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Larval food quantity affects the capacity of adult mosquitoes to transmit human malaria

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Adult traits of holometabolous insects are shaped by conditions experienced during larval development, which might impact interactions between adult insect hosts and parasites. However, the ecology of larval insects that vector disease remains poorly understood. Here, we used *Anopheles stephensi* mosquitoes and the human malaria parasite *Plasmodium falciparum*, to investigate whether larval conditions affect the capacity of adult mosquitoes to transmit malaria. We reared larvae in two groups; one group received a standard laboratory rearing diet, whereas the other received a reduced diet. Emerging adult females were then provided an infectious blood meal. We assessed mosquito longevity, parasite development rate and prevalence of infectious mosquitoes over time. Reduced larval food led to increased adult mortality and caused a delay in parasite development and a slowing in the rate at which parasites invaded the mosquito salivary glands, extending the time it took for mosquitoes to become infectious. Together, these effects increased transmission potential of mosquitoes in the high food regime by 260–330%. Such effects have not, to our knowledge, been shown previously for human malaria and highlight the importance of improving knowledge of larval ecology to better understand vector-borne disease transmission dynamics.

1. Introduction

The larvae of many insects that vector human disease exhibit very different ecologies from the adult vectors ultimately responsible for disease transmission. For example, while adult vectors often live in and around domestic dwellings, larval sandflies (vectors of leishmaniasis) inhabit subterranean habitats, such as rodent burrows, and larval mosquitoes (vectors of numerous diseases including malaria, filariasis, dengue, chikungunya and Zika virus) occupy aquatic habitats ranging from freshwater streams to stagnant water in drainage ditches or man-made containers [1]. For many holometabolous insects, conditions experienced during the larval stages can carry over to influence adult life-history traits [2–4]. However, the majority of vector biology research tends to focus directly on the adult vectors, and the potential for larval ecology to have indirect influence on disease transmission is relatively less well studied.

The most extensive research exploring the effects of larval condition on the vectorial capacity of adult mosquitoes derives from *Aedes*–arbovirus systems [5–11]. For example, in adult female *Aedes albopictus*, low larval rearing temperatures decrease dissemination of dengue virus, but increase susceptibility to chikungunya virus, in comparison with adults from warmer larval environments [5,8]. *Aedes albopictus* larvae that are competitively stressed, either intraspecifically (high larval density) or interspecifically (with *Aedes aegypti*) are more likely to become infected with Sindbis virus, whereas *Ae. aegypti* exhibits equivalent susceptibility, regardless of density or competitive species [7].

Additionally, reduced larval food quantity in *Ae. aegypti* has been shown to influence the interaction between humoral and cellular branches of the immune system in adult mosquitoes, which could also directly affect vector competence for a variety of pathogens [6].

A more limited number of studies in *Anopheles* spp., show similar effects of larval environment on life-history traits and vector competence [12–19]. In *Anopheles gambiae*, high larval densities and low larval food quantity hindered first mating success of emerging adult males [18]. In areas where larval competition between *An. gambiae* and *Anopheles arabiensis* occurs, it has been demonstrated that time to pupation is reduced for *An. gambiae* and extended for *An. arabiensis* compared with larval pools containing only one species [14]. The nature of local soils (organic carbon, nitrogen, and microbial content) has also been shown to influence larval development of *An. gambiae* and subsequent susceptibility of adult mosquitoes to *Plasmodium falciparum* infection [15,16]. We recently demonstrated that a reduction in larval mosquito food quantity significantly decreases vectorial capacity of *Anopheles stephensi* infected with a rodent malaria (*Plasmodium yoelii*). We noted significant effects of larval food quantity on adult life-history traits important to transmission, such as survival, length of gonotrophic cycle, and number of emerging adults; however, we observed no effect of nutrition on parasite prevalence [17].

In spite of this research, the role of larval ecology remains poorly integrated into understanding of vector-borne disease transmission, and for human malaria in particular, few studies have explored the net effect of larval condition on both adult and parasite traits that combine to determine ultimate transmission potential [17,19,20]. In this study, we investigate how differences in the quantity of food available to larval *An. stephensi* mosquitoes affect the capacity of adult mosquitoes to vector human malaria, *P. falciparum*. We found that reducing larval food quantity reduced adult longevity, increased the parasite extrinsic incubation period (EIP) and extended the time it took for mosquitoes to become infectious. Such effects have not, to our knowledge, been reported previously for human malaria but, given the variability in larval habitat quality in nature [21–25], could play a substantial role in transmission dynamics.

