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Supplementary Materials for

Clock genes and environmental cues coordinate *Anopheles* pheromone synthesis, swarming, and mating

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(available at science.sciencemag.org/content/371/6527/411/suppl/DC1)

MDAR Reproducibility Checklist (.pdf)
Movies S1 to S3 (.mp4)

Materials and Methods

Mosquito rearing

An. stephensi (Dutch strain) and *An. gambiae s.s.* (Keele strain) were reared using standard rearing procedures in standard laboratory conditions, at 27 ± 1 °C and 80 ± 5% relative humidity, with a 12 h/12 h light/dark cycle (11 h ~200 lux full light, 11 h darkness, 1 h dawn and 1 h dusk transitions). Larvae were fed on cat food pellets and ground fish food supplement. 400-500 newly emerged mosquitoes were placed in a mosquito cage (25 cm × 19 cm × 19 cm) and maintained on 10% (wt/vol) sucrose.

An. coluzzii used in the semi-field study was obtained from a colony established in September 2017 at the Institut de Recherche en Sciences de la Santé (IRSS) in Bobo Dioulasso. *An. coluzzii* gravid female mosquitoes were collected in human dwellings in Vallée du Kou (11°24"N; 4°24"W), a village located at 30 km north of Bobo-Dioulasso and surrounded by 1,200 ha of irrigated rice fields. Female mosquitoes were placed individually in oviposition cups containing tap water. After oviposition, mosquito species was molecularly identified by SINE-PCR (*42*) for detecting the insertion of *S200* X6.1, which is present in *An. coluzzii* and absent in *An. gambiae* (*s.l.*). The primers are shown in table S3. Larvae were fed with Tetramin Baby Fish Food (Tetrawerke, Melle, Germany). Early morning, the newly emerged males and females were separated. Mosquitoes were provided with 5% glucose solution. Insectarium conditions were 27 ± 0.5 °C, 70 ± 10% relative humidity, with a 12 h/12 h LD cycle (11 h ~200 lux full light, 11 h darkness, 1 h dawn and 1 h dusk transitions).

Field mosquito collection from swarms and inhabited houses.

Anopheles coluzzii mosquitoes were collected in July in Bobo-Dioulasso, Burkina Faso in 2011. Mosquitoes, mostly males, were collected in swarms using sweeping net during dusk. The indoor resting males with antennal fibrillae becoming erect were collected in inhabited houses using vacuum aspiration just prior to swarming time. The collected mosquitoes were placed in tubes containing RNAlater (Ambion, Austin, TX, USA) to prevent RNA degradation. Mosquito species was molecularly identified by SINE-PCR (*42*).

Laboratory reared mosquito sampling.

To test gene expression in different tissues, day-5 post emergence, male and female mosquitoes (n=20 per group) were dissected to collect different tissues including head, thorax, abdomen, midgut, hindgut, Malpighian tubules, ovary, testis and fat body. To examine the gene rhythmic expression in male heads at 3-4 d post emergence, 20 male mosquitoes were collected every 4 h for 48 h. To examine the gene rhythmic expression in dsRNA-treated mosquitoes, day-3 post emergence, cold-anesthetized four-day adult male mosquitoes were injected into hemocoel with 69 nl of a 3 µg/µl *tim* and *per* dsRNA solution, using a Nanoject II microinjector (Drummond). Control mosquitoes were injected with ds*GFP* dsRNA.

RNA isolation from mosquito heads or other tissues

Total RNA from 50 male mosquito heads was isolated using TRIzol Reagent (Invitrogen) and Phase Lock GelHeavy (Eppendorf) according to the manufacturer's instructions. To remove melanin pigments, the melanotic RNA was purified by overnight incubation at 4°C with the cationic detergent cetyl-trimethylammonium bromide (CTAB) and urea as previously described (*43*). The treated RNA was then suspended in 7 M guanidine hydrochloride to eliminate the CTAB by ionic exchange and precipitated by addition of two volumes of ethanol. The RNA was dissolved in RNase-free water.

Total RNA from other mosquito tissues except heads was isolated using RNAiso Plus (TaKaRa) and treated with Recombinant DNase I (TaKaRa) according to the manufacturer's instructions.

Microarray analysis

Probe sequences, microarray construction, probe preparation, and microarray hybridizations were performed as previously described (*44)* with some modifications. Both swarm male heads and indoor resting male heads were treated as experimental samples. Laboratory reared 2~6-day-old virgin *An. gambiae s.s.* male heads were used as reference samples (control). Three biological replicates were performed for each group. Cy5-labeled control and Cy3-labeled experimental cRNA probes were generated from 200 ng of RNA using Agilent Technologies Low Input Quick Amp Labeling Kit according to the manufacturer's instructions. Probe hybridization to the microarray slides was performed with 2 µg cRNA probes. Microarray slides were scanned using an Axon GenePix 4200AL scanner at 10-µm pixel size (Axon Instruments, Union City, California, USA). The scanned images were analyzed using feature extraction software (Agilent). gProcessedSignal and rProcessedSignal from Agilent feature extraction output files were imported into Partek and quantile normalized and log2 transformed. The normalized signal was further baseline corrected using the Cy5 labeled reference sample. Paired *t*-test was performed for each group and significant genes were detected using the criteria: $P < 0.01$ and the cutoff value for gene regulation at 0.8 in log2 scale (1.74 fold change) (*45*). The microarray data was assembled in the Minimum Information About a Microarray Experiment (MIAME)-compliant format and is available in the public Gene Expression Omnibus (GEO) database under accession GSE150971.

Real-time quantitative PCR Analysis.

First-strand cDNA was synthesized from total RNA using PrimeScript RT reagent Kit after removing genomic DNA (Takara) following the manufacturer's instructions. Gene expression was assessed by quantitative real time-PCR analysis with the Thermo Scientific PikoReal 96 Real-Time PCR system (Thermo Fisher) using the AceQ qPCR SYBR Green Master Mix (Vazyme). PCR involved an initial denaturation at 95℃ for 5 min, 40 cycles of 10 sec at 94°C, 30 sec at 60°C, and a final extension at 60°C for 30 sec. The housekeeping ribosomal protein S7 gene *AsS7* (ASTE004816) was used as an endogenous control. The primers are shown in Table S3.

dsRNA-mediated gene silencing in adult mosquitoes.

To produce the double-stranded RNA, the coding region fragments of *per*, *tim*, *desat1*, *ASTE000498*, *CPR30*, *CYP325G1* genes were amplified from *An. stephensi* or *An. gambiae s.s.* cDNA with forward and reverse primers containing the T7 promoter sequence at their 5' ends (TAATACGACTCACTATAGGG) (Table S3). The PCR products were purified with Cycle-Pure Kit (OMEGA) and used as the template to synthesize the dsRNA *in vitro* using the MEGAscript RNAi kit (Ambion, Life Technologies). The synthesized dsRNA was purified and eluted with the elution buffer supplied with the kit and concentrated to 3 μg/μl using a Microcon YM-100 filter (Millipore). The dsRNA for enhanced green fluorescent protein eGFP was synthesized and used as negative control for the non-specific dsRNA effects.

