

Disabling the Circadian Clock in the Yellow Fever Mosquito *Aedes Aegypti* Reduces Adult Life Span, Attraction to Host Odor and Mating Success

Vinaya Shetty (✉ vshetty@tamu.edu)

Texas A&M University

Jacob I Meyers

Texas A&M University

Ying Zhang

Texas A&M University

Christine Merlin

Texas A&M University

Michel A Slotman

Texas A&M University

Research Article

Keywords: Fever Mosquito, Host Odor, Mating Success

Posted Date: November 22nd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1029091/v1>

License:  This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Disabling the circadian clock in the yellow fever mosquito *Aedes aegypti* reduces adult life span, attraction to host odor and mating success

Vinaya Shetty^{1§*}, Jacob I Meyers^{1§}, Ying Zhang², Christine Merlin², Michel A Slotman¹

§ These authors contributed equally

¹ Department of Entomology, Texas A&M University, College Station, TX 77843

² Department of Biology, Texas A&M University, College Station, TX 77843

* Corresponding author: vshetty@tamu.edu (VS)

ABSTRACT

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, if expressed, 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.

INTRODUCTION

Eukaryotic organisms have endogenous 24-hour internal circadian clocks that assist them in optimizing their physiology and behavior to daily fluctuations in light, temperature, and resource availability[1]. 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-documented[2–8]. *Aedes aegypti*, the primary vector of numerous emerging vector-borne diseases including Yellow Fever, Dengue, Chikungunya, and more recently Zika virus[9] displays a small activity peak at the start of the light phase but is primarily active during the late afternoon hours[10]. The diurnal activity patterns of *Aedes aegypti* are found in flight activity, oviposition, host-seeking, and human landing/biting[11]. The endogenous circadian clock of mosquitoes regulates locomotor activity and blood feeding behavior[11–13], and is expected to regulate the timing of other behaviors such as host-seeking, mating and oviposition, although this has not been specifically tested[14].

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 hours[14]. The core circadian genes at the center of this feedback loop are *cycle*, *clock*, *period*, *timeless*, *pdp1*, and *cryptochrome-2*[5, 6, 15, 16]. In some insects, e.g. the monarch butterfly, *cycle* is now referred to as *bmall*[17]. This is because in these species, as in mosquitoes, *cycle* retains a C-terminal transactivation domain that is present in the mammalian orthologue *bmall* but is missing in the *Drosophila* orthologue *cycle*. Here, we refer to *Aedes aegypti cycle* (*AeCyc*) for consistency with the current genome annotation[8]. In addition to sustaining 24-hour rhythms, the circadian clock also regulates the rhythmic expression of a broad range of genes that drive circadian behaviors[18–21].

Research into circadian rhythms in *Drosophila* has made significant advances due to the vast repertoire of molecular and transgenic approaches available in this system. In *Drosophila*, knockout (KO) lines for the core circadian genes exhibit significant circadian locomotor arrhythmias[18, 19, 21, 22], as well as a wide range of physiological and behavioral deficits including immunity[14], learning and memory, feeding and metabolism[23], and visual and olfactory based behaviors[24]. Wildtype *D. melanogaster* locomotor behavior peaks at the beginning of the light phase and the beginning of the dark phase[22]. However, *cycle* KO results in arrhythmic, infrequent locomotor activity[25]. Wildtype *D. melanogaster* olfactory sensitivity

also follows a circadian pattern, with a distinct peak at four hours into the dark phase[24, 26]. *Cycle* KO abolishes this peak in olfactory sensitivity, reducing overall sensitivity[27].

In contrast to *Drosophila*, much less is known about how molecular clock disruption affects critical sensory and motor systems in mosquito disease vectors[14]. RNAi-mediated knockdown of *timeless* in *Ae. aegypti* caused a decrease in locomotor activity and an increase in the free-running period[13], but no other behavioral assays were performed in this study. In *Anopheles gambiae*, knocking down *timeless* or *cryptochrome 1* using RNAi increases blood feeding propensity[12]. Furthermore, the expression of these genes was affected by short light pulses[12]. In *Ae. aegypti* blood feeding reduces expression of the four critical clock genes *clock*, *cycle*, *timeless*, and *period*[13]. 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[27]. 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[14], 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 [28]. 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*'s 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* (Figure 1A and 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 F₁ mutant of this strain was backcrossed to wildtype *Ae aegypti* 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 of seven essential circadian clock genes: *AeCyc*, *AeClk*, *AePer*, *AeTim*, *AeCry1*, *AeCry2* and *AePdp1* at 4h 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 (Figure 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 (Figure 2A).

