Current estimates of malaria basic reproduction number underestimate parasite transmission efficiency due to multiple blood feeding

W. Robert Shaw[1*], Inga E. Holmdahl[2,3*], Maurice A. Itoe[1], Kristine Werling[1], Meghan Marquette[1], Douglas G. Paton[1], Naresh Singh[1], Caroline O. Buckee[2,3], Lauren M. Childs[4†], Flaminia Catteruccia[1†]

[1]Department of Immunology and Infectious Diseases, Harvard T.H. Chan School of Public Health, Boston, MA 02115, USA

[2]Department of Epidemiology, Harvard T.H. Chan School of Public Health, Boston, MA 02115, USA

[3]Center for Communicable Disease Dynamics, Harvard T.H. Chan School of Public Health, Boston, MA 02115, USA

[4]Department of Mathematics, Virginia Tech, Blacksburg, VA 24061, USA

*Equal contribution

[†]Correspondence: lchilds@vt.edu, +1-540-231-8265; fcatter@hsph.harvard.edu, +1-617-432-1773

Abstract

Background: Despite significant control efforts based on the large-scale distribution of insecticide treated nets, malaria cases have not declined in recent years. Mathematical models are used to predict the impact of these and other control interventions, but complex mosquito behaviors—such as repeated blood feedings—are generally not incorporated. Here, we examine how malaria transmission dynamics are impacted when infected mosquitoes feed a second time, as is typical in natural field settings.

Methods: We performed sporozoite time course analyses to measure the *Plasmodium falciparum* extrinsic incubation period (EIP) in *Anopheles gambiae* females blood fed once or twice. We incorporated these findings into a model of the basic reproduction number R_0, a measure of transmission potential, using current population and monthly mean temperature data across sub-Saharan Africa.

Findings: A second blood feed drastically shortens the EIP and increases the average R_0 by 12.2% (range: 10.2%-56.5%), impacting hundreds of millions of people. Moreover, parasite growth is further accelerated in mosquitoes with reduced reproductive capacity, which mimic genetic modifications currently proposed in population suppression gene drives.

Interpretation: Our findings suggest that transmission efficiency is systematically underestimated in malaria models, particularly in elimination settings. Due to a shorter EIP, younger mosquitoes and mosquitoes with reduced reproductive ability may provide a larger contribution to infection, with important consequences for insecticide-based interventions and gene drive strategies. These findings call for an urgent reassessment of current and future control campaigns.

Funding: Howard Hughes Medical Institute, Bill & Melinda Gates Foundation; National Institutes of Health; National Science Foundation.

Introduction

Malaria remains a devastating disease for tropical and subtropical regions, accounting for an estimated 405,000 deaths and 228 million cases in 2018 [1]. *Anopheles* mosquitoes transmit the causative *Plasmodium* malaria parasites, and vector control through long-lasting insecticide-treated bed nets (LLINs) and indoor residual spraying (IRS) has greatly decreased the malaria burden in recent decades.

Since the early 20th century, epidemiological models of malaria have been key to understanding which aspects of the transmission cycle should be targeted by malaria control to provide the most benefit [2,3]. Insecticidal interventions like LLINs are particularly effective in these models because they shorten the average lifespan of the mosquito, as well as lowering mosquito numbers and biting rates. As parasites require a significant amount of time to develop from sexual stages in the blood meal into infectious sporozoites in the salivary glands (a time period known as the extrinsic incubation period or EIP), decreasing mosquito survival curtails onward transmission to humans. The relationship between the EIP and the lifespan of the mosquito is therefore a key driver of malaria.

In most epidemiological studies the *Plasmodium falciparum* EIP is considered to last 12–14 days as determined through experimental laboratory infections [4]. This is similar to the expected lifespan of the *Anopheles* female, which although difficult to precisely measure due to the lack of reliable age markers, has been shown to be approximately 2-3 weeks [5,6]. When considered in the context of the basic reproduction number (R_0)—the average number of infections resulting from a first case [3]—the EIP has an exponential relationship with transmission, and small changes in this parameter have large effects on malaria [7]. Furthermore, the relationship between the mosquito mortality rate and the parasite EIP is such that changes in one can be compensated by changes in the other. Thus, shortened lifespan can be offset by faster parasite development and result in the same transmission potential.

The biological factors that influence parasite developmental rates are largely unknown. The EIP is dependent on environmental temperature and larval nutrition, with higher temperatures (up to a point) and plentiful nutrients available during larval stages accelerating parasite growth [8]. Our recent studies in *Anopheles gambiae* have shown the *P. falciparum* EIP is also dependent on mosquito oogenesis, a process largely orchestrated by the steroid hormone 20-hydroxyecdysone (20E) [9]. Parasite growth is faster in females with impaired oogenesis, detected by the presence of larger oocysts in the midgut and of infectious sporozoites in the salivary glands at earlier time points, reducing the EIP. Accelerated growth rates are likely mediated by nutrients available after blood feeding [10] that become more abundant when egg development is impaired [9].

Given this unexpected relationship between oogenesis and the *P. falciparum* EIP, the number of reproductive cycles a female mosquito completes may have important consequences for parasite transmission. There is a wealth of literature showing that many mosquito species, including *An. gambiae*, undergo multiple reproductive cycles of blood feeding, egg development and egg laying in their lifespan, which can be counted by ovarian dilatations [11,12]. Moreover, multiple feeds may

be required even within a single reproductive cycle due to interrupted feeding or to nutrient deprivation during the larval stage (pre-gravid behavior) [13]. Additional blood meals may therefore potentially influence oocyst growth and the EIP, as suggested by reports showing an additional feed can boost *P. falciparum* oocyst size [14] and salivary gland sporozoite numbers at a given time point [15].

Here, we examine how the parasite EIP is affected by multiple feedings, and the consequences of these effects in an epidemiological model of malaria transmission. By providing a second uninfected blood meal to females previously infected with *P. falciparum*, we show a striking boost in oocyst size and a substantially shortened EIP. A model-derived R_0 equation parameterized using these data predicts a sizeable underestimate of current malaria transmission potential across sub-Saharan Africa, with over 208 million people living in areas where multiple blood feeding raises average R_0 estimates by ≥15%. The speed of parasite development is further enhanced by a second feeding event in reproduction-defective females, suggesting that mosquito control strategies that reduce the reproductive output of *Anopheles* females—such as population suppression gene drives or chemical sterilants—could actually lead to increased parasite transmission. These data have important implications for generating accurate epidemiological models of malaria transmission and for better estimating the true impact of current and future mosquito control measures.

Methods

Rearing of Anopheles gambiae *mosquitoes*

Anopheles gambiae mosquitoes (wild-type G3 and transgenic strains) were reared in cages at 27°C, 70–80% humidity on a 12 h light:12 h dark cycle. Adults in colony cages were fed on 10% glucose solution *ad libitum* and weekly on human blood (Research Blood Components, Boston, MA). Males and females were mated in large cages.