For gene silencing, cold-anesthetized 3-day-old mosquitoes were injected with 69 nL of a 3 μg/μl dsRNA solution of each gene into the hemocoel using a Nanoject II micro injector (Drummond). Control mosquitoes were injected with ds*GFP*. Each treatment was replicated three times with 20 mosquitoes per replicate, and the experiments were repeated two or three times. The RNAi silencing efficiency in the mosquito head and other tissues after 3 days of injection was determined by qPCR analysis.

Mating behavior test in laboratory small cages

To test the role of *per* and *tim* during mating, *An. gambiae s.s.* and *An. stephensi* virgin males were injected in thoracic cavity with Per or Tim dsRNA. Virgin males were injected with eGFP dsRNA as control. Each treatment was replicated three times with 20 male mosquitoes per replicate, and the experiments were repeated three times. Three days later, 20 injected males were pooled with 20 virgin females in a small cage (25 cm × 19 cm × 19 cm) and maintained overnight in standard rearing conditions to allow for mating. The next day, female spermathecae were dissected and examined by microscopy for sperm insemination as an indicator of mating activity. Insemination rates or mating frequency was calculated as a percentage of the number of inseminated females divided by the number of dissected females.

Mating behavior test under semi-field conditions

To determine the role of *per* and *tim* in semi-field conditions, we performed swarming and mating experiments in Mosquito Spheres (*21*) located in the Vallée du Kou in the Bama district, Burkina Faso. A total of 150 *An. coluzzii* one-day-old virgin males were injected with either dsRNA for *per, tim* or *eGFP* (control). Three days post injection, ~100 injected males and 100 virgin females were released into a compartment of the Mosquito Sphere at 18:15 p.m. and maintained together to determine swarming and mating behavior under semi-field conditions. Swarming behavior was induced in the Mosquito Sphere by placing a swarm marker (1 m^2 black sheet) in the center of the chamber. Mosquito swarming was observed for ~40 minutes (until 19:15) and the following parameters were collected as previously described (*46*): swarm size (is the estimated number of males that participated in swarming activity), swarm height (maximum,

middle and minimum), and the number of mating couples. Females were recaptured with a mouth aspirator immediately after swarming and mating activity, provided with 5% glucose solution and taken to the laboratory to dissect the spermathecae for the presence of sperm, as an assessment of insemination status.

Locomotor activity recording

Mosquito flight activity was monitored with a Locomotor Activity Monitor 25 (LAM 25) system (TriKinetics, Waltham, MA, USA) by placing individual adult mosquitoes in 25 × 150 mm clear plastic tubes. Adult male mosquitoes were injected with *per* or *tim* dsRNA . Control mosquitoes were injected with ds*GFP*. Two days later, individual mosquitoes were introduced into a plastic tube with access to 10% sucrose provided. Mosquitoes were maintained at 27 ± 0.5 °C, 80 ± 5% relative humidity under a 12 h light/dark cycle. Flight locomotor activity per minute was monitored all day by recording breaks in a series of infrared beams.

Extraction of cuticular hydrocarbons.

Cuticular hydrocarbons were extracted from 80 mosquitos in 800 µL hexane for 5 min in 15 mL conical glass centrifuge tubes. Extracts were concentrated under a N2 stream. The concentrated extracts were immediately dissolved in 30 µL hexane before their injection into the high-resolution Agilent GS/TOF MS. All samples were analyzed within 48 h of preparation.

Gas Chromatograph/Quad-Time of Flight Mass Spectral analysis.

GC/MS analysis was performed on a HP-5MS column (Agilent, 30 m × 0.25 mm i.d., 0.25 µm film thickness, 5% phenyl methyl siloxane stationary phase) using an Agilent 7890B GC system coupled to a novel high-resolution Agilent 7200 Q-TOF MS. The GC oven temperature was programmed from an initial temperature of 80 °C held for 2 min, ramped at 20 °C/min to 200°C, and then 5 °C/min to 320 °C. The carrier gas was helium (purity > 99.999%) at a constant flow rate of 1.0 mL/min.

The QTOF Mass spectra were recorded at 5 scans per second with a mass-to-charge ratio 30 to 550 m/z mass acquisition ranges. Electron energy was kept at 70 eV, and the QTOFMS was operated in 2 GHz-EDR mode (2 GHz extended dynamic range) in order to extend the linear dynamic range. Data acquisition and evaluation were carried out using Agilent MassHunter Data Acquisition, Quantitative Analysis and Qualitative Analysis programs (version B07.00, Agilent Technologies, CA), respectively. The peaks were identified based upon comparison with standards and the National Institute of Standards and Technology library (NIST, version 14) library. The peak area of each CHC component was calculated as a proportion of the total area of all detected peaks for each sample. A student's *t*-test was used to compare differences in the relative percentages of each CHC component between mosquitoes injected with *dsdesat1* and ds*GFP* dsRNAs.

Effect of tricosane and heptacosane on mating activity

Tricosane and heptacosane were dissolved in *n*-hexane at a concentration of 75 µg/mL, and applied on the abdomen of two-day-old male *An. stephensi* mosquitoes using paintbrushes. The solvent *n*-hexane was used as control. Each treatment was replicated three times with 20 mosquitoes per replicate, and the mating activity assays were repeated three times. Treated male mosquitoes were maintained on 10% sucrose at 27 ± 0.5 °C and 80 ± 5% relative humidity, with a 12 h/12 h day-night cycle. Two days later, 20 treated male mosquitoes and 20 virgin female mosquitoes were introduced into a cage and allowed to mate overnight. Female spermathecae were then dissected and examined for insemination status. Insemination rates were calculated as a percentage of the number of inseminated females divided by the number of dissected females.

Effect of light and temperature on mosquito mating activity

To investigate the effect of temperature on mosquito mating activity, newly emerged male and female mosquitoes were separated and maintained at 27 °C and 80 ± 5% relative humidity under LD cycles. Three days later, males and females were mixed, and kept at 19 ℃, 27 ℃ and 34 ℃, respectively. Each treatment had three mosquito cups, and each cup contained 20 males and 20 females. Mosquitoes were allowed to mate overnight, and female spermathecae were then dissected and examined for insemination status.

To investigate the effect of light on mosquito mating activity, newly emerged male and female mosquitoes were separated and maintained at 27 °C and 80 ± 5% relative humidity under LD cycles. Three days later, the males and females were mixed, and maintained under both LD (light-off at night) and LL (light-on at night). Each treatment had three mosquito cups, and each cup contained 20 males and 20 females. The mosquitoes were allowed to mate overnight, and then female spermathecae were dissected and examined for insemination status.

Statistical analysis.

Statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software). Statistical significances were determined by Student's *t*-test or one-way ANOVA. A value of $P < 0.05$ was considered to be statistically significant.

Accession number. The microarray data was assembled in the Minimum Information About a Microarray Experiment (MIAME)-compliant format and is available in the public Gene Expression Omnibus (GEO) database under accession GSE150971.

