nature portfolio

Peer Review File

Asymptomatic school-aged children carry the majority of transmissible Plasmodium falciparum infections

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This analysis is a straightforward description of gametocyte prevalence in a large cohort population monitored monthly for one year. The study team used (approximately?) monthly active surveillance coupled with passive surveillance at local health facilities and tested participants for P. falciparum sexual and asexual stages at every contact.

The authors choose several related summary metrics – any gametocytes, more than one gametocyte event, gametocyte events above 1 per microliter or above 10 per microliter - and then correlated these with individual characteristics.

They find that children 6-16 years are more likely to have Pf infection and also more likely to have gametocytes. However, younger children have higher gametocyte densities and harbor proportionally more gametocytes over the study period. The latter finding is under-explored and sort of sidelined.

The age distribution of gametocytes and the age distribution of Pf infections has been well described. The longitudinal nature of the study represents a unique opportunity since most gametocyte studies are cross-sectional. However, most of their outcomes they have just translated the outcomes into cross-sectional measures. It would be more interesting to see gametocyte production in relationship to duration of infection, timing of symptomatic events, even household-level symptomatic events related to clustering of gametocytes.

Major comments
Ideally it would be useful to identify characteristics that correlate differently with overall infection versus gametocytes. I don't know if we've learned anything specific to gametocytes. If so, this needs to come out more clearly. Likewise for any gametocytes versus many instances of gametocytes or high versus low density gametocytemia – what is interesting is whether there are differences that can help us understand the system.

There is no direct causal relationship between housing construction or socioeconomic status and gametocyte prevalence or density. Although housing construction may be related to infection via exposure to infectious bites via more mosquito-permeable materials, it is preferable to use a more proximal explanatory variable such as frequency of Pf positive asexual episodes, recent asymptomatic/symptomatic episodes, mosquito density, net use, etc... It doesn't shed new light on the problem or identify exploitable differences to correlate gametocytes with wealth or housing – we can neither use this to target interventions nor understand the biological phenomenon. Likewise for education. Use of these variables is unfortunately rote in our field even when it adds little to the understanding.

Pf detection was done from blood extracted from filter paper dried blood spots but gametocyte detection was done from blood preserved in RNA reagent. If gametocyte detection was only done on samples which had a Pf positive DBS, is there a potential for missing information from low density infections given the lower efficiency of extraction from DBS?

Kids 6-14 have larger proportion of total parasites than they have proportion of total gametocytes. The final paragraph of the results indicates that young children have 40% of the gametocytes despite only representing 18% of the population. Perhaps more attention should be given to the finding of a significant reservoir in this group instead of only focusing on older children?

Fig 6a-d are very difficult to interpret. The labels cannot be read in all the boxes and the choice of variables is difficult to understand. The orientation of the comparisons changes between the panels which makes it impossible to visually identify differences in the distribution between panels. These figures don't contribute to the story.

The observation that "39% of individuals with multiple gametocyte-positive visits having high-density infections vs 8% of individuals with only a single gametocyte-positive
infection ($p<0.001$)" could be biased by missingness. The more observations you have about an individual, the more likely they are to have the characteristic you are testing for. Also, this information is not clear in Table 1 as indicated.

Is the statement 'limiting their [PCD] contribution to the infectious reservoir' well supported by the data? They may be rare, but the data presented here shows they more frequently harbored gametocytes and at higher density. It is not possible to measure their contribution to transmission. Based on the results of this study, I think it has to stop at comparing these two types of infections in terms of frequency and density. In the discussion, it could be speculated that they may not make a major contribution to the reservoir, but this is not directly supported by the results of the current study.

Fig 4 is not very useful for illustrating the point about household clustering since these are intentionally selected households with specific profiles. It is not easy to know how to extrapolate this to the rest of the households. I suggest finding a different way to visualize the household clustering.

Something is wrong with the xaxis of Fig 2. It goes from Jan to Nov but May is missing. However the text states that the study was from April 2019 to May 2020 so either the labeling is incorrect or the months are not presented in chronological order?

Minor comments
Use age range instead of 'school age' or 'SAC' especially in tables and figures.

Avoid using asymptomatic, chronic and low density interchangeably. Although infections are often all three of those, they are very different characteristics.

Use confidence intervals instead of p values in the text. Gives a more comprehensive understanding of the estimate than a pvalue.

