MOSQUITO BIOLOGY

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

Guandong Wang[1,2]*, Joel Vega-Rodríguez[3]*†, Abdoulaye Diabate[4]*, Jingnan Liu[5]*, Chunlai Cui[1,2], Charles Nignan[4], Ling Dong[1,2], Fang Li[1,2], Cheick Oumar Ouedrago[4], Abdoul Malik Bandaogo[4], Péguédwindé Simon Sawadogo[4], Hamidou Maiga[4], Thiago Luiz Alves e Silva[6], Tales Vicari Pascini[6], Sibao Wang[1,2]‡§, Marcelo Jacobs-Lorena[3]‡§

Anopheles mating is initiated by the swarming of males at dusk followed by females flying into the swarm. Here, we show that mosquito swarming and mating are coordinately guided by clock genes, light, and temperature. Transcriptome analysis shows up-regulation of the clock genes *period* (*per*) and *timeless* (*tim*) in the head of field-caught swarming *Anopheles coluzzii* males. Knockdown of *per* and *tim* expression affects *Anopheles gambiae s.s.* and *Anopheles stephensi* male mating in the laboratory, and it reduces male *An. coluzzii* swarming and mating under semifield conditions. Light and temperature affect mosquito mating, possibly by modulating *per* and/or *tim* expression. Moreover, the desaturase gene *desat1* is up-regulated and rhythmically expressed in the heads of swarming males and regulates the production of cuticular hydrocarbons, including heptacosane, which stimulates mating activity.

The malaria parasite, transmitted by *Anopheles* mosquitoes, infects more than 200 million people and causes nearly half a million deaths annually (*1*). In the absence of an effective vaccine, mosquito control using insecticide-treated nets and indoor residual spraying remains the most effective means of combating the disease (*2*). However, mosquito control is being affected by resistance to commonly used insecticides (*3*, *4*). Thus, there is an urgent need to develop alternative tools to control mosquito populations (*5*). Male mating behavior is a key aspect of the mosquito life cycle, and targeting this behavior has shown promise for vector control (*6*).

Males of numerous dipteran species, including many anopheline and culicine mosquitoes, form flight swarms that contain tens to thousands of males as a prerequisite for mating (*7*–*13*). Females fly into the swarm, where they select a male for copulation (*14*). After insemination, the female is generally not receptive to copulation for the remainder of the female's life (*15*).

Day-night cycle, light intensity, and temperature regulate the circadian rhythmicity of behavior and physiology of most organisms (*16*–*18*). For example, *Anopheles* mosquitoes swarm and mate daily at dusk (*8*). However, the molecular mechanisms that modulate mosquito swarming and mating behavior have yet to be clarified. Understanding mating biology is necessary to guide the development and implementation of vector control programs by means of the release of either conventional sterile or genetically engineered males (*19*). In this work, we undertook experiments in the laboratory and in near-field conditions to identify components of the circadian clock apparatus that regulate male swarming and mating behavior in *Anopheles* mosquitoes.

To identify genes involved in swarming and mating behaviors, we collected *Anopheles coluzzii* males at dusk from swarms and from houses (resting, nonswarming) in Vallée du Kou, Burkina Faso, and isolated RNA from their heads. *Anopheles gambiae s.s.* genome microarray analysis showed that 87 genes significantly differed (≥1.75-fold change, Student's *t* test, $P < 0.01$) between the two groups, including 45 up-regulated and 42 down-regulated genes in the heads of swarming versus nonswarming males (Fig. 1A and fig. S1). Notably, up-regulated genes in swarming males were overrepresented in the functional categories of circadian clock and metabolism, including two canonical clock genes—*period* (*per*) (AGAP001856) and *timeless* (*tim*) (AGAP006376)—and nine genes involved in carbohydrate, amino acid, or lipid metabolism (Fig. 1B). By contrast, all four differentially expressed genes (DEGs) encoding immunity were down-regulated in swarming male heads compared with nonswarming male heads (Fig. 1B). We used quantitative reverse transcription polymerase chain reaction (qRT-PCR) to validate DEGs associated with circadian clock and metabolism. Most genes tested had the expected directional changes (table S1). Collectively, global transcription analysis indicates that circadian clock and metabolism genes are associated with male swarming behavior.