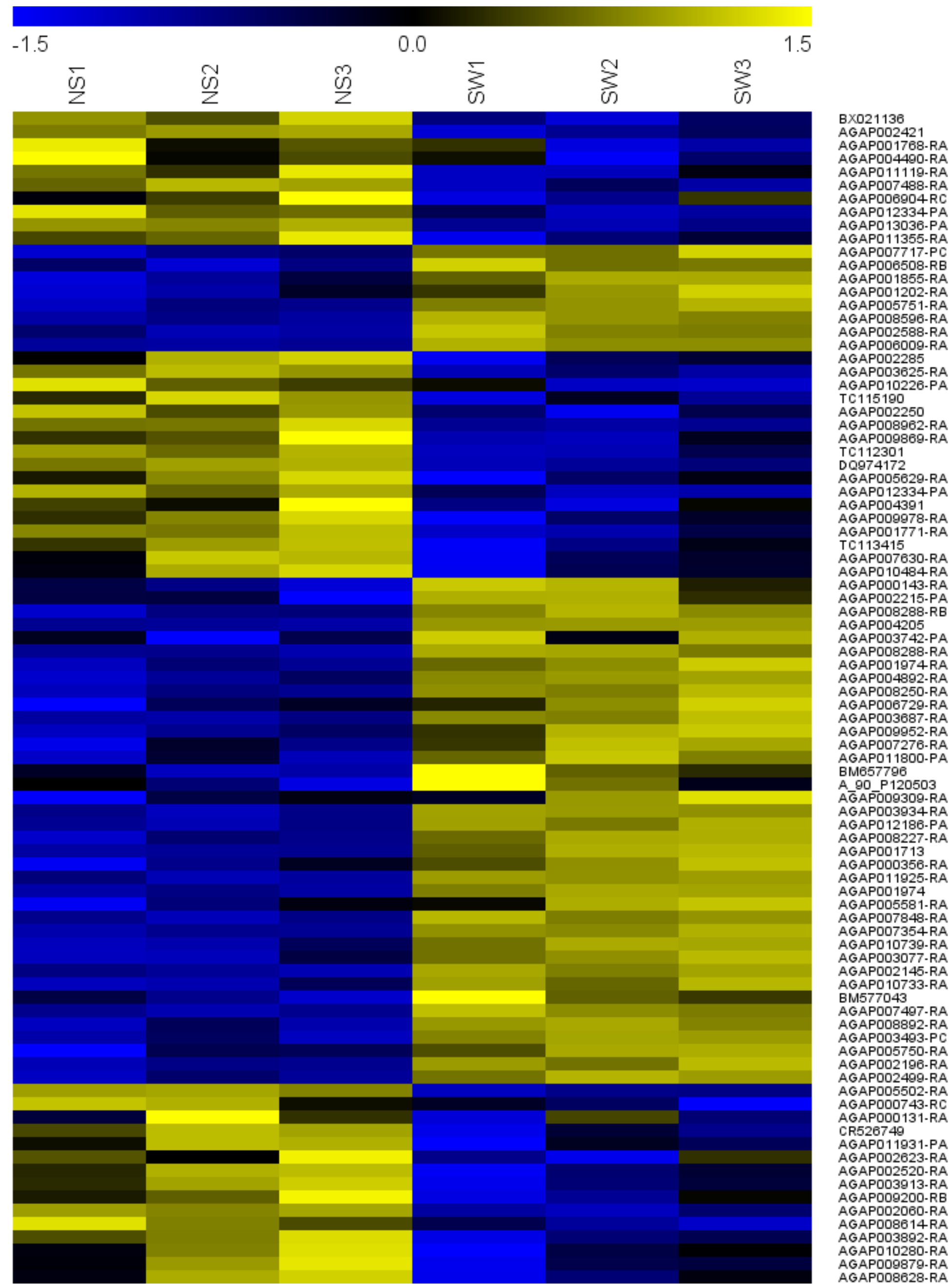


Fig. S1. Hierarchical cluster analysis of differentially expressed genes in male heads between swarming and non-swarming *An. coluzzii* mosquitoes. NSW, non-swarming; SW, swarming mosquitoes. Numbers following 'NSW' and 'SW' indicate results of independent biological repeats.

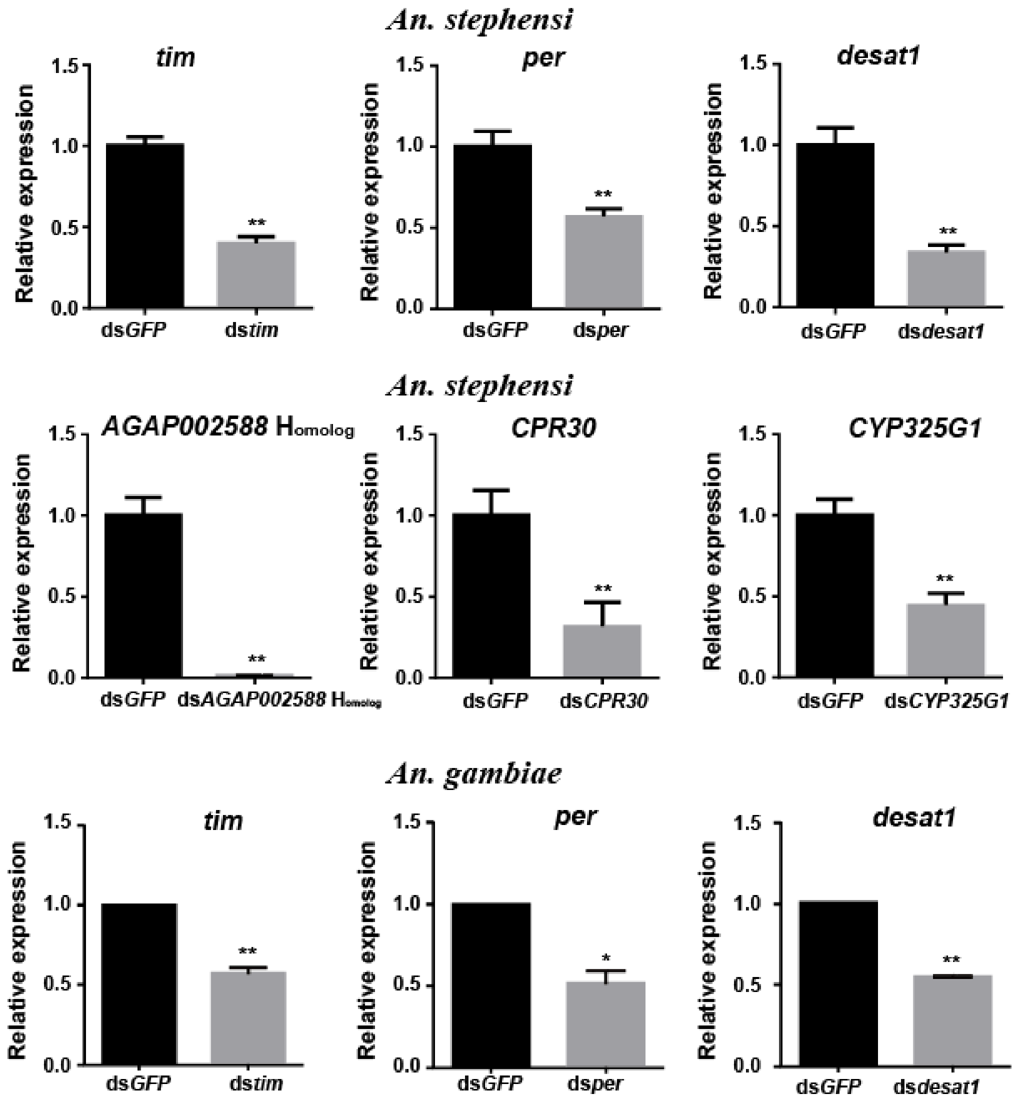
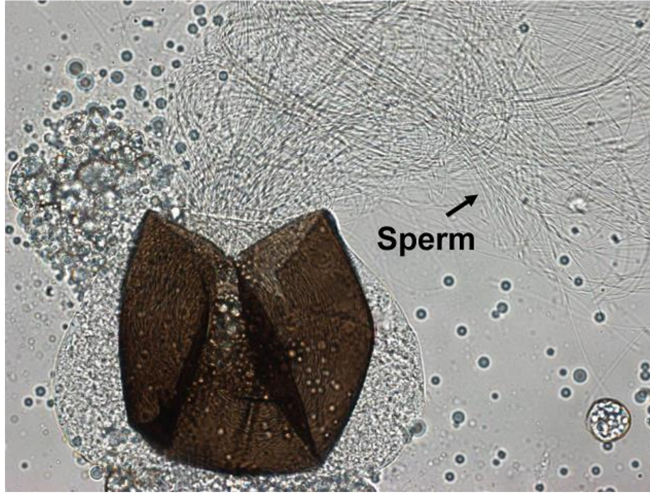


