Impact of disabled circadian clock on yellow fever mosquito Aedes aegypti fitness and behaviors

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Like other insects, Aedes aegypti displays strong daily patterns in host seeking and mating. Much of these behaviors are believed to be under the control of a circadian clock, an endogenous timekeeping mechanism relying on transcriptional/translational negative feedback loops that drive rhythmic physiology and behavior. To examine the connection between the circadian clock and various Ae. aegypti behaviors, we knocked out the core clock gene cycle using CRISPR/Cas9. We found that the rhythmic pattern and intensity of mRNA expression of seven circadian genes, including AeCyc−/−, were altered across the day/night cycle as well as in constant darkness conditions. We further show that the mutant CYC protein is incapable of forming a dimer with CLK to stimulate per expression and that the endogenous clock is disabled in AeCyc−/− mosquitoes. AeCyc−/− do not display the bimodal locomotor activity pattern of wild type, have a significantly reduced response to host odor, reduced egg hatching rates, delayed embryonic development and reduced adult survival and mating success. Surprisingly however, the propensity to blood feed in AeCyc−/− females is significantly higher than in wildtype females. Together with other recent work on the circadian clock control of key aspects of mosquito biology, our data on how cycle KO affects mosquito behavior and fitness provides a basis for further work into the pathways that connect the mosquito endogenous clock to its vector competence.

Eukaryotic organisms have endogenous 24-h internal circadian clocks that assist them in optimizing their physiology and behavior to daily fluctuations in light, temperature, and resource availability1. This has allowed many organisms to adapt to a temporal niche, displaying certain behaviors only during specific times of the light–dark (LD) cycle. This is true for mosquito disease vectors, in which circadian rhythms in behavior and gene expression is well-documented2–8. Aedes aegypti, the primary vector of numerous emerging vector-borne diseases including Yellow Fever, Dengue, Chikungunya, and more recently Zika virus8 displays a small activity peak at the start of the light phase but is primarily active during the late afternoon hours10. The diurnal activity patterns of Aedes aegypti are found in flight activity, oviposition, host-seeking, and human landing/biting11. The endogenous circadian clock of mosquitoes regulates locomotor activity and blood feeding behavior11–13, and is expected to regulate the timing of other behaviors such as host-seeking, mating and oviposition, although this has not been specifically tested14.

The mosquito circadian clock, which is entrained by the light:dark cycle, relies on two interlocked transcriptional/translational negative feedback loops that cycle every 24 h14. The core circadian genes at the center of this feedback loop are cycle, clock, period, timeless, pdp1, and cryptochrome-29,15,16. In some insects, e.g. the monarch butterfly, cycle is now referred to as bmal117. This is because in these species, as in mosquitoes, cycle retains a C-terminal transactivation domain that is present in the mammalian orthologue bmal1 but is missing in the Drosophila orthologue cycle. Here, we refer to Aedes aegypti cycle (AeCyc) for consistency with the current genome annotation15. In addition to sustaining 24-h rhythms, the circadian clock also regulates the rhythmic expression of a broad range of genes that drive circadian behaviors19–22. In Anopheles gambiae it was demonstrated that circadian dependent modulation of olfactory responses significantly influences the distinct behavioral responses in mosquitoes23.

However, much less is known about how molecular clock disruption affects critical sensory and motor systems in mosquito disease vectors14. RNAi-mediated knockdown of timeless in Ae. aegypti caused a decrease in locomotor activity and an increase in the free-running period13, but no other behavioral assays were performed in this study. In Anopheles gambiae, knocking down timeless or cryptochrome 1 using RNAi increases blood feeding propensity12. Furthermore, the expression of these genes was affected by short light pulses12. In Ae. aegypti blood
feeding expression of the four critical clock genes clock, cycle, timeless, and period\(^3\). Female mosquitoes stop responding to host cues after blood-feeding, and it has been hypothesized that suppression of endogenous clock genes is the mechanism through which this is achieved\(^4\). These studies demonstrate a connection between circadian clock genes and blood feeding behavior. Whether this is the case for other mosquito behaviors has not been established.

Given the pervasive regulation of Drosophila behaviors by the circadian clock and the conserved nature of the circadian clock between flies and mosquitoes\(^4\), a functional circadian clock is likely critical to the regulation of many mosquito behaviors as well. Studying genes controlling host-seeking and other behaviors is important, as they may be potential targets for future vector control\(^5\). To explore the connection between the endogenous clock and various mosquito behaviors, we used CRISPR/Cas9-mediated gene knockout of AeCycle to disable the endogenous circadian clock in Aedes aegypti. Disruption of the clock abolishes the characteristic circadian locomotor activity patterns and significantly reduces Ae. aegypti response to human host odor, mating success, but not blood feeding. Furthermore, it also delays larval development and reduces adult life span. These data show the pivotal role of the endogenous circadian clock in the biology of Ae. aegypti and opens the door to future work fully characterizing the function and role of the circadian clock in this important human disease vector.

Results

Generation of a AeCyc\(^{−/−}\) strain. CRISPR/Cas9-mediated targeted mutagenesis was used to generate a mutant bearing a 10 bp deletion in exon 5 of AeCyc (Fig. 1A,B). This deletion, in addition to causing a frame shift, resulted in a premature stop codon 30 bp upstream from the deletion. Any resulting protein would therefore include only the first 177 out of 767 CYCLE (CYC) amino acids, followed by 11 additional amino acids. Importantly, the truncated protein would contain the two helices necessary for DNA binding domain, but neither the transactivation domain, nor the two PAS domains that are thought to be responsible for dimerization with CLOCK. The F\(_1\) mutant of this strain was backcrossed to wildtype Aeegypti for four generations before a homozygous AeCyc\(^{−/−}\) strain was created.

