Quantification of anti-parasite and anti-disease immunity to malaria as a function of age and exposure

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Abstract

Fundamental gaps remain in our understanding of how immunity to malaria develops.

We used detailed clinical and entomological data from parallel cohort studies conducted across the malaria transmission spectrum in Uganda to quantify the development of immunity against symptomatic *P. falciparum* as a function of age and transmission intensity. We focus on: anti-parasite immunity (i.e; ability to control parasite densities) and anti-disease immunity (i.e; ability to tolerate higher parasite densities without fever).

Our findings suggest a strong effect of age on both types of immunity, not explained by cumulative-exposure. They also show an independent effect of exposure, where children living in moderate/high transmission settings develop immunity faster as transmission increases. Surprisingly, children in the lowest transmission setting appear to develop immunity more efficiently than those living in moderate transmission settings. Anti-parasite and anti-disease immunity develop in parallel, reducing the probability of experiencing symptomatic malaria upon each subsequent *P. falciparum* infection.
Introduction

The last decades have seen substantial declines in malaria transmission in sub-Saharan Africa that are largely attributable to increased access to effective control measures, including insecticide-treated bednets, indoor residual spraying of insecticide and artemisinin-based combination therapy (1,2). In settings where transmission has been low, increased access to effective control interventions opens the possibility for malaria elimination. In highly endemic settings, however, there are concerns around the potential impact of failing to sustain interventions that reduce but do not stop transmission. Short-term decreases in malaria incidence due to reductions in transmission could be offset over time by reductions in population immunity to malaria resulting from lower exposure to parasites (3-5).

Gradual acquisition of immunity against symptomatic malaria (also referred to as clinical immunity) is a key driver of the epidemiology of malaria in endemic settings, where the incidence of disease typically peaks in early childhood and then declines, while the prevalence of detectable asymptomatic parasitemia increases throughout childhood before declining in adulthood (6-12). While these epidemiologic patterns have been described across the transmission spectrum, there are still many fundamental gaps in our understanding of the factors driving the development of immunity, and of the independent roles of age and repeated infection. One reason it has been challenging to study immunity to malaria is that there are currently no agreed upon reliable and quantifiable immune correlates of protection that can be measured in epidemiological
studies(13,14). In addition, there are few available datasets that include both detailed clinical data and independent metrics of exposure at the individual level.

Here, we use data from three parallel cohort studies conducted across the spectrum of malaria transmission in Uganda to model and quantify the development of immunity against symptomatic malaria as a function of transmission intensity and age. A key strength of these studies is that they involved detailed clinical and entomological surveillance of all study households. We focus on two specific types of immunity: anti-parasite immunity (i.e; the ability to control parasite densities upon infection) and anti-disease immunity (i.e; the ability to tolerate higher parasite infections without developing objective fever), as they have been described as independent components of clinical immunity (15).

Results
The three cohorts enrolled a total of 1021 children aged 6 months to 10 years from 331 randomly chosen households across the three study sites. This analysis was limited to data from 773 children who experienced at least one patent *P. falciparum* infection between August 2011 and November 2014. Table 1 summarizes the general characteristics of the participants included in this analysis.

Participants living in Nagongera experienced the highest incidences of symptomatic malaria (median 2.6 episodes per person year), followed by those living in Kihihi (median 1.6 episodes per person year) and Walukuba (median 0.6 episodes per person year).
These incidences were consistent with results from monthly entomological surveys conducted in all cohort households, with significantly higher annual entomological inoculation rates (aEIR) recorded in Nagongera (median 51 infectious bites per year, range 10-582) as compared to Kihihi (median 8 infectious bites per year, range 4-47) and Walukuba (median 2 infectious bites per year, range 1-8).

Interestingly, prevalence of asymptomatic parasitemia did not follow this same relationship; the prevalence of asymptomatic parasitemia was highest in Nagongera, and prevalences in the lower transmission sites were similar.

**aEIR as a metric of individual exposure**

To assess whether entomological metrics were a good indicator of individual exposure to *P. falciparum*, we correlated the measured annual EIRs (aEIR) for each household (Figure 2a) with estimates of the average individual hazard of infection (Figure 2b). Individual hazards were estimated by fitting time-to-event models to the incidence data from each site. We found a significant correlation between these two independent metrics of exposure across sites ($R^2 = 0.47$, $p<0.001$). aEIR explained less of the variance between individuals within each site: Nagongera ($R^2 = 0.03$, $p=0.004$); Kihihi ($R^2 = 0.12$, $p<0.001$); Walukuba (0.01, $p=0.05$).

**Anti-parasite immunity**

Parasite densities developed upon infection decreased with increasing age in all settings and for both symptomatic (passive detection) and asymptomatic (detected during routine visits) infections. Despite the large variability in parasite densities recorded within and
between individuals, this trend is evident in the raw data (Figure 3a). A trend towards lower parasite densities was also observed among individuals living in settings with higher aEIRs (Nagongera), as compared to settings with lower aEIR (Kíhihi and Walukuba).