Fig. S2. Silencing efficiency of *tim, per*, *desat1, AGAP002588* Homolog*, CPR30* and *CYP325G1* in the head of *male An. stephensi* and *An. gambiae s.s.* mosquitoes (n = 20) injected with 70 ng of ds*GFP* or double-stranded RNA of the indicated genes. Error bars indicate SEM. $^{**}P < 0.01$.



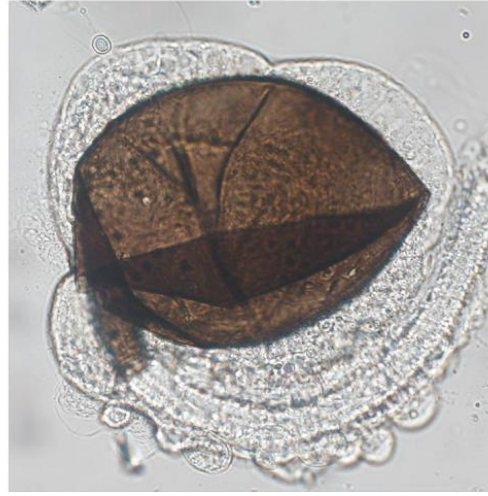


Fig. S3. Sperm in a mated female spermatheca. Arrow points to sperm.

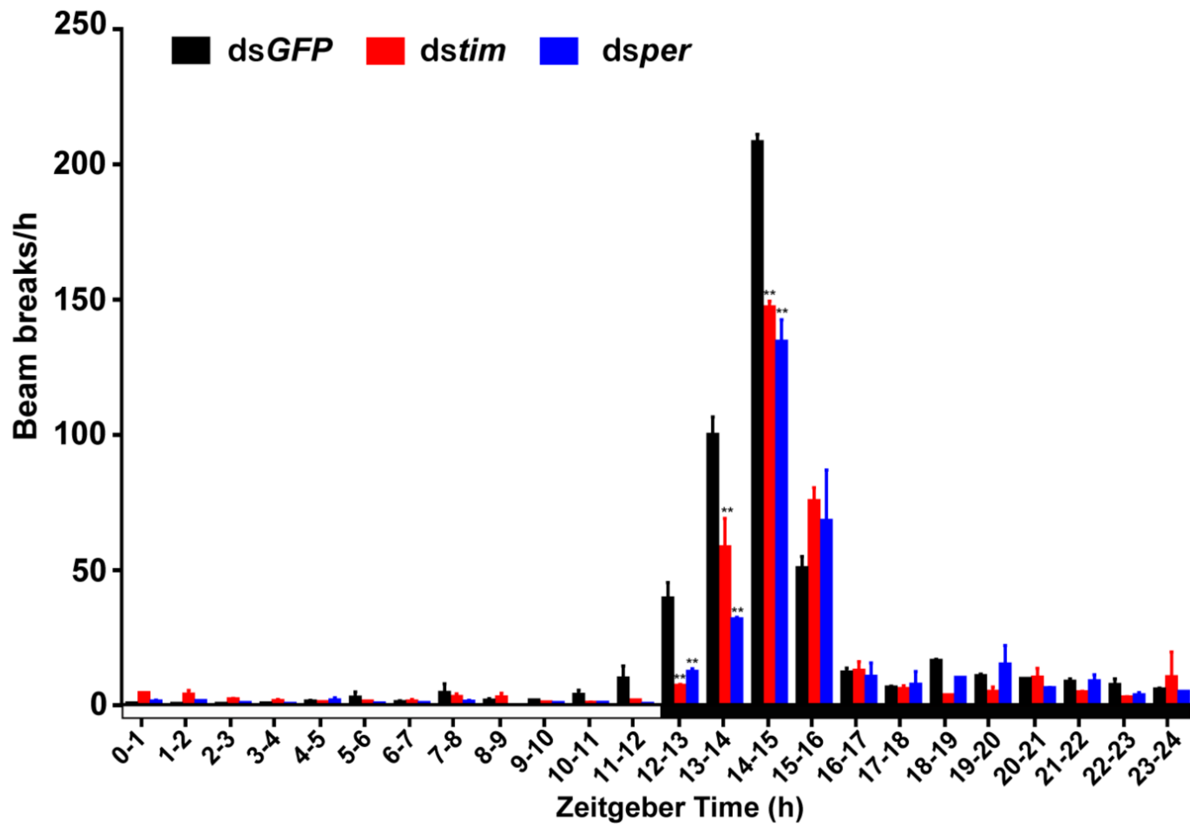


Fig. S4. *An. stephensi* virgin male mosquito cumulative flight activity. Twenty-four-hour distribution of mosquito flight activity measured by infrared beam breaks of *An. stephensi* virgin male mosquitoes injected with ds*GFP*, ds*tim*, ds*per* or ds*desat1* (Fig. 3C). The white and black bars below the graphs denote when lights were on and off, respectively. Values show the total activity within each hourly time bin (mean ± SEM) from 16 mosquitoes. $**P < 0.01$ (Student's t test). The experiments were repeated three times.

