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Title:

Thermal ecology of malaria transmission and the potential impact of behavioural resistance

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Abstract:

A number of studies report changes in the biting time of malaria mosquitoes following the introduction of long-lasting insecticide-treated bed nets (LLINs). Here, we explored whether timing of blood feeding interacts with environmental temperature to influence the vector competence of *Anopheles* mosquitoes for the human malaria parasite, *Plasmodium falciparum*. We found no effect of biting time on the proportion of mosquitoes that became infectious at constant temperature. However, the addition of realistic daily temperature fluctuation reduced the vector competence of mosquitoes feeding in the morning and increased the competence of those feeding in the early evening. A transmission dynamics model illustrates that such changes could have important implications for the epidemiological impact of "behavioural resistance". A shift in mosquito biting to the morning could reduce the transmission probability, and so poses little epidemiological risk. However, an increase in early evening biting could increase transmission not only because people are unprotected by bed nets, but also because there is a higher chance of blood-feeding mosquito becoming infectious.

Introduction

Wide scale use of long-lasting insecticide-treated bed nets (LLINS) and indoor residual insecticide sprays (IRS) has led to substantial declines in the global burden of malaria in recent years[1]. However, these gains are now threatened by the evolution of insecticide resistance[2-4]. Studies from many locations demonstrate both target site and metabolic resistance to be widespread in malaria vector populations[2-4]. In addition, there are growing reports of behavioural resistance, such as changes in mosquito biting behaviour (i.e. "anti-insecticide" behaviour), which reduce the probability of insecticide encounter and/or attenuate the efficacy of insecticides[5-8].

In principle, physiological mechanisms of resistance can be countered by switching classes of insecticide or using synergists to disrupt detoxification mechanisms[9-13]. However, behavioural resistance is potentially more insidious since changes in biting time (e.g. early evening biting before humans are protected under bed nets) and/or shifts in biting location (outdoor biting rather than indoors) could render whole classes of vector control tools ineffective[7,8,14]. Furthermore, even in the absence of behavioural or physiological resistance, typical biting patterns for many malaria vectors still span periods of the evening and morning, when effective coverage of bed nets is less[15]. This crepuscular biting behaviour contributes to 'residual transmission,' which is defined as the transmission that persists after achieving full universal coverage with an effective intervention such as LLINs, to which local vector populations are fully susceptible [15,16].

Vector competence describes the ability of an arthropod to become infected, allow replication, and ultimately transmit a pathogen[17]. Competence is determined by both genetic and environmental factors[17,18]. In order to become transmissible, malaria parasites go through multiple developmental stages within the mosquito, progressing from the gametocytes ingested in the blood meal, to gametes, the fertilized zygotes, the motile ookinetes that invade the mosquito midgut, the oocyst in which the parasite undergoes replication, and finally to the sporozoites that invade the salivary glands and can be passed onto a new host during a subsequent blood meal[19,20]. At present, little is known about whether biting time affects any of these processes, yet mosquitoes biting in the evening will tend to experience different

environmental temperatures and be at different points in their circadian cycle than mosquitoes that bite at midnight or in the morning. Mosquito gene expression is known to follow circadian rhythms[21-23]. Further, temperatures in many malaria endemic areas exceed 30°C during the day[24-27], and early parasite infection is known to be sensitive to high temperatures[28,29]. These extrinsic and intrinsic factors could have direct or indirect effects on parasite survival and establishment and hence, vector competence[22,28-30].

Here, we explore the effect of the temperature fluctuation and time of biting on vector competence of *Anopheles* mosquitoes. We find that while there is little apparent effect of circadian rhythm, diurnal temperature fluctuation leads to a significant increase in the vector competence of evening biting mosquitoes, but a decrease for morning biting mosquitoes. The changes in vector competence appear associated with high thermal sensitivity of the parasites during the initial infection process. These data suggest biting time could be a major driver of vectorial capacity.

Results and Discussion

Daily biting activity of malaria mosquitoes

We first reviewed the contemporary malaria control literature using PubMed to examine the biting activity of *Anopheles* mosquitoes. We identified 270 papers between 2000 and 2017 that referred to biting time of malaria vectors, with 42 papers providing measures of hourly biting activity. Peak biting time of most malaria vectors is generally considered to occur around 00:00-04:00h[15,31] and from these 42 papers, we identified 78 cases where biting conformed to this conventional pattern (Supplementary Table 1 and Supplementary Table 2). However, we identified 64 cases indicating a peak in biting time to occur before 22:00h (evening biting) and 9 cases indicating a peak in biting after 05:00h (morning biting) (Supplementary Table 1 and Supplementary Table 2). In about one third of those papers reporting altered biting time, there was a suggestion of behavioural change in response to the use of LLINs. Further, a number of these papers reported measures of environmental temperature. In the majority (N = 21), the mean temperatures were 25°C or above (overall mean = 26.9°C), while the remainder (N = 12) had a lower mean of 21.4°C (Supplementary Table 1). There were significantly more cases of evening biting

than morning biting overall (Chi-square test, $LR\text{-}\chi^2 = 46.68$, $df = 1$, $P < 0.0001$) regardless of temperature group (Fisher's exact test, $P = 0.171$) (Supplementary Table 2).

Effects of biting time and diurnal fluctuating temperature on vector competence

Having confirmed the potential for both morning and evening biting, we conducted a series of experiments to investigate whether biting time affected the potential for malaria vectors to become infected with the human malaria parasite, *Plasmodium falciparum*. We focused on the warmer temperature conditions as these were the most common in our literature search, are representative of high transmission settings, and are typical of conditions used in the majority of lab-based studies exploring human malaria-mosquito interactions. Accordingly, experiments were run on a 12:12h light:day cycle at either constant 27°C, or a more realistic mean temperature of 27°C with a Diurnal Temperature Range (DTR) of 10°C. Diurnal temperature ranges of 5-20°C are common across many malaria transmission settings[24,32,33] and so DTR of 10°C is a representative intermediate value. Adult female mosquitoes of the African malaria vector, *A. gambiae*, were given infected blood meals at one of three times of the day to capture the range of potential feeding times from the evening through to the morning: 18:00h, 00:00h, or 06:00h. These times of day equate to Zeitgeber Times of ZT12, ZT18 and ZT0, respectively, where ZT0 refers to the beginning of the daylight cycle. For these time-of-day experiments, mosquitoes were maintained in separate incubators in which the timers were offset so that the actual feeds took place simultaneously using the same parasite culture, but the mosquitoes were at different points in their diel cycle. Note also that for the temperature fluctuation treatments, the mosquitoes were fed at 27°C, and then returned to their individual incubators to follow their particular diurnal thermal trajectories (Supplementary Fig. 1; see later discussion).

We found significant interactions between temperature and time-of-day on different measures of infection (oocyst intensity: Generalized Linear Mixed effects Model [GLMM], $F = 9.75$, $df = 2$, $P < 0.0001$; oocyst prevalence: Generalized Linear Model [GLM], $LR\text{-}\chi^2 = 25.27$, $df = 2$, $P < 0.0001$; sporozoite prevalence: GLM, $LR\text{-}\chi^2 = 33.6$, $df = 2$, $P < 0.0001$; Supplementary Table 3). Specifically,

under the constant temperature regime, time-of-day did not affect oocyst intensity (i.e. number of oocyst in the midgut of infected mosquitoes), oocyst prevalence (i.e. proportion of mosquitoes infected), nor sporozoite prevalence (i.e. proportion of mosquitoes with sporozoites in their salivary glands and hence, potentially infectious) (post-hoc contrasts, $P > 0.05$; Fig. 1). In contrast, under more realistic fluctuating temperatures, there was a significant effect of time-of-day on oocyst intensity, oocyst prevalence, and most importantly, sporozoite prevalence (post-hoc contrasts, $P < 0.05$; Fig. 1). Each of these infection measures was highest in mosquitoes fed at 18:00h (ZT12) and lowest in those fed at 06:00h (ZT0) (Fig. 1). For the 06:00h treatment, there was an approximate 97% reduction in sporozoite prevalence relative to the 18:00h treatment, with <2% of mosquitoes potentially able to transmit parasites. In addition, oocyst intensity and sporozoite prevalence was also lower in the 00:00h (ZT18) treatment compared to both the 18:00h (ZT12) treatment in the fluctuating temperature regime, and 00:00h (ZT18) in the constant temperature regime (post-hoc contrasts, $P < 0.05$; Fig. 1), suggesting that normal peak biting time might result in lower infections in the field than indicated in lab studies, which are typically conducted under constant temperature conditions.

These results were corroborated for a second mosquito species, the Asian vector *A. stephensi*, in a slightly simplified version of the experiment in which we compared feeding at evening (18:00h [ZT12]) vs morning (05:00h [ZT23]), under both constant and fluctuating temperatures. We found significant interactions between temperature and time-of-day on different measures of infection (oocyst intensity: GLM, $LR\text{-}\chi^2 = 4.78$, $df = 1$, $P = 0.029$; oocyst prevalence: GLM, $LR\text{-}\chi^2 = 16.51$, $df = 1$, $P < 0.0001$; sporozoite prevalence: GLM, $LR\text{-}\chi^2 = 7.38$, $df = 1$, $P = 0.007$; Supplementary Table 4). Specifically, oocyst intensity, and oocyst and sporozoite prevalence were not affected by time-of-day at constant 27°C (post-hoc contrasts, $P > 0.05$), but all were significantly reduced when mosquitoes were fed in the morning under 27°C with a DTR of 10°C compared with the evening and morning feeds at constant temperatures (post-hoc contrasts, $P < 0.05$; Supplementary Fig. 3).

Effect of altered vector competence on malaria transmission potential

Our initial experiments suggest the potential for biting time to alter vector competence when daily temperatures fluctuate. To further explore the significance of these findings, we used a deterministic version of a transmission dynamics model of malaria[34-37] to illustrate the potential public health implications of changes in vector competence in the context of LLIN use. First, we examined the effects of differences in vector competence alone on malaria prevalence, considering feeding distribution for an anthropophilic and anthropophagic vector where most bites happen at midnight and indoors (i.e. 70% at midnight and 30% in the evening and morning[38]) and theoretical scenarios for shifts in biting time towards the evening (70% in the evening and 30% at midnight), or towards the morning (70% in the morning and 30% at midnight). Differences in mosquito behaviour could have a noticeable impact on disease endemicity, with evening biting increasing equilibrium infection prevalence and morning biting reducing prevalence (Fig. 2 and Supplementary Table 5). Adding LLINs at one net per two people (approximating universal coverage[39]) reduced infection prevalence, but the relative efficacy of LLINs was lower when biting is shifted towards the evening and greater when biting is shifted towards the morning, even when we assumed equivalent exposure to the LLINs for the different feeding behaviours (Fig. 2 and Supplementary Table 5). When we included the fact that evening and morning biters will likely experience reduced contact with LLINs (in the model, we halve the probability that biting takes place when people are in bed for the evening or morning biters), malaria prevalence increased overall, but the shift to evening biting resulted in the greatest prevalence and the lowest relative effectiveness of LLINs (Fig. 2 and Supplementary Table 5). Further mathematical modelling work is needed to better understand the full implications of this difference in human-to-mosquito transmission, though it will be impeded by a general lack of knowledge of mosquito behaviour and transmission ecology[40-42].

Determining the effect of temperature fluctuation on vector competence

In order to better understand the influence of temperature fluctuation on vector competence, we conducted a series of experiments to determine the thermal sensitivity of malaria parasite establishment. The focus on initial parasite establishment is justified since after 24h, all fluctuating temperature

169 treatments are essentially identical. First, we examined the effects of absolute temperature by feeding *A.*
170 *gambiae* and *A. stephensi* infected blood and maintaining them under constant temperatures of 27°C
171 (control), 30°C, or 32°C. We observed a decline in overall oocyst intensity (GLM, $LR\text{-}\chi^2 = 78.7$, $df = 1$, P
172 < 0.0001) and oocyst prevalence (GLM, $LR\text{-}\chi^2 = 36.9$, $df = 1$, $P < 0.0001$) at 30°C relative to 27°C for
173 both mosquito species, while no oocyst infections were observed at 32°C (Fig. 3a and Supplementary
174 Table 6). These data indicate that parasite establishment is constrained at temperatures that exceed 30°C.
175 Next, we examined the importance of duration of exposure to high temperatures by varying the period of
176 incubation at the permissive temperature of 27°C from 3 to 48h post blood meal, before moving
177 mosquitoes to the more constraining temperature of 30°C. In this case, overall infection levels were low
178 because the parasite culture had unexpectedly low gametocytemia. Nonetheless, we found that incubating
179 at 27°C for 12 to 24h led to a progressive recovery in oocyst intensity and oocyst prevalence rendering
180 the infections statistically not different to those observed in a cohort maintained at 27°C (post-hoc
181 contrasts, $P > 0.05$), while those mosquitoes transferred to 30°C before 12h showed no infections (Fig.
182 3b). Consistent with earlier work[28,29], these data indicate that transient exposure to temperatures > 30°C
183 can reduce vector competence via a negative effect on the initial stages of parasite development, and help
184 explain the time-of-day effects observed under fluctuating warm temperatures. That is, mosquitoes
185 feeding in the morning (i.e. 06:00h [ZT0]) have only 4h before temperatures exceed 30°C, while those
186 that feed at midnight or in the evening (i.e. 00:00h [ZT18] or 18:00h [ZT12]) have 10h and 16h at
187 permissive temperatures, respectively. As the duration of permissive temperatures increases, so does the
188 probability of parasite establishment.