We considered multiple candidate models to describe the association between parasite density, age and aEIR (Appendix 1). Models allowing smooth (non-linear) relationships with aEIR best fit the data. Models allowing for two-way interactions between age and aEIR also outperformed models that didn’t include interactions.

In moderate and high transmission settings (households with aEIR >5), increasing age and increasing exposure were independently and linearly associated with decreases in the parasite densities (Table 2). On average, parasite densities decreased by a factor of 0.76 (95%CI 0.75-0.77) for each additional year of age and by a factor of 0.73 (95%CI 0.70-0.76) for each two-fold increase in the aEIR. The relationship was less evident for the lower transmission households (aEIR<5). In these settings, there continued to be a decreasing (although smaller) association with age, but the expected parasite densities at any given age were equal or lower to those observed in the higher exposure (aEIR>10) settings.

Figures 4a and 5a present the predicted parasite densities, as a function of age and aEIR, according to the best fitting model. While an individual aged 1 year exposed to an aEIR of 10 is expected to develop a parasite density of 14610 parasites/μL (95%CI 5924–
36031 parasites/μL) upon infection, the expected parasite density goes down to 3237 parasites/μL (95% CI 1381–7586 parasites/μL) by age 10 years. In contrast, the expected parasite density in an individual living in a setting with aEIR of 150 will be similar at age 1 year (13071 parasites/μL (95% CI 5256–32503 parasites/μL)), but significantly lower by age 10 years (999 parasites/μL (95% CI 398–2508 parasites/μL)).

To test whether the observed associations with age could be explained by the cumulative exposure over a life time, we also fit models where, instead of adjusting for the aEIR, we adjusted for the cumulative number of infectious bites (i.e. the product of age and aEIR) (Figure 5-figure supplement 2). Results from these models are consistent with a smaller, yet independent effect of age on the development of anti-parasite immunity; for any given level of cumulative exposure, each additional year of life was associated with decreases in parasite densities by a factor of 0.82 (95% CI 0.81-0.85).

**Anti-disease immunity**

We define anti-disease immunity as the ability to tolerate a given parasite density without developing objective fever. Thus, we were interested in modeling temperatures recorded at specific parasite densities, as a function of age and aEIR. Consistent with models characterizing anti-parasite immunity, models including smooth effects and interactions fitted the data significantly better than simpler models.

As expected, we found a strong association between parasite densities and objective temperature (Figure 6-figure supplement 1). Increases in parasite densities above 1000
parasites/μL were associated with higher expected temperatures across ages and transmission settings. In addition, we found a negative association between objective temperature at a given parasite density and age (Figures 3b, 4b and 6). In moderate and high transmission settings (aEIR>5), the objective temperature at a given parasite density decreased on average by 0.08 °C (95%CI 0.07-0.10 °C) for each additional year of life (Table 2). Thus, while the expected temperature for a child aged 1 year living in a setting with aEIR of 10 with a parasite density of 40,000 would be 38.8 °C (95% CI 38.5-39.2 °C), the expected temperature would decrease to 37.6 °C (95% CI 37.3-38.0 °C) if the same child experienced the infection at age 10 years (Figures 4b and 6). This association was similar even when adjusting for cumulative exposure and for the differences in incidence of non-malarial fever across age-groups (Figure 5-figure supplement 5).

Similar to the anti-parasite immunity results described above, the observed association between exposure level and anti-disease immunity was less evident than the association with age (Figures 3b, 4b and 6). For moderate and high transmission settings (aEIR 5 to 300) there was a linear negative association between objective temperature at a given parasite density and aEIR. The objective temperature decreased by 0.07 °C (95%CI 0.05-0.10 °C) for each two-fold increase in aEIR. However, the relationship did not follow this trend for lower transmission settings (Table 2). Children living in the lowest transmission settings (aEIR 1 to 5) appeared to tolerate higher parasite densities than children living in moderate transmission settings (aEIR 5 to 10).
As an alternative way to characterize anti-disease immunity, we used our best fitting model to predict the fever threshold, defined as the minimum parasite density associated with objective fever (temperature $> 38^\circ C$), across levels of age and aEIR (figure 5b). This quantity is often referred to as the “pyrogenic density”. Results from this analysis show that, for settings with moderate and high transmission (aEIR>5), the fever threshold increases both with age and increasing exposure. Thus, while a 1 year old child living in a setting with aEIR of 10 presenting with a parasite density as low as 3747 parasites/μL (95%CI 777-11129 parasites/μL) will be expected to be febrile, children older than 6 years of age exposed to very high transmission (aEIR 150) might be afebrile even with parasite densities higher than 60000 parasites/μL.