Endogenous clock gene expression. Next, we examined the impact of Cyc KO on the relative expression and rhythmic appearance of seven essential circadian clock genes: AeCyc, AeClk, AePer, AeTim, AeCry1, AeCry2 and AePdp1 at 4 h intervals in light:dark conditions (ZT0 to ZT24). The mRNA expression study confirmed that the cyclical expression pattern of six clock genes is altered in AeCyc\(^{−/−}\), with the timing of peak expression shifted to different time points (Fig. 2A–G). Somewhat surprisingly, AeCyc\(^{−/−}\) mRNA was detected, and expression of this mutant gene did exhibit a cyclical expression pattern under LD conditions, with similar amplitude as in the wildtype strain but in antiphase. Interestingly, the cyclical expression pattern was similar between wildtype and AeCyc\(^{−/−}\) under DD conditions (Fig. 2A).

The other endogenous clock genes, with the exception of AeCry1, also showed a shift in the timing of peak expression under LD conditions (Fig. 2A–G). For AeCry1 the pattern was not very clear. Furthermore, while the expression of AeClk was enhanced in AeCyc\(^{−/−}\) under both LD and DD conditions, expression of AePer was much reduced in AeCyc\(^{−/−}\). In addition, AeCry1 and AePdp1 showed a marked peak in expression in both strains under DD conditions at ZT4 and ZT20, respectively. Overall, the changed cyclical expression pattern of these genes points towards a non-functional or disabled endogenous clock in AeCyc\(^{−/−}\).

Transcription assay. Because mutant AeCyc\(^{−/−}\) mRNA is expressed in AeCyc\(^{−/−}\) mosquitoes, we verified that AeCLK:AeCYC\(^{−/−}\) did not activate transcription in Drosophila schneider 2 (S2) cells co-expressed with monarch butterfly per E-boxes driving luciferase as a reporter, while AeCLK:AeCYC\(^{NT}\) does activate transcription (one-way ANOVA, p < 0.002) (Fig. 3). This is as expected if the truncated CYC protein was lacking the PAS domains necessary for dimerization with CLOCK. The control experiment also showed that AeCLK does not bind to endogenous Drosophila melanogaster DmCYC to activate luciferase transcription. These data indicate that AeCyc\(^{−/−}\) is non-functional, which should inactivate the endogenous circadian clock.

Circadian locomotor activity. To further confirm disruption of the circadian clock in AeCyc\(^{−/−}\), we compared circadian flight activity patterns between wildtype and AeCyc\(^{−/−}\) in a locomotor activity monitor. The movement of each mosquito was detected by the interruption of an infrared sensor on the monitor. Wildtype mosquitoes show the expected circadian activity pattern, with a small peak of activity at the start of the light phase (ZT0), and a pronounced activity peak towards the end of the light phase (ZT8-ZT12, Fig. 4A). During the dark phase (ZT12-ZT24) little activity was observed. During 72 h under DD conditions, the main activity peak between ZT8 and ZT12 was maintained, with an expected shift towards an earlier pattern of LD conditions (Fig. 4A). In contrast, AeCyc\(^{−/−}\) mosquitoes show constant activity throughout the light phase, with an overall significantly higher activity (74.5 beam breaks/hour on day 1 + 2) during this period compared to wildtype (41.6 beam breaks/hour on day 1 + 2, p < 0.001, Fig. 4B). Similarly to wildtype, AeCyc\(^{−/−}\) mosquitoes become largely inactive during the dark phase (5.4 beam breaks/hour, p = 0.731).

Importantly, in contrast to wildtype, AeCyc\(^{−/−}\) do not maintain a circadian locomotor activity pattern under DD conditions, showing low overall activity levels (10.8 beam breaks/hour) between ZT0 and ZT12 without any defined peak. Specifically, the level of activity between ZT0 to ZT12 is similar to that between ZT12 to ZT24 (10.6 beam breaks/hour). Furthermore, the level of activity displayed by AeCyc\(^{−/−}\) between ZT0 and ZT12 under DD conditions (10.8 beam breaks/hour) is significantly less than that of wildtype mosquitoes (28.4 beam breaks/hour, p < 0.0001). After restoring LD conditions following 72 h of DD conditions, both wildtype and AeCyc\(^{−/−}\) revert back to their respective LD activity patterns.
Life history parameters. The hatching rate for \( \text{AeCyc}^{-/-} \) was significantly lower than for wildtype \( \text{Ae. aegypti} \), \((0.55 \pm 0.18 \text{ vs. } 0.90 \pm 0.12, p = 0.0038, \text{Table 1})\). In addition, the average egg development time was significantly longer in \( \text{AeCyc}^{-/-} \) vs. wildtype eggs \((7.12 \pm 4.51 \text{ vs. } 2.03 \pm 0.04, t = -2.7663, df = 5.0006, p = 0.039)\).
Furthermore, fewer hatched larvae survived to the pupal stage in AeCyc−/− vs. WT (0.93 ± 0.06 vs. 0.99 ± 0.01, p = 0.038), although this difference was small compared to the hatching rates. In contrast to egg development, we found no significant difference between the larval development times between AeCyc−/− and wildtype (5.27 ± 0.13 vs. 5.23 ± 0.28, t = −0.30653, df = 7.217, p = 0.767).