Plasmodium falciparum (NF54 strain) was cultured as in [9] and is used under the permissions of a material transfer agreement from the laboratory of Carolina Barillas-Mury, National Institutes of Health, Bethesda, MD, USA.

P. falciparum *infections of* An. gambiae *mosquitoes*

Cages of mated female mosquitoes aged 4–6 days (d) were blood fed on ~320 µl *P. falciparum* culture for 30–60 min via heated membrane feeders and introduced into a custom-built glove box (Inert Technology, Amesbury MA). Feeding behavior was encouraged by starving mosquitoes of 10% glucose solution for 24 h and females not fully engorged were removed. Blood-fed mosquitoes were provided 10% glucose solution for 48 h and then given an oviposition site. Females were fed a second time 3 days after the initial infectious blood meal, using uninfected blood. Blood intake at the second blood meal was encouraged by providing an oviposition site 2 d post infectious blood meal (pIBM) and non-blood fed mosquitoes were removed. Females blood-fed twice and control mosquitoes fed once were provided 10% glucose solution until dissection. At dissection time points, mosquitoes were aspirated into 80% ethanol and transferred to 1X phosphate-buffered saline (PBS) (oocyst stages) or aspirated into ice-cold PBS

(sporozoites stages) and beheaded. At least 3 biological replicates of each infection were performed.

Oocyst counts and measurements: At 7 d pIBM, midguts were stained directly in 2 mM mercurochrome (Sigma-Aldrich, St. Louis, MO) for 12 min. Mercurochrome-stained midguts were imaged at 100X on an Olympus Inverted CKX41 microscope, and oocysts were counted and measured using scaled images in FIJI [16]. Burst oocysts were counted but excluded from oocyst size analysis. Mean oocyst size was calculated for each midgut to avoid pseudoreplication.

Oocyst staining: At 8 and 10 d pIBM, midguts were fixed in 4% formaldehyde for 30–40 min, permeabilized and blocked for 1 h in PBS, 0.1% Triton (PBS-T), 1% bovine serum albumin (BSA) at 22˚C, and stained with an anti-CSP mouse monoclonal 2A10 (BEI Resources)(1:350), followed by a goat anti-mouse-Alexa 488 secondary antibody (Molecular Probes)(1:1000). Samples were washed in PBS-T, stained with DAPI (1 µg/ml) and mounted in Vectashield with DAPI (Vector Laboratories, Burlingame, CA), and then imaged at 630X on a Zeiss Inverted Observer Z1 with Apotome2. Scaled images were processed in FIJI.

Sporozoite counts: Mosquitoes were decapitated and the salivary glands of individual females were collected in a small volume of PBS and disrupted using a handheld disposable pestle. Released sporozoites were spun at 8000 *g* for 10 min at 4˚C and resuspended in a known volume of PBS. Sporozoites in 0.1 µl were counted using a disposable hemocytometer at 200X magnification on an Olympus Inverted CKX41 microscope with phase-contrast microscopy and sporozoite totals for each mosquito were calculated.

Generation of Δzpg females

Zpg and Cas9-carrying mosquito strains were generated previously [9]. Zpg/Cas9 mutant females, hereafter Δ*zpg* mutants, were the F1 progeny of Zpg and Cas9 transgene homozygotes. Zpg controls and Δ*zpg* mutant females did not differ in their ability to take a blood meal.

Statistical analysis

Experimental data were analyzed using JMP 14 Pro statistical software and GraphPad Prism 8.0. JMP 14 Pro was used to construct statistical models to account for variation due to multiple factors in an experiment. Depending on the distribution of the data, a Generalized Linear Model (GLM) (Poisson) or a Standard Least Squares (SLS) Model (Normal) was used and non-significant interaction terms ($p>0.05$) were removed where possible and models with minimal AICc scores and their effect test outputs are reported (Tables S1 and S2). Graphpad Prism 8.0 was used to calculate logistic regression curves and Fisher's exact and χ^2 tests (Tables S3 and S4).

Modeling

We calculated the basic reproductive number, R_0, as a function of temperature and population as in [17] using their equation (2) reproduced here as

$$R_0(T) = \sqrt{\frac{a(T)^2 bc(T) e^{-\frac{\mu(T)}{PDR(T)}} EFD(T) p_{EA}(T) MDR(T)}{Nr\mu^3(T)}}.$$

The expression is based on temperature-dependent trait data from *Anopheles* species that vary with temperature including biting rate (a), vector competence (bc), adult mosquito mortality (μ), parasite development rate (PDR), egg-to-adult survival probability (p_{EA}), mosquito development rate (MDR) and eggs laid per female per day (EFD). Note that adult mortality is calculated through daily adult survival (p) via $p = e^{-\mu}$; parasite development rate is one over the extrinsic incubation period (EIP); and mosquito development rate is one over the larval development time (τ_{EA}). Additionally, human related quantities—human density (N) and recovery rate (r)—are not temperature-dependent. For computation we set r as 1/200 but this does not impact the final ratios as it cancels out. The temperature-dependent traits of biting rate, parasite development rate and mosquito development time are fit to Briere functions, represented by

$$cT(T - T_0)(T_m - T)^{\frac{1}{2}},$$

where c, T_0 and T_m are constants defined in [17]. The temperature-dependent traits vector competence, daily adult survival, egg-to-adult survival probability and eggs laid per female are fit to quadratic functions, represented by

$$qT^2 + rT + s,$$

where q, r, and s are constants defined in [17].

We then modified the basic reproductive number to incorporate a second blood feeding with reduced EIP, which we refer to as R_0^b given by

$$R_0^b(T) = \sqrt{\frac{a(T)^2 bc(T) e^{-\beta \frac{\mu(T)}{PDR(T)}} EFD(T) p_{EA}(T) MDR(T)}{Nr\mu^3(T)}},$$

where we scale EIP using the term β. We determine β as the relative reduction in EIP in the presence of a second blood feed: (β=8.63 (2BF)/10.88 (1BF)=0.793. We then consider the ratio of the modified R_0 to the original R_0 as

$$\frac{R_0^b}{R_0}.$$

To estimate the impact of the EIP reduction on transmission potential, we applied these R_0 functions to current population and monthly mean temperature data across Africa to estimate the percent change in R_0 under a scenario where mosquitoes blood feed while infected. We incorporated the distribution of the population to quantify the number of people living in regions experiencing particularly high changes in transmission estimates.