Reviewer #3

(Remarks to the Author)
The authors main observation is that school-going children harbor higher densities of gametocytes and hence are primary source of human-to-mosquito infection for transmission of malaria. They are also advocating for focus primary prevention of control and prevention measure for malaria on this group. While the observation is novel as this has been shown before, their study complements the similar observation and also present new methodologies that account for possible confounding effects for other variables.
The study is convincing and clear and so is the methodology.
The statistical methods described seems to suitable and mostly the work can be reproducible.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
My comments refer to the rebuttal letter

Comment 2 regarding longitudinal data
- Yes, they present incidence rate ratios, but I was thinking more of using the longitudinal data to look at continuous gametocyte carriage over time versus sporadic gametocyte detection, relationship between continuous asexual infection and gametocyte production, relationship between clinical episodes, treatment and gametocyte detection etc... You don't have to do genotyping to leverage the longitudinal nature of the data.
- Table 2 doesn't tell us anything about the time interval between detection and 'previously detected Pf parasitemia'.
- For table 4, this would be more interesting to look at whether the previous event was symptomatic before the event of detecting gametocytes. I don't think gametocytes are predictive of whether you will develop clinical symptoms but studies have shown that clinical symptoms are associated with lower gametocyte production. This is in supplemental table 6 but this seems more interesting to me than the Table 4 results.
- The interesting questions to answer longitudinally are 1) how many months after an individual is first infected do they start producing gametocytes? 2) do they produce gametocytes after a symptomatic episode/treatment? 3) Is gametocyte density proportional to lagged parasite density or does gametocyte density become independent of parasite density in very long-lived infections? 4) Do individuals with long-lived infections have intervals with and without gametocytes or is gametocyte production constant at the infection level? Maybe these questions are too detailed and move away from a population-level description of gametocyte production.

Major comment 1

- I wasn't suggesting that they delve into the biology of gametocyte production and transmission. Rather, if they are trying to characterize the population in order to enhance targeting of interventions, then three questions are relevant here 1) who are the individuals with gametocytes, 2) what type of gametocyte producer is most important to target (ie the frequent but low density versus the infrequent but high density) and 3) can we differentiate that type of producer based on observable characteristics. Even if the question of 'what type of gametocyte producer is most important' is too far into the transmission biology, some justification of why they are characterizing subpopulations of gametocytemic infections and whether observable characteristics (age, gender, household type, infection type) can help us target them is warranted.

Figure 5
- These are still very cumbersome and hard to interpret visually especially since comparison between the panels is the only way to understand the ACD/PCD dimension. I would suggest removing SES and having ACD and PCD on the same panel giving only 2 panels – total gametocytes and total parasites.

I suggest avoiding such statements as 'largest and most comprehensive study'. Qualify with 'one of the ...'

Version 2:

Reviewer comments:

Reviewer #4

(Remarks to the Author)
I was asked to comment in place of a reviewer who could not re-review. My overall impression is very positive: this is a valuable dataset and a well-written, interesting study. It has real potential to advance our understanding of how infectivity varies across age, season, care-seeking and intervention use. That said, I agree with the original reviewer that several methods are vulnerable to bias from the sampling framework. I suspect some of the remaining issues stems from a misunderstanding of what was requested and the good news is that most issues look readily fixable with small tweaks to analyses you already run. I've attached some simple pseudocode in an R script separately in the hope this will help to make things clearer.
For others (particularly spatial analyses) I think solving them would be quite complex analytically. For those, I recommended that the authors scale the complexity back and instead just let the visualisations of the data speak for themselves and have provided suggestions for those which, to me, would still make the paper highly valuable to the readership of nature communications medicine.

Major issues:
Dealing with differential sampling cadence
As the previous reviewer indicated a good proportion of this analysis is prone to bias due to unequal follow-up. In this dataset, children attend ACD more frequently than adults—especially men—so they have more chances to register at least one gametocyte-positive visit and to accumulate higher "total gametocyte abundance." In a simple counter-example (script provided), if under-5s, 5–14s, and ≥15s all have the same true per-visit prevalence but are sampled at different cadences (e.g., 8, 6, and 5 visits/person), then: (i) a chi-square on "ever ≥1 positive" becomes "significant" solely because the frequently sampled group has more opportunities to cross the ≥1 threshold; and (ii) summing densities across visits over-weights groups with more visits and longer persistence. Both statistics therefore conflate observation effort with transmission biology and will not recover the expected demographic contribution (e.g., 25/40/35) when true prevalence is equal (I've provided code showing this kind of bias using simulated data)