The adult emergence rate did not differ significantly between AeCyc−/− and wildtype Ae. aegypti (t = 0.8584, df = 10.008, p = 0.4108), but the survivorship of male and female AeCyc−/− adults was significantly lower than that of wildtype over a 30-day period (Fig. 5). Male AeCyc−/− were nearly twice as likely to die during this period than wildtype males (Hazard Ratio: 1.95 [1.28, 2.95], p < 0.001). Female AeCyc−/− had a significant similar reduction in survivorship (Hazard Ratio: 1.821 [1.346, 2.465], p = 0.00171).

**Mating success.** The mating success of AeCyc−/− and wildtype Ae. aegypti was assessed by determining insemination rates of five-day old virgin mosquitoes. Mating success was significantly lower in AeCyc−/− mosquitoes than in wildtype after 1 h of female-to-male exposure (42.0% vs 68.7%, p < 0.005, Fig. 6A). The insemination rate was not significant lower in AeCyc−/− females in the 24 h, 48 h and 72 h exposure experiments (Fig. 6B–D). Interestingly, the insemination rate following 1 h of male exposure was significantly lower in both reciprocal crosses than in wildtype crosses (48.0% vs 68.7%, p = 0.003 for ♀cyc−/−/♂wt; and 38.7% vs 68.7%, p = 0.002 for ♀wt/♂cyc−/−). No significant differences were observed after 24 h, 48 h or 72 h exposure. We also did not observe any differences between the two reciprocal crosses. Furthermore, insemination rates increased significantly in both AeCyc−/− and wildtype matings as time of exposure to males increased from 24 to 72 h (Huynh–Feldt: df = 2, F = 19.43, p = 0.001). Finally, although we did not quantify this measure, we note that AeCyc−/− females appeared to store less sperm, most of the sperm was only in one or two of the three spermathecal capsules.

**Attraction to human odor.** Next, we compared the attraction of AeCyc−/− and wildtype females to human odor (white worn socks) in a dual choice olfactometer assay at three different time points, ZT6, ZT12 and ZT18. ZT6 is in the middle of the light phase when wildtype females show low activity levels, ZT12 is just prior to the transition to the dark phase, when wildtype females are highly active, and ZT18 is in the middle of the dark phase. The sock was placed in one of the two collecting chambers connected to the odor ports in olfactometer, and the other collecting chamber was left empty.
Both AeCyc−/− and wildtype females show some response to host odor at each of the three time points (Fig. 4C). Interestingly, AeCyc−/− females are significantly less attracted to human odor than wildtype females at ZT12 and ZT18 (p = 0.001 and p = 0.022, respectively, Fig. 4C). These p-values remain significant following Holms-Bonferroni correction. AeCyc−/− females’ odor response was also lower at ZT6, but this difference was not significant. Furthermore, wildtype females are significantly more attracted to human odor at ZT12 than at ZT18 (p = 0.013). AeCyc−/− females appear to respond more to human odor at ZT6 than at ZT12 and ZT18 but the difference was not significant following Holms-Bonferroni correction.

Activation in olfactometer. To examine if differences in response to human odor can be explained by different activity levels of AeCyc−/− and wildtype females, we also recorded the proportion of females leaving the release cage of olfactometer (= “activation”). During ZT6 and ZT12 the activation rate of AeCyc−/− females was similar, with slightly higher activation in AeCyc−/− females (p > 0.005, Fig. 4D). Therefore, the lower odor response of AeCyc−/− females at ZT6 and ZT12 is not due to a lower activation rate. At ZT18, the activation rate was slightly smaller in AeCyc−/− females (Fig. 4D, p = 0.049), a difference that was not significant after Holms-Bonferroni correction. Not surprisingly, activity of both wildtype and AeCyc−/− females was significantly higher during the light phase (ZT6 and ZT12) compared to the dark phase (ZT18) (p < 0.005).

Blood feeding activity. To study the impact of cycle KO on blood feeding, we performed a time course analysis of blood feeding propensity during the LD cycle. The blood feeding propensity of AeCyc−/− females peaked during daytime compared to night and somewhat surprisingly, was significantly (p > 0.005) higher than that of wildtype females at ZT1, ZT5, ZT13 and ZT17 (Fig. 7, Table S3). At ZT9 however, when both have the highest feeding propensity, the difference was not significant (p = 0.10), although the trend was the same (AeCyc−/−: 75% vs. WT: 64%).

**Figure 3.** The mutant AeCyc lacking the PAS and transactivation domains is impaired in transcriptional activation in S2 cells. The monarch per E box luciferase reporter (dpPerEP_Luc; 10 ng) was used in presence (+) or absence (−) of wildtype AeClk, AeCycWT, AeCyc−/− expression plasmids (5 ng each). Firefly luciferase activity was computed relative to renilla luciferase activity. Each value is mean ± SEM of three replicates. One-way ANOVA, AeCLK:AeCYC−/− vs. AeCLK:AeCYCWT, *p ≤ 0.002.
Figure 4. (A, B) Circadian activity patterns in wildtype and AeCyc−/− mutants in both LD and DD conditions. (C) Attraction of wildtype and AeCyc−/− females to human odor in olfactometer. Proportion attracted = the proportion of mosquitoes in the arm of the olfactometer that contained the sock. (D) Comparing activation of wildtype and AeCyc−/− mutants in olfactometer. *activation* = the proportion of females leaving the release cage of olfactometer. *Statistically significant p ≤ 0.05.