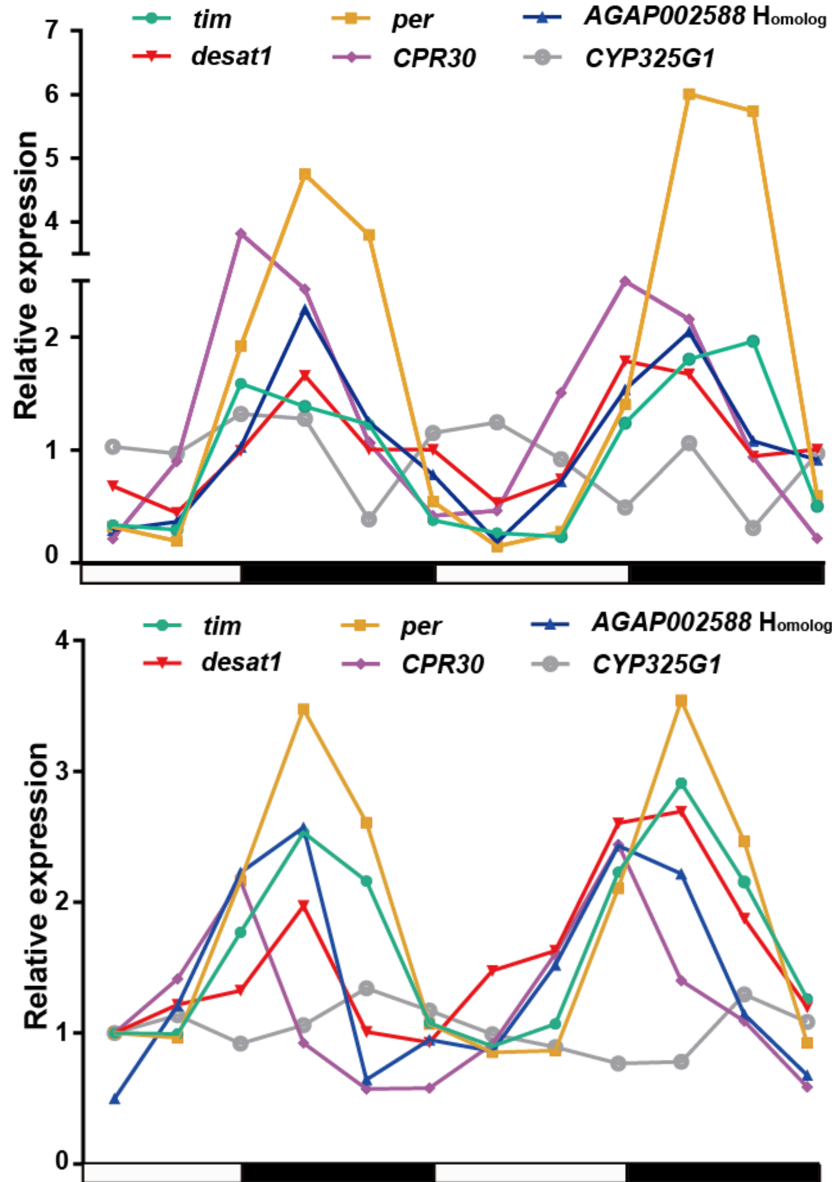


Fig. S5. Transcriptional profiles of differentially-expressed genes in the head of male *An. stephensi* mosquitoes under LD conditions. Male head expression profiles were determined by qPCR in mosquitoes maintained in 12/12 h LD cycles (white/black bars). The clock genes (*per*, *tim*), and the clock-controlled genes *desat1, AGAP002588* Homolog, *CPR30*, but not *CYP325G1*, are rhythmically expressed in the male mosquito head under LD cycles. The housekeeping gene *RPS7* (AsS7) was used as the internal control for qRT-PCR. Twenty mosquitoes were evaluated at each time point. Data were normalized to median fold change, and SD error bars were omitted for viewing purposes. The two panels show the results of two biological repeats.

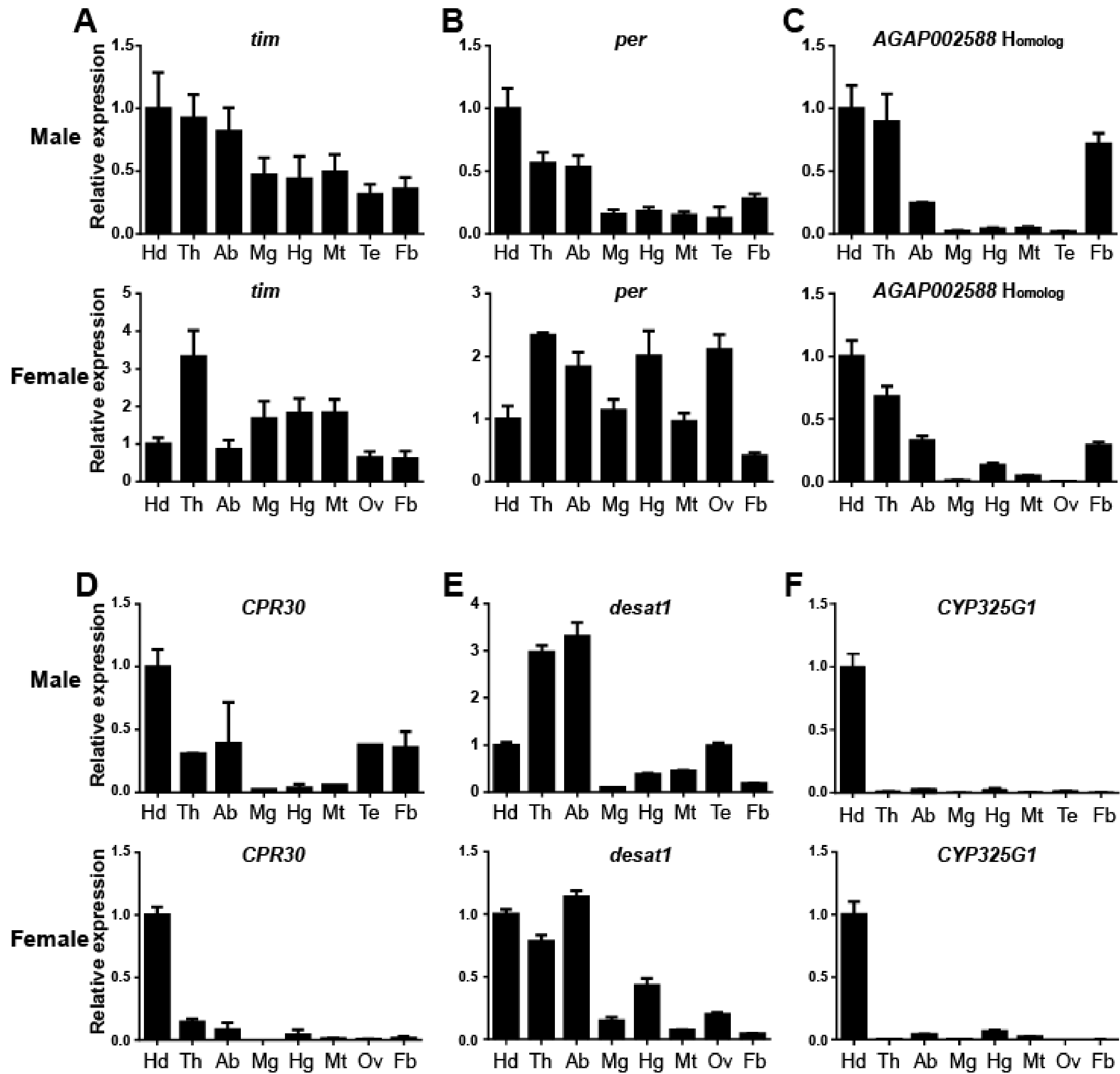


Fig. S6. Transcription levels of *per*, *tim, desat1, AGAP002588* Homolog*,* the cuticle protein gene *CPR30, and* the *P450* mitochondrial gene *CYP325G1* in tissues of virgin *An. stephensi*. males and females. Bars represent SEM. Hd, head; Th, thorax; Ab, abdomen; Mg, midgut; Hg, hindgut; Mt, Malpighian tubule; Te, testis; Ov, ovary; Fb, fat body.

