Figure supplement 6.

> *(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.*

We consider the quantity a census in that it is a total enumeration or count of infections in a given population sample and over a given time period. In this sense, it gives us a tangible notion of the size of the parasite population, in an ecological sense, distinct from the formal effective population size used in population genetics. Given the low overlap between *var* repertoires of parasites (as observed in monoclonal infections), the population size we have calculated translates to a diversity of strains or repertoires. But our focus here is in a measure of population size itself. The distinction between population size in terms of infection counts and effective population size from population genetics has been made before for pathogens (see for example Bedford et al. for the seasonal influenza virus and for the measles virus (Bedford et al., 2011)), and it is also clear in the ecological literature for non-pathogen populations (Palstra and Fraser, 2012).

We completely agree with the dependence of our quantity on sample size. We used it for comparisons across time of samples of the same depth, to describe the large population size characteristic of high transmission which persists across the IRS intervention. Of course, one would like to be able to use this quantity across studies that differ in sampling depth and the reviewer makes an insightful and useful suggestion. It is true that we can use mean MOI, and indeed there is a simple map between our population size and mean MOI (as we just need to divide or multiply by sample size, respectively) (Table supplement 7). We can go further, as with mean MOI we can presumably extrapolate to the full sample size of the host population,

or to the population size of another sample in another location. What is needed for this purpose is a stable mean MOI relative to sample size. We can show that indeed in our study mean MOI is stable in that way, by subsampling to different depths our original sample (Figure supplement 8 in the revised manuscript). We now include in the revision discussion of this point, which allows an extrapolation of the census population size to the whole population of hosts in the local area.

We have also clarified the time denominator: Given the typical duration of infection, we expect our population size to be representative of a per-generation measure_._

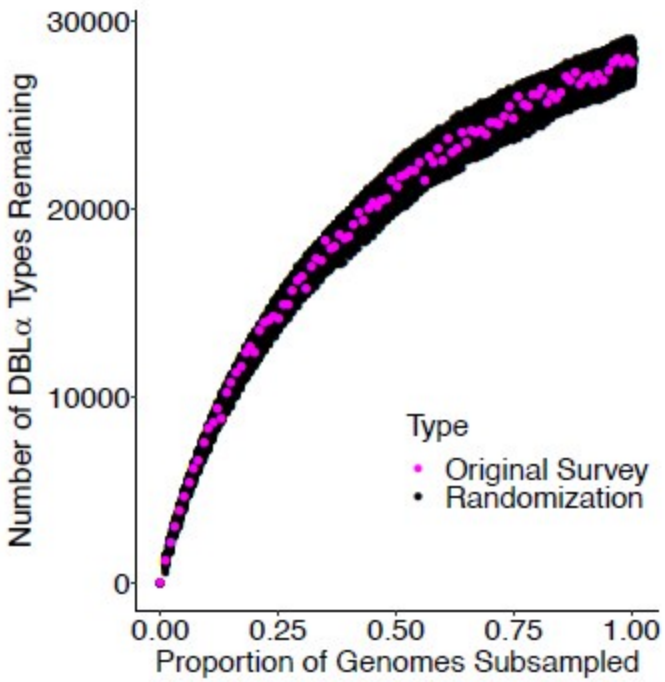
> *(3) The extraordinary diversity of DBLα presents challenges to analyzing the data. The authors explore the variability in repertoire richness and frequency over the course of the study, noting that richness rapidly declined following IRS and later rebounded, while the frequency of rare types increased, 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.*

We thank the reviewer raising this question which led us to consider whether the change in the number of DBLα types over the course of the study (and intervention) follows from simply sampling fewer *P. falciparum* cases. We interpreted this question as basically meaning that one can predict the former from the latter in a simple way, and that therefore, tracking the changes in DBLα type diversity would be unnecessary. A simple map would be for example a linear relationship (a given proportion of DBLα types lost given genomes lost), and even more trivially, a linear loss with a slope of one (same proportion). Note, however, that for such expectations, one needs to rely on some knowledge of strain structure and gene composition. In particular, we would need to assume a complete lack of overlap and no gene repeats in a given genome. We have previously shown that immune selection leads to selection for minimum overlap and distinct genes in repertoires at high transmission (see for example (He et al., 2018)) for theoretical and empirical evidence of both patterns). Also, since the size of the gene pool is very large, even random repertoires would lead to limited overlap (even though the empirical overlap is even smaller than that expected at random (Day et al., 2017)). Despite these conservators, we cannot a priori assume a pattern of complete non-overlap and distinct genes, and ignore plausible complexities introduced by the gene frequency distribution.

