Supporting Information

Paaijmans et al. 10.1073/pnas.1006422107

SI Materials and Methods

Temperature Regimens. All experiments were carried out in incubators (Percival Scientific Inc.), at 90 ± 5% relative humidity and a 12L:12D (Light:Dark) photoperiod. Temperature was either held constant or allowed to fluctuate around mean temperatures equivalent to the constant temperature treatments with a diurnal temperature fluctuation of ±6 °C (giving a DTR of 12 °C), using a realistic minimum-maximum temperature model (1) (Fig. S1). This model assumes temperature follows a sinusoidal pattern from sunrise to sunset and an exponential decrease from sunset to sunrise. The temperature was monitored closely with temperature loggers (TinyTalkII; Gemini) at 5- or 10-min intervals. Table S2 provides the actual measured daily mean temperatures and the average diurnal temperature ranges in the incubators.

To exclude the potential effect of incubator, incubator-programs were changed between experiments (e.g., an incubator running at a low but constant temperature was then programmed to fluctuate around a high mean temperature, etc).

Mosquito Rearing and Parasitology. *An. stephensi* were used throughout. Except where described otherwise, mosquitoes were reared as described in Bell et al. (2). We used genotype ER of *P. chabaudi chabaudi*, which was isolated from thicket rats and subsequently cloned (3). Parasites were grown in mice (Female C57BL/6 laboratory mice; Charles River Laboratories). For blood feeding, mice were anesthetized and mosquitoes allowed to feed for 30 min. Females that did not take a full blood-meal were discarded.

Extrinsic Incubation Period—Experiments 1 and 2

Parasite Growth Rate and Parasite Dissemination. ***Mosquito rearing.*** Larvae were reared in plastic trays (30 × 16 × 8.5 cm) filled with 1 L of distilled water at 27 ± 0.5 °C and a 12L:12D photoperiod. To reduce variation in adult size at emergence, larvae were reared at similar densities (400 larvae) per tray. Larvae were fed 0.3 mg of tropical fish food (Tetrafin) per larva per day, which was ground into a fine powder. Pupae were collected daily and transferred to holding cages and adults that emerged were fed ad libitum on a 10% glucose solution supplemented with 0.05% paraaminobenzoic acid (PABA). The female mosquitoes used in these bioassays were 2–5 d and kept at 26 ± 0.5 °C

Malaria infections. Females that successfully fed on malaria-infected mice were kept at 26 ± 0.5 °C and were fed ad libitum on a 10% glucose solution supplemented with 0.05% PABA. Two days postinfection, an oviposition medium was placed in the cages. Three days postinfection, all mosquitoes were pooled and subsequently distributed over experimental cardboard cups (diameter 7 cm), with 25–30 mosquitoes per cup. Cups were closed with netting and mosquitoes were fed ad libitum on a 10% glucose solution supplemented with 0.05% PABA via a soaked cotton pad. Cups were distributed over the different temperature treatments. The number of cups varied per temperature treatment according to the anticipated sampling schedule, as parasites will develop faster and parasites will disseminate sooner at warmer temperatures. The number of cups can be derived from Fig. 2; one data point equals one randomly selected cup from that temperature treatment.

Experiment 1. Mice were inoculated with 10^5 parasites. Mosquito feeds took place on day 13 after mice were infected (gametocytaemia, proportion of red blood cells infected with gametocytes 0.24–0.46%). After the initial 3 d at 26 °C, mosquitoes were exposed to a constant 16 °C or 26 °C, and a diurnal temperature range of 12 °C around these same means. Mosquitoes were exposed to the temperature treatments around 7:00 PM, when the fluctuating temperature equaled the daily mean temperature (Fig. S1)

Experiment 2. Mice were inoculated with 10^6 parasites. Mosquito feeds took place 12 d after mice were infected (gametocytaemia, proportion of red blood cells infected with gametocytes = 0.25–0.55%). After the initial 3 d at 26 °C, mosquitoes were exposed to a constant 18 °C or 24 °C (referred to as 18C and 24C, respectively), and a diurnal temperature range of 12 °C around these same means (referred to as 18F and 24F, respectively). Again, mosquitoes were exposed to the temperature treatments around 7:00 PM, when the fluctuating temperature equaled the daily mean temperature (Fig. S1).