Data

We used spatial raster files from multiple sources to map the change in R_0 across the continent of Africa. We obtained global monthly mean temperatures for the period 1970–2000 from WorldClim 2.0 [18]. We restricted analysis to the area of Africa where the predicted probability of *An. gambiae* and closely related species (*An. gambiae* complex) is greater than 5%, using predicted *Anopheles* distribution maps from the Malaria Atlas Project [19], under the assumption that the observed effects on the EIP are consistent in these species. Spatial human population

distribution for 2015 was obtained from WorldPop [20]. Monthly mean temperature and human population data were aggregated with bilinear resampling to match the projection of the *Anopheles* geographic extent data at a 5x5 km resolution.

R_0 projections

Using these data, we calculated R_0 and R_0^b for each 5x5 km grid cell for each month. We set a lower bound for human population of N=250 to avoid exponential growth of R_0 that occurs at very low population densities (**figure S1**). For areas with very low transmission, large ratio changes result from small absolute changes so we excluded data from months where the temperature is too high or too low to sustain transmission—i.e. $R_0^b < 1$—from both the maps and the following summary calculations. For each month, we took the ratio R_0^b/R_0 to show the monthly change in transmission potential under a multiple blood feeding scenario (**figure S2**). To estimate the maximum and minimum change, we selected the highest and lowest ratio observed for each location. To estimate the average impact, we took the mean of the monthly R_0 and R_0^b estimates over the year, excluding months where $R_0^b < 1$, and examined the ratio of the means.

Results

An additional blood meal accelerates *P. falciparum* development

To determine whether additional blood feeding events have an effect on parasite development, we fed *An. gambiae* females on a *P. falciparum* (NF54) culture, and at 3 days post infectious blood meal (pIBM) we provided them with a second, uninfected blood meal. A control group was instead maintained on sugar after the initial infectious feed (**figure 1A**). We dissected females 4 days later (7 d pIBM), and analyzed oocyst numbers and size in both groups. While we detected no effects on the prevalence or intensity of infection (**figure 1B**, prevalence shown in pie charts), we observed a striking increase in oocyst size in females that had been blood fed a second time (**figure 1C**, **figure 2A, B**). Mean oocyst size was 81% larger in doubly fed females (standard least squares (SLS) model: p<0.0001; #BF: p<0.0001, Table S1), suggesting increased growth rates following an additional blood meal. Moreover, these oocysts already showed increased DNA content (**figure 2A, B**) and release of mature sporozoites at an early time point (10 d pIBM)(**figure 2C**), by immunofluorescence microscopy. Together these data show that *P. falciparum* development is accelerated when females blood feed a second time.

Mosquitoes are infectious sooner following an additional blood meal

We next examined the effect of a second blood meal on the appearance of sporozoites, the infectious stage of parasite development, in the salivary glands. Following a similar protocol of a second blood meal at 3 d pIBM, we detected sporozoites as early as 7 d pIBM, and we observed a significant increase in sporozoite prevalence at early time points after infection (8 and 10 d pIBM) when most of the control females have no sporozoites in their glands (**figure 3A**). Doubly-fed mosquitoes were 15.0-fold and 7.7-fold, respectively, more likely to have sporozoites in their salivary glands at these time points (Fisher's exact test d8:p=0.0026; d10:p<0.0001). Moreover, the intensity of infection was also increased at 10 d pIBM (generalized linear model (GLM): p=0.0290, #BF:p=0.0085, Table S1)(**figure 3B**). These observations were not due to a change in

the prevalence or intensity of oocyst infection, which were both unaffected as described above (**figure 1B**). By 14 d pIBM singly and doubly-fed mosquitoes became equally infected (**figure 3A, B**), with sporozoite prevalence near 100% and no difference in infection intensity (GLM: p=0.0258, #BF:p=0.4032, Table S1).

Based on the above prevalence data we constructed EIP curves using logistic regression to describe how the infectious population changes over time, and calculated the EIP_{50}—the median time to infectiousness—for each of our treatment groups. A second blood feeding significantly shortened the $EIP_{50,}$ with mosquitoes becoming infectious 2.25 days earlier than females fed a single time (Z test: p<0.0001), corresponding to a 20.7% reduction (**figure 3C**). Given mosquitoes regularly take multiple (>3) blood meals in the field [5], this result infers that extensive transmission occurs much sooner and is mediated by considerably younger mosquitoes than previously thought, forcing a reevaluation of malaria transmission dynamics.

Modelled estimates of R_0 with a single blood meal underestimate transmission

Using these data, we mapped the consequence of a second blood meal on the distribution of R_0 across the continent of Africa, considering all locations where *An. gambiae* and closely related *Anopheles* vectors are present (as described in the methods). We chose a simple metric of transmission potential—the basic reproductive number R_0, calculated as a function of temperature and population as in [17]—to investigate the impact of revised estimates of the EIP on malaria transmission potential. For each 5x5 km grid cell of the map, we calculated both the standard R_0 using EIP_{50} estimates derived from a single blood feed [17] and the adjusted R_0 using the proportional reduction in EIP_{50} observed after two blood feeds in our experiments (20.7%). We then mapped the ratio of the adjusted to standard R_0 in each grid cell across Africa for each month of the year when there is active transmission.

The reduction in EIP_{50} leads to higher mean R_0 throughout the year, with an average increase of 12.2% across Africa (**figure 4A,** center panel). When analyzed as individual months, the greatest increase in R_0 ranges from 10.2–56.5%, depending on geographic location (**figure 4A**, right panel). Here, the largest proportional increases in R_0 are found where estimated transmission is lower, such as Southern Africa. Importantly, there is a minimum 10.2% rise in all mapped locations (**figure 4A,** left panel). Together, these results imply a significant underestimate of malaria transmission potential across the continent by current models.

To determine how many people are impacted by the change in transmission potential, we plotted how the R_0 increase is distributed across the human population considering the minimum, mean, and maximum mapped scenarios. For 21.6% of the population—equivalent to 208 million people—revising the EIP_{50} increases the average transmission potential by at least 15% (**figure 4B**, mean curve). The entire mapped population, over 950 million people, experiences at least a 10.2% increase in R_0 during periods of transmission (**figure 4B**, minimum curve). Furthermore, a quarter of the population experiences at least a 25% higher R_0 during one month of the year (**figure 4B**, maximum curve).