**Overall immunity against symptomatic malaria**

Finally, to characterize the association between age and aEIR on the overall risk of developing symptomatic malaria upon infection (i.e.; the combined effect of anti-parasite and anti-disease immunity), we fit a series of models where the outcome of each independent microscopically detectable infection (i.e.; symptomatic malaria or asymptomatic parasitemia) was modeled as a function of age and aEIR. Models allowing smooth relationships, with or without two-way interactions, fit the data equally well. Results from this analysis are consistent with results from the anti-parasite and anti-disease models (Figure 7). While young children living in low transmission settings (aEIR=5) develop symptomatic malaria in most their infections, the probability that an
infection results in symptomatic malaria decreases as a function of age and exposure. The expected probability of symptomatic disease for a child aged 1 year living in a setting with aEIR of 50 is 0.92 (95%CI 0.79-0.97), but it decreases to 0.51 (95%CI 0.29-0.73) by age 10 years.

**Impact of recent infection on immunity**

To assess whether recent *P.falciparum* infection was associated with different levels of anti-parasite and anti-disease immunity, we used data on the recent malaria history of each individual to fit models adjusted for number of *P.falciparum* positive visits in the past 3 and 6 months. We found no association between the number of recent malaria infections and our outcomes of interest (Appendix 2, figure 5-figure supplement 3).

**Development of anti-parasite and anti-disease immunity at the individual level**

Models that included random effects at the individual and household levels outperformed models that assumed independence of observations, consistent with large heterogeneity between individuals in the development of anti-parasite, anti-disease and overall immunity against symptomatic malaria. To illustrate this heterogeneity, we used the best fitting model to predict the trajectories of a subset of individuals with respect to anti-parasite and anti-disease immunity, as a function of age and aEIR (Figure 5-figure supplement 12).

**Sensitivity analyses**
Our main analyses include data from all visits regardless of their type (routine vs passive case detection). Thus, the expected values modeled here may be biased by the frequency of active vs passive episodes detected. In particular, it is possible that we have under-sampled the instances of asymptomatic infection, and thus, our estimates of the expected parasite densities may be an over-estimate of those present in the population. Similarly, it is also possible that consecutive asymptomatic infections represent persistent, rather than new infections. To address these limitations, we performed sensitivity analyses where we a) up-weighted the episodes of asymptomatic parasitemia, to account for potentially unobserved asymptomatic infections and b) included only “incident” asymptomatic infections, under the assumption that subsequent asymptomatic samples represented persistent (rather than new) infections. Results from these analyses were qualitatively identical to the main analysis reported here and are presented in the supplementary material (Figure 5 – figure supplement 6 and 7).

To explore whether differences in the prevalence of certain host genetic polymorphisms between sites could be driving some of our findings, we also performed sensitivity analyses limiting the dataset to those subjects without the sickle hemoglobin mutation (β globin E6V), known to protect against malaria (16,17). Even though the sample size of these analyses was smaller (observations from 155/773 individuals were excluded), results were unchanged qualitatively (Figure 5- figure supplement 8). Similarly, restricting the dataset to children without two other known polymorphisms (the α-thalassemia 3.7 kb deletion or glucose-6-phosphate dehydrogenase deficiency caused by the common African variant (G6PD A-)), had little effect on the results.
Discussion

Our findings illustrate how anti-parasite and anti-disease immunity develop gradually and in parallel, complementing each other in reducing the probability of experiencing symptomatic disease upon infection with *P. falciparum*. While anti-parasite immunity acts to restrict the parasite densities that develop upon each subsequent infection, anti-disease immunity increases the tolerance to high parasite densities. Thus, older children are less likely to develop symptomatic malaria upon infection both because they tolerate parasite densities better without developing fever, and because they are less likely to develop high parasite densities.

Our results indicate independent effects of age on the acquisition of both anti-parasite and anti-disease immunity. These independent age effects may reflect maturation of the immune system as well as other physiological changes that decrease the propensity to fever (15,18). Furthermore, our findings are consistent with independent effects of transmission intensity on the acquisition of these two types of immunity. While the results obtained for moderate and high transmission settings (aEIR >5) are consistent and expected, and suggest that immunity develops faster in settings where individuals get infected by *P. falciparum* more often, the results obtained for the lowest transmission settings are harder to reconcile. These results were largely driven by observations collected in the Walukuba site, and as such it is possible that site-specific characteristics may have driven them. Walukuba was previously a relatively high transmission rural area, but substantial decreases in transmission intensity have been observed since 2011,
likely due to urbanization. While our sensitivity analyses suggested that differences in the prevalence of three well characterized host-genetic polymorphisms between sites do not explain these discrepant results, it is still possible that other unmeasured site-specific characteristics may have driven them. Lower complexity of infection coupled with lower parasite diversity in Walukuba, for example, could cause this difference, as developing an effective immune response against fewer parasite strains may be much easier than developing immunity against a more diverse pool (19,20). Testing this hypothesis would require careful characterization of the complexity and diversity of infections in each of our cohort settings.