189 The mechanisms underlying the transient thermal sensitivity remain unclear. There could be
190 direct negative effects of temperature on parasite biology and/or indirect effects mediated via the
191 mosquito. Previous research has suggested that increased blood digestion rate at higher temperatures
192 could increase the quantity of midgut proteases, potentially reducing ookinete density in the mosquito
193 midgut[29]. Given the importance of elements of the innate immune response and certain components of the

midgut microbiome in determining susceptibility to infection, it is possible that these factors could also interact with temperature[43,44].

An additional observation is that the effects of high temperature appear to vary to some extent with oocyst intensity. For example, the data presented in Fig. 3a had the highest baseline intensities amongst our various experiments and in this case, reduction in oocyst prevalence at 30°C was not as high as when the baseline intensity was lower. To test the hypothesis that the negative effects of exposure to high temperature depend on infection intensity, we fed *A. gambiae* blood meals containing four different dilutions (1, 1/2, 1/4, or 1/10) of gametocytes to generate a range of infection loads, and then kept them at 27°C or 30°C. At 27°C, the oocyst prevalence varied from 84 to 52% across the dilution treatments, with median oocyst intensities ranging from nine down to one per mosquito (Supplementary Fig. 4). Incubation at 30°C reduced oocyst intensity and prevalence across the board (oocyst intensity: GLM, $LR\text{-}\chi^2 = 5.96$, $df = 1$, $P = 0.015$; oocyst prevalence: GLM, $LR\text{-}\chi^2 = 138$, $df = 1$, $P < 0.0001$; Supplementary Table 7). However, the percent reduction in oocyst prevalence was 73% in the highest oocyst intensity treatment and increased up to 96% in the lowest intensity treatment (Supplementary Fig. 4). Furthermore, when we plot percent reduction in oocyst prevalence due to high temperature against mean number of oocysts per mosquito for each of our experiments, we find that the impact of temperature declines as intensity of infection increases (Supplementary Fig. 4). Whether this pattern holds across a wider range of infection intensities is not known[45], but the effects could be important as natural infection intensity of *P. falciparum* tends to be lower than those observed in our experiments[46].

Assumptions and relevance to natural systems

The robustness of our results depends on a number of assumptions. First, we assume that in a fluctuating temperature environment, mosquitoes will generally track ambient temperature and not exhibit strong thermoregulatory behaviours that might limit exposure to the critical temperatures that impact parasite establishment. In order to investigate this, we adapted methods from a previous study[47] to examine the thermal avoidance behaviour of *A. gambiae* following a blood meal that is either infected or uninfected.

220 The approach exposes mosquitoes to temperatures that ramp gradually from 28 to >35°C and monitors the
221 time point at which mosquitoes escape the warmed microenvironment (Fig. 4a). We found no evidence
222 that mosquitoes were sensitive to temperatures of 30-32°C and only observed a thermal escape response
223 as temperatures approached 35°C (Fig. 4b). There were no differences between infected and uninfected
224 mosquitoes in escape response (Log-rank test; $\chi^2 = 1.25$, $df = 1$, $P = 0.264$) (dissection of mosquitoes from
225 this experiment revealed oocyst prevalence of 60-75% with 5.5-9 median oocyst intensity). The results
226 confirm that mosquitoes are behavioural unresponsive to temperatures that are critically damaging to
227 malaria parasite establishment.

228 Second, in our experiments the blood meal was administered at the mean temperature of 27°C
229 before mosquitoes were returned to their respective temperature treatments. This was done to standardize
230 blood feeding compliance and hence the proportion of mosquitoes acquiring parasites (note, blood
231 feeding frequency exceeded 95% in all experiments). It is also technically challenging to blood-feed
232 mosquitoes at different ambient temperatures for different temperature treatment groups using the same
233 parasite culture at the same time. In reality, mosquitoes have to feed at the prevailing ambient
234 temperatures. However, these prevailing temperatures for the different feeding times in the 27°C DTR
235 10°C regime vary from 22.6 to 28.5°C, so it is unlikely that these modest temperature differences would
236 impact feeding compliance or efficiency, especially when the blood meal itself is at 37°C and this has a
237 marked effect on mosquito body temperature during the feeding process[48,49]. To provide some
238 confirmation of this, we conducted a simple assay to compare the feeding efficiency of *A. gambiae*
239 mosquitoes at 21 and 27°C. We found no effect of temperature on feeding compliance (GLMM, $F = 1.39$,
240 $df = 1$, $P = 0.284$; Supplementary Table 8 and Supplementary Table 9). Furthermore, as part of a separate
241 investigation, we have conducted an experiment in which *A. gambiae* adults were maintained at 21°C, fed
242 at 27°C and then returned to 21°C. We found no difference in oocyst intensity, or oocyst or sporozoite
243 prevalence between mosquitoes transferred between 21 and 27°C, and those maintained at 27°C
244 throughout (post-hoc contrasts, $P > 0.05$; Supplementary Fig. 5). These data suggest that translocation
245 between temperatures for feeding, in and of itself, is unlikely to have a confounding effect.

246 Third, our study used standard laboratory mosquito and parasite strains, and it is possible that in
247 field settings, local adaptation could yield different patterns of thermal sensitivity for parasites in wildtype
248 mosquitoes[50]. Previous studies do indicate that infection with *P. falciparum* is possible above 32°C[51,52],
249 and there is a suggestion that naturally circulating parasites might exhibit higher thermal tolerance than
250 standard lab strains[53]. However, one study using parasite populations from naturally infected children in
251 Kenya found that while parasites could establish in mosquitoes exposed to 32°C in half (8/16) of the cases,
252 infections were eliminated in the other half[53]. Whether this pattern results from variation in parasite
253 thermal sensitivity between different infected hosts is not known. Our data, together with those of Bradley
254 *et al.*[54], suggest there could also be an influence of variation in gametocyte densities between hosts.
255 Nonetheless, the high thermal sensitivity of the early stages of malaria parasite infection is widely
256 observed in diverse systems, including human (*P. falciparum* and *P. vivax*), rodent (*P. chabaudi* and *P.*
257 *berghei*), and avian malaria (*P. relictum*)[28,29,32,55-57], so there is little reason to think the qualitative effects
258 we report are unique to our experimental system.
259 Finally, how the time-of-day effects that we report here ultimately impact malaria transmission
260 intensity in the field will likely vary with prevailing temperatures. If either the mean temperatures or the
261 extent of daily temperature variation limit exposure to temperatures above 30°C, there might be little
262 impact of biting time. However, there are extensive areas of malaria transmission in Africa where peak
263 daily temperatures exceed 30°C[24-27]. Furthermore, interactions with other traits could influence the net
264 impact on transmission. For example, it is generally assumed that malaria vectors feed at midnight to
265 exploit sleeping hosts and reduce biting-related mortality[15]. The extent to which feeding earlier in the
266 evening increases mortality rate or otherwise influences mosquito-to-human transmission and thus
267 vectorial capacity overall, is unknown. There could also be effects linked to circadian patterns in
268 gametocyte density[22] and the developmental rhythm of parasites[58]. Our experiments used cultured
269 parasites and we found little evidence for circadian effects in the mosquito in the absence of temperature
270 fluctuation. Recent work on rodent malaria, however, indicates that gametocytes are less infective in the
271 day than at night, but this reduced infectivity is partly offset by mosquitoes being more susceptible to

infection when they feed during the day[59] (though it should be noted that neither the mosquito or the rodent species used in these latter studies is the natural host, and the infection experiments were conducted under constant temperatures).

Conclusions

Overall, our empirical and theoretical findings have significant implications for basic understanding of malaria transmission since they suggest that not all mosquito bites are equivalent and that evening bites might contribute disproportionately to transmission. There is significant interest in how aspects of the innate immune system[60-62], or factors such as the midgut microbiome[63-65], can impact the capacity of mosquitoes to transmit malaria parasites. In the context of this research, it is noteworthy that ecological factors like daily variation in temperature and biting time can interact to render the same mosquitoes either completely susceptible, or essentially refractory. These results are not simply of academic interest as they also complicate the interpretation and significance of residual transmission and behavioural resistance. On the one hand, if mosquitoes feed in the morning, the reduced transmission potential of the mosquitoes could compensate for the lower contact rate with LLINs. Such an effect would represent an unexpected positive side effect of selection on mosquito life history[66]. On the other hand, if mosquitoes feed in the early evening, then not only will LLIN contact rate tend to be reduced, but the mosquitoes could be even more efficient vectors, exacerbating the epidemiological consequences of behavioural resistance. Finally, our results could have implications for the impacts of climate change. Previous research has demonstrated that transmission potential declines as mean temperature increases beyond the thermal optimum, and this is exacerbated by daily temperature variation[67]. The current data suggest that the upper thermal limit could further be influenced by the characteristics of local biting behaviour.

Materials and Methods

Characterization of biting behaviour in *Anopheles* mosquitoes in the literature

We used eight combinatorial search terms composed of 'biting', either of 'malaria' or 'Anopheles', and one of 'nets', 'bednets', 'ITNs', or 'LLINs' in PubMed for identifying literature that provided hourly biting time data (18:00-06:00h) generated by human landing catch methods or human baited bed net traps. Publication year was limited to 2000-2017 considering a marked increase for the malaria control efforts in sub-Saharan Africa since 2000[1]. Conventional peak biting time of *Anopheles* mosquitoes is generally known to occur between 00:00-04:00h[15,31], and studies have shown majority of people go to bed at 21:00-22:00h and get out of bed at 05:00-06:00h[7,68-73]. Accordingly, we considered cases of peak biting time before 22:00h (i.e. evening biting) or after 05:00h (i.e. morning biting) to be consistent with behavioural change.

Temperature data for the studies were either provided directly in the source literature or, if not presented, were estimated for the time and location of the study using Global Surface Summary of the Day (GSOD) provided by National Oceanic and Atmospheric Administration, Department of Commerce (https://data.noaa.gov/dataset/dataset/global-surface-summary-of-the-day-gsod), or Climte-Data.org (https://en.climate-data.org).

Mosquito transmission studies

Mosquitoes. *Anopheles gambiae* (G3, NIH) and *A. stephensi* (Liston, Walter Reed Army Institute of Research) mosquitoes were used throughout the experiments. Mosquitoes were reared under standard insectary conditions at 27°C±0.5°C, 80%±5% relative humidity, and a 12h:12h light-dark photo-period. Larval density and amount of larval food (ground TetraFin™; Tetra, Blacksburg, VA) were standardized to ensure uniform adult size. Adults were maintained on 10% glucose solution supplemented with 0.05% para-aminobenzoic acid (PABA). For the infectious feeds, 5-6-day-old female mosquitoes were randomly aspirated into cardboard cup containers that are covered with netting, and starved for approximately 6 hours before infectious feed. Individual containers contained 120-150 mosquitoes.

General procedures for infectious feeds and mosquito dissection. *In vitro* cultured *Plasmodium falciparum* (NF54 isolate, MR4) was provided by the Parasitology Core Lab

323 (http://www.parasitecore.org/) at John's Hopkins University. Gametocyte culture in stage four to five
324 (day 14 after gametocyte initiation) was transported overnight to Penn State in a sterile 50ml falcon tube
325 filled with fresh culture media. The culture tube was packaged in a Styrofoam box with heating pads to
326 keep the temperature at approximately 37°C during transport. Gametocyte-infected erythrocytes were
327 provided with fresh culture media on the day of arrival, and were maintained > 24 hours before the
328 infectious feed to allow additional maturation of gametocytes.

329 Mosquitoes were fed on day 16 post gametocyte initiation. The proportion of erythrocytes infected
330 with mature gametocytes (i.e. gametocytemia) generally ranged between 1-3% in the culture. An
331 infectious blood meal was prepared by mixing gametocyte infected erythrocytes with fresh human serum
332 and erythrocytes at 40% haematocrit on the day of blood feeding as previously described[74].
333 Gametocytemia in the blood meal was adjusted so that mosquitoes were infected at realistic infection
334 intensities (e.g., see Supplementary Fig. 4, and Bradly *et al*.[54]).

335 All infectious feeds were conducted in a walk-in environment controlled chamber. Glass bell jars
336 were uniformly covered with Parafilm to serve as membrane feeders and heated to 37°C with
337 continuously circulating water as previously described[74]. An appropriate amount of infectious blood (1-2
338 ml depending on the size of experiment but consistently the same amount within an experiment) was
339 pipetted into each bell jar. Containers of mosquitoes were randomly allocated to bell jars to minimize any
340 effect of position or feeder. Mosquitoes were fed for 20 min at 27°C, and > 95% mosquitoes were fully
341 engorged in all infectious feeds. Immediately after blood feeding, mosquitoes were placed into incubators
342 (Percival Scientific Inc., Perry, Iowa) with appropriate temperature treatment conditions (90%±5%
343 relative humidity, and 12h:12h light-dark photo-period) and provided daily with fresh 10% glucose
344 solution supplemented with 0.05% PABA. Mosquitoes were transferred and fed under red light as
345 appropriate to maintain light:dark cycles.