Effects of an additional blood meal are exacerbated in females with impaired egg development

As mosquito reproduction is a target of genetic and chemical control strategies currently in the pipeline [21-23], and we had previously shown that the parasite EIP is substantially shorter in all

instances where oogenesis is reduced [9], we went on to determine whether parasite growth could be further boosted after a second blood meal in females with impaired egg development. To this aim, we used genetically modified *An. gambiae* mutants that are mosaics of *zero population growth* (Δ*zpg*), a gene required for germline cell maintenance and therefore egg development [24]. After infecting Δ*zpg* or control females with a *P. falciparum* culture, we provided an additional blood meal to approximately half of each group to compare parasite development in both feeding regimes, as done above. Mean oocyst size was increased in Δ*zpg* females fed either once or twice when compared to controls (SLS model: $p<0.0001$; #BF: $p<0.0001$, genotype: $p<0.0001$, Table S1)(**figure 5A**), demonstrating that *P. falciparum* growth can be further boosted by a second blood meal in this background. Moreover, despite lower oocyst numbers (GLM: $p<0.0001$; genotype: $p<0.0001$, Table S1)(**figure 5B**), oocyst prevalence was not affected in Δ*zpg* mutants (**figure 5B** pie charts). Sporozoite intensity in the salivary glands at an early time point (10 d pIBM) was higher in both feeding regimes (GLM: $p<0.0001$; #BF: $p<0.0001$, genotype: $p=0.0013$, #BF x genotype: $p=0.0407$, Tables S1 and S2)(**figure 5C**). Although sporozoite prevalence at 10 d pIBM was only significantly higher in Δ*zpg* females fed once (**figure 5C** upper pie charts), cumulative prevalence across both single and double blood feedings was significantly increased (Fisher's exact test: $p<0.0001$) and these mutants were 3.9-fold more likely to be infected than control mosquitoes (odds ratio; 95% CI: 2.3–6.5 fold)(**figure 5C** lower pie charts). These results suggest that, even in cases where they may support lower oocyst loads, females with impaired egg development would be more effective at transmitting *P. falciparum* parasites under either blood feeding regime.

Discussion

The parasite EIP is a key parameter in malaria transmission dynamics. Given the relatively short *Anopheles* lifespan—estimated to be around 2–3 weeks depending on species and environmental conditions [5,6]—parasites with faster sporogonic development are more likely to be transmitted to the next human host [4]. Here, we demonstrate that multiple blood feedings significantly accelerate parasite development within the mosquito, shortening the time required for sporozoites to appear in the salivary glands. These findings are consistent with studies showing that a second blood meal increases sporozoite intensities [15,25,26], and with the recent observation of larger oocysts in females fed twice reported in *An. gambiae* infected with *P. falciparum* [14]. Our study provides a comprehensive assessment of the effects of a second blood meal on parasite developmental rates, conclusively linking additional blood meals to a shortened EIP.

Incorporating these results into a simple model shows that malaria transmission potential, estimated as R_0, may be significantly greater than previously appreciated. Indeed, with a scenario of multiple blood feeding resulting in a shorter EIP than with standard estimates, R_0 increases by a minimum of 10.2% across Africa, and up to 56.5% in specific locations. These are substantial effects, with hundreds of millions of people potentially impacted by these findings. The neglected contribution of multiple blood feeding may partly explain high malaria burdens in places and seasons when expected transmission potential is low [27]. Further, the most significant changes to modelled R_0 are in regions with relatively low transmission including Southern Africa, suggesting that malaria elimination efforts in these settings may be more challenging than anticipated.

Another key implication of a shortened EIP is that previous R_0 calculations may have overestimated the relevance of other parameters of malaria transmission, particularly daily mosquito survival. This is relevant for interventions such as LLINs, which impact standard R_0 estimates through the assumed reduction of the average lifespan of mosquitoes in a population. Given the importance of survival and EIP for modelling transmission dynamics, improved data on the nature of daily survival (e.g. constant vs age-dependent decrease) in natural mosquito populations is urgently needed.

One limitation of our work is the choice of the basic reproductive number as our metric. Although widely used, this metric considers mean-field dynamics, without accounting for known heterogeneities such as differences in mosquito lifespan, parasite strain competition and spatial variation [28]. While these heterogeneities play an important role in determining the precise level of transmission, we expect them to similarly affect our transmission estimates under both single and multiple blood feeds, as through our ratio calculation their contributions cancel out.

Another caveat is we measured the effects of an additional blood meal only in *An. gambiae* and we cannot exclude that the magnitude of the change will be different in other *Anopheles* vectors. Interestingly, however, in a recent study in the arboviral vector *Aedes aegypti*, a second blood meal shortened the dengue virus EIP [29], suggesting that exploiting mosquito resources may be a generalized mechanism for pathogens to plastically accelerate their development and improve their odds of transmission.

Considering females blood feed on average every 2–3 days [5], and thus may have taken >2 meals by the time they become infectious, our model might represent a conservative estimate of the impact of feeding behavior on the EIP. In support of this hypothesis, our data using Δ*zpg* mutants demonstrates that *P. falciparum* growth rates are not saturated with two blood feeds and that parasites are capable of undergoing even shorter developmental cycles in the mosquito vector. Additional resources provided by the second blood meal might be responsible for the accelerated parasite growth.

What are the implications of these findings for vector control strategies? Firstly, our data point towards females potentially contributing to malaria transmission from a younger age. Given the observed age-dependent mortality induced by insecticides, with higher resistance levels observed in younger mosquitoes [30,31], this observation implies that insecticide-based interventions (both LLINs and IRS) may be less impactful than predicted by current models. When combined with the observation that multiple blood feeding increases insecticide resistance in older females of a related species, *An. arabiensis* [32], our results suggest that mosquitoes feeding multiple times are more likely to survive to the point when they become infectious.

Secondly, results obtained with Δ*zpg* mutants confirm and expand previous findings that—due to a further increase in parasite growth rates—mosquitoes with reduced reproductive capacity have a significantly higher transmission potential, even in the face of possible lower oocyst numbers. These findings are particularly relevant for control strategies that tamper with mosquito reproduction, such as chemical sterilants [23] or genetically-engineered population suppression gene drives [21,22]. At a time when *Anopheles* gene drive strains are being tested in semi-field settings for their ability to suppress field mosquito populations, our data call for an

urgent and careful evaluation of whether these genetically-modified mosquitoes would contribute to more efficient malaria transmission while the drive is spreading.

Finally, our results emphasize that informed policy decisions on current and future malaria control strategies can only be built on thorough research into the fundamental factors affecting malaria transmission biology.

Acknowledgements
We thank Emily Lund, Kate Thornburg, and Emily Selland for help in mosquito rearing, and members of the Catteruccia laboratory for comments on the manuscript.

Funding sources
F.C. is funded by a Faculty Research Scholar Award by the Howard Hughes Medical Institute (HHMI) and the Bill & Melinda Gates Foundation (BMGF) (Grant ID: OPP1158190), and by the National Institutes of Health (NIH) (R01 AI124165, R01 AI104956). L.M.C. is supported by the National Science Foundation (NSF) (Grant ID: 1853495). The findings and conclusions within this publication are those of the authors and do not necessarily reflect positions or policies of the HHMI, the BMGF, or the NIH. The funders had no role in the study design or in the decision to publish.

Author contributions
W.R.S. and I.H. contributed to literature searches, study design, data collection, data analysis, data interpretation, figure creation, and writing; M.I., K.W., M.M., and D.P. contributed to study design, data collection, data analysis, and data interpretation; N.S. contributed to data collection; C. O. B. contributed to study design, data interpretation, figure creation, and writing; L. M. C. and F.C. contributed to study design, data analysis, data interpretation, figure creation, writing, and project supervision.