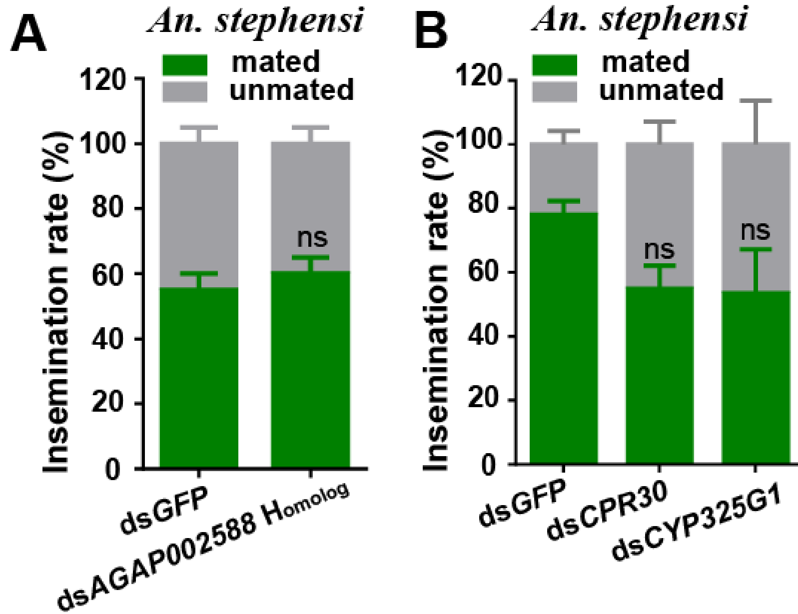


Fig. S7. Effect of *AGAP002588* Homolog, *CPR30* and *CYP325G1* knockdown on male mating activity. (A-B) Silencing of *AGAP002588* Homolog, *CPR30* or *CYP325G1* genes in virgin male mosquitoes does not significantly affect the rate of female insemination. Presence of sperm in the female spermathecae was examined 12 h after mosquito courtship. 20 female mosquitoes were evaluated for each group.

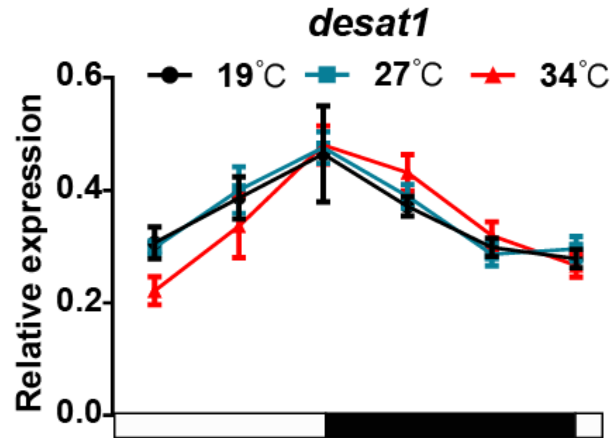


Fig. S8. Effect of temperature on *desat1* transcript abundance in the *An. stephensi* male head. Newly emerged male mosquitoes were maintained at 27 °C under LD cycling. Three days later, mosquitoes were switched to LD cycling at 19 °C, 27 °C and 34 °C. *desat1* transcript abundance in male heads was measured by qRT-PCR. Relative expression refers to *desat1/S7* values.

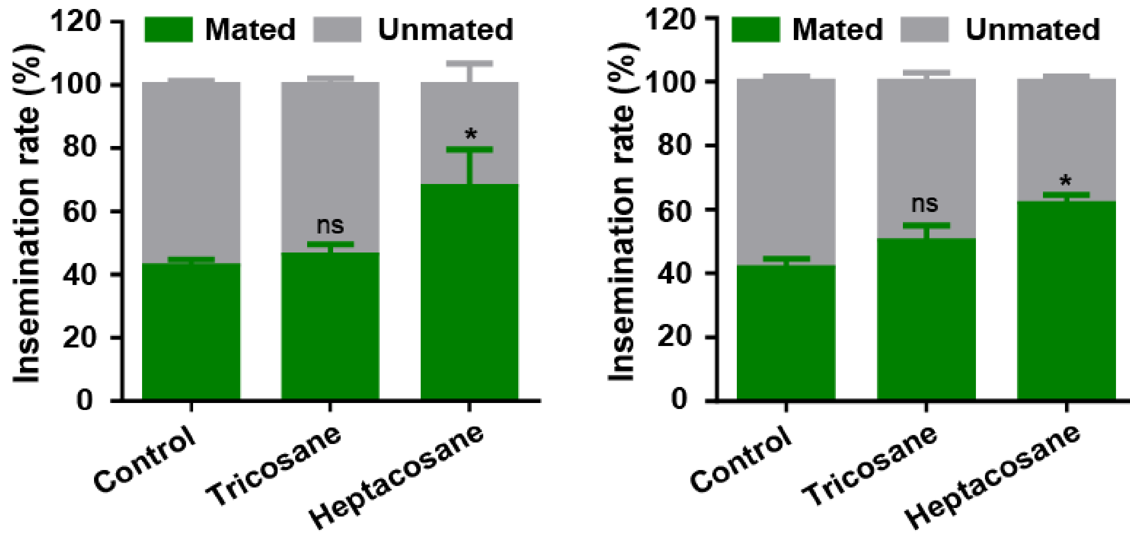


Fig. S9. Effect of cuticular hydrocarbons tricosane and heptacosane on mosquito mating activity. Perfuming *Anopheles* virgin males with tricosane does not alter mating activity, whereas males perfumed with heptacosane resulted in a significant increase of female insemination. This figure shows the results of two additional biological repeats of the experiment shown in Fig. 4D. Error bars indicate SEM. *$P < 0.05$ (Student's *t* test).