346 To determine vector competence, mosquitoes were randomly collected by aspirating into 95%
347 ethanol, and midguts and salivary glands were dissected in 1× phosphate-buffered saline solution under a
348 standard dissecting scope. Presence or absence of parasite infection was determined by examining

midguts and salivary glands, and oocysts in midguts were counted, using a compound microscope. To ensure correct scoring, oocysts and sporozoites were inspected under 40× magnification and cross-checked by a second person. Oocyst or sporozoite prevalence was calculated as the total number of infected mosquitoes divided by the total number of dissected mosquitoes by combining dissection data from given dissection days and replicated containers of mosquitoes for each treatment.

Experimental design for infection studies. For experiments examining the effect of time-of-day of blood meal and diurnal temperature fluctuation on vector competence, *Anopheles* mosquitoes were infected at different times of day and maintained at 27°C with a Diurnal Temperature Range of zero (i.e. DTR 0°C) or with a DTR of 10°C (i.e. 27°C±5°C; Supplementary Fig. 6). The Parton-Logan model was used for the fluctuating temperature regime that follows a sinusoidal progression and an exponential decay for the day and night cycle, respectively[32,75]. The air temperature of incubator (Percival Scientific Inc., Perry, Iowa) was monitored closely using HOBO data loggers (Onset Computer Corporation, Bourne, MA) at 5 min intervals, and the accuracy of temperature was maintained with the error range of ± 0.5°C. Prior to infections, pupae were collected and placed into separate incubators in which the clocks were offset so that adult mosquitoes emerged into environments that were staggered in terms of time-of-day. This enabled us to do the infectious feeds simultaneously using the same parasite culture, but with the mosquitoes at different points in their diel cycle. In the first experiment, approximately 300 *A. gambiae* mosquitoes were provided with infectious blood meals in two containers of mosquitoes (150 each) at 18:00h (ZT12), 00:00h (ZT18), or 06:00h (ZT0) and maintained at 27°C with DTR 0°C or DTR 10°C. For dissections, twenty mosquitoes were sampled daily (10 per container) on 7, 8, and 9 days post infection (dpi) for oocysts and 14, 15, and 16 dpi for sporozoites. Oocyst intensity, or oocyst or sporozoite prevalence were determined using dissection data from the three days (sample size of 60 per treatment). Similarly in the second experiment, approximately 150 *A. stephensi* mosquitoes were fed in a container at 18:00h (ZT12) or 23:00h (ZT23) and maintained at 27°C with DTR 0°C or DTR 10°C. Approximately 10 mosquitoes were sampled daily on 8-10 dpi for dissecting midguts to determine oocyst intensity or

375 prevalence, and on 13, 14, and 16 dpi for dissecting salivary glands to determine sporozoite prevalence. In
376 the third experiment, to determine the effects of blood feeding at different temperatures on blood feeding
377 compliance, approximately 240 *A. gambiae* mosquitoes were provided with infectious blood meals in two
378 containers (120 each) at 18:00h (ZT12), 00:00h (ZT18), or 06:00h (ZT0) and maintained at 21°C DTR
379 0°C. Similarly to the first experiment, pupae were collected and placed into separate incubators for each
380 time-of-day treatment at 21°C DTR 0°C prior to infections. Blood feeding compliance was measured by
381 scoring unfed mosquitoes to compare with that of mosquitoes in 27°C DTR 0°C treatment group (with
382 three times of day as treatments) where mosquitoes were kept at constant 27°C throughout and blood fed
383 at 27°C as described above. A separate batch of parasite culture was used for each experiment.
384 Effects of temperature treatment, mosquito species and/or gametocytemia on vector competence was
385 examined in a series of infection experiments. In each experiment, approximately 120 mosquitoes were
386 fed in a container (unless otherwise specified) with *P. falciparum* infected blood meals, and maintained at
387 appropriate temperature conditions. Approximately 10-15 mosquitoes were collected daily for generally
388 2-3 days to dissect midguts or salivary glands, unless otherwise specified. Dissection days were
389 determined by Detinova's parasite growth model[76] and data from pilot tests (data not shown) to ensure we
390 sampled when infection prevalence was at a maximum. For measures of vector competence, oocyst
391 intensity, or oocyst or sporozoite prevalence were determined by combining data among dissection days.
392 In the first experiment, infected *A. gambiae* and *A. stephensi* mosquitoes were maintained at 27°C, 30°C,
393 or 32°C to examine the effect of high temperature on vector competence. In the second experiment, to
394 examine the effect of high temperature on early parasite infection, *A. stephensi* mosquitoes were
395 incubated at 27°C for 3h, 6h, 12h, 24h, or 48h before moving them to 30°C. As a control group, infected
396 mosquitoes were maintained at 27°C. In the third experiment, to examine the effects of gametocytemia
397 and temperature interaction on vector competence, *A. stephensi* mosquitoes were fed blood meals with
398 varying gametocytemia dilutions (1, 1/2, 1/4, or 1/10) and maintained at 27°C or 30°C. An infectious
399 blood meal was prepared as described above, and serially diluted to generate blood meals with lower
400 gametocytemia while maintaining 40% haematocrit. In the fourth experiment, 240 *A. gambiae*

mosquitoes were fed in two containers (120 each) and kept at 21°C to examine the effect of transferring mosquitoes between different temperatures. Prior to the infection, pupae were collected and placed into the incubator at 21°C. As a control, mosquitoes were kept at 27°C throughout. Control and treatment mosquitoes were fed at 27°C (at 00:00h [ZT18]). A separate batch of parasite culture was used for each experiment, and mosquitoes were fed around 18:00h (ZT12) to standardize time-of-day of blood feeding, unless otherwise specified.

Thermal avoidance assay

A. gambiae mosquitoes were collected at pupal stage and adapted for > 5 days at 27°C DTR 10°C until blood feeding. Mosquitoes were fed with either *P. falciparum* infected or uninfected blood meals at 06:00h (ZT0) as described above, and maintained at 27°C until used for the behavioural assay. Three containers of mosquitoes were fed (100 mosquitoes per container) for the infected or uninfected groups, and mosquitoes from a container from each group were used for each round of assay. Infected and uninfected blood meals were prepared as described above, but gametocyte infected-erythrocytes were replaced with uninfected erythrocytes in the uninfected blood meal. The behavioural assay was conducted in an environmental chamber at 27°C±0.5°C with 80%±5% relative humidity using WHO insecticide bioassay tubes as described previously[47] (Supplementary Fig. 7). One side of the tube (the holding tube) was wrapped with plastic tubing with continuously circulating water heated by a water bath (WB05; PolyScience Inc., Niles, IL) to control the inner surface temperature of holding tube, while the temperature of escape tube was maintained at 28°C±0.5°C. Ten mosquitoes fully engorged with either infected or uninfected blood meals were introduced into a holding tube and acclimated at 28°C±0.5°C. The assay tubes were used in rotation by mosquito groups fed with either infected or uninfected blood meals within and between the assays. The gate between the holding and escape tubes was opened after 20 min of acclimation, and mosquitoes could then choose to move to the escape tube. The number of mosquitoes in the escape tube was recorded every 2 min. No mosquitoes in the escape tube returned ever to the holding tube during the entire assay period. The temperature of water bath was set to 32.6°C at the

time of gate was opened, which was equivalent to the maximum temperature in 27°C DTR 10°C treatment. The surface temperature of holding tube increased at the rate of approximately 0.23°C/min over 20 min and was maintained at 32.6°C±0.5°C for an additional 20 min. The temperature of the water bath was then set to 36°C to further examine the thermal behaviour. The surface temperature of holding tube increased at the rate of approximately 0.17°C/min over 20 min, and was maintained at 36°C±0.5°C for additional 20 min. The rates of temperature increase were comparable to that of Kirby and Lindsay[47]. The temperature of the two holding tubes in the treatment group was recorded at 5 sec intervals using thermocouple data loggers (SL500; MicroDAQ.com, Ltd., Contoocook, NH) throughout the experiments. Baseline activity of mosquitoes were monitored as a control by keeping the temperature of the holding and escape tubes at 28°C±5°C throughout the experiment and otherwise following the same methods as for the treatment group. A total of eight assay tubes were used for running the control and treatment groups (four each) at the same time, with two replicates for the mosquito group fed with infected or uninfected blood meals. Three rounds of assay were conducted between 4-10 hours post infection totalling six replicates for each mosquito group (see Supplementary Fig. 7 for experimental setup). Oocyst prevalence and intensity were determined on 8 dpi in total cohort of 60 mosquitoes (20 per container) fed with the same infectious blood meal and kept at 27°C.

Transmission dynamics model

A deterministic version of a transmission dynamics model of malaria[34-37] was adjusted and used to explore the potential public health implications of a theoretical change in mosquito infectivity driven by the timing of mosquito bites. The transmission model mechanistically tracked *P. falciparum* infection in people and mosquitoes. Susceptible people were exposed to infectious mosquito bites at a rate dependent on local mosquito density and infectivity. Mosquito dynamics described the effects of mosquito control and the resulting decline in egg laying[35]. Adult mosquitoes could be either susceptible to malaria, infected, or infectious to people.

Model adjustment. Susceptible (S_V), exposed (E_V) and infective (I_V) mosquitoes were present, and the dynamics of these were expressed as:

$$\frac{d}{dt}(S_V) = -inc_e - \mu_V S_V + \beta$$

$$\frac{d}{dt}(E_V) = inc_e - \mu_V E_V - inc_v$$

$$\frac{d}{dt}(I_V) = inc_v - \mu_V I_V$$

Where mosquito pupae emerged as adults at a rate β that was density dependent and died at a constant rate μ_V which was independent of infection status. The rate of exposure to infection with *Plasmodium falciparum* (inc_e) was dependent on the force of infections (FOI_V) such that:

$$inc_e = FOI_V \times S_V$$

The Force of Infection was the product of: i) the sum of infectivity from all infective people in the population, ii) the biting rate, which was allowed to vary such that some people could be bitten more than others; iii) the biting rates of mosquitoes, which was dependent on vector intervention categories (bed nets and/or indoor residual spraying may be used), and; iv) the age-specific force of infection, which was normalized so that people of different ages could contribute differently to transmission.

The rate that mosquitoes then became infectious (inc_v) was dependent on the proportion of mosquitoes that survived long enough to transmit infection onward. The extrinsic incubation period (EIP) is the number of days needed for sporozoites to be found in the salivary glands. The current model had a constant EIP of 11.5 days (to match experimental data).

$$inc_V = inc_e \times Surv$$

$$Surv = exp(-\mu_V \times EIP)$$

Based on the experimental observations, the transmission model was adjusted so that mosquitoes that exhibit either evening ($Mosq_{EV}$), midnight ($Mosq_{MD}$) or morning ($Mosq_{MN}$) biting behaviour had different rates of becoming infective (inc_V).

The differential equations describing the infection status of adult mosquitoes was adjusted such that:

$$\frac{d}{dt}(S_V) = -inc_e - \mu_V S_V + \beta$$

$$\frac{d}{dt}(E_V) = inc_e - \mu_V E_V - (Mosq_{EV} + Mosq_{MD} + Mosq_{MN}) \times inc_v$$

$$\frac{d}{dt}(I_V) = (Mosq_{EV} + Mosq_{MD} + Mosq_{MN}) \times inc_v - \mu_V I_V$$

The sum proportions of mosquitoes in the different feeding classes equalled to 1 but the conversion to sporozoite positive status was different for each group. Our empirical study show that about 65% of mosquitoes that bite in the evening became infective, compared to 28% that bite at midnight and 1.7% of those that bite in the morning. The conversion term, *tob*:

$$tob = (Mosq_{EV} + Mosq_{MD} + Mosq_{MN})$$

was parameterized to sum to 1 when all mosquitoes are feeding at midnight. The proportion of mosquitoes was adjusted given the ratio of 65%:28%:1.7% for evening, midnight and morning biters respectively, such that:

$$Mosq_{EV} = Prop_{EV} \times 2.32$$

$$Mosq_{MD} = Prop_{MD} \times 1$$

$$Mosq_{MN} = Prop_{MN} \times 0.061$$

Mosquito biting patterns. Evidence suggests that most mosquitoes actively search for blood-meals in the middle of the night and less so either in the evening or morning[69,72,77,78]. To reflect this, we considered a 'status quo' scenario that examines the proportion of mosquitoes feeding during the evening and morning to be 0.15 each whilst the proportion feeding at midnight is 0.7 (as per Table 1, run 1 and 4). We then explored what would happen if mosquito feeding patterns shifted toward evening (Table 1, run 2 and 5) or morning (Table 1, run 3 and 6).