To address the hypothesis that clock genes influence male swarming and mating activity, we silenced *per* and *tim* in *Anopheles stephensi* virgin males by systemic injection of double-stranded RNA (ds*per* or ds*tim*) (fig. S2). Injected males were allowed to mate with virgin females, and the presence of sperm in the female spermatheca was used as a mating indicator (fig. S3). Although swarming is not a common behavior in laboratory small cages, male *Anopheles* mosquitoes still exhibit swarm-like mating flight activity (*14*, *20*). We confirmed that *Anopheles* males display evening peak flight activity in laboratory cages and that knockdown of *per* or *tim* gene expression in *An. stephensi* males significantly reduces flying activity (fig. S4). Silencing of *per* or *tim* significantly affected male mating flight activity around Zeitgeber time (ZT) ZT13 to ZT15, when peak mating activity of double-stranded green fluorescent protein gene (ds*GFP*)–treated mosquitoes (control) occurs in small cages (fig. S4 and movies S1 and S2). Reduction in flight activity correlated with significant reduction of mating (female insemination rate) (Fig. 1, C and D). These results suggest that the two core clock genes *per* and *tim* regulate mating activity in *Anopheles* mosquitoes.

To determine the role of *per* and *tim* in mosquito swarming and mating activity in a semifield setting, we performed experiments in a large outdoor mosquito sphere in the Vallée du Kou in Burkina Faso. Locally captured and reared *An. coluzzii* virgin male mosquitoes were injected with double-stranded RNA encoding *per*, *tim*, or *GFP* as control. Three days later, the injected males and virgin females were released before sunset in compartments with a swarming marker (1-m^2 black cloth) (*21*), and swarming behavior was monitored for 1 hour. We observed a significant reduction in swarm maximum height and swarm size (number of swarming males) in male mosquitoes injected with ds*per* or ds*tim* (Fig. 1, E and F). Moreover, mating was significantly reduced in males injected with ds*per* (40% insemination) or ds*tim* (47% insemination) compared with male mosquitoes injected with ds*GFP* (70% insemination) (Fig. 1G). These reductions are consistent with those observed in a laboratory setting (Fig. 1C). Taken together, these results indicate that the circadian genes *per* and *tim* regulate male *Anopheles* swarming and mating activity.

[1]CAS Key Laboratory of Insect Developmental and Evolutionary Biology, CAS Center for Excellence in Molecular Plant Sciences, Shanghai Institute of Plant Physiology and Ecology, Chinese Academy of Sciences (CAS), Shanghai, China. [2]CAS Center for Excellence in Biotic Interactions, University of Chinese Academy of Sciences, Beijing, China. [3]Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health and Malaria Research Institute, Baltimore, MD, USA. [4]Institut de Recherche en Science de la Santé (IRSS), Bobo-Dioulasso, Burkina Faso. [5]School of Life Science and Technology, ShanghaiTech University, Shanghai, China. [6]Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD, USA.

*These authors contributed equally to this work. †Present address: Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD, USA. ‡These authors contributed equally to this work.

§Corresponding author. Email: sbwang@cemps.ac.cn (S.W.); ljacob13@jhu.edu (M.J.-L.)