Table 1. Comparison of life history traits between AeCyc−/− and wildtype Ae. aegypti mosquitoes. *Statistically significant p ≤ 0.05.
Discussion

Knocking out the cycle gene in Aedes aegypti disables its endogenous circadian clock, resulting in locomotor activity that is stimulated solely by the presence of light but that does not show the typical morning and evening peaks displayed by wildtypes. When light is present AeCyc−/− are indeed active throughout the light phase but revert to inactivity in darkness. Somewhat surprisingly, AeCyc−/− mRNA was detected in AeCyc−/− heads and did show a cyclical expression pattern. AeCyc−/− contains a premature stop codon and often such transcripts are removed through the nonsense mediated decay pathway (NMD), a process by which aberrant mRNAs that contain a premature stop codon are degraded. However, we demonstrated that if any AeCYC−/− were produced, it is incapable of forming a functional heterodimer with CLK. In Drosophila melanogaster, the CYC/CLK heterodimer is an essential component of the core circadian feedback loop that activates the transcription of both per and tim. Consistent with a recent finding that PER protein levels are much reduced in circadian neuronal circuits of Ae. aegypti whose circadian clock is disrupted by exposure to constant light conditions, we did find a much reduced expression of per under both LD and DD conditions in heads of AeCyc−/−.

However, the expression level of tim was similar between AeCyc−/− and wildtype but showed a different cyclical pattern. Furthermore, a study in Drosophila indicated that the low PER and TIM levels may be due to a lack of CYC:CLK heterodimer transcriptional regulators in the homozygous Cycle KO mutant strain.

The cyclical expression of other essential endogenous clock genes was disrupted in AeCyc−/− females as well. Clk expression was higher in AeCyc−/− compared to wildtype under both LD and DD conditions. In contrast, the expression of Cry1, which is sensitive to light and inhibits the formation of PER/TIM heterodimers, was similar between AeCyc−/− and wildtype under LD and DD conditions. A considerable portion of the transcriptome shows circadian expression patterns. Although the direct link between the expression of these and the essential clock genes is not clear, it is expected that disrupting the cyclical expression of endogenous clock genes would impact the expression of a variety of clock-controlled genes.

Studies in insects including Drosophila reported that circadian clock regulates the duration of preadult development. Several earlier studies have shown that pupation is gated in mosquitoes such as Anopheles gambiae and Aedes taeniorhynchus, and is regulated by the duration of the photoperiod via the secretion of the prothoracotrophic hormone that stimulates ecdysone secretion. Also, study showed that importance of the light cycle and the period gene in developmental time memory specifically pupation and adult emergence in fruit flies D. melanogaster is under circadian clock control. The environmental cycle and period allele both determines the time taken for each pre-adult developmental stages such as time taken for pupation. The significantly longer hatching time and lower hatching rates of AeCyc−/− indicates that this gene is involved in the proper timing and success of embryogenesis. The lower hatching rate is unlikely to be explained by the slightly lower insemination

Figure 5. Survivorship of AeCyc−/− and wildtype Ae. aegypti males and females. *Statistically significant p ≤ 0.05.
rate of \( \text{AeCyc}^{-/-} \). In addition, the pupation rate, and adult emergence is significantly reduced, although much less drastically than the hatching rate. The disruption of the circadian clock also impacts basic fitness parameters in Drosophila. Mutants lacking a functional clock have reduced egg production, as well as a reduced hatching rate\(^7\). Also, in immature stages of Drosophila (egg, larvae, pupae), null mutations in \( \text{period} \) increase development time under normal light/dark conditions\(^38\). Together with previous findings in Drosophila, where \( \text{period} \) mutants under prolonged or shortened circadian cycles have different development times from eggs to adults\(^39\). We also

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**Figure 6.** Insemination status of \( \text{AeCyc}^{-/-} \) (♂ & ♀) and wildtype (♂ & ♀) (Nonreciprocal) crossing, and reciprocal crossing groups \( \text{AeCyc}^{-/-} \)♂ and WT♀; \( \text{AeCyc}^{-/-} \)♀ and WT♂ mosquitoes after 1 h (A), 24 h (B), 48 h (C) and 72 h (D) of exposure to males for mating (copulation duration). Each value is mean ± SD of three replicates. *Statistically significant p ≤ 0.05.

**Figure 7.** \( \text{AeCyc}^{-/-} \) and wildtype \( \text{Ae. aegypti} \) blood-feeding behavior. Mosquitoes were allowed to feed for 20 min and a total percentage of blood fed mosquitoes (includes partially and fully fed) in each time point was scored and plotted. Error bars indicate the standard error. *Statistically significant p ≤ 0.05.
found that adult survival across a 30-day period (days 5 through 35) was reduced significantly, indicating that other developmental periods are also affected by the absence of a functional AeCyc as well, although to a lesser degree. Furthermore, cycle KO mutants in Drosophila also show a decrease in survivorship. Surprisingly however, this decrease in fitness was observed only in males and the sex-specific mechanisms driving this phenotypic difference is not understood. This is in contrast to our observation that both males and females AeCyc−/− have reduced survivorship. A potential explanation could be the difference in chromosomal sex determination between Drosophila and mosquitoes that Drosophila have heterogametic sex chromosomes, whereas Ae. aegypti do not.