2. Material and methods

(a) Mosquito rearing and experimental design

Anopheles stephensi larvae were collected from our laboratory colony at The Pennsylvania State University (this colony was initiated in April 2014 with eggs from a longstanding colony maintained at Walter Reed Army Institute of Research, Silver Spring, MD, USA). Newly hatched (less than 24 h old) first-instar larvae were transferred to plastic trays (36 × 20 × 13 cm) containing 1 l of distilled water at initial densities of 400 larvae per tray. Larvae were maintained on Tetrafin® fish food under standard insectary conditions (26°C ± 0.5°C, 80% humidity, and a 12 L : 12 D photoperiod).

From our collected first-instar larvae, we generated two colonies: (i) a ‘high’ food colony, receiving 0.6 mg of food per individual per day, which is consistent with our standard colony maintenance diet; and (ii) a ‘low’ food colony, receiving 0.2 mg of food per individual per day. The specific food treatments were chosen based on a series of pilot studies, with the goal of generating a ‘low food’ regime that still allowed for a

colony that would produce sufficient numbers of emerging adult mosquitoes to perform large-scale experimental infections (electronic supplementary material, figure S1). We maintained a constant concentration of food in each tray by filtering and replacing water daily before feeding. Each group consisted of 30 trays, with an initial population of 12 000 larvae in each colony.

To ensure that mosquitoes received an infectious blood meal at the same adult age, first-instar larvae from the low food treatment group were collected 3 days earlier than first-instar larvae from the high food treatment group (electronic supplementary material, figure S1). This was to adjust for the slower developmental time owing to low food availability, and to allow for an age-matched comparison of the groups at the same time points post-infection. Although the mosquitoes from the low food colony were chronologically older, because of the additional time spent in larval development, the adult females from each colony were of equal post-emergence age at the time of infection.

In each colony, pupae from each larval tray were distributed evenly across 10 mesh emergence cages (17.5 × 17.5 × 17.5 cm, BugDorm®, Taichung, Taiwan). Upon adult emergence, each cage was provided with cotton balls moistened with 10% glucose, replaced daily. Early emerging females (those emerged more than 5 days pre-infectious blood meal) in each cage were removed. Three to 5 days following peak female emergence, adult females from each emergence cage were evenly distributed across 8–10 cardboard cups (475 ml) for each colony, totalling 150 females per cup (electronic supplementary material, figure S2). Each cup was provided a blood meal of human blood infected with *P. falciparum*. Dead mosquitoes were counted daily in each cup, and subsamples of live mosquitoes were removed (details below) to assess the proportion of infectious mosquitoes. Owing to logistical constraints of maintaining tray-to-cage-to-cup replication within an experiment, we replicated the experiment twice through time (experimental block 1: eight cups per treatment; block 2: 10 cups per treatment; electronic supplementary material, figure S3).

As our experimental females are drawn from a large colony pool that has been mixed on two separate occasions from pupation to infection, we included wing length as a feedback signal to ensure our colonies were producing females that were significantly different between groups. During each experimental block, we removed 10 females from each emergence cage for both food groups at the same time as the allocation of females to infectious cups for blood meals. Wing lengths were measured to the nearest micrometre using CELLSENS imaging software (Olympus). Wing lengths were significantly different between food groups in both blocks (electronic supplementary material, figure S4), demonstrating that our colonies were indeed producing individuals of differing condition.

(b) *Plasmodium falciparum* culture and infection

In vitro cultures of *P. falciparum* strain NF54 (wild-type, Center for Infectious Disease Research, Seattle, WA, USA) were maintained in RPMI 1640 medium (25 mM HEPES, 2 mM L-glutamine), supplemented with 50 µM hypoxanthine and 10% human A+ serum (Valley Biomedical, Winchester, VA, USA). Culture was maintained in an atmosphere of 5% CO₂, 5% O₂ and 90% N₂. Parasite cells were then subcultured into

O+ human erythrocytes (Valley Biomedical). Gametocyte initiation occurred at 5% haematocrit and 0.8–1.0% mixed-stage parasitaemia. The culture was maintained for 17 days, and parasite cells were washed and provided fresh media daily.