Net contact. The degree of protection that a bed net can elicit depends on the proportion of bites received while a person is protected. Therefore, in the transmission model, the impact of bed nets was determined by the proportion of bites that happens when a person is in bed (Φ_B). Bed nets were modelled to impact the probability of mosquito from species *i* successfully biting (w_i), and the probability of repellence (a mosquito is reflected away by the intervention before biting) (z_i) following (1):

$$w_i = 1 - \varphi_B + \varphi_B s_N$$

$$z_i = \varphi_B r_N$$

Mosquitoes that successfully feed (s_N), die (d_N) or repeat a feeding attempt (r_N) in the presence of a bed net relative to the absence of a bed net were estimated using data from experimental hut trials that examined the entomological impact of LLINs[2,34]. People are usually not in bed at 18:00h and start getting up before 06:00h[79,80], thus the probability of LLIN contact varied by mosquito biting time (in reality, these proportions may vary night to night or person to person but in the absence of data, we simply assigned different estimates for Φ_B to each biting class):

$$\varphi_B = \left(\varphi_{B_{EV}} \times Prop_{EV} + \varphi_{B_{MD}} \times Prop_{MD} + \varphi_{B_{MN}} \times Prop_{MN}\right)$$

For the outputs in Table 1, $\varphi_{B_{EV}}$ and $\varphi_{B_{MN}}$ were defined as 0.445 (half the contact with bed nets of midnight feeding mosquitoes) whilst $\varphi_{B_{MD}}$ was parameterized to be 0.89. This reflected mosquito population that feeds principally at midnight (run 1 and 4), in the evening (runs 2 and 5) or in the morning (runs 3 and 6).

Model simulations. We explored i) how much biting time might affect estimates of prevalence in 2-10-year-old children in a theoretical high transmission setting (runs 1-3); and ii) what would happen if we altered the probability of exposure to LLINs for each of the biting classes (runs 4-6). For simplicity, we assumed that: i) the mosquito population is density dependent; ii) biting rates are constant between people; iii) that there are either no interventions, or 50% of people use bed nets, and; iv) there is no pyrethroid resistance in the mosquito population. The scenario was a high transmission, perennial setting such that without interventions prevalence in 2-10-year-old children is about 65%.

We estimated the relative efficacy of nets as:

$$Efficacy = \frac{(Prevalence_0 - Prevalence_N)}{Prevalence_0} \times 100$$

Where subscripts 0 and N represent the scenarios without or with bed nets, respectively.

Statistical analyses

The ratio of the number of cases where biting time shifted towards either evening or morning (Supplementary Table 1 and Supplementary Table 2) was compared to the expected ratio of 50% using chi-squire goodness-of-fit test. Fisher's exact test (two-tailed) was used to test if this ratio of evening and morning biting was different between the high and low temperature groups (Supplementary Table 1 and Supplementary Table 2).

533 Generalized Linear Models (GLM) were used to analyse oocyst intensity, oocyst or sporozoite
534 prevalence data for all infection studies, unless otherwise specified. Oocyst intensity data were analysed
535 with a negative binomial error structure with log link, except for a translocation experiment (reported in
536 Fig. 3b), which used Poisson error structure with log link. Oocyst or sporozoite prevalence data were
537 analysed with a binomial error structure with logit link. Model fit and distributions were determined based
538 on Akaike's Information Criterion (AIC) value and residual plots.

539 A Generalized Linear Mixed effects Model (GLMM) was used to examine the effects of time-of-
540 day of blood meal, temperature regime, and their interaction on oocyst intensity for the time-of-day
541 experiment using *A. gambiae* as each treatment group had two technically replicated containers of
542 mosquitoes. Container of mosquitoes was included as a random variable, and dissection day was
543 additionally included as a fixed variable in the model to account for any day effect. For prevalence data,
544 GLM was used for pooled data from two containers of mosquitoes after confirming no difference in
545 oocyst or sporozoite prevalence between the containers using Fisher's exact test (two-sided) within each
546 treatment group. This was because the variance of the random effect was estimated as zero (i.e. Hessian
547 matrix not positive definite) rendering validity of model uncertain when GLMM was used for prevalence
548 data[81,82]. Similarly to the model structure used in GLMM, time-of-day, temperature regime, and their
549 interaction were used as fixed variables in addition to dissection day. When a significant interaction effect
550 was observed between time-of-day and temperature regime, time-of-day groups were pairwise compared
551 for oocyst intensity, or oocyst or sporozoite prevalence within temperature regime groups and between
552 temperature regime groups for each time-of-day group, using post-hoc contrasts followed by Bonferroni
553 corrections.

554 For the constant or translocation experiments, GLM was used to examine the effects of temperature
555 treatments, mosquito species, gametocytemia and/or interactions on oocyst intensity, or oocyst or
556 sporozoite prevalence in each study. Treatment groups with zero infections were not included in the
557 analyses[81,82]. When one control group was compared to all other treatment groups, post-hoc contrasts
558 were used followed by Bonferroni corrections. To examine the effect of parasite intensity on the reduction

in infection prevalence at 30°C, a linear regression was used to examine the relationship between the mean oocyst intensity in 27°C control group and percent reduction in the oocyst prevalence at 30°C. The percent reduction was calculated as the reduced percentage in oocyst prevalence in the 30°C treatment relative to oocyst prevalence in the 27°C control using the data collected from the infection studies described above.

The escape probability of mosquitoes combined from six replicates was analysed using Kaplan Meier Log-rank test to examine the effects of parasite infection on the proportion of mosquitoes that escaped over time. Any mosquitoes that escaped within one minute after opening the gate were left-censored as it was considered a response to human disturbance. Mosquitoes that remained in the holding tube until the end of assay were right-censored.

The effects of temperature during blood feeding and time-of-day on the feeding compliance of *A. gambiae* mosquitoes (blood feeding success of individual mosquitoes) examined by using a GLMM (binomial error structure, logit link) as each treatment group had two technically replicated containers of mosquitoes. Temperature, time-of-day, and their interaction were included as fixed variables, in addition to container of mosquitoes as a random variable in the model. SPSS Statistics 25 (SPSS Incorporation, Chicago, IL) was used for all analyses.

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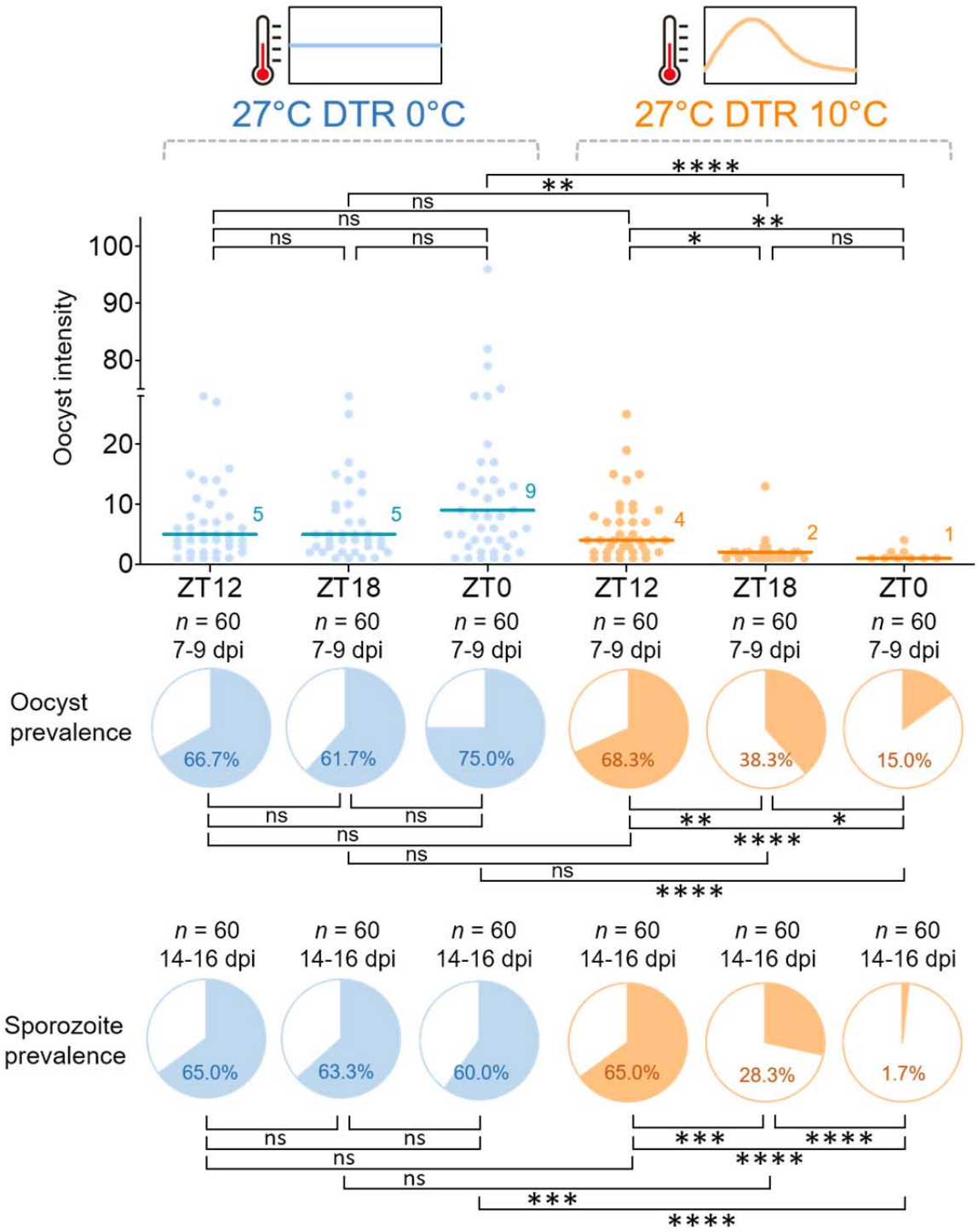

Figure 1. Effects of time-of-day of blood meal and diurnal temperature fluctuation on vector competence of *A. gambiae* mosquitoes infected with *P. falciparum* malaria. Mosquitoes were offered infected blood meals at a different time-of-day (18:00h [ZT12], 00:00h [ZT18], or 06:00h [ZT0]) and

801 kept under either constant (i.e. 27°C with a Diurnal Temperature Range [DTR] of 0°C) or fluctuating (i.e.
802 27°C with a DTR of 10°C) temperature regimes. The scatter plots show oocyst intensity, with the data
803 points representing the number of oocysts found in infected individual mosquitoes, and the horizontal
804 lines the median. The pie charts show oocyst or sporozoite prevalence calculated as the proportion of
805 infected mosquitoes revealed by dissection of midguts and salivary glands, respectively. Results of model
806 analyses to examine the effects of time-of-day and temperature regime on oocyst intensity, or oocyst or
807 sporozoite prevalence are reported in Supplementary Table 3. Asterisks indicate statistically significant
808 differences between treatments (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$; ns, not
809 significant at $P = 0.05$; P-values were Bonferroni corrected after pairwise comparisons). *n* indicates the
810 number of mosquitoes sampled from two technically replicated containers of mosquitoes. Twenty
811 mosquitoes were sampled daily (10 per container) for dissecting midguts on 7-9 days post infection (dpi)
812 or salivary glands on 14-16 dpi. Data for individual containers are reported in Supplementary Fig. 2.

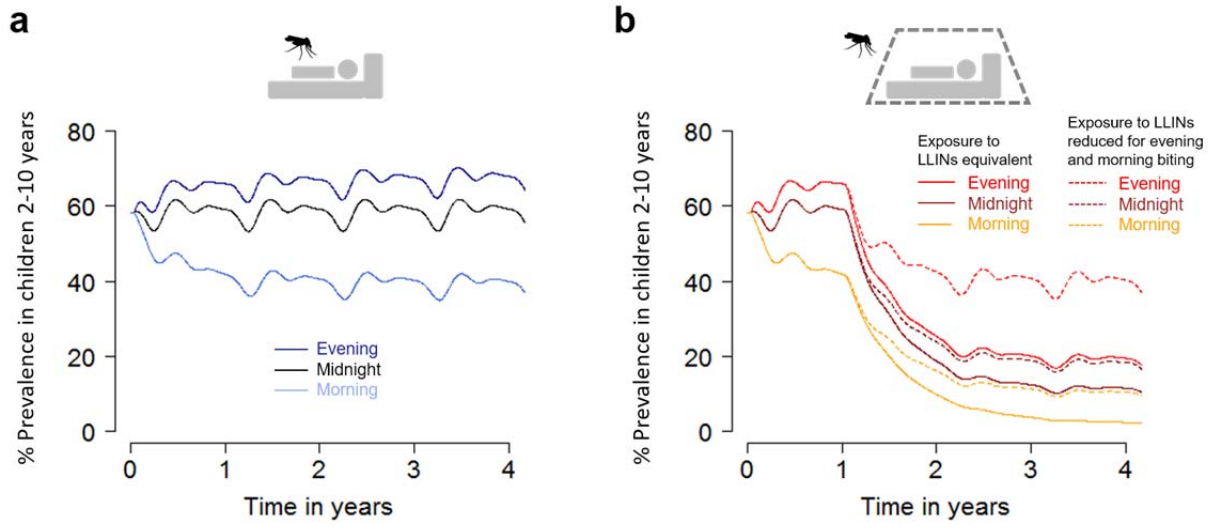

Figure 2. Epidemiological significance of altered vector competence. a, Effect of altered vector competence on malaria prevalence in a high transmission setting with mosquitoes biting predominantly in the evening (dark blue line, run 1 and 4 in Supplementary Table 5; increased vector competence), at midnight (black line, run 2 and 5 in Supplementary Table 5; unaltered vector competence) or in the morning (pale blue line, run 3 and 6 in Supplementary Table 5; reduced vector competence) in the absence of bed nets (LLINs). **b**, Impact of LLINs on malaria prevalence when mosquitoes bite predominantly in the evening (red line), at midnight (dark red line), or in the morning (orange line) with altered vector competence of mosquitoes only (run 1 - 3 in Supplementary Table 5; solid line), or with both altered vector competence of mosquitoes and reduced exposure to LLINs in the evening and morning biting mosquitoes (run 4 - 6 in Supplementary Table 5; dashed line).