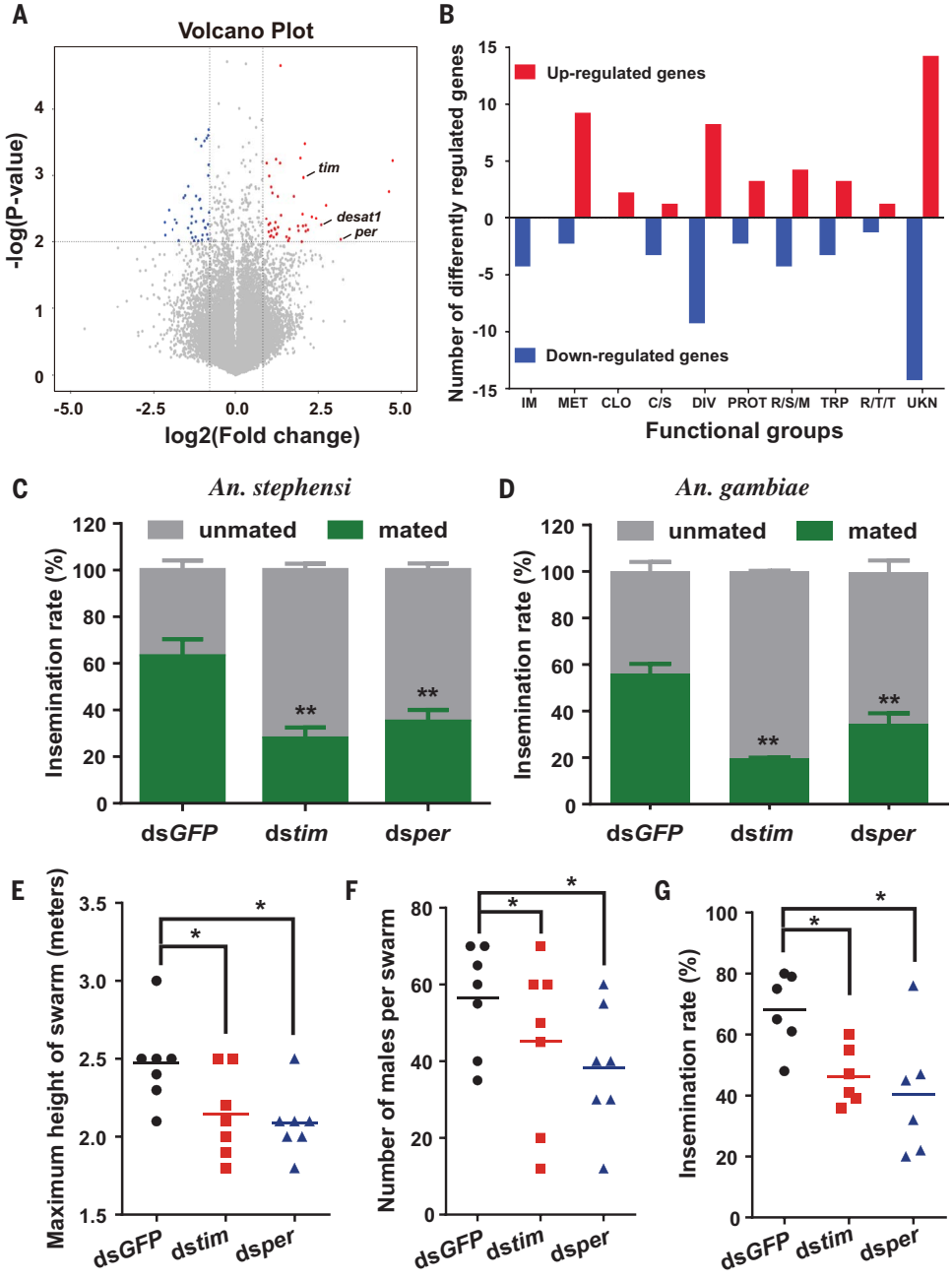

Fig. 1. Mosquito swarming and mating are regulated by the clock genes *per* and *tim*. (**A** and **B**) Transcriptome analysis of *An. coluzzii* head gene expression between swarming and nonswarming male mosquitoes. (A) Volcano plot of DEGs. Significantly up-regulated and down-regulated genes are marked with red and blue spots, respectively. (B) DEGs by gene ontology category in swarming male heads compared with non-swarming males. Differential gene expression was considered significant when the expression fold change (swarming versus nonswarming) was ≥0.75 on a log2 scale ($P < 0.01$). IM, immunity; MET, metabolism; CLO, clock; C/S, cytoskeletal and structural; DIV, diverse; PROT, proteolysis; R/S/M, oxidoreductive, stress-related, and mitochondrial; TRP, transport; R/T/T, replication, transcription, and translation; UKN, unknown function. (**C** and **D**) Silencing of *tim* and *per* in virgin *An. stephensi* (C) or *An. gambiae s.s.* (D) males causes a reduction in the rate of female insemination, as determined by light microscopy examination for the presence of sperm in the dissected female spermatheca (fig. S3). A total of 60 virgin males were injected with double-stranded RNA for each treatment. Error bars indicate SEMs. Similar results were obtained from two biological repeats. (**E** to **G**) Knockdown of *tim* or *per* in virgin *An. coluzzii* males affects the swarm maximum height (E), swarm size (number of swarming males per swarm) (F), and mating frequency (G) under semifield conditions (Burkina Faso). Each symbol denotes the result of an independent experiment ($n = 7$). Horizontal lines represent the means. Statistics were performed with Student's *t* test or one-way analysis of variance (ANOVA). *$P < 0.05$; **$P < 0.01$.