Prior to the administration of the infectious blood meal, females were starved for 18 h (imbibing only water) to ensure a maximum proportion of blood feeding. On the day the infectious feed was administered to mosquitoes, gametocyte cultures (approx. 8% gametocytaemia for each experimental block) were briefly centrifuged, and the supernatant was removed and discarded. Pelleted erythrocytes were diluted to 40% haematocrit using fresh A+ human serum and O+ human erythrocytes. The mixture was pipetted into glass bell jars fixed with a Parafilm membrane and connected by plastic tubing with continuously flowing water heated to 37°C. Each bell jar was filled with 2 ml of blood culture. Mosquitoes were given 20 min to fully engorge, after which the bell jars were removed, as the parasites in culture are no longer viable. In each colony, more than 90% of females across all cups were observed to have blood in their midguts in both experimental blocks. Following blood feeding, mosquitoes were maintained at 27°C ± 0.5°C, 85% humidity, and each cup was provided with cotton balls soaked with 10% glucose and 0.05% para-aminobenzoic acid in water, which were replaced daily.

(c) Parasite development and adult longevity

To estimate the effects of larval nutrition on parasite development rate, we assessed the daily proportion of mosquitoes that were infectious through days 9–16 following the infectious blood meal. To do this, we aspirated 8–10 mosquitoes from each replicate cup into absolute ethanol and dissected salivary glands in 0.01 M phosphate-buffered saline. Glands were ruptured and examined under a light microscope at 400× for the presence of sporozoites. Although not discussed in this study, data were also collected on parasite establishment within the mosquito midgut. We assessed both oocyst prevalence and number of oocysts per midgut via dissections from days 5 to 10 post-infection followed by observation under a light microscope (electronic supplementary material, figure S5 and tables S1 and S2).

To quantify effects of larval food quantity on adult survivorship post-infection, we counted dead mosquitoes in each replicate cup daily. For survival analysis, mosquitoes removed for dissections each day, and those still alive at the end of the experiment (day 16) were considered censored cases. We built a series of models with food and block as covariates, using the R package *flexsurv*. We compared exponential, Weibull and Gompertz distributions for survival, as these distributions have been commonly used to describe mosquito survival under laboratory conditions [26–28]. The best-fit model, which was a Gompertz curve described by larval food treatment and experimental block (electronic supplementary material, table S3), was chosen using Akaike's information criterion (AIC).

(d) Parasite dynamics and extrinsic incubation period

The EIP describes the time it takes for the parasite to develop in the mosquito from the initial parasite-infected blood feed through to the point at which sporozoites enter the salivary glands and the mosquito becomes infectious. The number of infectious mosquitoes in a cohort of mosquitoes is expected to

increase from zero to some maximum prevalence over several days [29–31], and defining EIP at different points along this cumulative curve has been shown to alter estimates of transmission intensity [29]. Whether the shape of this cumulative distribution is affected by larval diet is unknown.

To assess the effect of larval food quantity on parasite dynamics across the course of sporogony, we estimated how larval food treatment and experimental block influenced the daily mean values for sporozoite prevalence in a non-linear logistic regression model framework [29]. Here, the change in proportion of infectious mosquitoes over time is described by a sigmoidal curve with the given equation:

$$\text{proportion infectious} = \frac{g}{1 + e^{-k(x-t_m)'}}$$

where g is the maximum observed sporozoite prevalence over the course of the infection, k is the instantaneous rate of change, x is time post-infection (days) and t_m is the time at which change in the proportion of infectious mosquitoes is maximal. We constructed 24 competing models to describe how the dynamics of mosquito infection varied in response to food treatment and block effects (see the electronic supplementary material, table S4 for model parameter designations and electronic supplementary material, figures S7 and S8 for individual cup dynamics in each block). The largest model had 12 parameters—i.e. treatment, block and treatment × block interaction effects on g , t_m and k , whereas our null model contained only the three parameters g , k and t_m . We used the AIC to assess which model fitted our data best (electronic supplementary material, table S5).