To examine this insightful question, we simulated the loss of a given proportion of genomes from baseline in 2012 and examined the resulting loss of DBLα types. We specifically cumulated the loss of infections in individuals until it reached a given proportion (we can do this on the basis of the estimated individual MOI values). We repeated this procedure 500 times for each proportion, as the random selection of individual infection to be removed, introduces some variation. Figure 2 below shows that the relationship is nonlinear, and that one quantity is not a simple proportion of the other. For example, the loss of half the genomes does not result in the loss of half the DBLα types.

Author response image 1.

Non-linear relationship between the loss of DBLα types and the loss of a given proportion of genomes. The graph shows that the removal of parasite genomes from the population through intervention does not lead to the loss of the same proportion of DBLα types, as the initial removal of genomes involves the loss of rare DBLα types mostly whereas common DBLα types persist until a high proportion of genomes are lost. The survey data (pink dots) used for this subsampling analysis was sampled at the end of wet/high transmission season in Oct 2012 from Bongo District from northern Ghana. We used the Bayesian formulation of the _var_coding method proposed in this work to calculate the multiplicity of infection of each isolate to further obtain the total number of genomes. The randomized surveys (black dots) were obtained based on “curveball algorithm” (Strona et al., 2014) which keep isolate lengths and type frequency distribution.



We also investigated whether the resulting pattern changed significantly if we randomized the composition of the isolates. We performed such randomization with the “curveball algorithm” (Strona et al., 2014). This algorithm randomizes the presence-absence matrix with rows corresponding to the isolates and columns, to the different DBLα types; importantly, it preserves the DBLα type frequency and the length of isolates. We generated 500 randomizations and repeated the simulated loss of genomes as above. The data presented in Figure 2 above show that the pattern is similar to that obtained for the empirical data presented in this study in Ghana. We interpret this to mean that the number of genes is so large, that the reduced overlap relative to random due to immune selection (see (Day et al., 2017)) does not play a key role in this specific pattern.

> ***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.*

Lineages moving exclusively through a given type of host or “patch” are not a necessary requirement for enumerating the size of the total infections in such subset. It is true that what we have is a single parasite population, but we are enumerating for the season the respective size in host classes (children and adults). This is akin to enumerating subsets of a population in ecological settings where one has multiple habitat patches, with individuals able to move across patches.

Remaining mindful that the count is relative to sample size is an important point. Please see our response to comment (2) of reviewer 1, also for the choice of terminology. We prefer not to adopt “census count” as a census in our mind is a count, and we are not clear on the concept of lineage for these highly recombinant parasites. Also, census population size has been adopted already in the literature for both pathogens and non-pathogens, to make a distinction with the notion of effective population size in population genetics (see our response to reviewer 1) and is consistent with our usage as outlined in the introduction.

Thank you for the comment on an absolute number which would extrapolate to the whole host population. Please see again our response to comment (2) of reviewer 1, on how we can use mean MOI for this purpose once the sampling is sufficient for this quantity to become constant/stable with sampling effort.

> *(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.*

Again, thank you for the insightful comment. We provide this number as a separate quantity and not a distribution, although it is clearly related to the mean MOI of such distribution. It gives a tangible sense for the actual infection count (different from prevalence) from the perspective of the parasite population in the ecological sense. The “per host” notion which enables an extrapolation to any host population size for the purpose of a complete count, or for comparison with another study site, has been discussed in the above responses for reviewer 1 and now in the revision of the discussion.

> *(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?*

This is a very good point and one we now discuss further in our revision. There is no predefined upper bound one can present a priori. Intuitively, the approach to estimate MOI would appear to breakdown as overlap moves away from extremely low values, and therefore for locations with low transmission intensity. Interestingly, we have observed that this is not the case in our paper by Labbe et al. (Labbé et al., 2023) where we used model simulations in a gradient of three transmission intensities, from high to low values. The original _var_coding method performed well across the gradient. This robustness may arise from a nonlinear and fast transition from low to high overlap that is accompanied by MOI changing rapidly from primarily multiclonal (MOI > 1) to monoclonal (MOI = 1). This matter clearly needs to be investigated further, including ways to extend the estimation to explicitly include the distribution of overlap.

> *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.*

Based on the reviewer’s advice we have revised both Figure 4 and Figure 5, to include the missing uncertainty intervals. The specific approach for each quantity is described in the corresponding caption.