The good news is *I think* the authors already has everything needed to correct for this without adding complexity. First reframe the estimand from "probability of at least one positive during follow-up" to the per-sample (per-visit) probability of gametocyte detection. Take the visit-level logistic regression and translate the odds to probabilities for each stratum. These predicted probabilities can be multiplied by the demographic weights of each stratum to yield unbiased contributions (i.e., contribution = per-visit probability × population share). As the script shows, under equal prevalence this would then recovers the expected underlying demography; when prevalence truly differs, the contributions move accordingly. This approach transparently answers "who contributes how much?" while removing the sampling-cadence artifact. NB: Given repeated measures and observed over-dispersion, I recommend adding at least a household random intercept (and, if feasible, an individual random intercept). This can also then be used to predict/augment the missing data should the authors feel like going an extra step but I think for overall trends it isn't necessary.
Duration of gametocyte-detection
This seems to be based on the durations where individuals were gametocytaemic over two sequential visits? I don't think one this can really hold as individuals can be readily infected again between visits and regardless would be subject to biases associated with testing frequency (i.e. monthly visits will tend to produce monthly durations, if this weekly it would be often much shorter etc.).
Issues with odds of detection over time and PCD vs ACD contribution.
I think this possibly comes from a misinterpretation of what the previous reviewer but I don't think this approach is valid, at least for ACD. With scheduled monthly ACD, detection only occurs at visits, not continuously, so dividing "positive counts" by calendar time treats unsampled months as if they were observed. This metric also turns a single persistent infection into multiple "incidents" across months. In short, it mixes visit schedule, missed visits, and persistence with biology and is hard to

interpret so I would just frame everything to do with ACD around per-visit probability – one can include a fixed effect for month to tease apart temporal dynamics.
I also agree with the reviewer that the PCD and the ACD can't be compared in the way it has been. Instead, the authors could compare the prevalence by ACD (estimated using the above) to the cumulative incidence as a % of population in a given month which would likely give them something quite cool to talk about in terms of seasonal contribution. I agree with the reviewer about the need to caveat (or adjust) this based upon diverging gct density between ACD and PCD cases (see comment about the 'transmissibility metrics').
Spatial and temporal distribution
I agree with the reviewer that the nature of the data collection where things are samples in clusters makes this is a difficult dataset to apply standard spatial analyses to and I don't think the existing approach would stand much scrutiny from a geostatistician. I do think however, that this is a really cool dataset so one can get pretty far by just letting the data do the talking. I think a map showing gametocytaemia -or related metrics seem - for each study month would be really cool to see (potentially showing the PCD case counts somehow to see how asymptomatic dominates dry season?). I actually think Figure 4 is a really nice visualisation! However, I can see that if the spatial sampling framework isn't incorporated this would make it hard to interpret. Can you not just have a column for each of the 16 clusters? (or maybe this is what this is already and you've just missed a label?). I also think a really useful figure (at least for my work!) for each cluster would be https://en.wikipedia.org/wiki/Pareto_chart (i.e. rank each individual in descending order of contribution to infectivity (preferably with augmented data from the regression above so each individual has an observation each month)
Weighting infectivity
There is a somewhat arbitrary ranking of gametocyte density why not just use one of the well-defined relationships in the literature to convert into prob of transmission given fed upon from membrane feeding assays? Obviously comes with own set of assumptions but at least empirically derived.. e.g. https://elifesciences.org/articles/34463#fig2sdata2?
Minor issues:
"Prior cross-sectional surveys have compared gametocyte prevalence and density by age group with symptom status [17], but have not quantified the contribution of each group to the overall population-level transmission." – I might be missing some nuance here but I don't think it's first study to assess contribution to transmission longitudinally and it also arguably doesn't assess transmissibility as comprehensively as other CX studies using feeding assays. I think the topic is of sufficient interest regardless so suggest the authors be a bit more careful to say something that can't be argued with - it's possibly the largest?? Personally, I'd be more comfortable with one of the first?
– e.g. Andolina 2021: https://www.thelancet.com/journals/laninf/article/PIIS1473-3099(21)00072-4/fulltext

I'm aware this review process has already been quite an arduous one and am very happy for the authors to contact me directly should they need any further clarification around any of this or the related pseudocode.
Patrick Walker (Patrick.walker@imperial.ac.uk)

Version 3:

Reviewer comments:

Reviewer #4

(Remarks to the Author)
I thank the authors for dedicating so much effort to addressing my concerns. As stated in my original review, I think this is a really important and interesting dataset and am very happy to now say that I both think my concerns have been well addressed and that the additional analyses add even more to what is a very cool paper.

Dear reviewers,

Thank you for your thoughtful review and comments. We have responded to your feedback below (*in italics*):

Reviewer #1 (Remarks to the Author):

They find that children 6-16 years are more likely to have Pf infection and also more likely to have gametocytes. However, younger children have higher gametocyte densities and harbor proportionally more gametocytes over the study period. The latter finding is under-explored and sort of sidelined.