The circadian clock is intimately regulated by temperature and light fluctuations (*22*). To investigate the effect of temperature on mosquito mating, 3-day-old virgin male and female *An. stephensi* mosquitoes were pooled and kept overnight at 27°, 19°, or 34°C. We found that low temperature (19°C) and high temperature (34°C) significantly inhibited mosquito mating activity compared with the optimum temperature of 27°C (Fig. 2A).

We next examined whether light influences mosquito mating activity by comparing mating frequencies of mosquitoes that were maintained either in light-dark (LD) conditions (lights off at night) or light-light (LL) conditions (lights on at night). The mating frequency of mosquitoes in the LL condition was significantly lower than that of mosquitoes in the LD condition (Fig. 2B). These results suggest that adverse temperatures and continuous light strongly affect mosquito mating. Moreover, the rhythm of *per* and *tim* transcripts in *An. stephensi* male heads is strongly affected by changes in ambient temperature. Peak levels of *per* mRNA were higher at 19°C and lower at 34°C compared with those at 27°C (Fig. 2C). Contrastingly, the highest levels of *tim* expression were observed at 34°C with successive reduction at lower temperatures (Fig. 2D). Furthermore, the transcription abundance of *per* and *tim* in male heads was significantly lower under the continuous-light condition (Fig. 2, E and F), which indicates that prolonged exposure to LL conditions reduces the expression of *per* and *tim* in the evening.

Of 86 DEGs, one unknown gene (AGAP002588) and a cuticular protein gene *CPR30* (AGAP006009) were among the most up-regulated genes (~25-fold) in the heads of swarming males compared with nonswarming males (table S1). Additionally, an ortholog to the *Drosophila desaturase 1* (*desat1*) gene required for pheromone production (*desat1*, AGAP001713) and a P450 gene *CYP325G1* (AGAP002196) were also highly up-regulated in swarming male heads (table S1).