(e) Estimating transmission potential

One of the standard ways to characterize transmission potential is vectorial capacity, which describes the daily rate at which future infections arise from one infected human, given that all mosquitoes feeding on that human become infected [32]. However, vectorial capacity assumes there is a single fixed value for EIP and that there is a constant rate of daily adult mosquito survival, neither of which fit with our empirical data. Accordingly, we used an alternative measure, adapted from the work of Killeen *et al.* [33] to describe the transmission potential of a cohort of mosquitoes (see also [34] for analogous methods). In this framework, we assessed the number of mosquitoes that are both alive and infectious at any given time by overlaying our survival curves with our model fits for change in mosquito infectiousness over time. We extended our survival estimates to the point where just 1% of mosquitoes were predicted to be alive, as the Gompertz survival curves never reach zero. We follow the standard assumption that once sporozoites have invaded the salivary glands, a mosquito remains infectious for life [32,35,36].

This approach enables us to capture the interacting dynamics of sporogony and survival. The product of these two proportions (the area under the curve) represents the cumulative number of 'infectious mosquito days'. This value is then multiplied by the daily biting rate to provide an estimate of the probable number of infectious bites transmitted by a cohort of mosquitoes, assuming all blood meals are taken on humans (analogous to force of infection for a given mosquito cohort). Here, we illustrate our model results by assuming an initial cohort of 100 mosquitoes for each treatment group. For our estimate of daily biting rate, we followed convention by

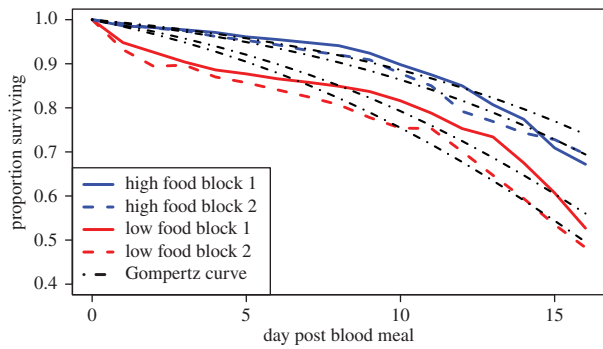


Figure 1. Proportion of mosquitoes surviving over time, with the predicted Gompertz distribution overlaid on raw survival data. Parameter estimates: shape (β) = 0.01184; high food block 1 rate (α) = 0.00632; low food block 1 rate (α) = 0.01214, high food block 2 rate (α) = 0.00765, low food block 2 rate (α) = 0.01470. The x -axis begins at 0.4 for ease of visualization.

taking the reciprocal of the gonotrophic cycle length [29,35,36] as *An. stephensi* have not been observed to take multiple blood meals per gonotrophic cycle in the field. We used empirically observed values of gonotrophic cycle length from a pilot experiment conducted with these same food regimes using the same stock colony (see the electronic supplementary material, figure S4 and also [17] for similar data).

3. Results

(a) Post-infectious longevity

Larval food treatment significantly affected daily survival of blood-fed adult females (figure 1). Median survival time predicted by our Gompertz function for females from the high food groups was 22.3 days (95% confidence interval (CI) = 21.2–23.5 days) and 20.8 days (95% CI = 19.9–21.8 days) for blocks 1 and 2, respectively, and 17.3 days (95% CI = 16.6–18.1 days) and 15.9 days (95% CI = 15.3–16.6 days) for females from the low food groups in blocks 1 and 2, respectively.

(b) Effects of treatment on the extrinsic incubation period and parasite dynamics

Our best-fit logistic regression model suggested important effects of larval food treatment on the cumulative distribution of infectious mosquitoes over time (figure 2 and table 1). This model was significantly better than the fully reduced model containing only the original terms g , k and t_m (electronic supplementary material, table S5).

We observed an overall reduction (significant in block 1) in the maximum proportion of infectious mosquitoes (g) in the reduced food treatment (figure 2). We also observed differences in the rates of parasite development between treatments with higher rates of change (k) in the high food treatment compared with the low food treatment, irrespective of asymptotic differences in maximum prevalence (figure 2 and table 1).