- *Despite a higher median gametocyte density, the distribution of gametocyte densities was not significantly different comparing young children to other groups, as such, it did not stand out as worth discussion. However, children 5-15 had a significantly higher frequency of high gametocyte density infections, which is discussed.*
- *We agree that children under 5 do harbor proportionally more gametocytes over the study period and we have edited the results and discussion to reflect this as follows:*
    - *Results: A proportionately higher fraction of gametocytes were detected among under-5 children, with 40% of gametocytes detected among under-5 children who represented only 18% of the census population.*
    - *Discussion: We additionally found that children under five carried a disproportionate number of gametocytes which were primarily detected at ACD visits, indicating an underappreciated burden of transmission-relevant asymptomatic infections in younger children.*

The age distribution of gametocytes and the age distribution of Pf infections has been well described. The longitudinal nature of the study represents a unique opportunity since most gametocyte studies are cross-sectional. However, most of their outcomes they have just translated the outcomes into cross-sectional measures. It would be more interesting to see gametocyte production in relationship to duration of infection, timing of symptomatic events, even household-level symptomatic events related to clustering of gametocytes.

- *We agree that it would be ideal to look at duration of gametocyte detection. Unfortunately we were unable to genotype the infections and thus cannot say for certain whether or not repeated gametocyte detection is due to the same infection or a new infection and, thus, cannot measure duration of detection. However, our results are not cross-sectional: Figure 3 presents incidence rate ratios for incidence of gametocyte detection over the study period. Figure 5 represents the cumulative longitudinal detection of gametocytes over time in the study given that the active case detection visits occurred at regular timepoints giving equal representation and the passive case detection visits (sick visits) occurred at their natural intervals. In this way, we were able to estimate the cumulative contribution of different groups to gametocytemia over an entire year. Likewise, Table 2 analyzes the data longitudinally, examining previous parasitemia and previous gametocytemia as predictors of subsequent detection of gametocytemia.*

- *For the timing of symptomatic events, we added an analysis to examine Pf parasitemia and gametocyte detection as predictors of subsequent symptomatic malaria (Table 4). We examined the relationship between any gametocyte or Pf detection and Pf or gametocyte detection at the previous visit and the risk of presenting with malaria symptoms at a subsequent visit. We found no association with previous infection (with or without gametocytes) and presenting later with clinical malaria.*
- *We appreciate your suggestion to examine if household level symptomatic events are related to clustering of gametocytes. We have added a new analysis examining whether or not household gametocytemia or parasitemia is associated with household symptomatic events. As expected, households with higher proportions of children under 5 in the household had significantly more sick visits. However, there was no association between occurrence of clinical malaria and either household levels of gametocytemia or household levels of Pf infection, with or without accounting for spatial autocorrelation.*

Major comments
Ideally it would be useful to identify characteristics that correlate differently with overall infection versus gametocytes. I don't know if we've learned anything specific to gametocytes. If so, this needs to come out more clearly. Likewise for any gametocytes versus many instances of gametocytes or high versus low density gametocytemia – what is interesting is whether there are differences that can help us understand the system.

- *We appreciate this comment and have struggled with the many ways in which our data can be utilized. In this manuscript we have deliberately chosen to focus on the population level distribution of gametocytemia as it relates to targeting interventions. The majority of prior analyses focus on production of gametocyte among the population with infection. However, understanding the distribution in the overall population is needed to understand the potential relative impact of targeting different demographic groups. We agree that additional analyses focused on the biology of infection and investment in gametocyte production is of interest. However, we believe that attempting to cover both of these topics in one manuscript complicates the message. We are conducting more detailed molecular analyses which will enhance forthcoming analyses of the infection-gametocyte system (see Vareta et al Malaria Journal April 2024 and subsequent analyses).*

There is no direct causal relationship between housing construction or socioeconomic status and gametocyte prevalence or density. Although housing construction may be related to infection via exposure to infectious bites via more mosquito-permeable materials, it is preferable to use a more proximal explanatory variable such as frequency of Pf positive asexual episodes, recent asymptomatic/symptomatic episodes, mosquito density, net use, etc...

- *Thank you for this suggestion. We examine net use throughout and, in Table 2, we examine the association between both recent and previous Pf positive asexual episodes as well as recent and previous gametocyte detection on having later gametocyte detection. We have expanded upon these findings in the discussion.*
- *On your suggestion, we have examined whether previous symptomatic malaria was associated with having later gametocyte positive infections. We found, as mentioned in the results, that individuals who have ever had symptomatic malaria are at decreased*

risk of subsequent gametocyte detection (OR = 0.57, 95%CI: 0.35, 0.93). We have added these results to the supplement (Supplemental Table 6).

Pf detection was done from blood extracted from filter paper dried blood spots but gametocyte detection was done from blood preserved in RNA reagent. If gametocyte detection was only done on samples which had a Pf positive DBS, is there a potential for missing information from low density infections given the lower efficiency of extraction from DBS?