While site specific characteristics may underlie the observed high levels of clinical immunity against malaria in the low transmission setting, it is also possible that this finding reflects biologically relevant differences in how immunity against malaria develops. For example, it has been hypothesized that immunity may develop optimally in individuals that are exposed at a low rate, and that more frequent infections may interfere with the development of robust immune responses (21,22). Answering this question will require further detailed studies across transmission settings, with careful characterization of both exposure and infection outcomes.

There are several limitations to this study. With a study design including routine visits every 3 months, we are likely to have missed several asymptomatic infections, particularly in the moderate and high transmission settings. Moreover, since infections were detected using microscopy, we were unable to detect sub-patent infections, and we lack knowledge about the genetic complexity of each infection. While it is possible that
the expected values modeled here (expected parasite density and fever threshold) were
biased by these sources of measurement error, sensitivity analyses suggest that the
relationships observed were robust. Secondly, while we found an independent association
between the average household aEIR and both anti-parasite and anti-disease immunity, it
is not clear that this is the most relevant metric of exposure for the development of
clinical immunity to malaria. Alternative metrics such as the number of discrete
infections, the number of “strains” seen or the total parasite-positive time might be more
relevant, but require the collection of additional data, including more frequent sampling.
Finally, while this study provides very detailed insight into how two types of clinical
immunity to malaria develop in endemic settings as a function of age and repeated
exposure, it says nothing about the duration of immunity.

Prior studies have tried to model the processes driving acquisition of clinical immunity
against malaria. However, these models have been generally informed by aggregated
epidemiological data (age-incidence and age-prevalence) which limits their capacity to
isolate the contributions of age and repeated exposure (3,6,23). Our results quantify how
anti-parasite and anti-disease immunity develop in children across the malaria
transmission spectrum, and they support strong independent effect of age and a perhaps
paradoxical effect of exposure. The methods proposed here to model anti-parasite and
anti-disease immunity may also provide a framework to select individuals with immune
and non-immune phenotypes for evaluations of immune correlates of protection.

Methods
Ethics Statement

The study protocol was reviewed and approved by the Makerere University School of Medicine Research and Ethics Committee (Identification numbers 2011-149 and 2011-167), the London School of Hygiene & Tropical Medicine Ethics Committee (Identification numbers 5943 and 5944), the Durham University School of Biological and Biomedical Sciences Ethics Committee (PRISM Entomology Uganda), the University of California, San Francisco, Committee on Human Research (Identification numbers 11-05539 and 11-05995) and the Uganda National Council for Science and Technology (Identification numbers HS-978 and HS-1019). All parents/guardians were asked to provide written informed consent at the time of enrollment.

Data

We used data from three parallel cohort studies conducted in Uganda in sub-counties chosen to represent varied malaria transmission(24). Walukuba, in Jinja district, is a peri-urban area near Lake Victoria that has the lowest transmission among the three (annual entomological inoculation rate (aEIR) estimated to be 2.8(24)). Kihiihi, in Kanungu district, is a rural area in southwestern Uganda characterized by moderate transmission (aEIR=32). Nagongera, Tororo district, is a rural area in southeastern Uganda with the highest transmission (aEIR=310)(24,25). Details on how the study households and participants were selected has been described elsewhere(24). Briefly, all households were enumerated, and then approximately 100 households were selected at random from each site. Between August and September 2011, all children from these households aged between 6 months and 10 years who met eligibility criteria were invited to participate. As
the cohorts were dynamic, additional children from participating households were invited
to participate if they became eligible while the study was ongoing. Unless participants
were withdrawn from the study either voluntarily or because they failed to comply with
study visits, they were followed-up until they reached 11 years of age. Children from 31
randomly selected additional households were enrolled between August and October
2013 to replace households in which all study participants had been withdrawn. For this
analysis, we used data collected from visits between August 2011 and November 2014.
The studies included passive and active follow-up of participants. Parents/guardians were
encouraged to bring their children to designated study clinics for any illness. All medical
care was provided free of charge, and participants were reimbursed for transportation
costs. All children who reported fever in the previous 24 hours or were febrile at the time
of the visit (tympanic temperature > 38.0°C) were tested for malaria infection with a
thick blood smear. Light microscopy was performed by an experienced laboratory
technician who was not involved in direct patient care and verified by a second
technician. Parasite density was calculated by counting the number of asexual parasites
per 200 leukocytes (or per 500 leukocytes, if the count was <10 asexual parasites/200
leukocytes), assuming a leukocyte count of 8,000/µl. A blood smear was considered
negative when no asexual parasites were found after examination of 100 high power
fields.
If the smear was positive, the patient was diagnosed with symptomatic malaria and
received treatment with artemether-lumefantrine (AL), the recommended first-line
treatment in Uganda. Episodes of complicated or recurrent malaria occurring within 14
days of therapy were treated with quinine. In addition, routine evaluations were
performed every three months, including testing for asymptomatic parasitemia using thick blood smears.