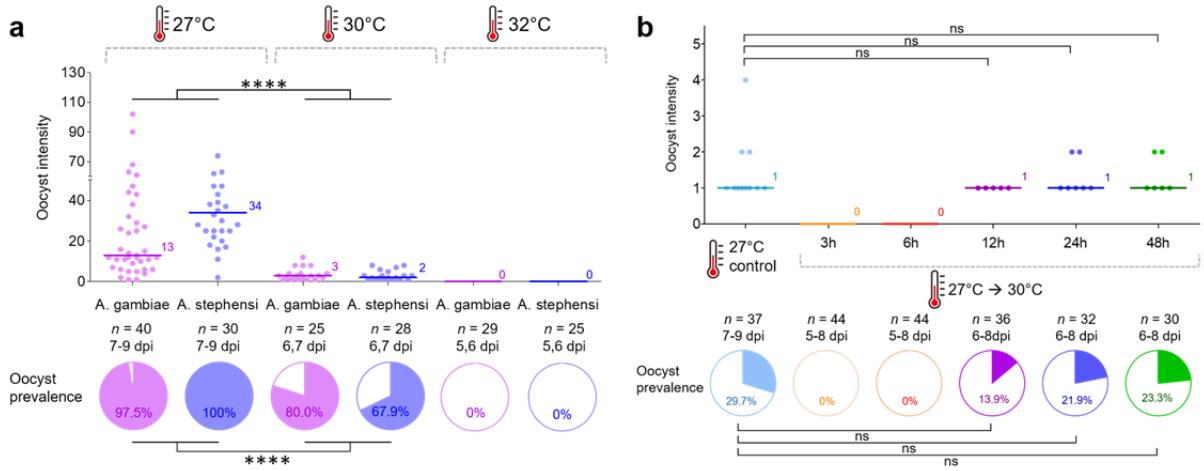

Figure 3. Effect of exposure to high temperatures on vector competence of *Anopheles* mosquitoes infected with *P. falciparum* malaria. **a**, *A. gambiae* and *A. stephensi* mosquitoes were kept at 27°C, 30°C, or 32°C following an infectious blood meal. Results of GLMs to examine the effects of temperature treatment and mosquito species on oocyst intensity or prevalence are reported in Supplementary Table 6. Asterisks indicate statistically significant differences between treatment groups (**** $P < 0.0001$). **b**, *A. stephensi* mosquitoes were incubated at 27°C for various periods of time ranging from 3 to 48h following an infectious blood meal, before being transferred to 30°C. Control mosquitoes were kept at 27°C throughout. The control group was compared with each treatment group with > 0 infection using GLM with pairwise post-hoc contrasts followed Bonferroni corrections for *P*-values (ns, not significant at $P = 0.05$). For **(a)** and **(b)**, the scatter plots show oocyst intensity, with the data points representing the number of oocysts found in individual mosquitoes, and the horizontal lines the median. The pie charts show oocyst or sporozoite prevalence calculated as the proportion of infected mosquitoes revealed by dissection of midguts and salivary glands, respectively. *n* indicates the number of mosquitoes sampled per treatment group (dpi = days post infection).

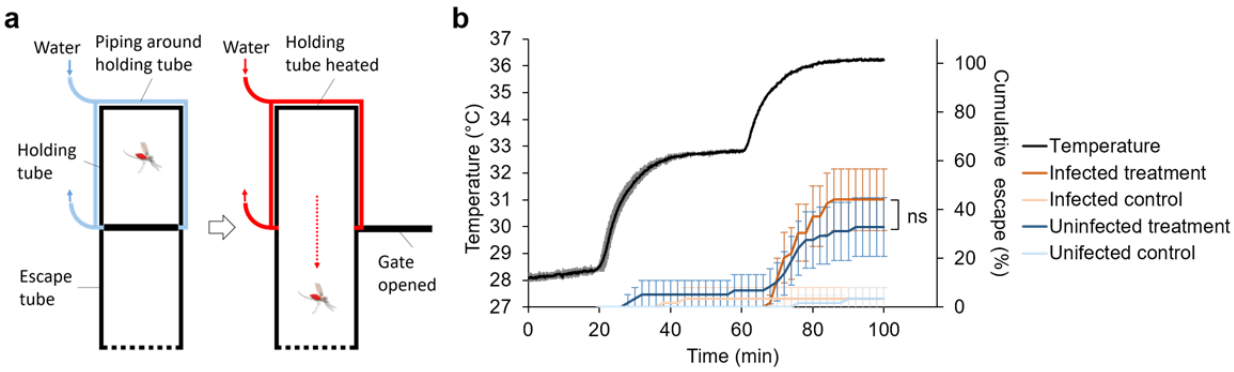

Figure 4. Behavioural assay to investigate thermal avoidance behaviour of *A. gambiae* mosquitoes following a blood meal. a, Diagram of the behavioural assay. The apparatus comprises two clear Perspex tubes joined by a sliding gate. One tube (the holding tube) is wrapped in plastic piping through which water is circulated. Infected or uninfected blood-fed mosquitoes (blood fed at 06:00h [ZT0]) are introduced into the holding tube and after a period of acclimation, the water is gradually heated from 28-36°C, and the sliding gate opened. The rate at which the mosquitoes leave the holding tube and enter the adjacent escape tube is recorded. For a control, the water is maintained at constant 28°C to measure baseline movement rates across the assay period for both infected and uninfected mosquitoes. **b**, Cumulative escape rate of infected and uninfected *A. gambiae* mosquitoes (error bars = 95% confidence intervals) in relation to temperature in the holding tube. The black line shows mean temperature with standard deviation (grey lines) in the holding tube in the ramping temperature treatment from three replicate runs. There were six replicates in total for each of the four mosquito groups (infected or uninfected, with either ramping temperature or constant temperature). Log-rank test was used to compare escape probability between the treatment groups (ns, not significant at $P = 0.05$).

Supplementary Information for

Thermal ecology of malaria transmission and the potential impact of behavioural resistance

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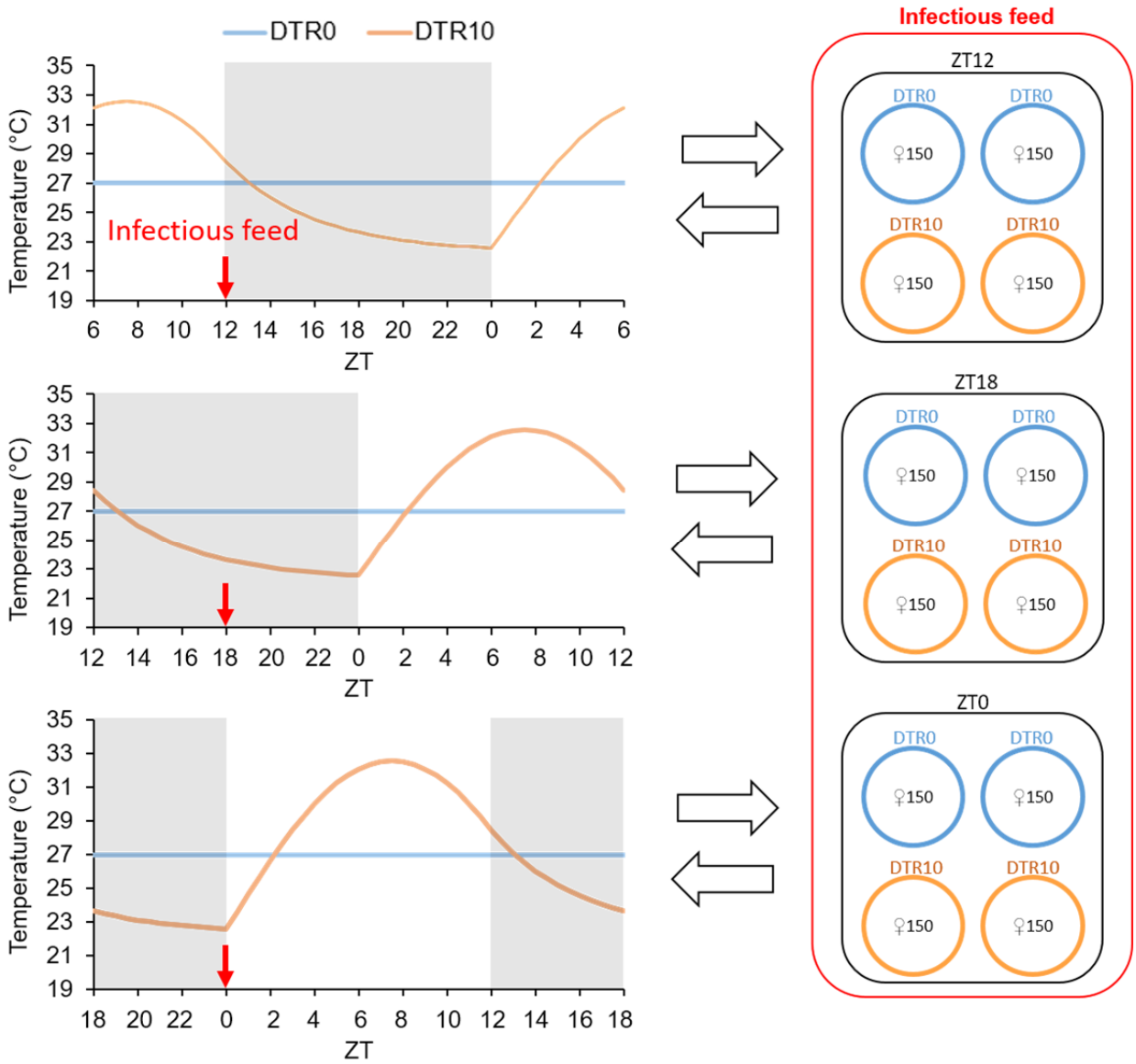
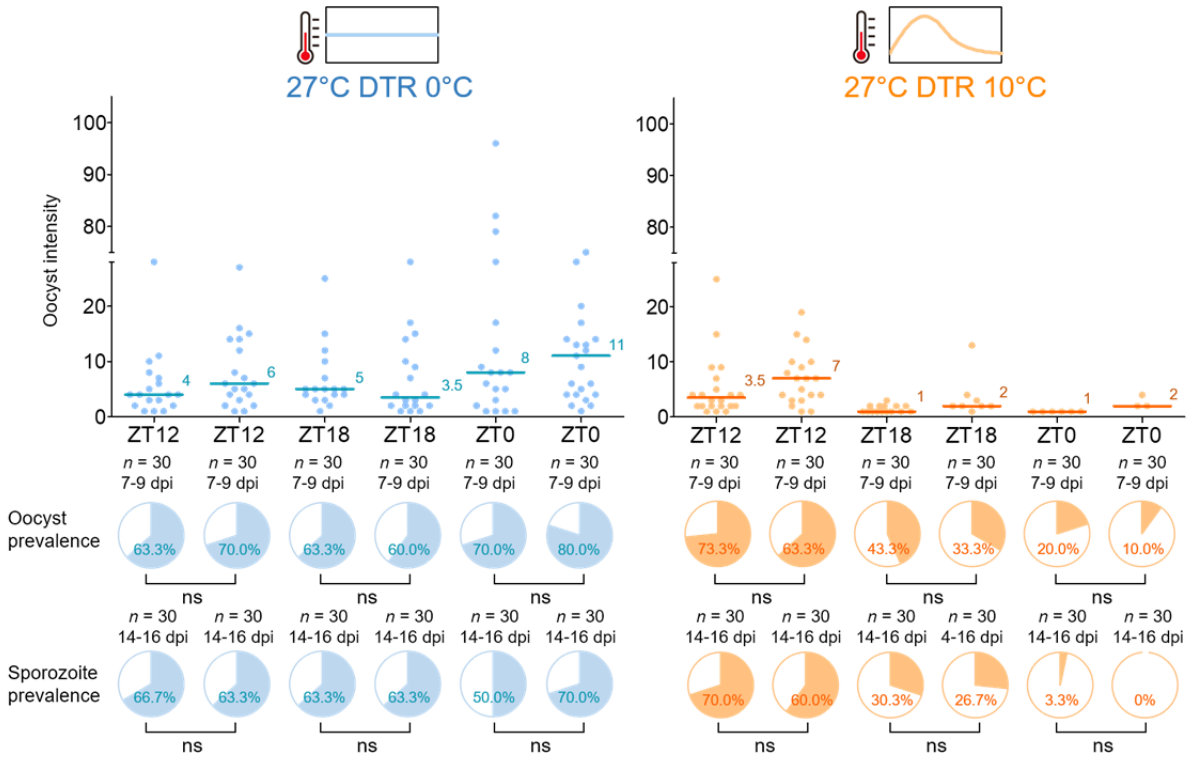
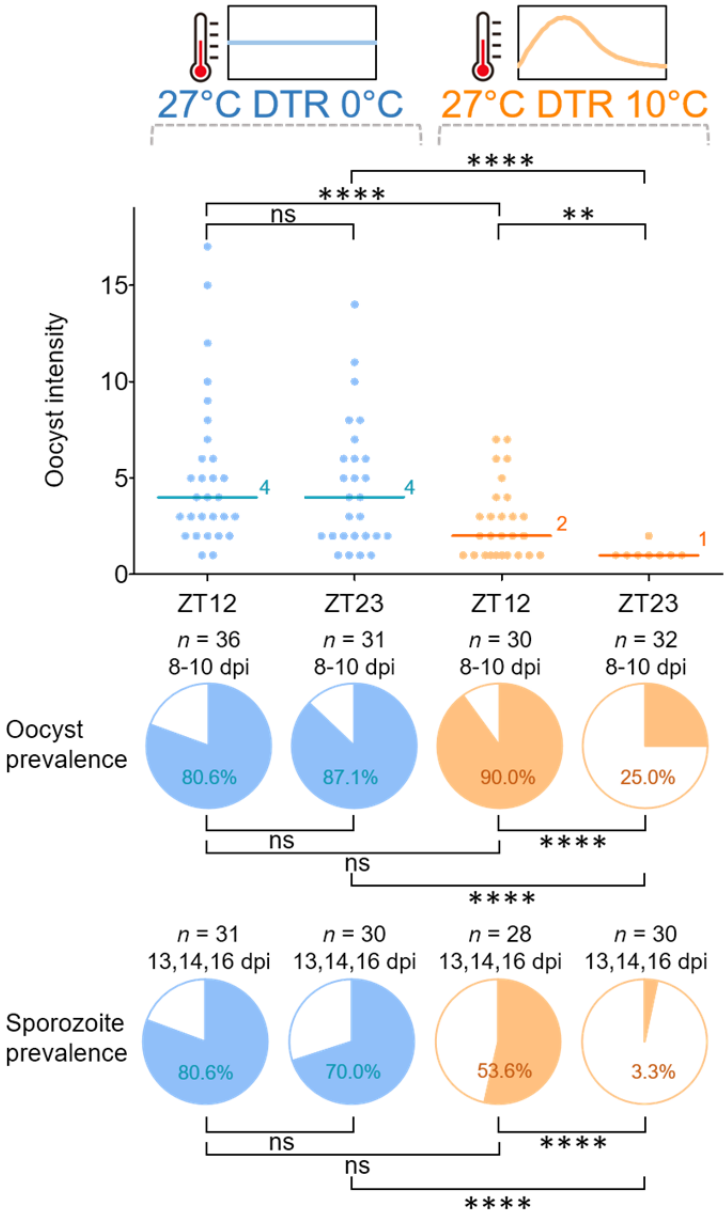