Mosquito dissections. To determine parasite development rates, one cup was chosen randomly from each temperature treatment at certain time intervals (Fig. 2) and the midguts from 20 to 25 mosquitoes were dissected using a standard dissecting microscope. After counting the total number of oocysts, the midgut was stored in 20 μL of 1× PBS in a −20 °C freezer. To determine completion of the extrinsic incubation period, it was noted when sporozoites were present in the hemocoel.

Quantifying parasite growth kinetics. Parasite numbers present on the midguts of mosquitoes were determined using qPCR. DNA was extracted from individual mosquito midguts using the E.Z.N.A MicroElute Genomic DNA kit (Omega Bio-Tek), eluted in a total volume of 20 μL and stored at −80 °C until quantification. Quantitative standards for *P. chabaudi* were obtained by extracting DNA from a known number of infected murine red blood cells (each infected rbc harboring a single ring stage parasite that equals a single parasite genome) using the BloodPrep kit (Applied Biosystems) on the ABI Prism 6100 Nucleic Acid Prep Station. DNA was eluted in a total volume of 200 μL, aliquoted, and stored at −80 °C. Both extraction procedures were performed according to manufacturer's instructions.

A "generic" real-time quantitative PCR assay for counting *Plasmodium* parasites was developed by Bell et al. (2); the PCR primers and a minor grove-binder (MGB) probe, targeting the *Plasmodium chabaudi chabaudi msp-1* gene, were designed using Primer Express (Applied Biosystems) software.

The qPCRs were performed on an Applied Biosystems 7500 Fast Real-Time PCR System with an initial denaturation of 95 °C for 20 s followed by 40 cycles of denaturation at 95 °C for 3 s and annealing/extension at 60 °C for 30 s. Two microliters of DNA was included in a 25-μL volume PCR with the following components: 1.5 μL each of forward and reverse primer, both at a final concentration of 300 nM; 12.5 μL of 2× PerfeCTa (Quanta Biosciences, Inc.) qPCR FastMix, Low Rox; 1 μL of MGB probe at a final concentration of 200 nM; and 6.5 μL of sterile water.

Absolute quantification of experimental samples was determined by comparing threshold cycle numbers against a standard curve. A series of quantification standards were generated from serial dilutions of a thawed *P. chabaudi* DNA aliquot. Three replicates of each DNA standard (covering five orders of magnitude from 10^6 to 10^2 parasite genomes) were included in each quantitative PCR run.

Quantitative PCR counts the number of genomes per midgut. A single genome will at a certain stage during parasite development become a single sporozoite. Parasite growth was assessed per mosquito sample by dividing the total number of parasites on the midgut by the number of oocysts present on the midgut.

Data analysis. Because oocysts counts of >100/midgut tend to become unreliable (oocysts are often grouped together and/or on top of each other), therefore increasing the margin for errors, only midguts with ≤100 oocysts were included in the analysis (excluding 16 mosquitoes). Quantitative PCR data of day 21 in the 18F treatment are missing due to an erroneous DNA extraction.

Data were analyzed using IBM SPSS software (version 18; SPSS Inc.). Difference in growth rate was assessed by comparing sporozoite numbers per oocyst between the "constant" and "fluctuating" temperature treatment for each mean temperature, on the day the first oocysts had completed development and sporozoites disseminated, using an ANOVA. Sporozoite numbers were square-root transformed to meet the homogeneity of variance and normality assumptions.

Differences in numbers of mosquitoes with disseminated sporozoites between the constant and fluctuating temperature treatment for each mean temperature were tested with χ^2 tests. Data from the first day onwards at which sporozoites were observed in the constant and/or the fluctuating temperature treatment were included in the analysis.

Additional data. To ensure similar infection burdens between the constant and fluctuating temperature treatment, malaria prevalence (i.e., percentage of mosquitoes with oocysts on the midguts) and the number of oocysts per midgut was compared for each mean temperature. In both cases data from day 6 until the day at which disseminated sporozoites were observed in the constant and/or the fluctuating temperature treatment were included in the analysis.