> ***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.*

Census population size is complementary to parasite prevalence where the former gives a measure of the "parasite population size", and the latter describes the "proportion of infected hosts". The reason we see similar trends for the "*genetic information*" (i.e., census population size) and "*age-specific parasite prevalence*" is because we identify all samples for _var_coding based on the microscopy (i.e., all microscopy positive *P. falciparum* isolates). But what is more relevant here is the relative percentage change in parasite prevalence and census population size following the IRS intervention. To make this point clearer in the revised manuscript we have updated Figure 4 and included additional panels plotting this percentage change from the 2012 baseline, for both census population size and prevalence (Figure 4EF). Overall, we see a greater percentage change in 2014 (and 2015), relative to the 2012 baseline, for census parasite population size vs. parasite prevalence (Figure 4EF) as a consequence of the significant changes in distributions of MOI following the IRS intervention (Figure 3). As discussed in the Results following the deployment of IRS in 2014 census population size decreased by 72.5% relative to the 2012 baseline survey (pre-IRS) whereas parasite prevalence only decreased by 54.5%.

With respect to the reviewer's comment on "practicalities and cost", _var_coding has been used to successfully amplify *P. falciparum* DNA collected as DBS that have been stored for more than 5-years from both clinical and lower density asymptomatic infection, without the additional step and added cost of sWGA ($8 to $32 USD per isolates, for costing estimates see (LaVerriere et al., 2022; Tessema et al., 2020)), which is currently required by other molecular surveillance methods (Jacob et al., 2021; LaVerriere et al., 2022; Oyola et al., 2016). _Var_coding involves a single PCR per isolate using degenerate primers, where a large number of isolates can be multiplexed into a single pool for amplicon sequencing. Thus, the overall costs for incorporating molecular surveillance with _var_coding are mainly driven by the number of PCRs/clean-ups, the number samples indexed per sequencing run, and the NGS technology used (discussed in more detail in our publication Ghansah et al. (Ghansah et al., 2023)). Previous work has shown that _var_coding can be use both locally and globally for molecular surveillance, without the need to be customized or updated, thus it can be fairly easily deployed in malaria endemic regions (Chen et al., 2011; Day et al., 2017; Rougeron et al., 2017; Ruybal-Pesántez et al., 2022, 2021; Tonkin-Hill et al., 2021).

> *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.*

Thank you for the overall positive assessment. On addressing the "issues with a section of the methods" and "some of the more definitive statements given the evidence supporting them", it is impossible to do so however, without an explicit indication of which methods and statements the reviewer is referring to. Hopefully, the answers to the detailed comments and questions of reviewers 1 and 2 address any methodological concerns (i.e., in the Materials and Methods and Results). To the issue of "*definitive statements*", etc. we are unable to respond without further information.

> ***Recommendations For The Authors:***

> ***Reviewer #1 (Recommendations For The Authors):***
>
> *Line 273: there is a reference to a figure which supports the empirical distribution of repertoire given MOI = 1, but the figure does not appear to exist.*

We now included the correct figure for the repertoire size distribution as Figure supplement 3 (previously published in Labbé et al (Labbé et al., 2023)). This figure was accidently forgotten when the manuscript was submitted for review, we thank the reviewer for bringing this to our attention.

> *Line 299: while this likely makes little difference, an insignificant result from a Kolmogorov-Smirnov test doesn't tell you if the distributions are the same, it only means there is not enough evidence to determine they are different (i.e. fail to reject the null). Also, what does the "mean MOI difference" column in supplementary table 3 mean?*

The mean MOI difference is the difference in the mean value between the pairwise comparison of the true population-level MOI distribution, that of the population-level MOI estimates from either pooling the maximum *a posteriori* (MAP) estimates per individual host or the mixture distribution, or that of the population-level MOI estimates from different prior choices. This is now clarified as requested in the Table supplements 3 - 6.

> *Figure 4: how are the confidence intervals for the estimated number of var repertoires calculated? Also should include horizontal error bars for prevalence measures.*

The confidence intervals were calculated based on a bootstrap approach. We re-sampled 10,000 replicates from the original population-level MOI distribution with replacement. Each resampled replicate is the same size as the original sample. We then derive the 95% CI based on the distribution of the mean MOI of those resampled replicates. This is now clarified as requested in the Figure 4 caption (as well as Table supplement 7 footnotes). In addition, we have also updated Figure 4AB and have included the 95% CI for all measures for clarity.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *- I would like to see a plot like Supplemental Figure 8 for the upsA DBLa repertoire size.*

The upsA repertoire size for each survey and by age group has now been provided as requested in Figure supplement 5AB.