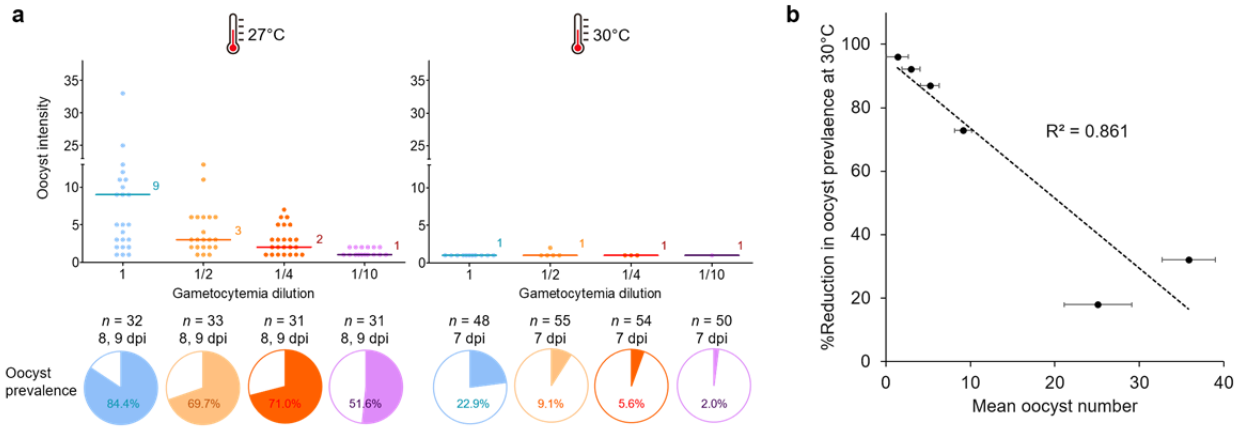
Supplementary Figure 1. Experimental design for infectious feeds. Adult mosquitoes were acclimated in separate incubators set at either constant (i.e. 27°C with a Diurnal Temperature Range [DTR] of 0°C) or fluctuating (i.e. 27°C with a DTR of 10°C) temperature regimes with a timer offset for each time-of-day treatment so that infectious blood feeding took place simultaneously using the same parasite infected blood meals, but the mosquitoes themselves were at different points in their diel cycle (18:00h [ZT12], 00:00h [ZT18], or 06:00h [ZT0]). Feeding took place in an environmental chamber set at 27°C and then blood fed mosquitoes were immediately moved back to their respective incubators. Each treatment group had 300 female mosquitoes in two containers (150 each).



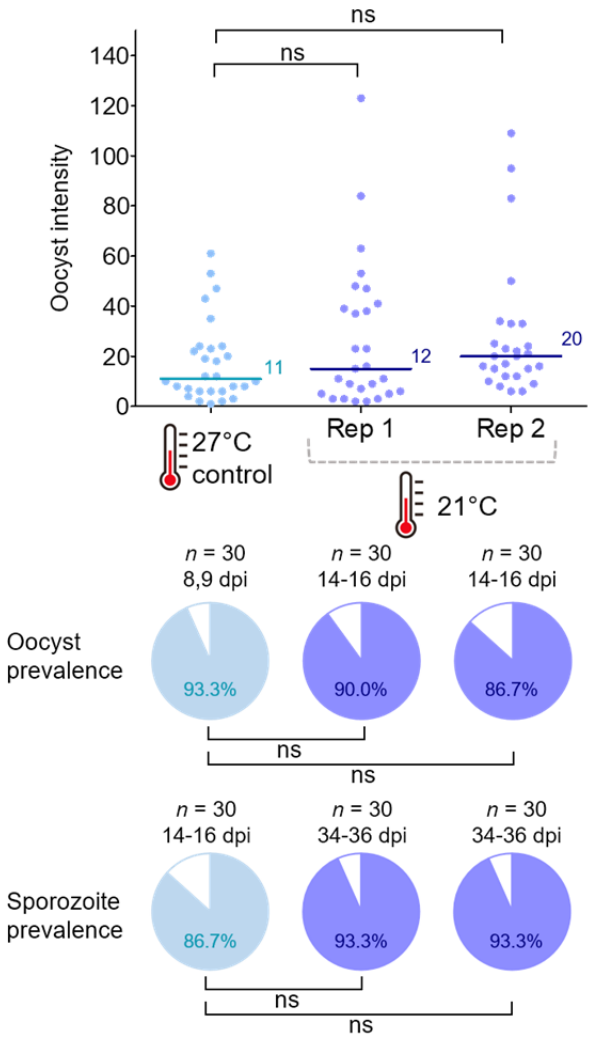
Supplementary Figure 2. Effects of time-of-day of blood meal and diurnal fluctuating temperature on vector competence of *A. gambiae* mosquitoes infected with *P. falciparum* malaria presented by two technically replicated containers of mosquitoes per treatment group. Mosquitoes were offered infected blood meals at a different time-of-day (18:00h [ZT12], 00:00h [ZT18], or 06:00h [ZT0]) and kept under either constant (i.e. 27°C with a Diurnal Temperature Range [DTR] of 0°C) or fluctuating (27°C with a DTR of 10°C) temperature regimes. The scatter plots show oocyst intensity, with the data points representing the number of oocysts found in individual mosquitoes, and the horizontal lines median oocyst number. The pie charts show oocyst or sporozoite prevalence calculated as the proportion of infected mosquitoes revealed by dissection of midguts and salivary glands, respectively. *n* indicates the number of mosquito sample per container. Ten mosquitoes were sampled daily from each container for dissecting midguts on 7-9 days post infection (dpi) or salivary glands on 14-16 dpi. After confirming no difference in oocyst or sporozoite prevalence between two containers of mosquitoes in each treatment group (Fisher's exact test, two-sided, $P > 0.05$), pooled data were used in GLM analysis (see Fig. 1 and Supplementary Table 3).



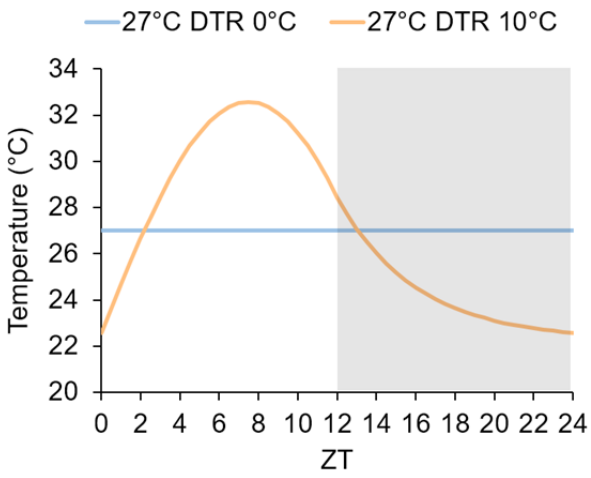
Supplementary Figure 3. Effects of time-of-day of blood meal and fluctuating temperature on vector competence of *A. stephensi* infected with *P. falciparum.* Mosquitoes were infected at a different time-of-day (18:00h [ZT12] or 05:00h [ZT23]), and kept under constant (i.e. 27°C with a Diurnal Temperature Range [DTR] of 0°C) or fluctuating (27°C with a DTR of 10°C) temperature regimes. The scatter plots show oocyst intensity, with the data points representing the number of oocysts found in individual mosquitoes, and the horizontal lines the median. The pie charts show oocyst or sporozoite prevalence calculated as the proportion of infected mosquitoes revealed by dissection of midguts and salivary glands, respectively. Results of GLMs to examine the effects of time-of-day and temperature regime on oocyst intensity, or oocyst or sporozoite prevalence are reported in Supplementary Table 4. *n* indicates the number of mosquito sample per treatment group. Approximately 10 mosquitoes were sampled daily for dissecting midguts on 8-10 days post infection (dpi) or salivary glands on 13, 14, and 16 dpi. Asterisks represent statistically significant difference (** $P < 0.01$, **** $P < 0.0001$; *P*-values were Bonferroni corrected after pairwise comparisons).