There was no significant difference in the malaria prevalence or in the number of oocysts per midgut between the constant and fluctuating temperature treatment. Malaria prevalence in the mosquitoes was 53% (16 °C; $\chi^2 = 0.57$, $P = 0.75$), 79% (18 °C; $\chi^2 = 0.22$, $P = 0.90$), 83% (24 °C; $\chi^2 = 2.35$, $P = 0.31$), and 62% (26 °C; $\chi^2 = 0.86$, $P = 0.65$).

There was no significant difference in oocysts numbers between the constant and fluctuating temperature regimens (16 °C, Mann–Whitney U test, $P = 0.87$; 18 °C, $P = 0.91$; 24 °C, $P = 0.40$; 26 °C, $P = 0.52$), indicating similar infection burdens. The mean numbers of oocysts per midgut were 8.3 ± 1.1 (16 °C), 25.5 ± 2.9 (18 °C), 28.8 ± 3.1 (24 °C) and 9.4 ± 1.5 (26 °C).

Extrinsic Incubation Period—Experiment 3

Early Sporogony. ***Bioassay.*** Mice were inoculated with 10^6 parasites and mosquito feeds took place on day 12 postinfection (gametocytaemia = 0.56–0.67%). Approximately 300 female mosquitoes, 2–3 d, were selected and placed in a single cage. They were allowed to feed on four infectious mice for 20 min. Immediately after the blood-feed, fully engorged mosquitoes were divided over four cages (16 × 16 × 16 cm; 50 mosquitoes per cage) and directly exposed to the various temperature treatments (one cage per treatment). Mosquitoes were exposed to the temperature treatments around 7:00 PM, when the fluctuating temperature equaled the daily mean temperature (Fig. S1). Mosquitoes were kept at a constant 18 °C and 24 °C and at equivalent mean temperatures but with a diurnal temperature range of approximately 12 °C. Mosquitoes were fed ad libitum on a 10% glucose solution supplemented with 0.05% PABA. Two days postfeeding an oviposition medium was added to the cages. From each cage, 20–25 mosquitoes were dissected (dissections 18C and 18F on day 14, 24C and 24F on day 9). Quantifying the number of oocysts per midgut (microscopy) and total parasite number per midgut (qPCR) was performed as described above.

Data analysis and additional data. Because, as described above, oocysts counts of >100/midgut tend to become unreliable, the higher numbers observed during this experiment are likely to be underestimates. For accuracy, only midguts with <100 oocysts were used in the growth analysis (qPCR). Data were analyzed using IBM SPSS software (version 18; SPSS Inc.). Oocyst numbers per midgut between the constant and fluctuating temperature treatment for each mean temperature were compared using a negative binomial generalized linear model with a log link function.

Difference in growth rate was assessed by comparing sporozoite numbers per oocyst between the constant and fluctuating temperature treatment for each mean temperature, on the day the first sporozoites disseminated, using ANOVA. Sporozoite numbers were log-transformed to meet the homogeneity of variance and normality assumptions. All mosquitoes were infected.

Larval Development and Survival

Bioassay. *An. stephensi* were selected from a tray containing >10,000 larvae (first instars; <24 h) and transferred to transparent plastic cups, containing 3 cm of distilled water (diameter 7 cm; 115 mL of water). A small water volume was chosen to ensure water temperature tracked the temperature in the incubators. Rearing occurred at (*i*) three different densities (0.5, 1, and 2 larvae/cm^2, or 18, 36, and 72 larvae per cup, respectively) and (*ii*) four different temperature regimens (at constant 20 °C and 27 °C and a diurnal temperature range of 12 °C around these same means), but (*iii*) under similar food conditions: 0.3 mg of tropical fish food (Tetrafin) per larva per day, ground into fine powder. The lowest density (0.5 larvae/cm^2) had 15 replicates per temperature treatment to account for a lower number of emerging adults. The two other densities had 10 replicates per temperature treatment. Living larvae and pupae were counted and removed from the cups daily, after which cups were cleaned and water was replaced to prevent scum formation and accumulation of metabolites that may be toxic to the larvae (4). They were placed back and food was added to the water. Emerged mosquitoes were counted and removed. When the pupae were observed, the cup was covered with netting (1-mm mesh size) for the remainder of the experiment.