- *We agree that there is potential for missing information about gametocytes due to the differential sample/assay sensitivity and the sample selection strategy we used. However, we anticipate that undetected gametocytes are rare, of very low density, and would likely be detected more often in 5-15 year olds based on the distribution of the detected infections. Thus, these undetected gametocyte-containing infections would likely only strengthen the associations we describe.*
- *We added this point to the discussion as follows: Lastly, we only tested for gametocytes in visits that participants had previously tested positive for Pf from dried blood spots. Given the lower sensitivity of DNA detection from dried blood spots compared to RNA detection from whole blood, it is possible we may have missed some low-density Pf infections which were thus never tested for gametocytes, however this would lead to also missing very low density gametocyte-containing infections and is unlikely to substantially bias our results.*

Kids 6-14 have larger proportion of total parasites than they have proportion of total gametocytes. The final paragraph of the results indicates that young children have 40% of the gametocytes despite only representing 18% of the population. Perhaps more attention should be given to the finding of a significant reservoir in this group instead of only focusing on older children?

- *We added emphasis on this finding in the discussion: We additionally found that children under five carried a disproportionate number of gametocytes which were primarily detected at ACD visits, indicating an underappreciated burden of transmission-relevant asymptomatic infections in younger children.*

Fig 6a-d are very difficult to interpret. The labels cannot be read in all the boxes and the choice of variables is difficult to understand. The orientation of the comparisons changes between the panels which makes it impossible to visually identify differences in the distribution between panels. These figures don't contribute to the story.

- *Thank you for this comment, we have attempted to make these figures easier to follow and understand and hope they are now clearer to the reader as we believe they are an important part of the story. These figures (Now Figure 5) indicate the cumulative number of gametocytes detected in the entire population over the entire course of the study period, including all visits. They represent the total load of transmission-relevant parasites in the population longitudinally, and allow us to see how each group may in*

theory contribute to transmission over time, instead of relying on a single time point to examine the distribution of gametocytes. These figures capture both the frequency of visits, the density of gametocyte-containing infections, and the repeated detections of gametocytes in all individuals.

The observation that “39% of individuals with multiple gametocyte-positive visits having high-density infections vs 8% of individuals with only a single gametocyte-positive infection ($p<0.001$)” could be biased by missingness. The more observations you have about an individual, the more likely they are to have the characteristic you are testing for. Also, this information is not clear in Table 1 as indicated.

- *We appreciate this comment and have repeated this analysis using logistic regression while adjusting for the number of visits a participant had in the study. We have updated the text to reflect the change: These high gametocyte density infections were clustered among individuals who had multiple gametocyte-positive visits, with 39% of individuals with multiple gametocyte-positive visits having high-density infections vs 8% of individuals with only a single gametocyte-positive infection (aOR = 6.7, 95% CI = 3.1, 16.2) after adjusting for number of study visits.*

Is the statement ‘limiting their [PCD] contribution to the infectious reservoir’ well supported by the data? They may be rare, but the data presented here shows they more frequently harbored gametocytes and at higher density. It is not possible to measure their contribution to transmission. Based on the results of this study, I think it has to stop at comparing these two types of infections in terms of frequency and density. In the discussion, it could be speculated that they may not make a major contribution to the reservoir, but this is not directly supported by the results of the current study.

- *Again, thank you for this comment which we believe has two distinct components. One is terminology regarding contribution to the ‘infectious reservoir’ vs the frequency and density of gametocyte-containing infections. Here we agree that the data we are presenting is on gametocytes and not contribution transmission. We have rephrased our statement to clarify this. However, the second point on the relative contribution of infections detected at passive case detection, we respectfully disagree, looking at the total gametocytes in the population over the complete study which incorporates regular active follow-up and the natural occurrence of episodes of symptomatic infections captured at PCD (Figure 5), only 6% of gametocytes were detected at symptomatic sick visits, indicating clearly that gametocyte clearance via treatment of symptomatic episodes seeking treatment would clear a negligible fraction of gametocytes in the population. We understand that Figure 5 was not well designed previously which may have contributed to this confusion, but hopefully the improved Figure gets the message across better.*

Fig 4 is not very useful for illustrating the point about household clustering since these are

intentionally selected households with specific profiles. It is not easy to know how to extrapolate this to the rest of the households. I suggest finding a different way to visualize the household clustering.

- *We acknowledge your point and have made a new figure (Figure 2) to illustrate household clustering, hopefully this is a clearer visualization.*

Something is wrong with the x-axis of Fig 2. It goes from Jan to Nov but May is missing. However the text states that the study was from April 2019 to May 2020 so either the labeling is incorrect or the months are not presented in chronological order?