Entomological surveys were also conducted every month at all study households. During these surveys, mosquitoes were collected using miniature CDC light traps (Model 512; John W. Hock Company). Established taxonomic keys were used to identify female *Anopheles* mosquitoes. Individual mosquitoes were tested for sporozoites using an ELISA technique (25). All female *Anopheles* mosquitoes captured in Walukuba and Kihhi were tested; in Nagongera testing was limited to 50 randomly selected female *Anopheles* mosquitoes per household per night due to the large numbers collected. Therefore, for each household and/or site it was possible to calculate multiple entomological metrics, including the average human biting rate (average number of female *Anopheles* mosquitoes caught in a household per day), the average sporozoite rate (the average proportion of mosquitos that tested positive for *Plasmodium falciparum*) and the entomological inoculation rate (EIR, the product of the household human biting rate and the site sporozoite rate).

**Statistical analyses**

The purpose of these analyses was to model and quantify the development of immunity against symptomatic malaria, as a function of age and exposure, measured by the household EIR. We modeled two specific types of immunity that have been previously described as components of immunity to malaria. We defined anti-parasite immunity as the ability to
control parasite densities upon infection and anti-disease immunity as the ability to
tolerate parasite infections without developing objective fever. Thus, for models of anti-
parasite immunity, the outcome of interest was the parasite density recorded (using thick
blood smear) at each parasite positive study visit. For models of anti-disease immunity,
the outcome of interest was the objective temperature recorded during parasite positive
visits, conditional on the parasite density. In addition, we also modeled overall immunity
against symptomatic malaria. For these analyses, the outcome of interest was the
probability of presenting with fever given infection (parasite positivity).

In order to model the association between the outcomes and covariates of interest we
used generalized additive models (gams). Gams provide a good framework, as they allow
for smooth non-linear relationships. Details on the specific models explored are provided
in the supplementary material (Appendix 1). In summary, the models followed the
following form.

1) Anti-parasite immunity

$$\text{Log}_{10}(\text{Parasite density})_{ijk} = f(\text{age}_{ijk}, \text{Log}_2aEIR_j) + u_i + \gamma_j$$

2) Anti-Disease immunity

$$\text{Temperature}_{ijk} = f(\text{age}_{ijk}, \text{Log}_2aEIR_j, \text{Log}_{10}\text{Parasite density}_{ijk}) + u_i + \gamma_j$$

3) Overall immunity against symptomatic malaria
\[ P(\text{symptomatic malaria upon infection})_{ijk} = f(\text{age}_{ijk}, \log_2 aEIR_j) + u_i + \gamma_j \]

Where \(i\) is an index for individuals, \(j\) for households and \(k\) for specific visits. Thus, \(\text{age}_{ijk}\) represents the age of child \(i\) from household \(j\) during visit \(k\), and \(aEIR_j\) represents the average annual EIR recorded for household \(j\). We included the EIR as an average (time-invariant) covariate, as we were interested in modeling the impact of the average exposure to malaria over time on the development of clinical immunity. Therefore, our model implicitly assumes that malaria transmission has been relatively stable at these three sites. To account for lack of independence, all models included random effects at the individual (\(u_i\)) and household (\(\gamma_i\)) levels.

All of our primary analyses included the full dataset. However, since results were consistent with a non-monotonic relationship between \(aEIR\) and the outcomes of interest, we also fit models stratified by \(aEIR\) (\(aEIR \geq 5\) vs. \(aEIR < 5\)). All models were fitted in the R statistical framework using package mgcv(26). Best fitting models were selected based on Akaike’s Informaiton Criterion, but changes in the percent deviance explained are also presented.

**Code and data availability**

All the data used for these analyses as well as the R code used to reproduce the main study findings are available at https://github.com/isabelrodbar/immunity(27). Complete
data from the 3 cohort studies are available in the ClinEpiDB website (https://clinepidb.org/ce/app).

Acknowledgements

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Competing interests

None

References


Tables and figures

Table 1: Characteristics of the study participants

<table>
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<th>Characteristic</th>
<th>Nagongera</th>
<th>Kihhi</th>
<th>Walukuba</th>
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<tbody>
<tr>
<td>Number of households</td>
<td>106</td>
<td>100</td>
<td>76</td>
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<tr>
<td>Number of children</td>
<td>329</td>
<td>305</td>
<td>139</td>
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<tr>
<td>Female, n (%)</td>
<td>151(46)</td>
<td>150 (49)</td>
<td>66 (47)</td>
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<tr>
<td>Mean age at enrollment, years (sd)</td>
<td>4.4 (2.7)</td>
<td>4.6 (2.6)</td>
<td>4.3 (2.6)</td>
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<td>Mean follow up time, months (range)</td>
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<td>24.4 (0.8, 38.8)</td>
<td>22.1 (2.3, 3.9)</td>
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</table>

Symptomatic malaria

Symptomatic Malaria episodes, n
Median number of symptomatic malaria episodes/child, n (range)
Median incidence of symptomatic malaria episodes ppy (range)

Asymptomatic parasitemia

Asymptomatic parasitemia episodes, n
Median number of asymptomatic parasitemia episode/child, n (range)
Median prevalence of asymptomatic parasitemia (range)