As shown in figure 2, the logistic model indicated that the initial release of sporozoites within the population (which we characterize as time to 10% of maximum infectiousness) occurred at 10.3 days post-blood meal in the high food

treatment in both blocks 1 and 2. For the low food treatment, 10% infectiousness also occurred at day 10.3 and day 11 in blocks 1 and 2, respectively. As the infection proliferated within the population, the differences in parasite development between the two treatment groups increased. The time to 50% of maximum infectiousness (the median incubation period, indicated by the inflection point) occurred at 10.9 and 10.7 days for blocks 1 and 2 in the high food treatments, and 12.1 days for the low food treatments in both experimental blocks. The time to 90% infection (i.e. approaching the maximum prevalence, g) was 11.4 and 11.2 days in blocks 1 and 2 for the high food treatment but 13.7 and 12.9 days for the low food treatment.

(c) Estimating treatment effects on transmission potential

Using our metric of transmission potential, we see a clear difference in the number of cumulative infectious mosquito days between the two larval food groups (area under the curve indicated in figure 3). Assuming an initial cohort of 100 mosquitoes, the resulting values represent the probable number of infectious bites transmitted for that cohort until only 1% of the population is remaining. After multiplying the number of cumulative infectious mosquito days by daily biting rate, we observe transmission potential values for the high food group of 247 and 186 infectious bites for blocks 1 and 2, respectively. For the low food groups, we observe values of 74 and 71 infectious bites for blocks 1 and 2, respectively. Overall, transmission potential of mosquitoes in the high food regime was increased by 260–330% relative to the low food group (table 2).

4. Discussion

Numerous studies have demonstrated that larval conditions in holometabolous insects can carry over to affect adult life-history traits. The aim of the current experiments was to test the hypothesis that differences in larval food quantities can affect the potential of adult *An. stephensi* mosquitoes to vector the human malaria parasite *P. falciparum*. Our results show that changes in larval food can lead to substantial changes in the force of infection of a cohort of mosquitoes (table 2).

One effect of variation in larval food was an impact on daily adult survival. This effect is not surprising given that adult survival has been shown to be directly correlated to larval nutritional status in many mosquito species [11,13,17,36,37], including uninfected *Anopheles* species [37,38], and in *Anopheles* adults infected with rodent malaria species [13,17].

Perhaps less expected was the clear influence of larval food quantity on parasite development within adult mosquitoes. Although we did not note a significant difference in parasite prevalence between food treatments in our second experimental block, the effects of larval nutrition on the inflection point and instantaneous rate of change of our model curves describing parasite growth kinetics were consistent between blocks. This result suggests a robust effect of larval food on parasite development. Further, the effect of larval diet on the time taken to reach maximum infectiousness suggests an influence of larval nutrition during energetically demanding stages of oocyst maturation.

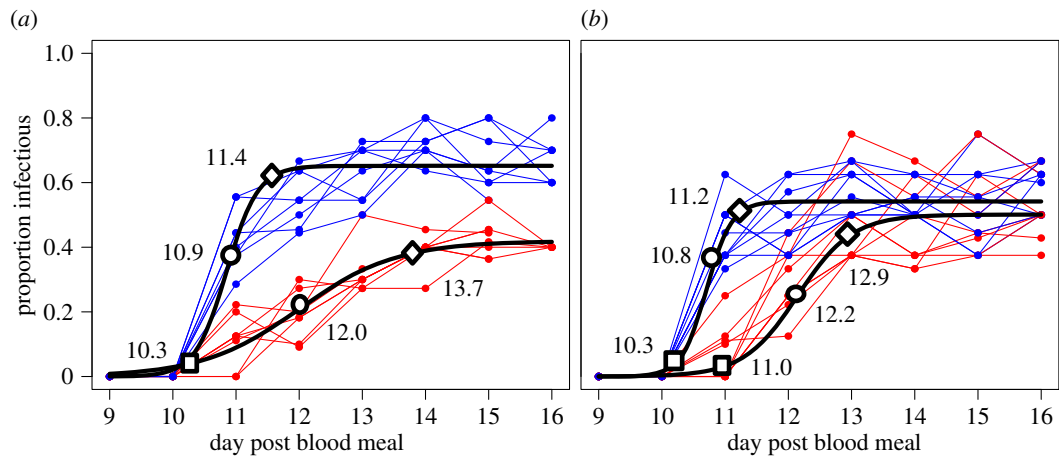


Figure 2. Raw data for sporozoite prevalence over time for (a) block 1 and (b) block 2; (coloured points, blue = high food, red = low food) fitted with our best-fit binary logistic regression model (black line). Lines connecting points represent separate experimental cups within each treatment. To illustrate the change in differences between groups through time, shapes along the black line represent time to 10% (open squares), 50% (open circles) and 90% (open diamonds) of maximum infectiousness. The numbers beside each shape are the corresponding days to reach that point.