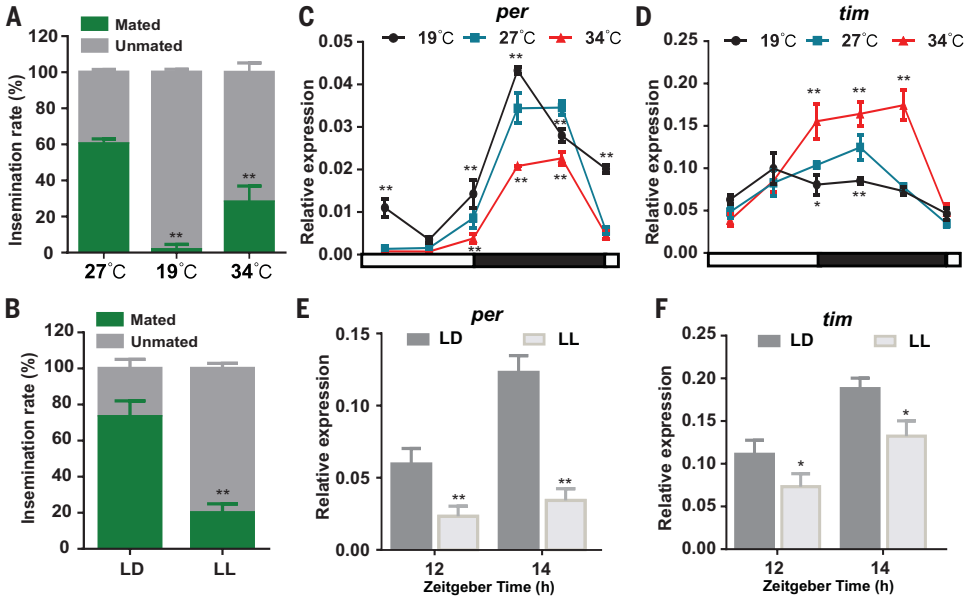

Fig. 2. Temperature and light affect mating activity. Newly emerged male and female mosquitoes were kept separately for 3 days at 27°C under 12-hour–12-hour LD cycling before performing the experiment. (**A**) Temperature affects mosquito mating activity. Males and females were pooled; kept overnight at 19°, 27°, or 34°C; and female insemination rates were determined. (**B**) Light inhibits mosquito mating activity. Males and females were pooled, kept in LD or LL conditions, and female insemination rates were determined. (**C** and **D**) Effect of temperature on *per* (C) and *tim* (D) transcript abundance in male *An. stephensi* heads under LD cycling. Mosquitoes were kept under LD cycles at 19°, 27°, or 34°C. *per* and *tim* transcript rhythm in male heads was measured by qRT-PCR. The housekeeping gene *RPS7* (*AsS7*) was used as an internal reference in qRT-PCR assays. (**E** and **F**) Effect of prolonged light on *per* (E) and *tim* (F) transcript abundance in male *An. stephensi* heads. Male mosquitoes were maintained for 3 days at 27°C under LD cycling and then either kept in the LD condition or switched to the LL condition. Mosquitoes were collected at ZT12 (lights off) and ZT14 (2 hours after lights off) for detection of *per* and *tim* expression levels. Error bars indicate SEMs. *$P < 0.05$; **$P < 0.01$ (Student's *t* test). Similar results were obtained from two biological repeats.