- *We have removed this figure and are now using a single new figure (Figure 2) to show both individual and household clustering.*

Minor comments
Use age range instead of 'school age' or 'SAC' especially in tables and figures.

- *This has been fixed in all tables and figures.*

Avoid using asymptomatic, chronic and low density interchangeably. Although infections are often all three of those, they are very different characteristics.

- *We reviewed our use of these terms to ensure we are using precisely.*

Use confidence intervals instead of p values in the text. Gives a more comprehensive understanding of the estimate than a p-value.

- *The authors agree with the reviewer's sentiment and have replaced p-values with regression coefficients and 95% CIs where relevant. The p-values that remain in the text are from chi-squared tests, where confidence intervals would not be relevant.*

Reviewer #2 (Remarks to the Author):

The authors main observation is that school-going children harbor higher densities of gametocytes and hence are primary source of human-to-mosquito infection for transmission of malaria. They are also advocating for focus primary prevention of control and prevention measure for malaria on this group. While the observation is novel as this has been shown before, their study complements the similar observation and also present new methodologies that account for possible confounding effects for other variables.
The study is convincing and clear and so is the methodology.
The statistical methods described seems to suitable and mostly the work can be reproducible.

- *The authors thank you for your review and comments.*

Dear Reviewer,

Thank you for your careful and thoughtful comments. We agree with you that there are many approaches to analyzing this robust, observational dataset. However, we also recognize that it is not possible to include them all in a single manuscript. We have done our best to include your suggestions, but maintain a clear focus in our analyses. We hope that you will find this new version suitable for publication. Our specific responses to your remarks are below:

Reviewer #1 (Remarks to the Author):

My comments refer to the rebuttal letter

Comment 2 regarding longitudinal data
- Yes, they present incidence rate ratios, but I was thinking more of using the longitudinal data to look at continuous gametocyte carriage over time versus sporadic gametocyte detection,

- *We present this visually in Figure 2 – in order to further explore this topic, we have additionally added the distribution of sequential visits to Table 1 and added some text to indicate how frequently gametocytes are detected at sequential visits and for how long, on average,: "and 91/216 (42%) had gametocytes detected at sequential visits with a mean of 2.6 gametocyte-positive visits per person, a maximum of 10 gametocyte-positive observations for any one individual and a maximum duration of persistent gametocyte detection of 355 days (Median: 64 days, IQR: 34 – 116 days) with individuals in Ntaja having significantly longer duration of sequential gametocyte detection (p=0.04), but no other variables were associated with differences in duration"*

relationship between continuous asexual infection and gametocyte production, relationship between clinical episodes, treatment and gametocyte detection etc...

- *Analyses addressing the relationship between clinical episodes and gametocyte detection were previously included in the supplement but are now moved to the main text of the results in table 2. While it is somewhat unclear exactly what you would like to see, we additionally attempted to examine the relationship between timing of asexual detection and gametocyte detection: Among the 216 individuals with gametocytes detected, 128 (59%) had gametocytes detected on the same date as their first detected Pf. infection, with a mean of 56 days and a maximum of 346 days between first Pf. detection and subsequent gametocyte detection, with no difference by age, symptoms, or any other characteristics.*

- Table 2 doesn't tell us anything about the time interval between detection and 'previously detected Pf parasitemia'.

  - *Thank you for this comment – we have calculated the mean time interval between Pf parasitemia detection and subsequent gametocyte detection by age and whether or not the Pf infection was symptomatic. "Among the 216 individuals with gametocytes detected, 128 (59%) had gametocytes detected on the same date as their first detected Pf. infection, with a mean of 56 days and a maximum of 346 days between first Pf. detection and subsequent gametocyte detection, with no difference by age or any other characteristics. "*

- For table 4, this would be more interesting to look at whether the previous event was symptomatic before the event of detecting gametocytes. I don't think gametocytes are predictive of whether you will develop clinical symptoms but studies have shown that clinical symptoms are associated with lower gametocyte production. This is in supplemental table 6 but this seems more interesting to me than the Table 4 results.

  - *Thank you for this assessment, we have moved the data from supplemental Table 6 to the main results into Table 2.*

- The interesting questions to answer longitudinally are 1) how many months after an individual is first infected do they start producing gametocytes?

    - *Thank you for this question, we have calculated the time between first Pf detection and gametocyte detection. As most individuals with Pf infection do not ever have gametocytes detected, this analysis was restricted to individuals with gametoctyes. "Among the 216 individuals with gametocytes detected, 128 (59%) had gametocytes detected on the same date as their first detected Pf. infection, with a mean of 56 days and a maximum of 346 days between first Pf. detection and subsequent gametocyte detection, with no difference by age or any other characteristics. "*

2) do they produce gametocytes after a symptomatic episode/treatment?