Figure legends

Figure 1. A second blood meal increases oocyst size
(A) Mated females were infected with *P. falciparum* and then provided either a second uninfected blood meal 3 days post infectious blood meal (d pIBM)(two red circles) or maintained on sugar (one red circle). Infection outcomes in both groups were determined at 7–14 d pIBM (arrows). (B) Oocyst prevalence (P, pie charts) (Fisher's exact) and intensity (Generalized Linear Model (GLM) with Poisson distribution) are not affected by a second blood meal at 3 d pIBM. (C) Oocyst size at 7 d pIBM is significantly increased in females fed twice (Standard Least Squares (SLS) model with Normal distribution). n=sample size. See Tables S1 and S3 for details of statistical models.

Figure 2. A second blood meal accelerates oocyst development
(A–B) Immunofluorescence assay of oocysts from females (A) fed once (one red circle) or (B) fed twice (two red circles) at 8 d pIBM. (C) Oocyst from a female fed twice showing the release of mature sporozoites at 10 d pIBM. Sporozoites are labelled with circumsporozoite protein CSP (magenta) and DNA is stained with DAPI (gray). Scale bar=10 µm

Figure 3. Mosquitoes are infectious sooner following a second blood meal
(A and B) Salivary glands of females fed twice (blue, two red circles) show (A) a significantly higher prevalence (P, pie charts) of sporozoites at 8 and 10 d pIBM (Fisher's exact) and (B) significantly more sporozoites at 10 d pIBM (GLM, Poisson) than females fed once (orange, one red circle). There is no difference in either sporozoite prevalence or intensity at a later time point (14 d pIBM). (C) The EIP_{50} (time to 50% infectious – dotted line; values also shown ± s.e.) of females fed a second time (blue line) is reduced by 2.25 days (20.7%) compared with controls fed once (orange line), as determined from the sporozoite prevalence data shown in (A). Logistic curve fit (line), EIP_{50} ± s.e. (shaded), z test, $Z_{0.05}$=5.74, p<0.0001. n=sample size. See Tables S1 and S2 for details of statistical models.

Figure 4. Relative R_0 values across Africa show increase in malaria transmission potential
(A) Ratios between R_0 using the standard estimates of EIP_{50} (derived from a single blood feed) to R_0^b using revised estimates of EIP_{50} (derived from two blood feeds). Standard models underestimate R_0 by at least 10.2%, with no areas unchanged (minimum, left). In these same regions, the average change over the year using the shortened EIP ranges from a 10.2% to 56.4% increase, with a mean of 12.2% (mean, center). Some regions see much greater changes during at least one month of the year, ranging from 10.2% to 56.5% (maximum, right). (B) Distribution of R_0 increases across the affected population (950 million people). For 21.6% of the population living in these areas—equivalent to 208 million people—revising the EIP parameter increases the modelled average transmission potential by 15% or more (area right of pink dotted line, and below mean line). A quarter of the population experiences at least a 25% higher R_0 during one month of the year (area right of purple dotted line, and below maximum line). Note the logarithmic y-axis.

Figure 5. Parasite developmental rates are further enhanced in eggless mosquitoes
(A) Oocysts are significantly larger in Δ*zpg* mutant females at 7 d pIBM after both one or two blood meals (SLS model, Normal) compared to *Zpg* controls (Cntrl). (B) Oocyst intensities are lower in Δ*zpg* mutant females compared to *Zpg* controls (GLM, Poisson), whereas oocyst prevalence (P, pie charts) (chi-squared test, χ^2=6.488, d.f.=3) is unaffected. (C) Salivary glands sporozoite numbers are increased in Δ*zpg* mutant females at 10 d pIBM after either one or two blood meals (GLM, Poisson), with sporozoite prevalence (P, upper pie charts) also increased in the Δ*zpg* mutant background (chi-squared test, χ^2=81.43, d.f.=3), significantly at the first blood meal. Pooling data shows higher sporozoite prevalence in Δ*zpg* mutant population (P, lower pie charts) (Fisher's exact). n=sample size. See Tables S1–S4 for details of statistical models.

Supplementary Figure 1. Baseline R_0 values vary with the size of population considered. We set a lower population bound of N=250 (dotted line) to avoid exponential growth in R_0 that occurs at very low population densities due to our formula structure. This cutoff reflects the changing shape of this function across different temperature values, demonstrated using temperatures that allow for higher (25˚C), medium (30˚C) and lower (20˚C) transmission. R_0 estimates are highest closer to the optimal temperature of 25˚C [17].

Supplementary Figure 2. Monthly changes in R_0 under a multiple blood feeding scenario. We calculated the monthly changes in R_0 by taking the ratio R_0^b/R_0 using the mean temperature of each month for each grid point. For areas with very low transmission, large ratio changes are the result of small absolute changes so these maps are restricted to exclude areas where R_0^b is less than 1. The restricted data points shown here are used to create the summary maps in **figure 4**.

References

1. WHO. World Malaria Report. Geneva: World Health Organization, 2019.
2. Ross R. Some Quantitative Studies in Epidemiology. *Nature* 1911; **87**: 446-67.
3. Macdonald G. The analysis of equilibrium in malaria. *Trop Dis Bull* 1952; **49**(9): 813-29.
4. Macdonald G. Epidemiological basis of malaria control. *Bull World Health Organ* 1956; **15**(3-5): 613-26.
5. Gillies MT, Wilkes TJ. A study of the age-composition of populations of *Anopheles gambiae Giles* and *A. funestus Giles* in North-Eastern Tanzania. *Bulletin of Entomological Research* 1965; **56**(2): 237-62.
6. Costantini C, Li SG, Della Torre A, Sagnon N, Coluzzi M, Taylor CE. Density, survival and dispersal of Anopheles gambiae complex mosquitoes in a west African Sudan savanna village. *Med Vet Entomol* 1996; **10**(3): 203-19.
7. Smith DL, Battle KE, Hay SI, Barker CM, Scott TW, McKenzie FE. Ross, macdonald, and a theory for the dynamics and control of mosquito-transmitted pathogens. *PLoS Pathog* 2012; **8**(4): e1002588.
8. Ohm JR, Baldini F, Barreaux P, et al. Rethinking the extrinsic incubation period of malaria parasites. *Parasit Vectors* 2018; **11**(1): 178.
9. Werling K, Shaw WR, Itoe MA, et al. Steroid hormone function controls non-competitive *Plasmodium* development in *Anopheles*. *Cell* 2019; **(in press)**.
10. Costa G, Gildenhard M, Eldering M, et al. Non-competitive resource exploitation within mosquito shapes within-host malaria infectivity and virulence. *Nat Commun* 2018; **9**(1): 3474.
11. Detinova TS. Age-grouping Methods in Diptera of Medical Importance with Special Reference to Some Vectors of Malaria. Geneva, 1962.
12. Clements AN. The Biology of Mosquitoes. London: Chapman & Hall; 1992.
13. Scott TW, Takken W. Feeding strategies of anthropophilic mosquitoes result in increased risk of pathogen transmission. *Trends Parasitol* 2012; **28**(3): 114-21.
14. Kwon H, Reynolds RA, Simões ML, Dimopoulos G, Smith RC. Malaria parasite immune evasion and adaptation to its mosquito host is influenced by the acquisition of multiple blood meals. *bioRxiv* 2019: 1-22.
15. Ponnudurai T, Lensen AH, van Gemert GJ, Bensink MP, Bolmer M, Meuwissen JH. Sporozoite load of mosquitoes infected with Plasmodium falciparum. *Trans R Soc Trop Med Hyg* 1989; **83**(1): 67-70.
16. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods* 2012; **9**(7): 676-82.