Household malaria exposure

Household aEIR, median (range)
Table 2: Results of linear models quantifying the association between age, aEIR and immunity outcomes

<table>
<thead>
<tr>
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<th>All data</th>
<th>aEIR ≥ 5 (n= 5047)</th>
<th>aEIR &lt; 5 (n= 593)</th>
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<td><strong>Anti-parasite immunity</strong></td>
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<tr>
<td>Age (years)</td>
<td>0.78 (0.77, 0.79)</td>
<td>0.76 (0.75, 0.77)</td>
<td>0.87 (0.83, 0.90)</td>
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<td>Log₂ aEIR</td>
<td>0.82 (0.79, 0.84)</td>
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<td><strong>Anti-disease immunity</strong>*</td>
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<tr>
<td>Age (years)</td>
<td>-0.07 (-0.06,-0.08)</td>
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<td>Log₂ aEIR</td>
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<td>-0.07 (-0.05,-0.1)</td>
<td>0.27 (0.11, 0.44)</td>
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<tr>
<td><strong>Overall immunity against symptomatic malaria</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.78 (0.75, 0.82)</td>
<td>0.77 (0.74, 0.80)</td>
<td>0.90 (0.83, 0.99)</td>
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<td>Log₂ aEIR</td>
<td>0.91 (0.74, 1.13)</td>
<td>0.62 (0.48, 0.80)</td>
<td>3.83 (1.39, 10.6)</td>
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* Model adjusted as well for Log parasite density
Figure legends

Figure 1: Incidence of malaria per person year (ppy) (a) and prevalence of asymptomatic parasitemia (b) in the three study sites as a function of age, modeled using generalized additive models (GAMS). Shaded areas represent 95% confidence bounds.

Figure 2: a. Distribution of the average annual entomological inoculation rate (aEIR) experienced by the study households in the three study sites. b. Correlation between the measured aEIRs and the estimated individual hazards of infection.

Figure 3: Trends in parasite densities (a) recorded during symptomatic (passive surveillance) infections and routine (active surveillance) visits as a function of age (left) and aEIR (right); and trends in the objective temperature (b) recorded during visits in which participants were found to have a parasite density between 50,000 parasites/μL and 200,000 parasites/μL, as a function of age (left) and aEIR (right). Each point represents a measurement obtained during a study visit. The median and interquartile range are shown in black.

Figure 4: Results from models quantifying anti-parasite immunity (a), anti-disease immunity (b) and overall immunity against symptomatic malaria (c). Each plot shows, for specific ages and aEIRs, the expected parasite density (/μL) (a), objective temperature given a density of 40,000 parasites/μL (b) and the probability of developing symptomatic malaria upon infection (c), estimated using the best fitting model. 95% confidence intervals of the estimates are also shown.

Figure 5. Results of the best fitting models quantifying anti-parasite (a) and anti-disease immunity (b). These results are similar to those presented in Figure 4, but for the full range of ages and aEIRs included in the data. Panel a. shows expected parasite densities (parasites/μL, log 10) upon infection for different ages and levels of exposure (aEIR). Panel b. shows the “fever threshold” or “pyrogenic density”, the minimum parasite densities (parasites/μL, log 10) associated with fever (temperature 38°C or greater), again as a function of age and exposure. Confidence bounds are presented in Figure 5 –figure supplement 1.

Figure 6. Results of the best model quantifying anti-disease immunity. Each panel shows how the expected objective temperature (°C) varies as a function of age and parasite density, for different transmission settings. a. aEIR=2; b. aEIR=10; c. aEIR=50; d. aEIR=200. Contours indicating the fever threshold (38°C) are also shown. Confidence bounds for these plots are presented in Figure 6 –figure supplement 1.

Figure 7. Results of the best model quantifying overall immunity against symptomatic malaria (upon microscopically detectable infection), as a function of age and exposure (aEIR). Colors represent the expected probability of developing symptomatic malaria upon infection. Confidence bounds for these plots are presented in Figure 7 –figure supplement 1.
Legends supplementary materials

Figure 5-figure supplement 1
Confidence bounds of best fitting model quantifying anti-parasite immunity. Left panel (a) is equivalent to Figure 5a, and shows the expected parasite densities (log 10, parasites/μL) after infection at different ages and levels of exposure (aEIR). Panel b. shows the lower and upper 95% confidence bounds. Panel c. shows the width of the confidence intervals (2*1.96*standard error) for different levels of age and exposure.

Figure 5-figure supplement 2
Adjusting for cumulative exposure
To further explore the independent contribution of age on the development of anti-parasite and anti-disease immunity, we fit models where, instead of adjusting for aEIR, we explicitly adjusted for the cumulative aEIR (cumEIR) as a metric of cumulative exposure. Cumulative aEIR was calculated as the product of age and aEIR. We fit the following models.