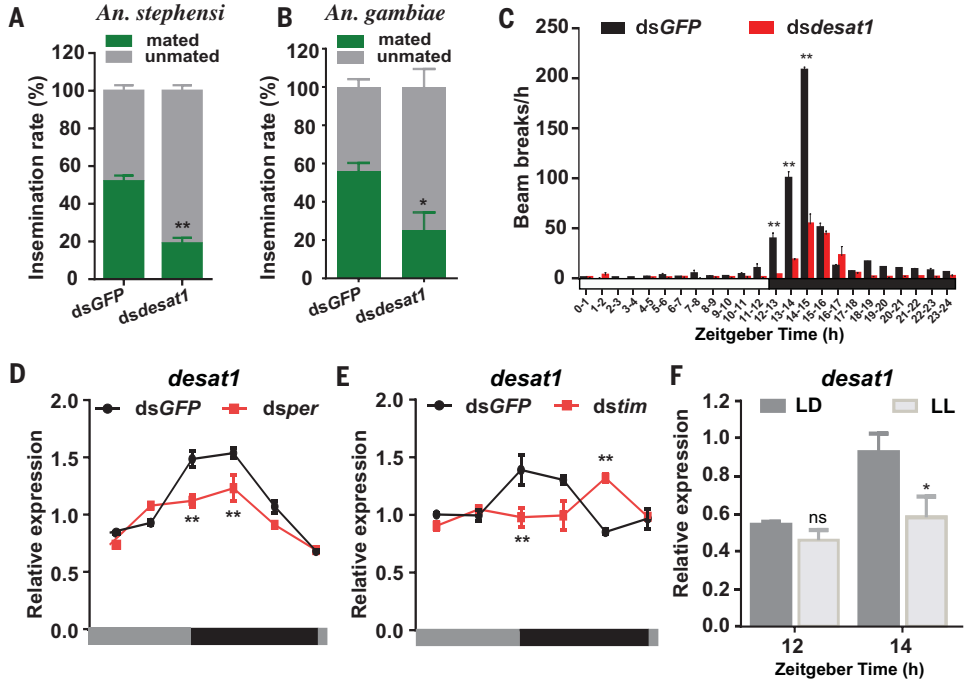

Fig. 3. *Desat1* regulates male flight activity and mating. (**A** and **B**) Silencing *desat1* expression in *An. stephensi* (A) and *An. gambiae s.s.* (B) virgin males causes reduction of female insemination. (**C**) Flight activity of *An. stephensi* virgin male mosquitoes injected with ds*GFP* (black) or ds*desat1*(red) and maintained under 12-hour–12-hour LD cycles. Values show the total activity within each hourly time bin (mean ± SEM) of 16 mosquitoes in one treatment. The white and black bar below the graph denotes when lights were on and off, respectively. Lights on occurred at ZT0 and lights off occurred at ZT12. (**D** and **E**) Silencing *per* (D) or *tim* (E) influences the expression pattern of *destal1* in *An. stephensi* male heads maintained under DD (constant dark) condition. The housekeeping gene *RPS7* (*AsS7*) was used as the internal control for qRT-PCR. Data were normalized to median fold change. Circadian day and night are indicated by the horizontal gray and black (indicating subjective day and subjective night, respectively) bars below the charts. (**F**) Effect of prolonged light on *desat1* transcript abundance in male *An. stephensi* heads. After being maintained at 27°C under LD cycling for 3 days, male mosquitoes were either kept in the LD condition or switched to the LL condition. Mosquitoes were collected at ZT12 (lights off) and ZT14 (2 hours after lights off) for detection of *desat1* expression levels. Error bars indicate SEMs. ns, not significant; *$P < 0.05$; **$P < 0.01$ (Student's *t* test). Similar results were obtained from three experimental repeats.

The expression of *desat1*, the AGAP002588 gene homolog, and *CPR30*—but not *CYP325G1*—exhibited robust, high-amplitude rhythm in synchrony with the two canonical clock genes *per* and *tim* (fig. S5). The expression patterns of these DEGs in different mosquito tissues is presented in fig. S6.

We next determined whether these genes play any role in mosquito mating. Knockdown of *desat1* expression in *An. stephensi* and *An. gambiae s.s.* virgin males significantly reduced female insemination rates (Fig. 3, A and B) and markedly reduced male mating flight activity (Fig. 3C and movie S3), whereas silencing of the AGAP002588 gene homolog, *CPR30*, and *CYP325G1* did not affect female insemination rate (fig. S7). Moreover, we found that knockdown of *per* or *tim* significantly altered the amplitude or rhythm of *desat1* expression in *An. stephensi* male heads under a dark-dark (DD) (constant dark) condition