17. Mordecai EA, Paaijmans KP, Johnson LR, et al. Optimal temperature for malaria transmission is dramatically lower than previously predicted. *Ecol Lett* 2013; **16**(1): 22-30.
18. Fick SE, Hijmans RJ. WorldClim 2: new 1-km spatial resolution climate surfaces for global land areas. *International Journal of Climatology* 2017; **37**(12): 4302-15.
19. Wiebe A, Longbottom J, Gleave K, et al. Geographical distributions of African malaria vector sibling species and evidence for insecticide resistance. *Malar J* 2017; **16**(1): 85.
20. WorldPop.org. www.worldpop.org.
21. Hammond A, Galizi R, Kyrou K, et al. A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector Anopheles gambiae. *Nat Biotechnol* 2016; **34**(1): 78-83.
22. Kyrou K, Hammond AM, Galizi R, et al. A CRISPR-Cas9 gene drive targeting doublesex causes complete population suppression in caged Anopheles gambiae mosquitoes. *Nat Biotechnol* 2018; **36**(11): 1062-6.
23. Tiono AB, Ouedraogo A, Ouattara D, et al. Efficacy of Olyset Duo, a bednet containing pyriproxyfen and permethrin, versus a permethrin-only net against clinical malaria in an area with highly pyrethroid-resistant vectors in rural Burkina Faso: a cluster-randomised controlled trial. *Lancet* 2018; **392**(10147): 569-80.
24. Tazuke SI, Schulz C, Gilboa L, et al. A germline-specific gap junction protein required for survival of differentiating early germ cells. *Development* 2002; **129**(10): 2529-39.
25. Meredith JM, Basu S, Nimmo DD, et al. Site-specific integration and expression of an anti-malarial gene in transgenic Anopheles gambiae significantly reduces Plasmodium infections. *PLoS One* 2011; **6**(1): e14587.
26. Stone WJ, Eldering M, van Gemert GJ, et al. The relevance and applicability of oocyst prevalence as a read-out for mosquito feeding assays. *Sci Rep* 2013; **3**: 3418.
27. Beier JC, Killeen GF, Githure JI. Short report: entomologic inoculation rates and Plasmodium falciparum malaria prevalence in Africa. *Am J Trop Med Hyg* 1999; **61**(1): 109-13.
28. Gupta S, Trenholme K, Anderson RM, Day KP. Antigenic diversity and the transmission dynamics of Plasmodium falciparum. *Science* 1994; **263**(5149): 961-3.
29. Armstrong PM, Ehrlich HY, Magalhaes T, et al. Successive blood meals enhance virus dissemination within mosquitoes and increase transmission potential. *Nat Microbiol* 2020; **5**(2): 239-47.
30. Rowland M, Hemingway J. Changes in malathion resistance with age in *Anopheles stephensi* from Pakistan. *Pesticide Biochemistry and Physiology* 1987; **28**(2): 239-47.
31. Lines JD, Nassor NS. DDT resistance in Anopheles gambiae declines with mosquito age. *Med Vet Entomol* 1991; **5**(3): 261-5.
32. Oliver SV, Brooke BD. The effect of multiple blood-feeding on the longevity and insecticide resistant phenotype in the major malaria vector Anopheles arabiensis (Diptera: Culicidae). *Parasit Vectors* 2014; **7**: 390.

Figure 1

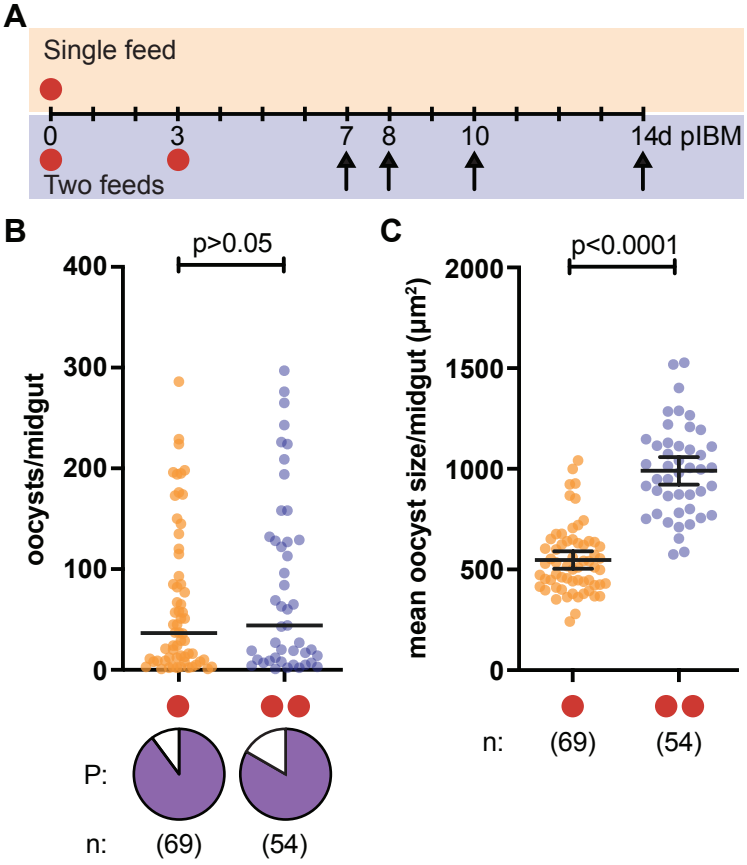

A
Single feed
0
3
7
8
10
14d pIBM
Two feeds
B
p>0.05
oocysts/midgut
400
300
200
100
0
P:
n:
(69)
(54)
C
p<0.0001
mean oocyst size/midgut (µm²)
2000
1500
1000
500
0
n:
(69)
(54)