Anti-parasite immunity
\[ \log_{10}(Parasite\ density)_{ijk} = f(age_{ijk}, \log_{2}cumEIR_{ijk}) + u_i + \gamma_j \]

Anti-disease immunity
\[ Temperature_{ijk} = f(age_{ijk}, \log_{2}cumEIR_{ijk}, \log_{10}Parasite\ density_{ijk}) + u_i + \gamma_j \]

Figure shows the results of these models. These results are similar to those presented in Figure 5, but adjusted for cumulative aEIR rather than for EIR. Left panel shows expected parasite densities (log 10, parasites/μL) after infection at different ages and levels of cumulative exposure. Right panel shows the expected fever thresholds (parasite densities required to develop a temperature 38°C or greater).

Figure 5-figure supplement 3
Adjusting for the number of infections in the past 3 months: Results of models
quantifying anti-parasite (left) and anti-disease immunity (right). These results are similar to those presented in Figure 5, but adjusted for the number of P.falciparum positive visits in the last 3 months. Left panel shows expected parasite densities (log 10, parasites/μL) after infection at different ages and levels of exposure (aEIR). Right panel shows the expected fever thresholds (parasite densities required to develop a temperature 38°C or greater). Supplementary file 2 shows the estimated coefficients for these and additional models exploring the impact of recent exposure.

Figure 5-figure supplement 4
Adjusting for the probability of non-malaria fevers
Since it is known that the probability of developing fever (not due to malaria) decreases with age, we performed sensitivity analyses where we adjusted for the probability of non-malaria fevers.
Using data from instances when participants consulted for fever and/or were objectively febrile but were found to be smear negative, we fit a model to estimate the monthly probability of non-malaria fever as a function of age. This model was specified as:

\[
\log \text{Odds}(\text{non malaria fever})_{ij} = f(\text{age}_{ij})
\]

where \(i\) is an index for individuals and \(j\) is an index for month. The model also included a random effect to account for clustering within individuals.

Figure shows the distribution of estimated probabilities of non-malaria for the different age-groups in the dataset.

We then used this model to predict the probability of non-malaria fever for each individual at each time point, and re-estimated our anti-parasite and anti-disease models adjusting for this probability (on the logit scale). See Figure 5-figure supplement 5.

**Figure 5-figure supplement 5**

**Adjusting for the probability of non-malaria fevers**

Results of models quantifying anti-parasite (left) and anti-disease immunity (right). Left panel shows expected parasite densities (log 10, parasites/μL) after infection at different ages and levels of exposure (aEIR). Right panel shows the expected fever thresholds (parasite densities required to develop a temperature 38°C or greater). These results are similar to those presented in Figure 5, but adjusting for the probability of non-malaria fever experienced by the different age-groups. See Figure 5-figure supplement 4.

**Figure 5-figure supplement 6**

**Adjusting for the probability of observation**

Since the study design only included “routine” samples collections every 3 months, it is likely that we missed several events of asymptomatic infection, particularly in the moderate and high transmission settings. Moreover, since infections were detected using microscopy, we lack knowledge about the complexity of each infection. To account for the lower probability of observing asymptomatic, as compared to symptomatic infections, we conducted sensitivity analyses where we up-weighted asymptomatic observations to account for potentially missed infections. Weights were calculated as the expected number of infections experienced by the participant during the period of time comprised between the last visit (where the participant was tested for parasites) and the current visit. The expected number of infections (\(M\)) were derived using estimates of the average daily individual hazard of infection (\(\lambda\)) as

\[
M = D(1 - \exp(-\lambda))
\]

where \(D\) is the number of days between the last visit when the participant was tested and \(1 - \exp(-\lambda)\) is the daily probability of infection.

Figure shows results of models quantifying anti-parasite (left) and anti-disease immunity (right). Left panel shows expected parasite densities (log 10, parasites/μL) after infection at different ages and levels of exposure (aEIR). Right panel shows the expected fever
thresholds (parasite densities required to develop a temperature 38°C or greater). These results are similar to those presented in Figure 5, but weighted for the probability of observation of asymptomatic infections.

Figure 5-figure supplement 7
Limiting the analysis to “incident” infections
Since we don’t have data on the genotypes of parasites, it is also possible that consecutive asymptomatic infections represent persistent, rather than new infections. To assess the potential impact of including these persistent infections in the analyses, we conducted sensitivity analyses where we limited the dataset to “incident” infections. We considered the following samples as belonging to “Incident” infections.
- All instances of symptomatic malaria infection not preceded by an asymptomatic infection within 10 days
- All instances of asymptomatic infection when:
  - The previous blood smear (from routine or active surveillance) was negative
  - The previous blood smear (from routine surveillance) was positive, but the participant received malaria treatment between that visit and the current one

Limiting the analyses to incident infections implied excluding 700/1431 instances of asymptomatic parasitemia.