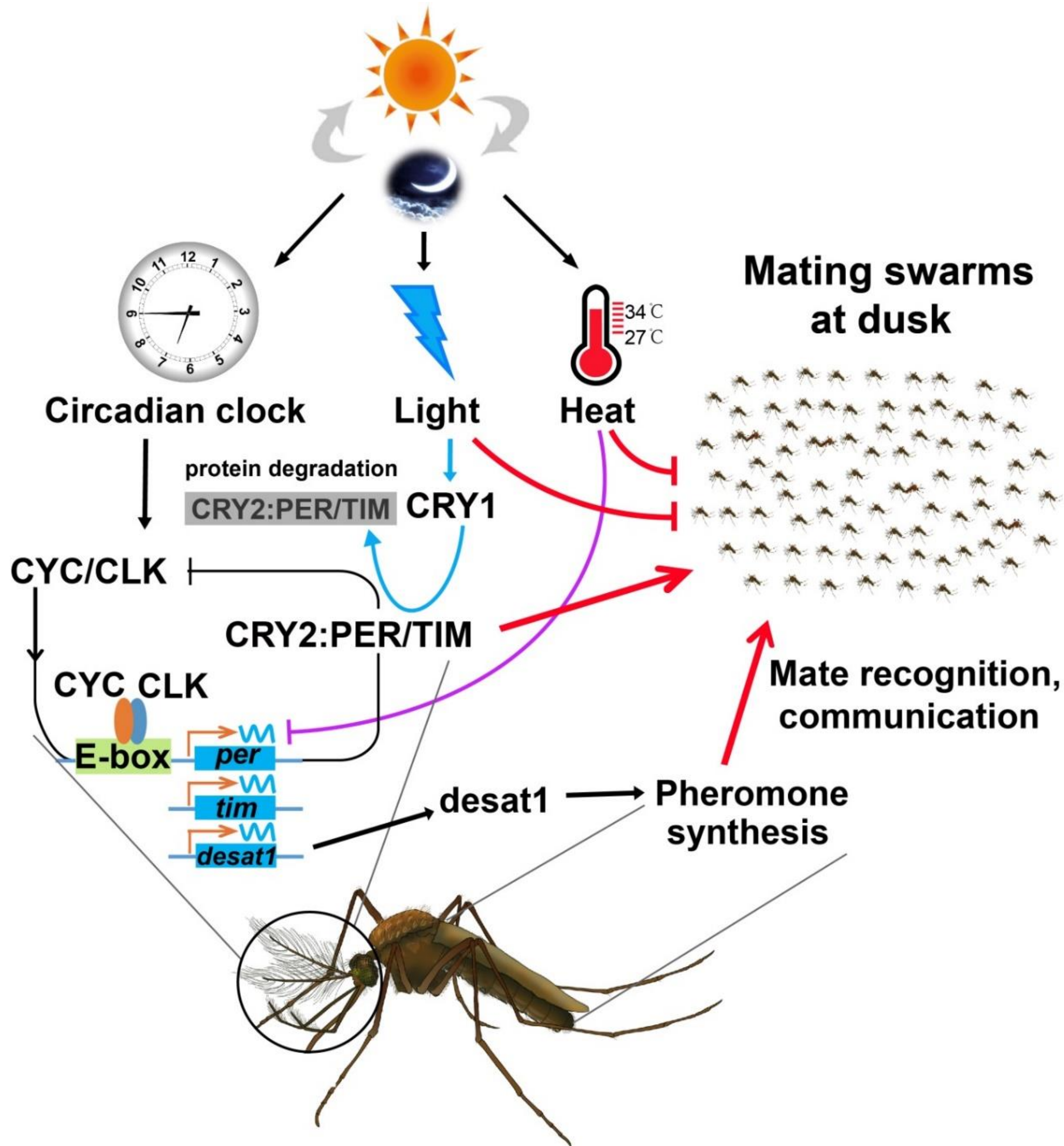


Fig. S10. A schematic model of mosquito swarming and mating showing that these activities are governed by the clock genes, light and temperature. The molecular feedback loop is formed by the negative feedback of PER and TIM on their own transcription (*17*). The clock genes *per* and *tim* are markedly upregulated in the head of swarming male mosquitoes and modulate male swarming and mating activity. The *desat1* gene, which is upregulated and rhythmically expressed in male heads, regulates the production of cuticular hydrocarbons. Of these, heptacosane enhances mating activity. Moreover, temperature and light affect mating activity and are important environmental cues for entraining the circadian clock in *Anopheles* mosquitoes. Light is perceived by the blue-light-sensitive photoreceptor Cryptochrome CRY1 that resets the molecular oscillator by triggering the light-induced degradation of TIM, PER and CRY2 (*22*).

Table S1. Quantitative real-time RT-PCR validation of the microarray analysis.

Gene ID	Fold change array data (SW/NSW)	Fold change by qRT-PCR	Gene function
AGAP008288	4.26	4.81	TIM (Timeless)
AGAP001856	9.31	6.43	PER (Period)
AGAP001202	4.15	2.49	organic cation transporter
AGAP008892	4.62	3.77	unknown
AGAP007497	4.97	2.89	CuSOD1 (copper-zinc superoxide dismutase 1)
AGAP001713	6.08	5.88	desat1 (acyl-CoA desaturase 1)
AGAP003493	3.79	3.59	sugar transporter
AGAP003934	4.09	2.91	cln3/battenin
AGAP000356	4.08	2.21	venom allergen
AGAP006009	25.14	9.69	CPR30 (cuticle protein)
AGAP002215	2.33	1.11	HEAT repeat-containing protein 5B-like
AGAP002588	26.80	37.88	unknown
AGAP008596	5.45	5.82	long-chain-fatty-acid--CoA ligase ACSBG
AGAP002196	2.17	1.36	CYP325G1 (cytochrome P450)
AGAP011925	3.28	6.23	patatin-like phospholipase domain containing 5
AGAP012334	0.43	0.46	high mobility group protein D, isoform C
AGAP001774	0.22	1.56	allatostatin receptor
AGAP005751	6.67	3.59	glucosyl/glucuronosyl transferase

Table S2. Identity of cuticular hydrocarbon peaks of male adult *Anopheles stephensi* mosquitoes (related to Fig. 6A).

Peak No.	Retention time	Full compound name	Peak No.	Retention time	Full compound name
1	10.569	cis-vaccenic acid	**9**	21.503	nonacosane
2	10.704	n-hexadecanoid acid	**10**	21.882	2-methylnonadecane
3	13.965	tricosane	**11**	22.667	triacontane
4	14.888	tetracosane	**12**	23.907	hentriacontanol
5	16.377	pentacosane	**13**	25.409	dotriacontane
6	17.634	hexacosane	**14**	26.904	tritriacontane
7	18.985	heptacosane	**15**	29.077	tetratriacontane
8	20.179	octacosane	**16**	31.126	hexatriacontane

Table S3. Primers used in this study.