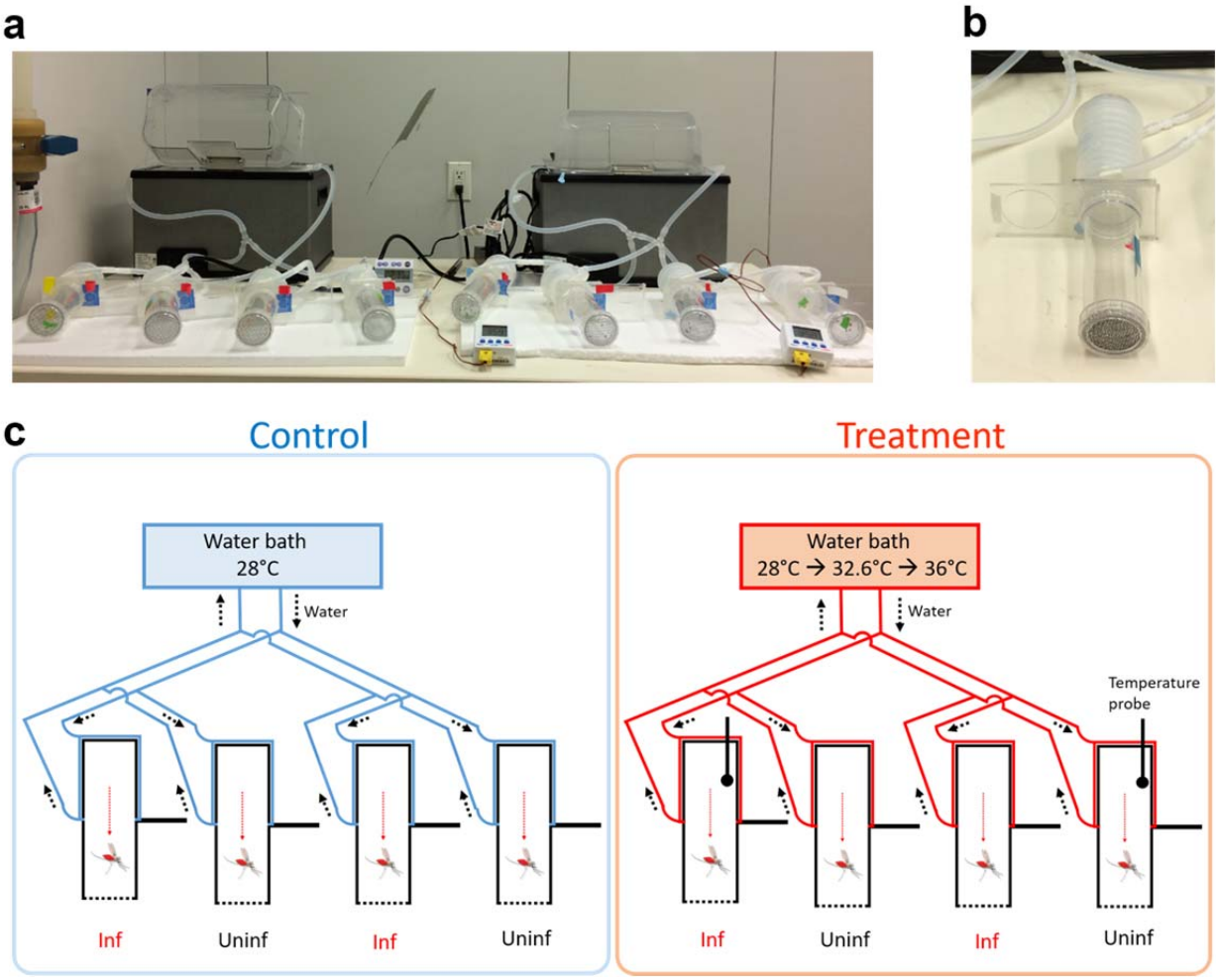
Supplementary Figure 4. Effects of gametocytemia and temperature on vector competence of *A. stephensi* mosquitoes infected with *P. falciparum* malaria. **a,** Mosquitoes were fed on blood meals with serially diluted gametocytemia (1, 1/2, 1/4, or 1/10) and kept at 27°C or 30°C to examine the effects of high temperature interacting with gametocytemia on oocyst infections. The scatter plots show oocyst intensity, with the data points representing the number of oocysts found in individual mosquitoes, and the horizontal lines the median. The pie charts show oocyst or sporozoite prevalence calculated as the proportion of infected mosquitoes revealed by dissection of midguts and salivary glands, respectively. Results of GLMs to examine the effects of gametocytemia and temperature treatment on oocyst intensity or oocyst prevalence are reported in Supplementary Table 7. *n* indicates the number of mosquito sample per treatment group (dpi = days post infection). **b,** Relationship between percent reduction in oocyst prevalence due to exposure to 30°C and mean oocyst intensity (error bars = SEM). Percent reduction represents reduced percentage in oocyst prevalence in the 30°C treatment relative to oocyst prevalence in the 27°C control. Oocyst prevalence and intensity data were derived from experiments reported in Fig. 3a and Supplementary Fig. 4a. Dashed line indicates linear regression line ($F_{1, 4} = 24.78$, $P = 0.008$)



Supplementary Figure 5. Effect of transferring mosquitoes between 21°C and 27°C on vector competence of *A. gambiae* mosquitoes infected with *P. falciparum* malaria. Treatment mosquitoes in two replicate containers were kept at 21°C, blood fed at 27°C, and moved back to 21°C, while control mosquitoes were kept at 27°C throughout and blood fed at 27°C. The scatter plots show oocyst intensity, with the data points representing the number of oocysts found in individual mosquitoes, and the horizontal lines the median. The pie charts show oocyst or sporozoite prevalence calculated as the proportion of infected mosquitoes revealed by dissection of midguts and salivary glands, respectively. GLM was used to compare control to each replicate container of mosquitoes with pairwise post-hoc contrasts followed by Bonferroni corrections (ns, not significant at $P = 0.05$). *n* indicates the number of mosquito sample per treatment (dpi = days post infection).



Supplementary Figure 6. Plots of temperature treatments used in the current study showing 27°C with a Diurnal Temperature Range (DTR) of 0°C or 10°C. The Parton-Logan model was used for the diurnal fluctuating temperature regime that follows a sinusoidal progression and an exponential decay for the day and night cycle, respectively. Shaded areas indicate scotophase.



Supplementary Figure 7. Experimental setup for thermal avoidance assay. Pictures of (**a**) water linked to multiple tubes and (**b**) individual assay tubes. **c**, A schematic diagram of the experimental setup. Total eight assay tubes were used (four for control and four for treatment group) in an assay run, with a total three rounds of assay. Mosquitoes fed with parasite infected (Inf) or uninfected (Uninf) blood meals were introduced into tubes, and the treatments were rotated between the assay rounds.

Supplementary Table 1. Biting activity profile for *Anopheles* mosquitoes identified to exhibit evening, midnight, or morning biting time in 42 published studies. Biting activities were categorized into 'evening', 'midnight', or 'morning' biting group with peak biting observed before 22:00h, between 22:00 and 05:00h, or after 05:00h, respectively. Studies were grouped into high or low temperature environment (divided by double line in the table)

Year[ref.]	Country	Mosquito species	Description on biting activity	Peak biting time	Temperature (°C)
2017[1]	Cameroon	*A. gambiae* s.l.	Peak between 00:00 - 02:00 (In+Out), Garoua	Midnight	29.5
			Peak between 22:00 - 00:00 (In+Out), Mayo Oulo	Midnight	
			Peak between 00:00 - 02:00 (In+Out), Pitoa	Midnight	
		A. rufipes	Peak between 20:00 - 22:00 (In+Out), Garoua	Evening	
			Peak between 00:00 - 02:00 (In+Out), Mayo Oulo	Midnight	
			Peak between 00:00 - 02:00 (In+Out), Pitoa	Midnight	
2012[2]	Benin	*A. funestus*	Peak between 05:00 - 06:00 (In+Out), Lokohoue, 2011	Morning	28.7
2009[3]	Chad	*A. arabiensis*	Peak between 01:00 - 02:00 (In+Out)	Midnight	27.9
		An. pharoensis	Peak between 21:00 - 22:00 (In+Out)	Evening	
		A. funestus	Peak between 03:00 - 04:00 (In+Out)	Midnight	
		A. ziemanni	Peak between 18:00 - 19:00 (In+Out)	Evening	
2017[4]	Indonesia	*A. vagus*	Peak between 21:00 - 22:00 (In+Out)	Evening	27.8
		A. sundaicus	Peak between 21:00 - 22:00 (In+Out)	Evening	
		A. subpictus	Peak between 23:00 - 00:00 (In+Out)	Midnight	
		A. indefnitus	Peak between 23:00 - 00:00 (In+Out)	Midnight	
		A. peditaeniatus	Peak between 00:00 - 01:00 (In+Out)	Midnight	
		A. nigerrimus	Peak between 20:00 - 21:00 (In+Out)	Evening	
2011[5]	Solomon Islands	*A. farauti*	Peak between 18:00 - 19:00 (In), Pala, Dec 2010	Evening	27.1
			Peak between 19:00 - 21:00 (Out), Pala, Dec 2010	Evening	
2007[6]	Tanzania	*A. gambiae* s.s.	Peak between 01:00 - 02:00 (In)	Midnight	27.0
		A. gambiae s.s.	Peak between 01:00 - 02:00 (Out)	Midnight	
		A. arabiensis	Peak between 20:00 - 21:00 (In)	Evening	
		A. arabiensis	Peak between 22:00 - 23:00 (Out)	Midnight	
2008[7]	French Guiana	*A. darlingi*	Peak between 22:30 - 23:30 (Out), Twenké	Midnight	27.0
			Peak between 22:30 - 23:30 (Out), Taluéne	Midnight	
			Peak between 05:30 - 06:30 (Out), Cayodé	Morning	
2012[8]	Suriname	*A. darlingi*	Peak between 05:00 - 06:00 (In), Drietabiki	Morning	27.0
			Peak between 04:00 - 05:00 (Out), Drietabiki	Midnight	
			Peak between 01:00 - 02:00 (In), Jamaica	Midnight	
			Peak between 01:00 - 02:00 (Out), Jamaica	Midnight	
2014[9]	Senegal	*A. funestus*	Peak between 08:00 - 09:00 (In+Out)	Morning	27.0
2004[10]	Eritrea	*A. gambiae* s.l.	Peak between 02:00 - 03:00 (In), Gash-barka	Midnight	26.8
			Peak between 22:00 - 23:00 (Out), Gash-barka	Midnight	
			Peak between 02:00 - 03:00 (In), Debub	Midnight	
			Peak between 21:00 - 22:00 (Out), Debub	Evening	

			Peak between 01:00 - 02:00 (In), Anseba	Midnight	
			Peak between 21:00 - 22:00 (Out), Anseba	Evening	
2012[11]	Cameroon	*A. gabmiae* s.l.	Peak between 01:00 - 02:00 (Out)	Midnight	26.8
2017[12]	Papua New Guinea	*A. farauti* 4	peak between 19:00 - 20:00 (Out), Kokofine, 2011	Evening	26.7
			peak between 20:00 - 21:00 (Out), Mauno, 2011	Evening	
2005[13]	Bolivia	*A. darlingi*	Peak between 20:00 - 21:00 (Out)	Evening	26.6
2011[14]	Solomon Islands	*A. farauti*	Peak between 18:00 - 19:00 (In)	Evening	26.5
			Peak between 19:00 - 20:00 (Out)	Evening	
		A. solomonis	Peak between 18:00 - 19:00 (In)	Evening	
			Peak between 18:00 - 19:00 (Out)	Evening	
2017[15]	Tanzania	*An. arabiensis*	Peak between 20:00 - 21:00 (Out)	Evening	26.5
		An. funestus	Peak between 05:00 - 06:00 (Out)	Morning	
2008[16]	Ghana	*A. gambiae* s.l.	Peak between 02:00 - 03:00 (Out), Dzorwulu	Midnight	26.4
			Peak between 03:00 - 04:00 (Out), Kaneshie	Midnight	
			Peak between 02:00 - 03:00 (Out), Korle Bu	Midnight	
			Peak between 02:00 - 03:00 (Out), Kotobabi	Midnight	
			Peak between 02:00 - 03:00 (Out), La	Midnight	
			Peak between 03:00 - 04:00 (Out), Ushertown	Midnight	
2013[17]	Uganda	*A. gambiae* s.l.	Peak between 04:00 - 05:00 (In+Out), Bugabula	Midnight	26.2
			Peak between 02:00 - 03:00 (In+Out), Budiope	Midnight	
		A. funestus	Peak between 04:00 - 05:00 (In+Out), Budiope	Midnight	
			Peak between 05:00 - 06:00 (In+Out), Bugabula	Morning	
2015[18]	Peru	*A. darlingi*	Peak between 21:00 - 22:00 (Out), Riverine	Evening	26.1
			Peak between 22:00 - 23:00 (Out), Highway	Midnight	
2015[19]	Peru	*A. darlingi*	Peak between 18:00 - 19:00 (Out), San José de Lupuna, April 2011	Evening	26.1
			Peak between 23:00 - 00:00 (Out), Villa del Buen Pastor, April 2011	Midnight	
			Peak between 22:00 - 23:00 (Out), Cahuide, May 2012	Midnight	
2009[20]	Colombia	*A. darlingi*	Peak between 18:00 - 19:00 (In)	Evening	26.0
			Peak between 20:00 - 21:00 (Out)	Evening	
		A. oswaldoi	Peak between 18:00 - 19:00 (Out)	Evening	
			Peak between 18:00 - 19:00 (Out)	Evening	
2014[21]	Solomon Islands	*A. farauti*	Peak between 19:00 - 20:00 (In)	Evening	26.0
			Peak between 19:00 - 20:00 (Out)	Evening	
2015[22]	Equatorial Guinea	*Anopheles* spp.	Peak between 03:00 - 04:00 (In)	Midnight	26.0
			Peak between 03:00 - 04:00 (Out)	Midnight	
2016[23]	Solomon Islands	*A. farauti* s.s.	Peak between 18:00 - 19:00 (In)	Evening	26.0
			Peak between 18:00 - 19:00 (Out)	Evening	
2012[24]	Zambia	*A. funestus*	Peak between 03:00 - 04:00 (In), LLINs alone	Midnight	25.7
			Peak between 05:00 - 06:00 (Out), LLINs alone	Morning	
			Peak between 03:00 - 04:00 (In), LLINs + IRS	Midnight	
			Peak between 00:00 - 01:00 (In), LLINs + IRS	Midnight	
		A. quadriannulatus	Peak between 19:00 - 20:00 (In), LLINs alone	Evening	