Figure 2

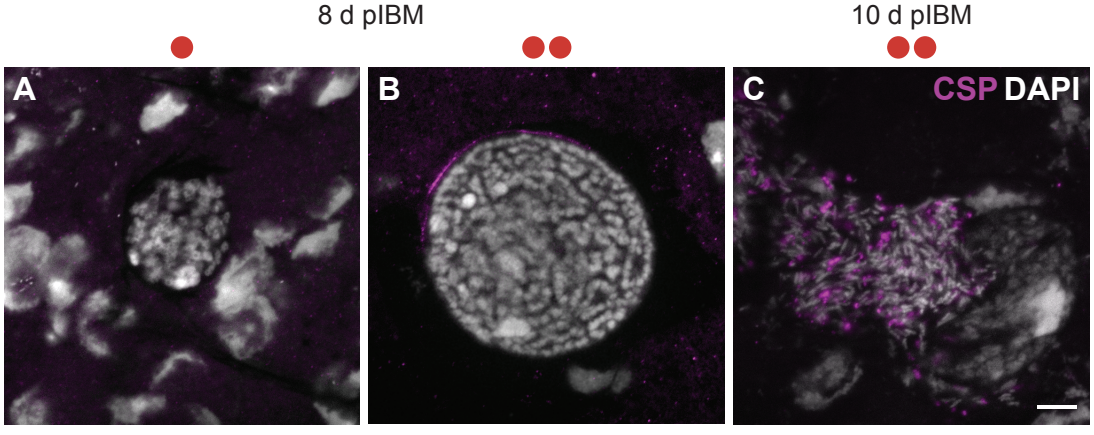


Figure 3

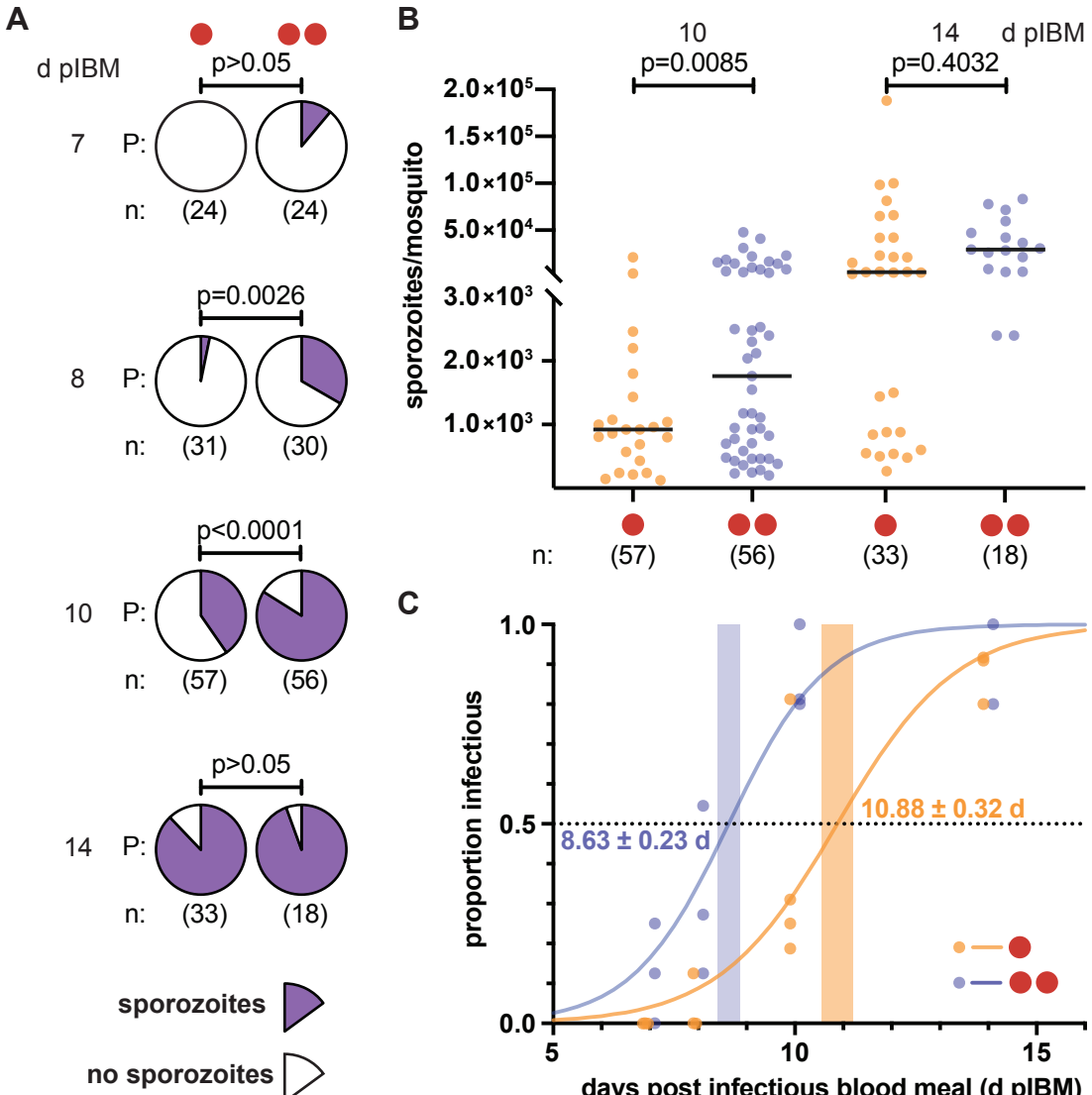

A
d pIBM
p>0.05
7
P:
n:
(24)
(24)
p=0.0026
8
P:
n:
(31)
(30)
p<0.0001
10
P:
n:
(57)
(56)
p>0.05
14
P:
n:
(33)
(18)
sporozoites
no sporozoites
B
10
p=0.0085
14
p=0.4032
d pIBM
sporozoites/mosquito
2.0×10⁵
1.5×10⁵
1.0×10⁵
5.0×10⁴
3.0×10³
2.0×10³
1.0×10³
n:
(57)
(56)
(33)
(18)
C
proportion infectious
1.0
0.5
0.0
8.63 ± 0.23 d
10.88 ± 0.32 d
5
10
15
days post infectious blood meal (d pIBM)