Importantly, similar cycle or clock knockouts in D. melanogaster resulting in arrhythmic, infrequent locomotor activity14–43, AeCyc−/− also display several behavioral deficiencies compared to wildtype mosquitoes. AeCyc−/− are active throughout the photophase, without showing the bimodal activity pattern typical of wildtype, and become inactive under dark conditions, even when these are imposed for an extended period. This further demonstrates that AeCyc−/− no longer have a functioning endogenous circadian clock. While AeCyc−/− show activity throughout the light phase, their activity level is highest at the start and declines slowly until this phase ends. Possibly this decline is related to diminishing energy reserves across this period of high activity. This activity pattern indicates that light provides a strong stimulus and that the mosquito’s response to this stimulus is strongly modulated by the clock. This is consistent with previous work which showed that Ae. taeniorhynchus exposed to constant LL conditions, and whose clock therefore has been disrupted, display irregular excessive outburst of activity44. Previous work also showed that the temporary RNAi-mediated knockdown of timeless in Ae. aegypti, one of the central clock genes, caused a temporary decrease in regular diurnal peak activity patterns45. Although this RNAi approach had only a transient effect, results appear to be consistent with our observation.

An additional important behavioral deficiency of AeCyc−/− females is the 65% reduction in their response to human odor during peak activity hours, despite the fact that both were activated to the same degree in the olfactometer. Olfaction is essential for many behaviors in mosquitoes (e.g., blood and sugar feeding, mating, and oviposition) and is itself under circadian control in insects42,45. In Ae. aegypti, antennal sensitivity to some host odors is highest towards the end of the light phase, although for others sensitivity peaks during midday46. In An. gambiae, odorant binding proteins are thought to be involved in modulating temporal changes in odorant sensitivity, enabling the olfactory system to dictate the circadian niche5.

Odor responses in Drosophila antennae are controlled autonomously by circadian clocks present within olfactory sensory neurons (OSNs)42,47. GPRK2 has been identified as a key regulator of olfactory receptor (OR) function and olfactory responses, and GPRK2 levels are under the control of circadian molecular pacemakers located within the OSNs44,45. Several olfaction genes in Aedes and Anopheles show circadian expression patterns, indicating that they may be under control of the endogenous circadian clock5,46. Also, a study in An. gambiae revealed that circadian dependent expression of antennal proteins involved in modulating of temporal changes in odorant sensitivity23. Thus, disrupting the circadian clock should alter the expression of olfactory genes, resulting in impaired odor response. Olfactory responses are likely modulated both by peripheral and centralized processes46, and whether the reduced odor response observed here is due to a reduced antennal response or due to higher order processes in the cerebral ganglion remains to be determined. Future studies using RNAseq and electroantennogram analyses will be necessary to answer this question.

In addition to having a reduced response to host odor and impaired locomotor activity, AeCyc−/− also show a lower mating efficiency when given a short mating opportunity time (i.e. within an hour), which likely reflects their mating opportunity in the wild. Under these conditions, the insemination rate of females in crosses involving both AeCyc−/− males and females appeared to store lower quantities of sperm, which could suggest that either the duration of copulation was reduced or that males produce less sperm. Given that when females were allowed to mate by the clock that light provides a strong stimulus and that the mosquito’s response to this stimulus is strongly modulated by the clock. This is consistent with previous work which showed that Ae. taeniorhynchus exposed to constant LL conditions, and whose clock therefore has been disrupted, display irregular excessive outburst of activity44. Previous work also showed that the temporary RNAi-mediated knockdown of timeless in Ae. aegypti, one of the central clock genes, caused a temporary decrease in regular diurnal peak activity patterns45. Although this RNAi approach had only a transient effect, results appear to be consistent with our observation.

Interestingly, AeCyc−/− females and males contributed equally to the reduction in the insemination rate, but their effects were not additive. That is, crosses in which only one of the sexes were AeCyc−/− had a similar insemination rate as crosses in which both males and females belonged to AeCyc−/−. In addition, we noticed that AeCyc−/− females appeared to store lower quantities of sperm, which could suggest that either the duration of copulation was reduced or that males produce less sperm. Given that when females were allowed to mate for an extended period (i.e. 24 h, 48 h, and 72 h), no difference in insemination rate was observed between AeCyc−/− and wildtype, the lower sperm storage phenotype we observed in AeCyc−/− within an hour could be due to a delayed start in sperm production. However, AeCyc−/− mutant females appeared to store relatively less sperm, predominantly only in one or two spermaticheal capsules compared to wildtype females in the extended mating experiments as well. This could explain the reduced hatching rates and lowered fitness.