  - *This was in the supplement but has been moved to the results and is now in table 2.*

3) Is gametocyte density proportional to lagged parasite density or does gametocyte density become independent of parasite density in very long-lived infections?

- *We thank you for this suggestion, it is unfortunately unclear how you want us to explore this question. Note that the vast majority of parasitemia episodes are not associated with any gametocyte detection and, likewise, lagged parasitemia for a given gametocyte detection is extremely likely to be zero. It is thus challenging to understand what the reviewer is asking for here.*

4) Do individuals with long-lived infections have intervals with and without gametocytes or is gametocyte production constant at the infection level? Maybe these questions are too detailed and move away from a population-level description of gametocyte production.

- *It is not clear how we would analyze this; it would be purely descriptive of a few observed individuals. As the reviewer notes, this moves away from a population-level assessment of gametocyte carriage. Thus, we have not added additional analyses to address this/these questions.*

Major comment 1
- I wasn't suggesting that they delve into the biology of gametocyte production and transmission. Rather, if they are trying to characterize the population in order to enhance targeting of interventions, then three questions are relevant here 1) who are the individuals with gametocytes, 2) what type of gametocyte producer is most important to target (ie the frequent but low density versus the infrequent but high density) and 3) can we differentiate that type of producer based on observable characteristics. Even if the question of 'what type of gametocyte producer is most important' is too far into the transmission biology, some justification of why they are characterizing subpopulations of gametocytemic infections and whether observable characteristics (age, gender, household type, infection type) can help us target them is warranted.

- *Thank you, we agree that these are the relevant questions and also that this paper can't answer question 2 (what type of gametocyte producer is most important to target?). The answer to this question comes from which gametocyte producers (both infection and host characteristics) are most infectious to mosquitos. What we know about this is primarily that infectiousness to mosquitos is driven by gametocyte density. Thus, in this paper, we look at predictors of ever*

having gametocytes, ever having high density gametocytes, incidence of any gametocytes, incidence of gametocytes, and total gametocyte burden (Figure 5 represents the summation of gametocytes over time getting at some of the reviewers questions on multiple visits with low density gametocytemia vs a single episode of high density gametocytemias). Without the ability to apply a metric of infectiousness to these infections (such a metric isn't available in the literature), we can't determine their relative contribution to transmission. Thus, in this paper, we are assessing patterns of predictors of each of these outcomes to determine the observable characteristics that help us target interventions to reduce gametocyte carriage at a population level.

-

Figure 5

- These are still very cumbersome and hard to interpret visually especially since comparison between the panels is the only way to understand the ACD/PCD dimension. I would suggest removing SES and having ACD and PCD on the same panel giving only 2 panels – total gametocytes and total parasites.

- *Thank you for this suggestion, we have removed the SES variable to simplify.*
- *All data from ACD and PCD are currently shown on the same panels (Figure 5b and 5d) – Panels 5a and 5c are included in order to show the gametocytes of net users versus non-net users. Panels 5a and 5c are restricted to ACD visits as net use was not asked about during PCD visits.*

I suggest avoiding such statements as 'largest and most comprehensive study'. Qualify with 'one of the ...'

- *Thank you for this comment, this has been fixed.*

Dear Dr. Walker,

Thank you for your helpful review and comments, and particularly, thank you for meeting with me to discuss your comments as it was extremely helpful. Hopefully we have been able to successfully address your concerns. We have included responses to your comments below, in italics:

Reviewer Comments:

For others (particularly spatial analyses) I think solving them would be quite complex analytically. For those, I recommended that the authors scale the complexity back and instead just let the visualisations of the data speak for themselves and have provided suggestions for those which, to me, would still make the paper highly valuable to the readership of nature communications medicine.

- *We appreciate you comment and agree, we have removed the spatial analysis.*

Major issues:
Dealing with differential sampling cadence. First reframe the estimand from “probability of at least one positive during follow-up” to the per-sample (per-visit) probability of gametocyte detection. Take the visit-level logistic regression and translate the odds to probabilities for each stratum. These predicted probabilities can be multiplied by the demographic weights of each stratum to yield unbiased contributions (i.e., contribution = per-visit probability × population share). NB: Given repeated measures and observed over-dispersion, I recommend adding at least a household random intercept (and, if feasible, an individual random intercept). This can also then be used to predict/augment the missing data should the authors feel like going an extra step but I think for overall trends it isn’t necessary.