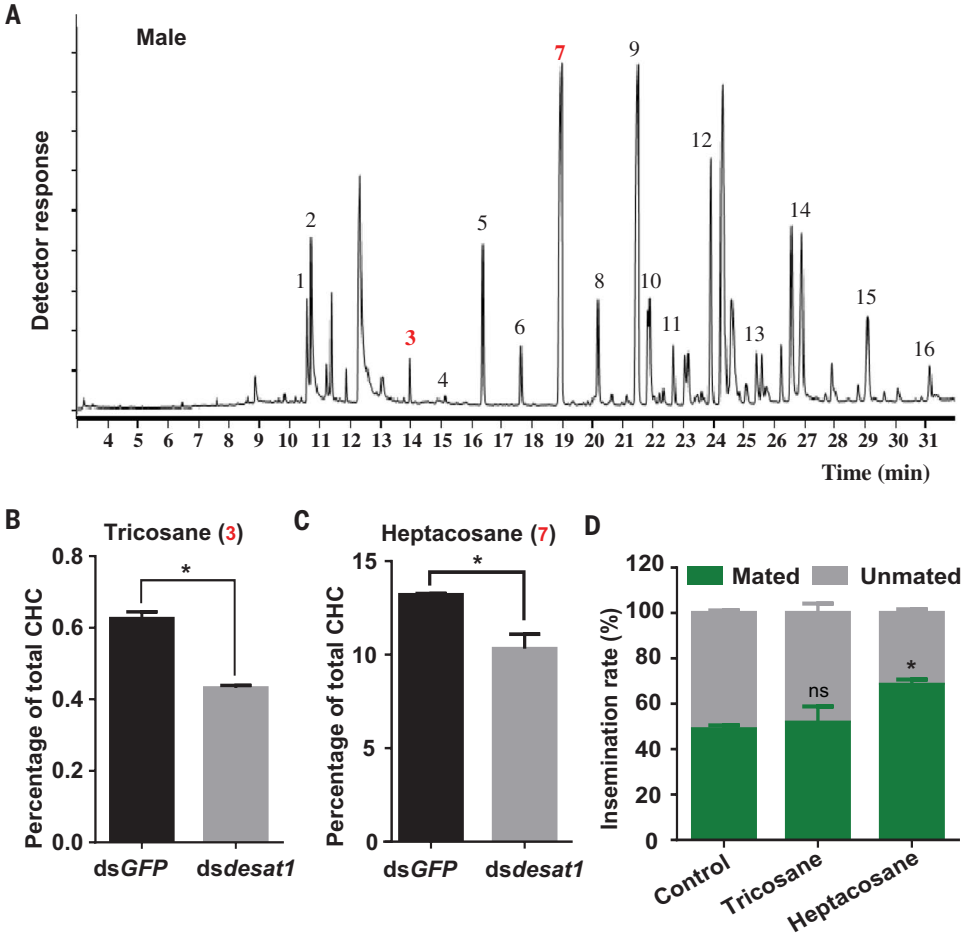

Fig. 4. Silencing of *desat1* changes the CHC profile of male mosquitoes. (**A**) Representative CHC profile of virgin male *An. stephensi* mosquitoes. Numbers above the peaks correspond to peak numbers given in table S2. (**B** and **C**) Comparison of CHCs between mosquitoes injected with ds*GFP* and ds*desat1*. Silencing of *desat1* resulted in a significant reduction of the relative percentage of tricosane (peak 3) (B) and heptacosane (peak 7) (C). (**D**) Effect of the CHCs tricosane and heptacosane on mosquito mating activity. Perfuming *Anopheles* virgin males with tricosane did not alter mating activity, whereas perfuming males with heptacosane significantly increased female insemination. Error bars indicate SEMs. *$P < 0.05$ (Student's *t* test).

(Fig. 3, C and D), which indicates that *desat1* is a clock-controlled gene.

We next investigated whether temperature and light affect the expression of *desat1*. We found that *desat1* transcript rhythm in the *An. stephensi* male heads is not affected by changes in ambient temperature (fig. S8), but under prolonged light exposure, *desat1* transcript abundance decreases significantly (Fig. 3F). These results indicate that light exposure in the evening inhibits *desat1* expression, which may be related to the inhibition of mosquito mating by light (Fig. 2B).

Many dipterans use cuticular hydrocarbons (CHCs) as sex pheromones for species and sex recognition and courtship (*23*–*25*), but little is known about the possible role of CHCs in mosquito mating and chemical communication. In *Drosophila*, *desat1* regulates the biosynthesis of CHCs (*26*), several of which function as sex pheromones (*27*). To examine whether *desat1* regulates the biosynthesis of *Anopheles* CHCs, we determined the hydrocarbon profile by gas chromatography of cuticular extracts of virgin male *An. stephensi* whole body. Sixteen major CHCs were identified (Fig. 4A and table S2). Silencing of *desat1* resulted in significant reduction of tricosane and heptacosane representation (Fig. 4, B and C), which indicates that *desat1*, a circadian regulated gene, is involved in the production of *Anopheles* CHCs. We next examined the role of tricosane and heptacosane in *Anopheles* mating activity and found that perfuming *Anopheles* males with tricosane did not alter mating activity, whereas perfuming males with heptacosane lead to a significant increase in the rate of female insemination (Fig. 4D and fig. S9). These results suggest that heptacosane enhances the interaction between males and courting females.