Data Analysis and Additional Data. Per experimental unit (cup) and per temperature treatment (constant or fluctuating temperature), the development time (mean time to adult), and survival (percentage of larvae that reached the adult stage) were calculated. Data were analyzed using IBM SPSS software (version 18; SPSS Inc.). Univariate ANOVA was used to test for the effect of "temperature treatment," "density" and for an interaction between them on "development time" and "survival." Maximal models were fitted first and, beginning with higher order interactions; nonsignificant terms were sequentially removed in a process of backward elimination to generate minimal models. Out of the initial 270 larvae at the 18C treatment (lowest larval density) only two mosquitoes emerged. The development times of these two mosquitoes were excluded from the analysis. To meet homogeneity of variance and normality assumptions, mortality data were arcsine transformed.

Density affected immature development time at 20 °C ($F_{1,36} = 28$; $P < 0.001$) and 27 °C ($F_{2,66} = 76$; $P < 0.001$). Density affected immature survivorship at 20 °C ($F_{2,64} = 98$; $P < 0.001$) and 27 °C ($F_{2,64} = 6$; $P = 0.01$). There was an interaction between temperature treatment and density on survival at both 20 °C ($F_{2,64} = 4$, $P = 0.02$) and 27 °C ($F_{2,64} = 4$, $P = 0.02$).

Mosquito Survival

Bioassay. Two- and 3-d female mosquitoes were allowed to feed on naïve mice for 30 min, after which they were pooled and distributed over experimental cardboard cups (diameter 7 cm), approximately 25 mosquitoes per cup. Cups contained an oviposition medium and mosquitoes were fed ad libitum on a 10% glucose solution supplemented with 0.05% PABA via a soaked cotton pad. Cups were distributed over the different temperature treatments, four replicates per temperature treatment. Mortality was scored twice daily during the first 2 wk, due to another ongoing experiment, and daily afterward. Dead mosquitoes were removed from the cups.

Data Analysis. Difference in mosquito survival between constant and fluctuating temperature treatments was analyzed using Kaplan-Meier survival analysis and the log rank tests (IBM SPSS software, version 18; SPSS Inc.).

Mosquito Gonotrophic Cycle

Bioassay. Approximately 300 female mosquitoes, 3–4 d, were allowed to blood feed for 30 min, after which engorged females were placed individually in plastic 5-mL tubes (diameter 1.5 cm, height 6 cm). Tubes were closed with netting and mosquitoes were fed ad libitum on a 10% glucose solution supplemented with 0.05% PABA via a soaked cotton pad. All tubes contained 1 mL of distilled water and a small filter-paper cone (height 2 cm) as oviposition medium. After the feed, mosquitoes were distributed over the four temperature treatments (18C/F and 24C/F). Tubes were monitored daily for eggs, and the length of the gonotrophic cycle (time between blood meal and oviposition) was recorded.

Data Analysis. Data were analyzed using IBM SPSS software (version 18; SPSS Inc.). Difference in cycle length between constant and fluctuating temperature treatments was analyzed using nonparametric Mann–Whitney *U* tests.

1. Parton WJ, Logan JA (1981) A model for diurnal variation in soil and air temperature. *Agric Meterol* 23:205–216.
2. Bell AS, Blanford S, Jenkins N, Thomas MB, Read AF (2009) Real-time quantitative PCR for analysis of candidate fungal biopesticides against malaria: Technique validation and first applications. *J Invertebr Pathol* 100:160–168.
3. Beale GJ, Carter R, Walliker D (1978) In *Rodent Malaria*, eds Killick-Kendrick R, Peters W (Academic Press, London), pp 213–245.
4. Bayoh MN, Lindsay SW (2003) Effect of temperature on the development of the aquatic stages of *Anopheles gambiae* sensu stricto (Diptera: Culicidae). *Bull Entomol Res* 93:375–381.