Recent work in Anopheles demonstrated the direct connection between the endogenous circadian clock and mating behavior48. Knockdown of both per and tim in An. gambiae and An. stephensi reduces swarming behavior and insemination rates in the lab and under semi-field conditions. Furthermore, per and tim expression was shown to be higher in swarming vs non-swarming field-collected mosquitoes48. Per and tim also control the circadian rhythms of female mating activity in Drosophila melanogaster49. Moreover, gene transfer experiments implicate per in the species-specific behaviors of locomotor activity, love song rhythms, and time of mating50. An. stephensi swarming behavior is strongest when per and tim expression peaks49, which is not the case in Aedes aegypti, as shown in this study. While per expression is much reduced overall in AeCyc−/− compared to wildtype, at least in female heads, tim expression is actually increased during the time the one-hour experiment was conducted. It is therefore not clear if the difference in expression level of these two genes could be responsible for the reduced mating success we observed. Another explanation might lie in the observation that mosquito antennae possess autonomous circadian clocks that could control circadian rhythms of olfactory response42. Because it has been suggested that Aedes aegypti males produce volatiles that attract females51, the sensitivity of AeCyc−/− antennae to these compounds could be affected as well.
Finally, while the response to host odors and mating efficiency were reduced, the blood-feeding propensity of the AeCyc−/− females was significantly higher than that of wildtype females throughout much of the day. The only exception was around ZT9 when the difference was not significant. Wildtype Aedes aegypti are at their most active at this time and their blood feeding propensity was highest at this point as well. Previously, it was shown that knockdown of tim expression reduces blood-feeding behavior in Aedes aegypti, which seemed to suggest that deregulating the circadian clock reduces blood feeding propensity. This is in contrast to our observation. However, RNAi silencing of tim, cry1 and clk in An. gambiae also increased blood feeding propensity. As with the other behaviors it is not clear through which pathways the circadian clock controls blood feeding in mosquitoes, although a several other genes that impact blood feeding, such as odorant binding proteins, takeout, and others have been shown to be under circadian control.

Conclusions

Here we demonstrated the effect of disabling the endogenous circadian clock by knocking out the cycle gene on developmental processes, lifespan and essential behaviors in Aedes aegypti. Cycle knockout alter the cyclical expression patterns of several clock genes, with the most effect on per expression that becomes completely arrhythmic. Interestingly, AeCyc−/− mutants maintain a diel activity throughout the light phase, but this too is strongly affected, as the characteristic bimodal activity peaks early and late during the light phase seen in wildtype are lost in AeCyc−/− mutants. The largest impact of cycle knock out on life history traits is on embryonic development, as AeCyc−/− have a much delayed hatching rate. Not surprisingly, various circadian behaviors were impacted by cycle KO as well. AeCyc−/− show a reduced response to host odors, reduced mating success, but an increase in blood feeding propensity. Together with other recent work on the circadian clock of mosquitoes, this work contributes to elucidate the pathways through which the circadian clock controls mosquito behavior. Future studies aiming at understanding the potential impact of cycle and other clock genes KO on metabolism, insecticide susceptibility and vector competence, could provide important insights on the biology of this important disease vector to ultimately deploy potential control strategies that takes into account time-of-day parameters.

Methods

All experiments methods were performed according to relevant guidelines and regulations for animal use and laboratory practices, including environmental health, occupational safety, and biosafety. All studies and facilities were approved by the Institutional Biosafety Committee (IBCG# 2018-029) of Texas A&M University.

Mosquito rearing. Aedes aegypti (Liverpool strain) were maintained at 27 °C, 60–70% relative humidity (RH) on a 12:12 h light/dark cycle (this includes 1 h dawn and 1 h dusk transitions). Eggs were hatched in deionized water, and larvae were fed ground Tetramin® fish food daily. Adults were provided with cotton balls soaked in a 10% sucrose solution. Colony mosquitoes were fed once a week on defibrinated calf or sheep blood (Hemostat Laboratories) fed through an artificial membrane feeder.

Ae. aegypti cycle KO mutant generation. Six sgRNAs targeting exons 3 and 5 of cycle were designed using CHOPCHOPv2. These sgRNAs were generated by in vitro transcription following Bassett et al. sgRNA cutting efficiency was tested in vitro on purified PCR product of the cycle target region using the Cas9 manufacturer’s protocol (PNA Bio). An sgRNA for targeting sequence TCAGTTACACAGGCGCAACTACGGC C in exon 5 (AAEL002049-RD) was selected for further injection. An injection solution was prepared by combining 100 ng/µL of sgRNA and 200 ng/µL of Cas9 in RNase-free water. This mixture was incubated at 37 °C for 20 min, and centrifuged at 4 °C for 30 min at 14,000×g. The injection mixture was kept on ice protected from direct light until the injection.

To collect embryos for injection, adult female Ae. aegypti were blood-fed defibrinated calf blood and maintained in the insectary without access to egg paper. After four days, ~ 30 adult female Ae. aegypti were placed into a 50 mL conical tube containing a wet cotton ball and a disc of wet filter paper and placed in the dark for 25 min to lay eggs. Freshly laid embryos were transferred to a clean wet filter paper and aligned with a paintbrush. Aligned embryos were transferred to coverslip by double-sided masking tape and covered in emersion oil for injection.

Needles were fabricated from borosilicate capillaries (1 mm × 0.5 mm × 10 cm) using the P-1000 Pipette Fuller and beveled using the BV-10 Micropipette Beveler (Sutter Instrument). Injections were performed using a Pneumatic Picopump (World Precision Instruments) and visualized with the DinoXcope (Dunwell Technologies Inc.). Following injection, embryos were rinsed with distilled water and kept on wet filter paper for four days in the insectary before being placed in a water basin for hatching. Surviving larvae were raised to adulthood as previously described.

DNA from surviving adults was extracted using the NucleoSpin Tissue Kit (Macherey–Nagel) from a single leg using the Phire Animal Tissue Direct PCR Kit (Thermo-Fisher Scientific). PCR was conducted on extracted DNA using GoTaq® Flexi PCR Kit (Promega) or the Phire Animal Tissue Direct PCR Kit (Thermo-Fisher Scientific) using a fluorescent forward primer for fragment analysis. Fragment analysis was conduct on a 3500 Genetic Analyzer (Applied Biosystems) with the GeneScan™ 500 LIZ™ size standard (Thermo-Fisher Scientific) using a fluorescent forward primer for fragment analysis. Fragment analysis was conduct on a 3500 Genetic Analyzer (Applied Biosystems) with the GeneScan™ 500 LIZ™ size standard (Thermo-Fisher Scientific) and analyzed in Geneious (Biomatters) to detect indels at the CRISPR/Cas9 target site. Confirmed mutants were outcrossed with wildtype mates for four generations before heterozygous individuals were inter-mated to generate the homozygous AeCyc−/− knockout line.