> *- Supplemental Table 2 is cut off in the pdf.*

We have now resolved this issue so that the Table supplement 2 is no longer cut off.

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *The manuscript terms the phrase "census population size". To me, the census is all about the number of individuals, not necessarily their diversity. I appreciate that there is no simple term for this, and I imagine the authors have considered many alternatives, but could it be clearer to say the "genetic census population size"? For example, I found the short title not particularly descriptive "Impact of IRS and SMC on census population size", which certainly didn't make me think of parasite diversity.*

Please see our response to comment (2) of reviewer 1. We prefer not to add “genetic” to the phrase as the distinction from effective population size from population genetics is important, and the quantity we are after is an ecological one.

> *The authors do not currently say much about the potential biases in the genetic data and how this might influence results. It seems likely that because (i) patients with sub-microscopic parasitaemia were not sampled and (ii) because a moderate number of (likely low density) samples failed to generate genetic data, that the observed MOI is an overestimate. I'd be interested to hear the authors' thoughts about how this could be overcome or taken into account in the future.*

We thank the reviewer for this this comment and agree that this is an interesting area for further consideration. However, based on research from the Day Lab that is currently under review (Tan et al. 2024, *under review*), the estimated MOI using the Bayesian approach is likely not an "overestimate" but rather an "underestimate". In this research by Tan et al. (2024) isolate MOI was estimated and compared using different initial whole blood volumes (e.g., 1, 10, 50, 100 uL) for the gDNA extraction. Using _var_coding and comparing these different volumes it was found that MOI was significantly "underestimated" when small blood volumes were used for the gDNA extraction, i.e., there was a ~3-fold increase in median MOI between 1µL and 100µL blood. Ultimately these findings will allow us to make computational corrections so that more accurate estimates of MOI can be obtained from the DBS in the future.

> *The authors do not make much of LLIN use and for me, this can explain some of the trends. The first survey was conducted soon after a mass distribution whereas the last was done at least a year after (when fewer people would have been using the nets which are older and less effective). We have also seen a rise in pyrethroid resistance in the mosquito populations of the area which could further diminish the LLIN activity. This difference in LLIN efficacy between the first and last survey could explain similar prevalence, yet lower diversity (in Figures 4B/5). However, it also might mean that statements such as Line 478 "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" need to be tapered as the higher prevalence observed in this age group could be caused by lower LLIN efficacy at the time of the last survey, not loss of immunity (though both could be true).*

We thank the reviewer for this question and agree that (i) LLIN usage and (ii) pyrethroid resistance are important factors to consider.

(i) Over the course of this study self-reported LLIN usage the previous night remained high across all age groups in each of the surveys (≥ 83.5%), in fact more participants reported sleeping under an LLIN in 2017 (96.8%) following the discontinuation of IRS compared to the 2012 baseline survey (89.1%). This increase in LLIN usage in 2017 is likely a result of several factors including a rebound in the local vector population making LLINs necessary again, increased community education and/or awareness on the importance of using LLINs, among others. Information on the LLINs (i.e., PermaNet 2.0, Olyset, or DawaPlus 2.0) distributed and participant reported usage the previous night has now been included in the Materials and Methods as requested by the reviewer.

(ii) As to the reviewer's question on increased in pyrethroid resistance in Ghana over the study period, research undertaken by our entomology collaborators (Noguchi Memorial Insftute for Medical Research: Profs. S. Dadzie and M. Appawu; and Navrongo Health Research Centre: Dr. V. Asoala) has shown that pyrethroid resistance is a major problem across the country, including the Upper East Region. Preliminary studies from Bongo District (2013 - 2015), were undertaken to monitor for mutations in the voltage gated sodium channel gene that have been associated with knockdown resistance to pyrethroids and DDT in West Africa (*kdr-w*). Through this analysis the homozygote resistance *kdr-w* allele (RR) was found in 90% of *An. gambiae* s.s. samples tested from Bongo, providing evidence of high pyrethroid

resistance in Bongo District dating back to 2013, i.e., prior to the IRS intervention (S. Dadzie, M. Appawu, personal communication). Although we do not have data in Bongo District on *kdr-w* from 2017 (i.e., post-IRS), we can hypothesize that pyrethroid resistance likely did not decline in the area, given the widespread deployment and use of LLINs.