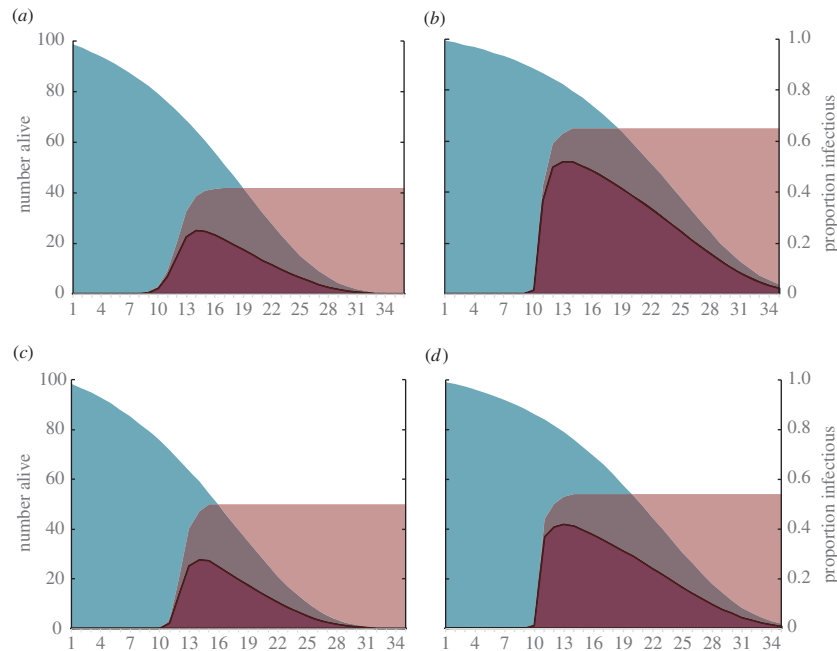


Figure 3. Area curves for rates of survival (blue) and infection (pink) for each block and treatment combination: (a) low food, block 1; (b) high food block 1; (c) low food block 2; and (d) high food block 2. Purple areas represent the product of the two curves (i.e. the number of mosquitoes alive and infectious).

Table 1. Estimates for best-fit logistic model parameter values describing the kinetics of parasite development within the adult mosquitoes for each block and treatment combination.

| parameter | low food block 1 | high food block 1 | low food block 2 | high food block 2 |
|----------------------|------------------|-------------------|------------------|-------------------|
| asymptote (g) | 0.4189 | 0.6519 | 0.5009 | 0.5416 |
| rate (k) | -1.2967 | -4.1039 | -2.3244 | -5.0417 |
| inflection (t_m) | 12.0064 | 10.8519 | 12.1496 | 10.7087 |

There currently is limited knowledge on what nutrients *Plasmodium* parasites use from their mosquito hosts. *Plasmodium*-infected mosquitoes have been shown to contain significantly lower amounts of amino acids, glucose and sterols compared with non-infected, blood-fed conspecifics,

suggesting that parasite development may influence the composition and quantity of mosquito reserves [39–41]. *Plasmodium* spp. are also known to scavenge fatty acids from their mosquito and vertebrate hosts, despite their ability to synthesize fatty acids de novo [40,42], and laboratory

Table 2. Calculations of transmission potential for each food treatment and block combination, and the relative increase in transmission potential from low food to high food.

| treatment | block | cumulative infectious mosquito days | biting rate | transmission potential | increase (%) |
|-----------|-------|-------------------------------------|-------------|------------------------|--------------|
| high food | 1 | 718.32 | 0.344 | 247.102 | 330 |
| low food | 1 | 260.79 | 0.283 | 73.804 | |
| high food | 2 | 539.59 | 0.344 | 185.619 | 260 |
| low food | 2 | 251.70 | 0.283 | 71.231 | |

observations of *Aedes* and *Anopheles* spp. infected with avian and rodent malaria, respectively, show that the mosquito lipoprotein lipophorin is essential for the development and maturation of oocysts [43–45]. Finally, numerous studies indicate that poor larval diet drastically reduces the relative amount of proteins, glycogen stores and total lipids per microgram of adult body mass in *Anopheles* and *Aedes* species [13,46–50]. Hence, it is possible that differences in larval food quantity result in variation in levels of compounds essential to oocyst maturation in adult female mosquitoes.