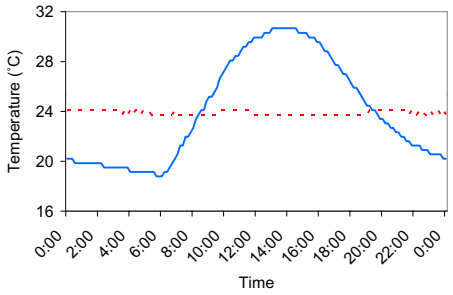

Fig. S1. Actual recorded temperatures in two incubators over 1 d. One incubator was programmed to keep a constant 24 °C (dotted red line) and one to fluctuate 12 °C around the same mean temperature of 24 °C (solid blue line).

Table S1. Numbers of oocysts per midgut and numbers of sporozoites per oocyst in female *An. stephensi* mosquitoes that were exposed to constant or fluctuating temperature regimens

	Oocysts per midgut		Sporozoites per oocyst	
	Mean ± SEM	*n*	Mean ± SEM	*n*
18C	382.4 ± 76.6	25	362.1 ± 46.1	7
18F	337.5 ± 67.6	25	940.0 ± 219.1	7
24C	217.9 ± 43.7	25	3265.9 ± 859.6	8
24F	55.6 ± 12.5	20	915.7 ± 145.4	15

Mosquitoes were exposed to a constant 18 °C (18C) and 24 °C (24C), and to a mean temperatures of 18 °C and 24 °C but with a diurnal temperature range of 12 °C (18F and 24F, respectively). Dissections were carried out on day 9 (18C/18F) or 14 (24C/24F) postinfection.

Table S2. Actual measured daily mean temperatures (±SD) and the average diurnal temperature ranges (DTRs ± SD) in the incubators

	Constant	Fluctuation	
	Mean, °C	Mean, °C	DTR, °C
Extrinsic incubation period, first experiment			
16 °C (*n* = 28 d)	16.2 ± 0.13	16.1 ± 0.09	11.7 ± 0.53
26 °C (*n* = 12 d)	26.0 ± 0.09	26.1 ± 0.16	12.1 ± 0.43
	Constant	Fluctuation	
	Mean, °C	Mean, °C	DTR, °C
Extrinsic incubation period, second experiment			
18 °C (*n* = 26 d)	17.7 ± 0.20	17.7 ± 0.13	12.4 ± 0.39
24 °C (*n* = 18 d)	23.9 ± 0.10	24.1 ± 0.21	11.8 ± 0.33
	Constant	Fluctuation	
	Mean, °C	Mean, °C	DTR, °C
Extrinsic incubation period, third experiment			
18 °C (*n* = 15 d)	17.8 ± 0.17	17.8 ± 0.22	12.7 ± 0.27
24 °C (*n* = 10 d)	24.0 ± 0.09	24.1 ± 0.10	11.6 ± 0.25
	Constant	Fluctuation	
	Mean, °C	Mean, °C	DTR, °C
Immature mosquito development and survival			
20 °C (*n* = 29 d)	20.0 ± 0.14	20.1 ± 0.15	12.7 ± 0.28
27 °C (*n* = 17 d)	27.0 ± 0.11	27.2 ± 0.08	12.4 ± 0.22
	Constant	Fluctuation	
	Mean, °C	Mean, °C	DTR, °C
Mosquito survival			
18 °C	17.8 ± 0.20*	17.9 ± 0.21[†]	12.4 ± 0.34
24 °C	24.0 ± 0.14[‡]	24.0 ± 0.22[§]	11.7 ± 0.29
	Constant	Fluctuation	
	Mean, °C	Mean, °C	DTR, °C
Mosquito gonotrophic cycle			
18 °C (*n* = 18 d)	17.8 ± 0.16	17.8 ± 0.22	12.7 ± 0.30
24 °C (*n* = 11 d)	24.0 ± 0.09	24.2 ± 0.11	11.7 ± 0.25

*68 d.
[†]84 d, 5 d missing.
[‡]63 d.
[§]62 d, 1 d missing.