Thus, given this information that (i) self-reported LLIN usage remained high in all surveys (≥ 83.5%), and that (ii) there was evidence of high pyrethroid resistance in 2013 (i.e., *kdr-w* (RR) _~_90%), the rebound in prevalence observed for the older age groups (i.e., adolescents and adults) in 2017 is therefore best explained by a loss of immunity.

> *I must confess I got a little lost with some of the Bayesian model section methods and the figure supplements. Line 272 reads "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)." This does not appear correct as this figure is measuring kl divergence. If this is not a mistake in graph ordering please consider explaining the rationale for why this graph is being used to justify your point.*

We now included the correct figure for the repertoire size distribution as Figure supplement 3 (previously published in Labbé et al (Labbé et al., 2023)). This figure was accidently forgotten when the manuscript was submitted for review, we thank the reviewer for bringing our attention to this matter. We hope that the inclusion of this Figure as well as a more detailed description of the Bayesian approach helps to makes this section in the Materials and Methods clearer for the reader.

> *I was somewhat surprised that the choice of prior for estimating the MOI distribution at the population level did not make much difference. To me, the negative binomial distribution makes much more sense. I was left wondering, as you are only measuring MOI in positive individuals, whether you used zero truncated Poisson and zero truncated negative binomial distributions, and if not, whether this was a cause of a lack of difference between uniform and other priors.*

Thank you for the relevant question. We have indeed considered different priors and the robustness of our estimates to this choice and have now better described this in the text. We focused on individuals who had a confirmed microscopic asymptomatic *P. falciparum* infection for our MOI estimation, as median *P. falciparum* densities were overall low in this population during each survey (i.e., median ≤ 520 parasites/μL, see Table supplement 1). Thus, we used either a uniform prior excluding zero or a zero truncated negative binomial distribution when exploring the impact of priors on the final population-level MOI distribution. A uniform prior and a zero-truncated negative binomial distribution with parameters within the range typical of high-transmission endemic regions (higher mean MOI with tails around higher MOI values) produce similar MOI estimates at both the individual and population level. However, when setting the parameter range of the zero-truncated negative binomial to be of those in low transmission endemic regions where the empirical MOI distribution centers around mono-clonal infections with the majority of MOI = 1 or 2 (mean MOI » 1.5, no tail around higher MOI values), the final population-level MOI distribution does deviate more from that assuming the aforementioned prior and parameter choices. The final individual- and population-level MOI estimates are not sensitive to the specifics of the prior MOI distribution as long as this distribution captures the tail around higher MOI values with above-zero probability.

> *The high MOI in children <5yrs in 2017 (immediately after SMC) is very interesting. Any thoughts on how/why?*

This result indicates that although the prevalence of asymptomatic *P. falciparum* infections remained significantly lower for the younger children targeted by SMC in 2017 compared 2012, they still carried multiclonal infections, as the reviewer has pointed out (Figure 3B). Importantly this upward shift in the MOI distributions (and median MOI) was observed in all age groups in 2017, not just the younger children, and provides evidence that transmission intensity in Bongo has rebounded in 2017, 32-months a er the discontinuation of IRS. This increase in MOI for younger children at first glance may seem to be surprising, but instead likely shows the limitations of SMC to clear and/or supress the establishment of newly acquired infections, particularly at the end of the transmission season following the final cycle of SMC (i.e., end of September 2017 in Bongo District; *NMEP/GHS, personal communication*) when the posttreatment prophylactic effects of SMC would have waned (Chotsiri et al., 2022).

> *Line 521 in the penultimate paragraph says "we have analysed only low density…." should this not be "moderate" density, as low density infections might not be detected? The density range itself is not reported in the manuscript so could be added.*

In Table supplement 1 we have provided the median, including the inter-quartile range, across each survey by age group. For the revision we have now provided the density min-max range, as requested by the reviewer. Finally, we have revised the statement in the discussion so that it now reads "….we have analysed low- to moderate-density, chronic asymptomatic infections (see Table supplement 1)……".

> *Data availability - From the text the full breakdown of the epidemiological survey does not appear to be available, just a summary of defined age bounds in the SI. Provision of these data (with associated covariates such as parasite density and host characteristics linked to genetic samples) would facilitate more in-depth secondary analyses.*

To address this question, we have updated the "Data availability statement" section with the following statement: "All data associated with this study are available in the main text, the Supporting Information, or upon reasonable request for research purposes to the corresponding author, Prof. Karen Day (karen.day@unimelb.edu.au)."

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