			Peak between 20:00 - 21:00 (Out), LLINs alone	Evening	
			Peak between 20:00 - 21:00 (In), LLINs + IRS	Evening	
			Peak between 21:00 - 22:00 (Out), LLINs + IRS	Evening	
2011[25]	Equatorial Guinea	*A. gambiae* s.s.	Peak between 23:00 - 00:00 (In)	Midnight	24.6
			Peak between 23:00 - 00:00 (Out)	Midnight	
2011[26]	Indonesia	*A. aconitus*	Peak between 22:00 - 23:00 (In+Out)	Midnight	24.1
		A. vagus	Peak between 19:00 - 20:00 (In+Out), West Timor	Evening	
		A. barbirostris	Peak between 01:00 - 02:00 (In+Out)	Midnight	
		A. vagus	Peak between 02:00 - 03:00 (In+Out), Java	Midnight	
		A. subpictus	Peak between 22:00 - 23:00 (In+Out), West Timor	Midnight	
2007[27]	Venezuela	*A. darlingi*	Peak between 01:00 - 02:00 (In)	Midnight	24.0
2012[28]	Iran	*A. culcifacies*	Peak between 23:00 - 00:00 (In+Out)	Midnight	23.5
		A. fluviatilis	Peak between 22:00 - 23:00 (In+Out)	Midnight	
		A. stephensi	Peak between 19:00 - 20:00 (In+Out)	Evening	
2011[29]	Tanzania	*A. gambiae* s.l.	Peak between 00:00 - 01:00 (In), 2009	Midnight	23.3
			Peak between 22:00 - 23:00 (Out), 2009	Midnight	
		A. funestus	Peak between 20:00 - 21:00 (In), 2009	Evening	
			Peak between 22:00 - 23:00 (Out), 2009	Midnight	
2005[30]	India	*A. baimaii*	Peak between 22:00 - 23:00 (In)	Midnight	23.2
2001[31]	Kenya	*A. gambiae* s.l.	Peak between 23:00 - 00:00 (Out), bed net village	Midnight	23.0
			Peak between 23:00 - 00:00 (Out), control village	Midnight	
		A. funestus	Peak between 22:00 - 23:00 (Out), bed net village	Midnight	
			Peak between 23:00 - 00:00 (Out), control village	Midnight	
2000[32]	Mozambique	*A. arabiensis*	Peak between 01:00 - 02:00 (In)	Midnight	22.8
			Peak between 23:00 - 00:00 (Out)	Midnight	
		A. funestus	Peak between 02:00 - 03:00 (In)	Midnight	
			Peak between 02:00 - 03:00 (Out)	Midnight	
2015[33]	Uganda	*A. gambiae* s.l.	Peak between 19:00 - 20:00 (In+Out), Engari, rainy season	Evening	22.3
			Peak between 19:00 - 20:00 (In+Out), Engari, dry season	Evening	
			Peak between 19:00 - 20:00 (In+Out), Kigorogoro, dry season	Evening	
2015[34]	Kenya	*A. gambiae* s.l.	Peak between 22:00 - 00:00 (In)	Midnight	22.1
			Peak between 18:00 - 20:00 (Out)	Evening	
		A. funestus	Peak between 18:00 - 20:00 (In)	Evening	
			Peak between 18:00 - 20:00 (Out)	Evening	
2006[35]	Tanzania	*A. gambiae* s.l.	Peak between 05:00 - 06:00 (In), Lupiro 2004	Morning	21.8
			Peak between 23:00 - 00:00 (Out), Lupiro 2004	Midnight	
2014[36]	Kenya	*A. gambiae* s.s.	Peak between 05:00 - 06:00 (In), Asembo 2011	Morning	21.8
			Peak between 01:00 - 02:00 (Out), Asembo 2011	Midnight	
		A. arabiensis	Peak between 01:00 - 02:00 (In), Asembo 2011	Midnight	
			Peak between 03:00 - 04:00 (Out), Asembo 2011	Midnight	

		A. funestus	Peak between 01:00 - 02:00 (In), Asembo 2011	Midnight	
			Peak between 01:00 - 02:00 (Out), Asembo 2011	Midnight	
2015[37]	Madagascar	*A. coustani*	Peak between 22:00 - 23:00 (In)	Midnight	21.3
			Peak between 19:00 - 21:00 (Out)	Evening	
		A. mascarensis	Peak between 01:00 - 02:00 (In)	Midnight	
			Peak between 02:00 - 03:00 (Out)	Midnight	
		A. funestus	Peak between 21:00 - 22:00 (In)	Evening	
			Peak between 02:00 - 03:00 (Out)	Midnight	
		A. arabiensis	Peak between 04:00 - 05:00 (In)	Midnight	
			Peak between 00:00 - 01:00 (Out)	Midnight	
2016[38]	Ethiopia	*A. arabiensis*	Peak between 00:00 - 01:00 (In)	Midnight	21.1
			Peak between 21:00 - 22:00 (Out)	Evening	
		A. pharoensis	Peak between 19:00 - 20:00 (In)	Evening	
			Peak between 19:00 - 20:00 (Out)	Evening	
		A. ziemanni	Peak between 19:00 - 20:00 (In)	Evening	
			Peak between 19:00 - 20:00 (Out)	Evening	
		A. funestus s.l.	Peak between 23:00 - 00:00 (In)	Midnight	
			Peak between 21:00 - 22:00 (Out)	Evening	
2010[39]	Ethiopia	*A. arabiensis*	Peak between 19:00 - 20:00 (In)	Evening	20.0
			Peak between 18:00 - 19:00 (Out)	Evening	
		A. pharoensis	Peak between 20:00 - 21:00 (In)	Evening	
			Peak between 19:00 - 20:00 (Out)	Evening	
		A. coustani	Peak between 18:00 - 19:00 (In)	Evening	
			Peak between 18:00 - 19:00 (Out)	Evening	
2010[40]	Zambia	*A. arabiensis*	Peak between 24:00 - 01:00 (In)	Midnight	19.9
			Peak between 01:00 - 02:00 (Out)	Midnight	
2016[41]	Ethiopia	*A. gambiae* s.l.	Peak between 19:00 - 20:00 (In)	Evening	18.0
			Peak between 19:00 - 20:00 (Out)	Evening	
		A. coustani s.l.	Peak between 21:00 - 22:00 (In)	Evening	
			Peak between 19:00 - 20:00 (Out)	Evening	
		A. pharoensis	Peak between 19:00 - 20:00 (In)	Evening	
			Peak between 20:00 - 21:00 (Out)	Evening	
2012[42]	Ethiopia	*A. arabiensis*	Peak between 19:00 - 20:00 (In)	Evening	17.4
			Peak between 19:00 - 20:00 (Out)	Evening	

In: peak biting observed for indoor biting.
Out: peak biting observed for outdoor biting.
In+Out: peak biting observed for combined data of indoor and outdoor biting.
[a]If a subset of data showed a shift in biting time in each study, the data set was described for the details such as study sites, year, and/or intervention methods (e.g., long-lasting insecticide-treated bed nets (LLINs), indoor residual spray (IRS), etc.).

Supplementary Table 2. Summary of biting activity profile from Supplementary Table 1

Biting time	No. cases (%) by temperature measures	
	High (25°C or above)	Low (< 25°C)
Evening	33 (21.9)	31 (20.5)
Midnight	40 (26.5)	38 (25.2)
Morning	7 (4.6)	2 (1.3)

Supplementary Tables 3. Model analyses examining the effects of time-of-day (18:00h [ZT12], 00:00h [ZT18], and 06:00h [ZT0]) and temperature regime (27°C DTR 0°C and 27°C DTR 10°C) on oocyst intensity (GLMM), or oocyst or sporozoite prevalence (GLM) in *A. gambiae* (See Fig. 1 and Supplementary Fig. 2)

		Oocyst intensity		Oocyst prevalence		Sporozoite prevalence	
Effect	*df*	*F*	*P*	*LR*-χ^2	*P*	*LR*-χ^2	*P*
Time[a]	2	3.82	0.024	14.03	< 0.001	42.52	< 0.0001
DTR[b]	1	48.50	< 0.0001	28.51	< 0.0001	48.53	< 0.0001
Time × DTR	2	9.75	< 0.0001	25.27	< 0.0001	33.6	< 0.0001
Day[c]	2	5.15	0.007	0.57	0.753	0.38	0.828

LR-χ^2: Likelihood ratio chi-square value.
[a]Time-of-day.
[b]Diurnal temperature range.
[c]Dissection day (day post infection).

Supplementary Table 4. GLMs examining the effects of time-of-day (18:00h [ZT12] and 05:00h [ZT23]) and temperature regime (27°C DTR 0°C and 27°C DTR 10°C) on oocyst intensity, or oocyst or sporozoite prevalence in *A. stephensi* (see Supplementary Fig. 3)

		Oocyst intensity		Oocyst prevalence		Sporozoite prevalence	
Effect	*df*	*LR*-χ^2	*P*	*LR*-χ^2	*P*	*LR*-χ^2	*P*
Time[a]	1	9.31	0.002	8.17	0.004	16.01	< 0.0001
DTR[b]	1	45.64	<0.0001	4.93	0.026	33.29	< 0.0001
Time × DTR	1	4.78	0.029	16.51	< 0.0001	7.38	0.007
Day[c]	2	10.65	0.005	2.35	0.31	0.80	0.672

LR-χ^2: Likelihood ratio chi-square value.
[a]Time-of-day.
[b]Diurnal temperature range.
[c]Dissection day (day post infection).

Supplementary Table 5. Outputs from a malaria transmission dynamics model illustrating the potential effect of altered vector competence in mosquitoes biting in the evening (EV; increased vector competence), at midnight (MD; unaltered vector competence), or morning (MN; reduced vector competence) on malaria prevalence and efficacy of bed nets (LLINs). Post bed net prevalence estimates are taken 6 years after they were introduced at 50% coverage (and redistributed at year 3) (See Fig. 2)

Run	Proportion of mosquitoes biting during different periods of the night			Proportion of bites received in bed			Prevalence (%) in 2-10-year-old children		Estimated efficacy of LLINs (% relative reduction in prevalence)
	EV	MD	MN	EV	MD	MN	Without LLINs	With LLINs	
1	0.7	0.3	0	0.89†	0.89†	0.89†	68.2	19.4	71.6
2	0.15	0.7	0.15	0.89†	0.89†	0.89†	59.3	11.7	80.3
3	0	0.3	0.7	0.89†	0.89†	0.89†	39.5	1.8	95.3
4	0.7	0.3	0	0.45	0.89†	0.45	68.2	39.5	42.0
5	0.15	0.7	0.15	0.45	0.89†	0.45	59.3	18.4	69.0
6	0	0.3	0.7	0.45	0.89†	0.45	39.5	10.8	72.8

†See references[43,44].

Supplementary Table 6. GLMs examining the effects of mosquito species (*A. gambiae* and *A. stephensi*) and/or temperature treatment (27°C and 30°C) on oocyst intensity or oocyst prevalence (see Fig. 3a). Oocyst prevalence data were pooled within each temperature treatment group after confirming no difference between two species (Fisher's exact test, two-sided, $P > 0.05$) to ensure model validity[45,46]

		Oocyst intensity		Oocyst prevalence	
Effect	*df*	$LR\text{-}\chi^2$	*P*	$LR\text{-}\chi^2$	*P*
Species	1	0.23	0.632	NA	NA
Temperature	1	78.7	< 0.0001	36.9	< 0.0001
Species × Temperature	1	1.29	0.256	NA	NA

$LR\text{-}\chi^2$: Likelihood ratio chi-square value.

Supplementary Table 7. GLMs examining the effects of gametocytemia dilutions (1, 1/2, 1/4, and 1/10) and temperature treatment (27°C and 30°C) on oocyst intensity or oocyst prevalence in *A. stephensi* (see Supplementary Fig. 4a)

		Oocyst intensity		Oocyst prevalence	
Effect	*df*	*LR*-χ^2	*P*	*LR*-χ^2	*P*
Gametocytemia	3	2.48	0.479	20.3	< 0.0001
Temperature	1	5.96	0.015	138	< 0.0001
Gametocytemia × Temperature	1	2.72	0.438	1.33	0.724

LR-χ^2: Likelihood ratio chi-square value.

Supplementary Table 8. Blood feeding compliance of *A. gambiae* mosquitoes fed at either 27°C or 21°C. Mosquitoes were kept at either 27°C DTR 0°C or 21°C DTR 0°C and fed infectious blood meals at a different time-of-day (18:00h [ZT12], 00:00h [ZT18], or 06:00h [ZT0]) at their corresponding temperature (i.e. either 27°C or 21°C). Data for feeding compliance at 27°C were obtained from the infectious feed reported in Fig. 1 and Fig. S2 (i.e. 27°C DTR 0°C treatment group), and data for feeding compliance at 21°C were obtained from a separate infectious feed. GLMM examining the effects of temperature and time-of-day on the feeding compliance is reported in Supplementary Table 9

Blood feeding temperature	Time-of-day	No. fed	No. total	% fed
27°C	ZT12	138	146	94.5
		138	142	97.2
	ZT18	145	147	98.6
		130	133	97.7
	ZT0	140	143	97.9
		141	145	97.2
21°C	ZT12	112	119	94.1
		108	118	91.5
	ZT18	115	118	97.5
		108	117	92.3
	ZT0	115	115	100.0
		113	117	96.6

Supplementary Table 9. GLMM examining the effects of blood feeding temperature (27°C and 21°C) and time-of-day (18:00h [ZT12], 00:00h [ZT18], and 06:00h [ZT0]) on feeding compliance (See Supplementary Table 8)

		Feeding compliance	
Effect	*df*	*F*	*P*
Temperature	1	1.39	0.284
Time-of-day	2	2.95	0.128
Temperature × Time-of-day	2	1.14	0.381

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