Endogenous clock gene expression. The expression of seven essential clock genes, cycle (AeCyc), clock (AeClk), period (AePer), timeless (AeTim), cryptochrome-1 (AeCry1), cryptochrome-2 (AeCry2) and par domain
protein 1 (AePdp1), was examined by qRT-PCR from heads of AeCyc−/− and wildtype mosquitoes collected at four-hour time intervals across a single light/dark (LD) cycle, as well as across 24 h of darkness (DD) after entrainment to LD cycles (Table S1). LD and DD cycles both are recorded as Zeitgeber Time (ZT), in LD cycle particularly ZT0 being defined as the end of the 1 h dawn transition and the beginning of the full light cycle, and ZT12 defined as the time of lights off at the end of the dusk transition. mRNA was extracted from 10 female heads per replicate using the Dynabeads® mRNA DIRECTTTM Micro Purification Kit (Thermo Fisher Scientific). RNA quantity and quality were assessed on a NanoDrop spectrophotometer, and RNA Pico LabChip runs on an Agilent BioAnalyzer 2100. qRT-PCR of AeCyc RNA quantity and quality were assessed on a NanoDrop spectrophotometer, and RNA Pico LabChip runs on an Agilent BioAnalyzer 2100. qRT-PCR of AeCyc, AePer, AeTim, AeCry1, AeCry2 and AePdp1 were performed on 3 replicates using SYBR Green One-Step Real-Time RT-PCR (Thermo Fisher Scientific) on the Bio-Rad CFX96 Real-Time System. Normalization of genes calculated relative to ribosomal protein S6, which has previously shown to have constitutive expression across the light/dark cycles in Ae. aegypti58.

Luciferase transcriptional assay. The pGL-dpPer4Ep and pAC5.1V5/HisA control plasmids were provided by Zhu et al.38 and pAC-RENilla-Luc control plasmid was provided by McDonald et al.56. pAC plasmids containing wildtype AeCyc, AeCycWT, and AeCyc−/−, were generated by PCR amplification from cDNA generated from the wildtype or mutant AeCyc mosquito lines that were subsequently subcloned into pAC5.1V5/HisA. Wildtype AeCyc was subcloned between Kpn1 and Xho1, wildtype AeCyc was subcloned between EcoRI and Xbal, and the mutant AeCyc was subcloned between EcoRI and Xho1. Details of primers used for the cloning are provided in Table S2.

S2 cells were maintained at 25 °C in Schneider's Drosophila medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco) and 100 U/mL penicillin and streptomycin (Gibco). Transient transfections were performed as previously described39,40 in 12-well plates using 10 ng/well of dpPer4Ep-Luc as a reporter and 30 ng/well pAC-RENilla-Luc as a normalization control. S2 cells were co-transfected with 5 ng/well of pAC plasmids expressing wildtype AeCyc, wildtype AeCyc and/or the mutant AeCyc. For luciferase assay, cells were lysed with 50 μl of 1X Passive lysis buffer (Promega). Firefly and renilla luciferase activities were quantified with a Dual-Luciferase reporter assay system (Promega) using 5 μl of cell protein lysate on a VICTOR3 V Multilabel Plate Counter (PerkinElmer). Firefly luciferase activity was computed relative to renilla luciferase activity.

Locomotor activity assay. Males and females were raised together for 5 days and then briefly knocked down by cooling at 4 °C and individually placed into glass vials. One end of the glass vial was blocked with a cotton ball and the second end of the glass vial was blocked with a plastic tube containing 10% sugar water and a cotton ball. The activity monitor was placed inside an incubator with an DEnM Environmental Monitor (Trikinetics) kept at 27 °C and 60–70% relative humidity. Locomotor activity of wildtype and AeCyc−/− mosquitoes that were subsequently subcloned into pAC5.1V5/HisA. Wildtype AeCyc was subcloned between Kpn1 and Xho1, wildtype AeCyc was subcloned between EcoRI and Xbal, and the mutant AeCyc was subcloned between EcoRI and Xho1. Details of primers used for the cloning are provided in Table S2.

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Measurement of life history parameters. Egg hatching. Mosquito eggs were collected from 25 five-days old wildtype and AeCyc−/− females they were mated with respective genotype males. Freshly laid eggs were collected, and 25 eggs were randomly chosen and placed in water containing fish food for hatching. This was performed for six replicates. Newly hatched larvae were counted for 30 days.

Pupation rate. Six replicates of 25 freshly hatched first instar wildtype and AeCyc−/− larvae (L1) were collected and placed into a small container with 25 mL of water containing fish food ad libitum. The number of pupae was recorded daily.

Adult emergence. Six replicates of 25 new wildtype and AeCyc−/− pupae were collected from wildtype and AeCyc−/− pans. Each replicate was placed into a small plastic cup with water in an adult cage. The number of emerged adults was recorded daily.