Figure 4

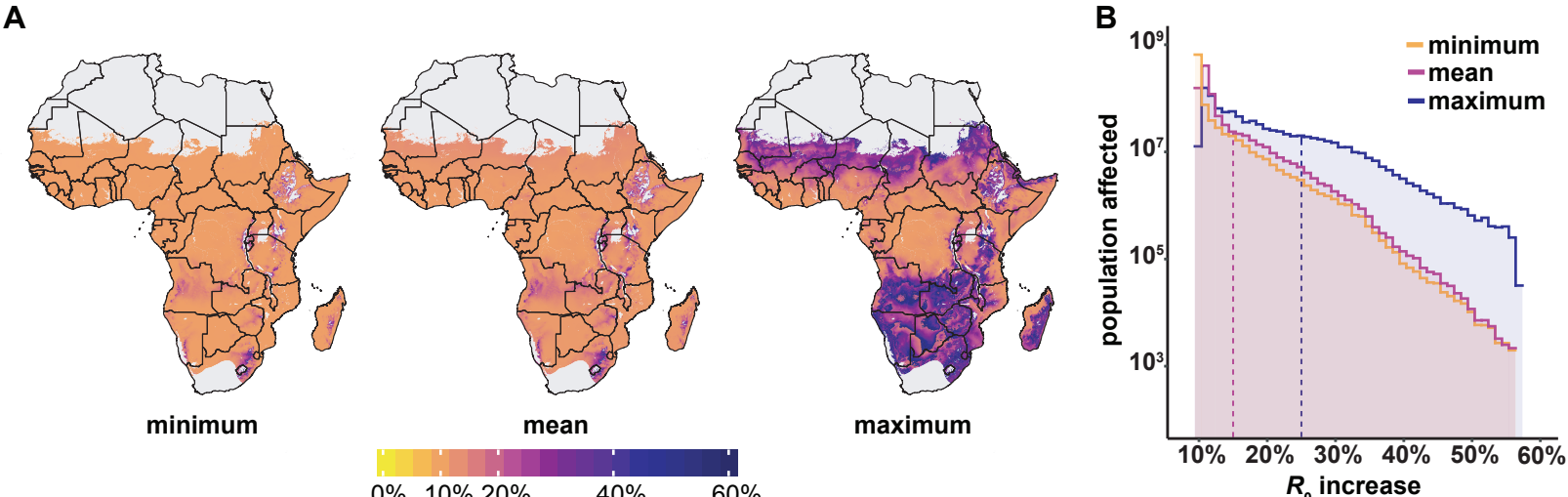
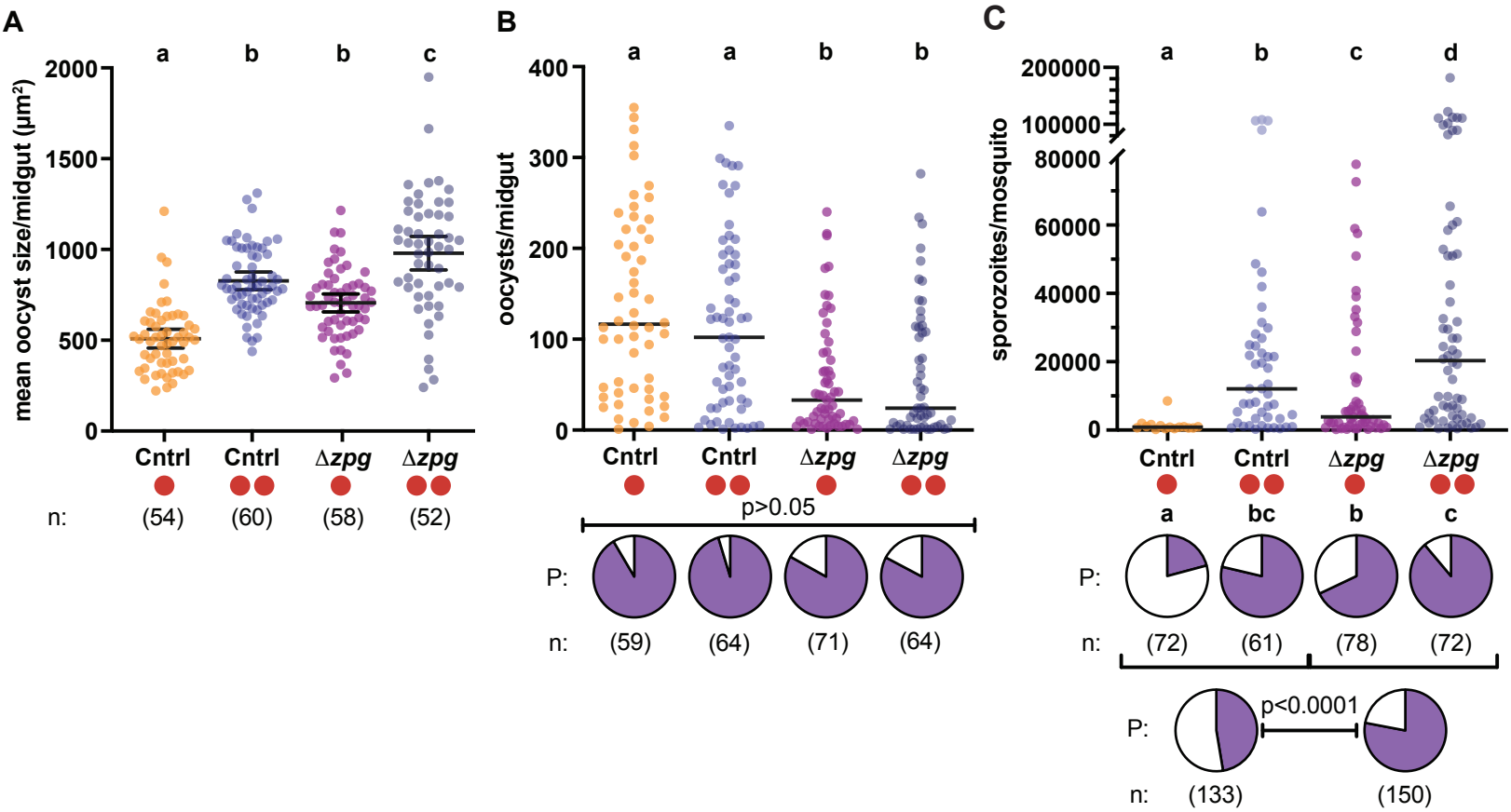

A
minimum
mean
maximum
0%
10%
20%
40%
60%
B
minimum
mean
maximum
population affected
10^9
10^7
10^5
10^3
10%
20%
30%
40%
50%
60%
R_0 increase

Figure 5


A
a
b
b
c
mean oocyst size/midgut (µm²)
2000
1500
1000
500
0
Cntrl
Cntrl
Δzpg
Δzpg
n:
(54)
(60)
(58)
(52)
B
a
a
b
b
oocysts/midgut
400
300
200
100
0
Cntrl
Cntrl
Δzpg
Δzpg
p>0.05
P:
n:
(59)
(64)
(71)
(64)
C
a
b
c
d
sporozoites/mosquito
200000
100000
80000
60000
40000
20000
0
Cntrl
Cntrl
Δzpg
Δzpg
a
bc
b
c
P:
n:
(72)
(61)
(78)
(72)
p<0.0001
P:
n:
(133)
(150)