Understanding mosquito mating biology is central to any successful genetic-control strategies. Our transcriptome analysis showed enrichment of up-regulated metabolism and circadian clock genes and down-regulated immune genes in swarming mosquitoes, as swarming is an energy-consuming process accompanied by increased metabolic activity that occurs at dusk.

The circadian clock is cell-autonomous and consists of a series of interlocking transcriptional feedback loops requiring *per* and *tim*. Our work shows that these core clock genes are markedly up-regulated in swarming male mosquitoes compared with nonswarming males, and knocking down their expression affects male swarming (lowering swarm height and size) and mating in the laboratory and in semifield conditions.

Light has been shown to influence mosquito blood feeding, gene expression, fecundity, and reproductive barrier (*14*, *28*–*33*). Our study shows that adverse temperature (lower or higher) and increased light exposure during dark periods inhibit mating activity and influence the expression of *per* and *tim*, which indicates that the two factors interact to regulate the timing of male swarming. In *Drosophila*, temperature regulates the levels and activity of PER and TIM by modulating the genes' splicing patterns (*34*, *35*). Similarly, our study shows that the *per* and *tim* transcript levels in mosquitoes are affected by ambient temperatures. Therefore, light and temperature together entrain the circadian clock, influencing the programming of mosquito swarming and mating (fig. S10).

In many dipteran species, sex pheromones—together with visual, tactile, and acoustic cues—play important roles in courtship behavior preceding mating (*36*–*38*). CHCs act as sex pheromones in mate recognition and chemical communication for many insect genera (*23*, *27*). In *Drosophila*, these CHC pheromones are synthesized by *desat1* in secretory cells

called oenocytes in the abdominal epidermis, and they are then deposited on the cuticle (*23*, *26*). No *Anopheles* pheromone has been identified (*39*). Notably, we found in mating bioassays that perfuming virgin males with the C27 linear heptacosane promotes mosquito mating activity. Heptacosane is known to be a contact sex pheromone that facilitates mating activity of the tea weevil *Myllocerinus aurolineatus* (*40*). We found that *desat1* is a clock-controlled gene in mosquitoes and that light inhibits its transcription in the evening, reducing the production of CHCs and mating activity. A previous study has shown that the abundance of CHCs, including heptacosane (nC27), in male *Drosophila* varies in response to light and time of day (*41*), which indicates that male CHC profiles are a dynamic trait.

This work provides mechanistic insights on the molecular, chemical, and environmental factors regulating *Anopheles* swarming and mating behaviors. It contributes to a better understanding of mating mechanisms, which might lead to vector control strategies that target insect reproductive behavior.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S10
Tables S1 to S3
References (*42*–*46*)
MDAR Reproducibility Checklist
Movies S1 to S3

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Guandong Wang, Joel Vega-Rodrguez, Abdoulaye Diabate, Jingnan Liu, Chunlai Cui, Charles Nignan, Ling Dong, Fang Li, Cheick Oumar Ouedrago, Abdoul Malik Bandaogo, Pgudwind Simon Sawadogo, Hamidou Maiga, Thiago Luiz Alves e Silva, Tales Vicari Pascini, Sibao Wang, and Marcelo Jacobs-Lorena

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Cuticular pheromone circadian regulation

Several species of anopheline mosquitoes are important malaria vectors in Africa. Male mosquitoes show species-specific swarming behaviors at certain times of the day to attract females for mating. Wang *et al.* found that transcriptional patterns of metabolic and immune function genes apparently showed a diurnal rhythm that correlated with the physiological demands of mating flight swarming (see the Perspective by Manoukis). By altering temperature and light regimes and by knocking out the master genes *period* and *timeless* for circadian clock regulation, the authors disrupted mating flight behavior in a combination of cage experiments and enclosed field conditions. Knocking out the rhythmically expressed desaturase enzyme reduced cuticular hydrocarbon pheromone production and limited mating success. These key interacting components of the diurnal regulation of mosquito mating behavior are potential targets for alternative malaria control strategies.

Science, this issue p. 411; see also p. 340