Longevity/survivorship. For each wildtype and AeCyc−/− genotype, males and females were raised together for 5 days and allowed to mate. Mosquitoes were then briefly knocked down by cooling at 4 °C and five replicates of 30 adult male or female wildtype or AeCyc−/− mutants were placed into adult bins, and survivorship was recorded for 30 days.

Mating success. Males and females were separated during the pupal phase. Three replicates of 50 five-days old adult females and males were placed in 30 × 30 × 30 cm cages and allowed to mate for 1 h, 24 h, 48 h, and 72 h. The 1 h experiment was conducted towards the end of the light phase, i.e., during the peak in locomotor activity of wildtype Ae. aegypti. Insemination experiments were performed both within wildtype and AeCyc−/− lines, as well between the two strains. At the end of each experiment, mosquitoes were killed and their spermathecae examined at 400X magnification to determine insemination status.

Attraction to human odor. Adult female mosquitoes were raised in the presence of males for five days. One day prior to wind tunnel experiments, four replicates of 50 female mosquitoes were briefly knocked down by cooling at 4 °C and 50 females were sorted into the release chamber. A cotton ball soaked in 10% sugar water...
was provided after sorting. Twelve hours before the wind tunnel experiment, the cotton ball soaked in sugar water was replaced with a cotton ball soaked in fresh water. Three hours prior to olfactometer experiments, the holding chamber containing mosquitoes was moved from the insectary to the wind tunnel room, which was kept at 27–30 °C and 70–80 RH. To provide a human odor source, a white sock worn for two days by a volunteer was incubated at 37 °C for 3 h.

The dual choice olfactometer measures 6ft × 2.5ft × 2.5ft. Warm humidified air was released into the olfactometer through two odor ports at a speed of ~ 0.5 m/s. The incubated sock was placed in one of the two collecting chambers connected to the odor ports, and the other collecting chamber was left empty. Position of the sock in left vs right collection chamber was switched between experimental days. CO2 (5%) was released into collecting chambers connected to the odor ports, and the other collecting chamber was left empty. Position of the sock

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7. Rund, S. S. C., Gentile, J. E. & Duffield, G. E. Extensive circadian and light regulation of the transcriptome in the malaria mosquito
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wildtype females at different time points. Luciferase assay data was analyzed using One-Way ANOVA by compar-
ing activity were compared between wildtype and
AeCyc−/− were used as the response variable. Egg hatching rate, pupation rate, emergence rate and mosquito circadian
behavior at different time points during both LD and DD cycles. Luciferase assay data was analyzed using One-Way ANOVA by compar-
ing AeCyc−/− vs. AeClk:AeCyc−/−. Mosquito activity and host-seeking behavior in the wind tunnel were analyzed by generalized linear mixed models. Data were fit to a binomial distribution. Replicates were used as a random variable. Genotype was used as the independent variable and the level of activity and responsiveness were used as the response variable. Egg hatching rate, pupation rate, emergence rate and mosquito circadian activity were compared between wildtype and AeCyc−/− mutants using the Welch two sample t-test. Survivorship was analyzed using the Cox proportional hazards regression model using the R package "survival". Blood feeding behavior at different time points during LD cycle was statistically analyzed using Student t-tests. All the statisti-
cal analyses were performed using R software (version 3.6.3).

Blood feeding activity. To assess the blood feeding behavior of the AeCyc−/− mutant mosquitoes, a series of assays were performed in LD conditions. The blood-feeding assays were repeated at least four independent time points, with three replicates (20 mosquitoes in each replicate). 5–6 days old, inseminated females of both wildtype and AeCyc−/− mutants were allowed to blood feed at four-hour intervals across the light/dark condition (i.e., ZT1, ZT5, ZT9, ZT13 and ZT17). Defibrinated sheep blood at 37 °C, which we routinely use for colony maintenance, was provided through an artificial membrane feeder. Mosquitoes were allowed to blood feed for 20 min, after which they were knocked down by cooling at 4 °C and scored as nonblood-fed, partially blood-fed, or fully engorged under a microscope. A total percentage of blood-fed AeCyc−/− females were compared with wildtype females at different time points.

Statistical analysis. Endogenous clock gene expression data were analyzed using One-Way ANOVA and Post Hoc Bonferroni tests were used for pair-wise comparisons between AeCyc−/− and wildtype at different time points during both LD and DD cycles. Luciferase assay data was analyzed using One-Way ANOVA by compar-
ing AeClk:AeCyc−/− vs. AeClk:AeCyc−/−. Mosquito activity and host-seeking behavior in the wind tunnel were
analyzed by generalized linear mixed models. Data were fit to a binomial distribution. Replicates were used as a random variable. Genotype was used as the independent variable and the level of activity and responsiveness were used as the response variable. Egg hatching rate, pupation rate, emergence rate and mosquito circadian activity were compared between wildtype and AeCyc−/− mutants using the Welch two sample t-test. Survivorship was analyzed using the Cox proportional hazards regression model using the R package "survival". Blood feeding behavior at different time points during LD cycle was statistically analyzed using Student t-tests. All the statisti-
cal analyses were performed using R software (version 3.6.3).

Data availability
All the data pertaining to knockout line creation, gene expressions, all the behavioral studies and statistical analysis were included in the supplementary pages S1.

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**Author contributions**

V.S. designed and conducted experiments, analyzed data and co-wrote the manuscript. J.I.M. co-designed the study, designed and conducted experiments, analyzed data and contributed to manuscript preparation. Y.Z. conducted experiments. C.M. designed and supervised experiments and edited manuscript. M.A.S. co-designed and supervised the study and co-wrote the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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