Research in other insect-parasite systems indicates that host nutritional status can affect parasite development. For example, in bumblebees infected with the trypanosome *Crithidia bombi*, starving adult bees of pollen resulted in significantly decreased parasite intensity. Additionally, the life cycle of *C. bombi* is normally synchronized in healthy bees, but in starved bees, parasite populations were observed to comprise a mix of parasite life cycle stages [51,52]. Similar dynamics have been observed in triatomine bugs infected with *Trypanosoma cruzi* (the causative agent of Chagas' disease in humans), with evidence that quality and quantity of blood meals can directly influence parasite development [53,54]. These findings contrast with a recent study on *Ae. aegypti*, which showed no impact of larval rearing condition on the EIP of dengue virus in spite of clear effects on life-history traits such as adult longevity [55].

Our approach to describing overall transmission potential (force of infection for a given mosquito cohort) better captures the dynamic interaction of sporogony and survival than conventional static metrics such as vectorial capacity [32,35,56,57]. All derivations of the vectorial capacity equation assume a single value for EIP for a particular mosquito population or environment (typically the first time point that mosquitoes become infectious). However, our data clearly show sporogony to follow a distribution that cannot be simply represented by a single time point (see [34] for analogous patterns in dissemination of dengue virus in *Aedes aegypti*). Additionally, mosquito survival is typically characterized using an exponential function that assumes a constant rate of survival per day. Our data show mortality to follow an age-dependent Gompertz distribution, a result consistent with numerous studies on survival of caged *Anopheles*, *Culex* and *Aedes* species [26–28,58,59]. We note, however, that we did not follow adult survival until all mosquitoes were dead and so cannot confirm the complete shape of the survival function empirically. Additionally, mortality in the field is likely to be dependent on many factors, including temperature, rainfall, humidity, predation and habitat (e.g. rural or urban) [21–25,59–62].

We acknowledge that our results derive from laboratory-adapted strains of mosquito and parasite. In the field, we might expect local strains to vary in terms of key life-history traits [63]. The use of the standard mass-rearing larval diet of fish food could also influence parasite dynamics. However, what constitutes an 'optimal' diet in the field is unclear as larval habitats are diverse [21–25,64,65]. Furthermore, the effects of varying nutrition ought to be fundamental: it has been demonstrated in multiple studies that varying nutrition will affect size, longevity, fecundity, gonotrophic cycle and other life-history traits [13,17,46,66–68], so there is little reason to assume the qualitative alterations in traits we observe are an artefact of the laboratory system alone. Consistent with this argument, seasonal quality of larval habitats has been shown to influence adult body size in field populations of *Anopheles* species [68,69]. Natural variation in body size of field-collected *An. gambiae* has also been shown to influence probability of eventual sporozoite infection [70]. The same is true for other disease vectors, with the frequency of dengue infection increasing with female body size in *Ae. aegypti* [71].

Recent years have seen a substantial body of research exploring potential effects of environmental drivers, such as temperature, on malaria transmission [29,72–77]. Our results demonstrate that variation in larval nutrition can impact transmission potential with equivalent effect size to typical variation in environmental temperature (including predicted effects of climate change), yet the role of larval ecology in both current and future disease dynamics has been largely overlooked to date. That the effects we observe are substantial provides important motivation for future research to examine these processes with field strains under field conditions. Quantifying how larval traits feed through to impact malaria transmission could improve understanding of disease dynamics and inform the development of improved vector control strategies.

Data accessibility. All relevant data and R scripts are available at <http://dx.doi.org/10.5061/dryad.d1b7h>.

Authors' contributions. L.L.M.S., C.C.M. and M.B.T. designed and conceptualized the experiment; L.L.M.S. and R.J.T. performed the experiment and data collection; L.L.M.S., G.R.J. and C.C.M. analysed the data; L.L.M.S., C.C.M. and M.B.T. wrote the manuscript.

Competing interests. All authors declare that they have no financial or non-financial competing interests.

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