Primers	5'-3' sequences	Applification
S200 X6.1F	TCGCCTTAGACCTTGCGTTA	Diagnostic PCR
S200 X6.1R	CGCTTCAAGAATTCGAGATAC	detection of *S200* X6.1 locus
AG-S7F	AGAACCAGCAGACCACCATC	qPCR of *AgS7*
AG-S7R	GCTGCAAACTTCGGCTATTC	
AGAP002588F	TATTCTGCCCACTGGAATCA	qPCR of *AGAP002588*
AGAP002588R	CAACGTGTGAATGCCACATA	
AGAP005751F	TGTTGATAAGGGCGTTCTTG	qPCR of *AGAP005751*
AGAP005751R	TGTGGAGGTTCCGATATTGA	
AGAP007497F	CCCGTACATCAATCTGTTCG	qPCR of *AGAP007497*
AGAP007497R	GGAAGGAGATCGGGTTGTTA	
AGAP008892F	GTGGTGAAAGAGGTCCCAGT	qPCR of *AGAP008892*
AGAP008892R	AACATTTCCGCTACCACCTC	
AGAP001202F	GCCAGAGCTTCATTTCCTTC	qPCR of *AGAP001202*
AGAP001202R	TAGCCTCCTCGAGTGTGTTG	
AGAP003934F	CAACACGTTCTATCGCATCC	qPCR of *AGAP003934*
AGAP003934R	CACCGGTAGATCACAGATCG	
AGAP000356F	GGCCGGATGATACAGAAGAT	qPCR of *AGAP000356*
AGAP000356R	CTGCCAAATTCGCTAACTCA	
AGAP001774F	AGACATGCAGCTTCCTCGAA	qPCR of *AGAP001774*
AGAP001774R	CCACATGTACAGGACGCTGA	
AGAP002215F	CCAGTCGCTCAACTTCATCG	qPCR of *AGAP002215*
AGAP002215R	CGATCAGTGGCGTAATCTGC	
AGAP003934F	GCGATTGGTTTACAGGGCAA	qPCR of *AGAP003934*
AGAP003934R	GCACAAACACCCGAACAGAT	
AGAP008596F	CCATCTCGAACGCACAGAAG	qPCR of *AGAP008596*
AGAP008596R	CTTGTTCTTTTCCGCCACGA	
AGAP011925F	AGACAGCTTGCAAAAGAGGC	qPCR of *AGAP011925*
AGAP011925R	ACGAGTTGGAAAGGGAGGAG	
AGAP012334F	ATGTTCGGGGCCTTACTTCA	qPCR of *AGAP012334*
AGAP012334R	GGAATACTGGGACTGGGGAC	
AGAP006009F	TCCGTGTGTACCGTCTAACC	qPCR of *CRP30*
AGAP006009R	GCTTCCATCGACGTTGTTGT	
AGAP002196F	TCTCGAGAAGGCAGACGTTT	qPCR of CYP325G1
AGAP002196R	AAAGATCGCCACAAAGCTGG	
dsAG-perF	TAATACGACTCACTATAGGGTACCGTACA ATCCCGAGTGC	dsRNA synthesis of *Ag-per* (*AGAP001856*)
dsAG-perR	TAATACGACTCACTATAGGGGGTGGTGGA CATTTTGTGTG	
AG-perF	CGATAGCGGACGTGTTTGC	qPCR of *Ag-per*
AG-perR	AGCGTGTCGTCGGAGAATTG	(*AGAP001856*)
dsAG-timF	TAATACGACTCACTATAGGGAACCCGCAG ATCAACAGC	dsRNA synthesis of *Ag-tim* (*AGAP008288*)
dsAG-timR	TAATACGACTCACTATAGGGGGCACGATG TCGTTCTTGAC	
AG-timF	ACATAGTGACGCTCGTGCAGTAC	qPCR of *Ag-tim*
AG-timR	GTCCTCTGGTTTGGGCGAAT	(*AGAP008288*)

AS-S7R	AGCGCGGTCTCTTCTGCTTGT	qPCR of *As-S7*
AS-S7F	TCGGTTCCAAGGTGATCAAAGC	
dsAS-timF	TAATACGACTCACTATAGGGCTATGCGAG CAGTTGGAACA	dsRNA synthesis of *As-tim*
dsAS-timR	TAATACGACTCACTATAGGGCAGTATGCT CGGCTGAAACA	
AS-timF	GTTTCAGCCGAGCATACTGA	qPCR of *As-tim*
AS-timR	CAATATCCGGTAGCGTTGTG	
dsAS-perF	TAATACGACTCACTATAGGGACGACCGTC AACTACGAACC	dsRNA synthesis of *As-per*
dsAS-perR	TAATACGACTCACTATAGGGAAGGGCGAT ACAAACACGTC	
AS-perF	CTAGAGTTCGTGATCGGCAA	qPCR of *As-per*
AS-perR	CACCATGTCGGATGGTTTAG	
dsASTE000498F	TAATACGACTCACTATAGGGATGTTCCATT TGTCCGTTCT	dsRNA synthesis of *ASTE000498*
dsASTE000498R	TAATACGACTCACTATAGGGTTATGCCAC ACGGCCGATCG	
ASTE000498F	CAAGATTGTAGCGGTGGTTG	qPCR of *ASTE000498*
ASTE000498R	ATTGTGCTTAAGCGATCGTG	
dsdesat1F	TAATACGACTCACTATAGGGTTCCAACGC AAGCATTACATGATT	dsRNA synthesis of *desat1*
dsdesat1R	TAATACGACTCACTATAGGGACTGGTCCT GATCCTTGTCGC	
desat1F	ACGTGATTGCCTTCGGTTACC	qPCR of *desat1*
desat1R	AGCCCGGATACAACGTACAG	
dsCYP325G1-F	TAATACGACTCACTATAGGGCTGATTTGG ATGGTCCTCGTGA	dsRNA synthesis of *CYP325G1*
dsCYP325G1-R	TAATACGACTCACTATAGGGCCATGCTCA TTCTGGAGGTTCA	
CYP325G1F	CGACGCTACAGTCCTCCAAGC	qPCR of *CYP325G1*
CYP325G1R	CGTTAATGCGGACGTTTCGTT	
dsCPR30F	TAATACGACTCACTATAGGGTTTGCATTTG TGTGCCTGTTGG	dsRNA synthesis of *CPR30*
dsCPR30R	TAATACGACTCACTATAGGGGCTTCACCA TTGGACGGTATCC	
CPR30F	ATGAAGTTTGTCGCTGTATTTGC	qPCR of *CPR30*
CPR30R	CTTCTGACCGTCCGATTGTTCG	
dsGFPF	TAATACGACTCACTATAGGGAGATGGTGA GCAAGGGCGAGGAGCTGT	dsRNA synthesis of *GFP*
dsGFPR	TAATACGACTCACTATAGGGAGTTACTTGT ACAGCTCGTCCATGCCG	

Note: As, *Anopheles stephensi*; Ag, *An. gambiae s.s.*.

Movie S1.

Silencing of *per* affects male mating flight activity around ZT13-14, when peak mating activity of ds*GFP*-treated *An. stephensi* mosquitoes occurs in the small cage.

Movie S2.

Silencing of *tim* affects male mating flight activity around ZT13-14, when peak mating activity of ds*GFP*-treated *An. stephensi* mosquitoes occurs in the small cage.

Movie S3.

Silencing of *desat1* affects male mating flight activity around ZT13-14, when peak mating activity of ds*GFP*-treated *An. stephensi* mosquitoes occurs in the small cage.

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