Figure shows results of models quantifying anti-parasite (left) and anti-disease immunity (right). Left panel shows expected parasite densities (log 10, parasites/µL) after infection at different ages and levels of exposure (aEIR). Right panel shows the expected fever thresholds (parasite densities required to develop a temperature 38°C or greater). These results are similar to those presented in Figure 5, but limiting the dataset to “incident” infections.

Figure 5-figure supplement 8
Limiting the analysis to individuals without the sickle hemoglobin mutation (β globin E6V)
This analysis only included data from 618/773 individuals without the sickle hemoglobin mutation (β globin E6V).

Figure shows results of models quantifying anti-parasite (left) and anti-disease immunity (right). Left panel shows expected parasite densities (log 10, parasites/µL) after infection at different ages and levels of exposure (aEIR). Right panel shows the expected fever thresholds (parasite densities required to develop a temperature 38°C or greater). These results are similar to those presented in Figure 5, but limited to children without the sickle hemoglobin mutation (β globin E6V).
Figure 5-figure supplement 9
**Limiting the analysis to individuals from Tororo and Kanungu**
This analysis only included data from 634/773 individuals living in Tororo (Nagongera) and Kanungu (Kihihi).

Figure shows results of models quantifying anti-parasite (left) and anti-disease immunity (right). Left panel shows expected parasite densities \((\log_{10}, \text{parasites/μL})\) after infection at different ages and levels of exposure (aEIR). Right panel shows the expected fever thresholds (parasite densities required to develop a temperature 38°C or greater). These results are similar to those presented in Figure 5, but limited to children living in Tororo (Nagongera) and Kanungu (Kihihi).

Figure 5-figure supplement 10
**Limiting the analysis to individuals living in settings with aEIR ≥ 5**
This analysis only included data from 554/773 individuals living in households with aEIR >5.

Figure shows results of models quantifying anti-parasite (left) and anti-disease immunity (right). Left panel shows expected parasite densities \((\log_{10}, \text{parasites/μL})\) after infection at different ages and levels of exposure (aEIR). Right panel shows the expected fever thresholds (parasite densities required to develop a temperature 38°C or greater). These results are similar to those presented in Figure 5, but limited to children living in households with aEIR ≥ 5.

Figure 5-figure supplement 11
**Using a metric of aEIR that only includes prior observations**
In our main analysis, the metric of exposure used was the mean aEIR over the whole study period, as a proxy of the mean exposure that each participant has experienced over their life-time.

We performed a sensitivity analysis where instead, aEIR is calculated as the mean aEIR up to each point in time.

This analysis only excluded data from 344/5640 observations where we didn’t have prior exposure information.

Figure shows results of models quantifying anti-parasite (left) and anti-disease immunity (right). Left panel shows expected parasite densities \((\log_{10}, \text{parasites/μL})\) after infection at different ages and levels of exposure (aEIR). Right panel shows the expected fever thresholds (parasite densities required to develop a temperature 38°C or greater). These results are similar to those presented in Figure 5, but with an alternative metric of aEIR as described above.

Figure 5-figure supplement 12
**Predicted individual trajectories of anti-disease and anti-parasite immunity**
Figure showing predicted individual trajectories in the development of anti-parasite (a.) and anti-disease (b.) for a sample of study participants from each study site. a. shows expected parasite densities (parasites/μL, log 10) after infection at different ages and levels of exposure (aEIR). b. shows the expected fever thresholds (parasite densities
required to develop a temperature 38°C or greater). Each line represents the predicted trajectory, but the solid portion represents the period of time when the individual contributed to the dataset.

**Figure 5-figure supplement 13**

**Model checks**
Residual plots and response vs. fitted plots for the best fitting models (Models AP4 and AD4).

**Figure 6-figure supplement 1**
Association between parasite density (parasites/μL) and objective temperature (in °C)

**Figure 6-figure supplement 2**
Confidence bounds of best fitting model quantifying anti-disease immunity. Left panel (a) is equivalent to Figure 6c, and shows how the expected objective temperature (°C) varies as a function of age and parasite density, in a setting with aEIR of 50. Panel b. shows the lower and upper 95% confidence bounds. Panel c. shows the width of the confidence intervals (2*1.96*standard error) for different levels of age and exposure.

**Figure 7-figure supplement 1**
Confidence bounds of best fitting model quantifying overall immunity against symptomatic malaria. Left panel (a) is equivalent to Figure 7. Colors represent the expected probability of developing symptomatic malaria upon infection, as a function of age and exposure. Panel b. shows the lower and upper 95% confidence bounds. Panel c. shows the width of the confidence intervals (2*1.96*standard error), on the logit scale, for different levels of age and exposure.

**Appendix 1**
Detailed description of the models fitted.

**Appendix 2**
Results of models adjusted for metrics of recent exposure.
**Figure a.** Parasite density (parasites/μL) vs. Age (years).

**Figure b.** Temperature vs. Parasite density vs. Age (years).

**Figure c.** Probability of symptomatic malaria infection vs. Age (years).

- **aEIR** values: 2, 5, 10, 50, 200.