- *Thank you! This is a great suggestion, we have added this estimate to Table 1.*

Duration of gametocyte-detection
This seems to be based on the durations where individuals were gametocytaemic over two sequential visits? I don’t think one this can really hold as individuals can be readily infected again between visits and regardless would be subject to biases associated with testing frequency (i.e. monthly visits will tend to produce monthly durations, if this weekly it would be often much shorter etc.).

- *Thank you for your comment, we agree with the points you have made and have decided to remove the duration analysis as we do not have appropriate data to address this question correctly.*

Issues with odds of detection over time and PCD vs ACD contribution.
I think this possibly comes from a misinterpretation of what the previous reviewer but I don't think this approach is valid, at least for ACD. With scheduled monthly ACD, detection only occurs at visits, not continuously, so dividing "positive counts" by calendar time treats unsampled months as if they were observed. This metric also turns a single persistent infection into multiple "incidents" across months. In short, it mixes visit schedule, missed visits, and persistence with biology and is hard to interpret so I would just frame everything to do with ACD around per-visit probability – one can include a fixed effect for month to tease apart temporal dynamics.

- *You makes a good point here, we have redone the incidence rate analysis in Figure 3 and recalculated incidence rates using the number of participant visits instead of the participant follow-up time.*

I also agree with the reviewer that the PCD and the ACD can't be compared in the way it has been. Instead, the authors could compare the prevalence by ACD (estimated using the above) to the cumulative incidence as a % of population in a given month which would likely give them something quite cool to talk about in terms of seasonal contribution. I agree with the reviewer about the need to caveat (or adjust) this based upon diverging gct density between ACD and PCD cases (see comment about the 'transmissibility metrics').

- *We see the problem and have removed the ACD/PCD comparison from Figure 5. We have created a new figure (Figure 6) that compares the number of gametocytes detected at an individuals average seasonal ACD or PCD visit, thus normalizing for the frequent repeated ACD visits and the difference in number of ACD visits between individuals.*

Spatial and temporal distribution
I agree with the reviewer that the nature of the data collection where things are samples in clusters makes this is a difficult dataset to apply standard spatial analyses to and I don't think the existing approach would stand much scrutiny from a geostatistician. I do think however, that this is a really cool dataset so one can get pretty far by just letting the data do the talking. I think a map showing gametocytaemia -or related metrics seem - for each study month would be really cool to see (potentially showing the PCD case counts somehow to see how asymptomatic dominates dry season?). I actually think Figure 4 is a really nice visualisation! However, I can see that if the spatial sampling framework isn't incorporated this would make it hard to interpret. Can you not just have a column for each of the 16 clusters? (or maybe this is what this is already and you've

just missed a label?). I also think a really useful figure (at least for my work!) for each cluster would be https://en.wikipedia.org/wiki/Pareto_chart (i.e. rank each individual in descending order of contribution to infectivity (preferably with augmented data from the regression above so each individual has an observation each month)

- *Thank you for these comments, we agree with them and have tried to adapt accordingly. We have removed the spatial analysis and added columns for sample clusters in Figure 2 to indicate the spatial clustering of household gametocytemia.*

Weighting infectivity
There is a somewhat arbitrary ranking of gametocyte density why not just use one of the well-defined relationships in the literature to convert into prob of transmission given fed upon from membrane feeding assays? Obviously comes with own set of assumptions but at least empirically derived.. e.g. https://elifesciences.org/articles/34463#fig2sdata2?

- *Thank you for the comment, we have used thresholds associated with the probability of transmission from "Churcher, T.S., et al., Predicting mosquito infection from Plasmodium falciparum gametocyte density and estimating the reservoir of infection. Elife, 2013.* ***2****: p. e00626". As we discussed with you, we reviewed in detail the calculations underlying the curves presented in the more recent manuscript you suggest. However, this manuscript uses a location specific term for interpreting the gametocyte sex ratio relationships with infectivity. The location specific term is not available for Malawi and the transmission setting differs from the locations in the manuscript.*

Minor issues:
"Prior cross-sectional surveys have compared gametocyte prevalence and density by age group with symptom status [17], but have not quantified the contribution of each group to the overall population-level transmission." – I might be missing some nuance here but I don't think it's first study to assess contribution to transmission longitudinally and it also arguably doesn't assess transmissibility as comprehensively as other CX studies using feeding assays. I think the topic is of sufficient interest regardless so suggest the authors be a bit more careful to say something that can't be argued with - it's possibly the largest?? Personally, I'd be more comfortable with one of the first? – e.g. Andolina 2021: https://www.thelancet.com/journals/laninf/article/PIIS1473-3099(21)00072-4/fulltext

- *We cite Andolina et al. We have edited this sentence to say, "Prior cross-sectional surveys have compared gametocyte prevalence and density by age group with symptom*

status [17], but few have quantified the contribution of each group to the overall population-level transmission."