The other endogenous clock genes, with the exception of *AeCry1*, also showed a shift in the timing of peak expression under LD conditions (Figure 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 dysfunctional 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 per E-boxes driving luciferase as a reporter. As expected if the truncated CYC protein was lacking the PAS domains necessary for dimerization with CLOCK, we found that while AeCLK:AeCYC^{WT} activates transcription of the reporter, AeCLK:AeCYC^{-/-} does not (one-way ANOVA, *p*<0.002) (Figure 3). 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 dysfunctional, 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. 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, Figure 4A). During the dark phase (ZT12-ZT24) little activity was observed. During 72 hours under DD conditions, the main activity peak between ZT8 and ZT12 was maintained, with an expected shift towards an earlier (Figure 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, $p < 0.001$, Figure 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 hours of DD conditions, both wildtype and *AeCyc*^{-/-} revert back to their respective LD activity patterns.

Life history parameters

The hatching rate for *AeCyc*^{-/-} was significantly lower than for wildtype *Ae. aegypti*, (0.55 ± 0.18 vs. 0.90 ± 0.12 , $p = 0.0038$, Table 1). In addition, the average egg development time was significantly longer in *AeCyc*^{-/-} vs. wildtype eggs (7.12 ± 4.51 vs. 2.03 ± 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 different 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 (Figure 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 hour of female-to-male exposure (42.0% vs 68.7%, $p < 0.005$, Figure 6A). The insemination rate was not significant lower in *AeCyc*^{-/-} females in the 24h, 48h and 72h exposure experiments (Figure 6B, C & D). Interestingly, the insemination rate following 1 hour 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 24h, 48h or 72h 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 24h to 72h (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, predominantly using only 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 in an 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.

Both *AeCyc*^{-/-} and wildtype females show some response to host odor at each of the three time points (Figure 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, Figure 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*^{-/-} and wildtype females was similar, with slightly higher activation in *AeCyc*^{-/-} females (p>0.005, figure 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 (figure 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 (Figure 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%).

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[29]. 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*[30]. 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[31], 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 [25].

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[32], was similar between *AeCyc*^{-/-} and wildtype under LD and DD conditions. A considerable portion of the transcriptome shows circadian expression patterns[6, 8]. 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[33]. Several earlier studies have shown that pupation is gated in mosquitoes such as *Anopheles gambiae*[34] and *Aedes taeniorhynchus*[35], and is regulated by the duration of the photoperiod via the secretion of the prothoracicotrophic hormone that stimulates ecdysone secretion[36]. Also, it 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[37, 38]. The environmental factors with circadian clock (eg: light input pathways regulate the period gene expression) determines the stage of

development and duration of time (width of the gate) within a cycle available for an insect to emerge into an adult[39]. 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 rate of *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[39]. Also, in immature stages of *Drosophila* (egg, larvae, pupae), null mutations in *period* increase development time under normal light/dark conditions[40]. Together with previous findings in *Drosophila*, where *period* mutants under prolonged or shortened circadian cycles have different development times from eggs to adults[41]. We also 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 decreased fitness was observed only in males and the sex-specific mechanisms driving this phenotypic difference is not understood[42]. This is in contrast to our observation that both males and female *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 activity[43][26][44], *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 activity[45]. 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 patterns[13]. 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 and other insects (e.g., blood and sugar feeding, mating, and oviposition) and is itself under circadian control in insects[26, 46]. In *Ae. aegypti*, antennal sensitivity to some host odors is highest towards the end of the light phase, although for others sensitivity peaks during midday[47]. 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 niche[7].

Odor responses in *Drosophila* antennae are controlled autonomously by circadian clocks present within olfactory sensory neurons (OSNs)[24, 26]. 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 OSNs[45, 46]. Several olfaction genes in *Aedes* and *Anopheles* show circadian expression patterns, indicating that they may be under control of the endogenous circadian clock[7, 8, 47]. 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 processes[47], 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 sexes of *AeCyc*^{-/-} was reduced between 30.1% to 43.7%. These one-hour mating experiments were performed during the last part of the light phase, when wildtype show peak activity levels but *AeCyc*^{-/-} mutants showed lower activity. It is thus possible that this may explain some of the observed differences.

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. 24h, 48h, and 72h), 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 spermathecal 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 behavior[48]. 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 mosquitoes. *Per* and *tim* also control the circadian rhythms of female mating activity in *Drosophila melanogaster*[49]. Moreover, gene transfer experiments implicate *per* in the species-specific behaviors of locomotor activity, love song rhythms, and time of mating[31, 50]. *An. stephensi* swarming behavior is strongest when *per* and *tim* expression peaks[48], 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 response[26]. Because it has been suggested that *Aedes aegypti* males produce volatiles that attract females[48], 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 of the 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*[13], 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[13]. 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[13].

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 (IBC# 2018-029) of Texas A&M University.

Mosquito Rearing

Ae. aegypti (Liverpool strain) were maintained at 27°C, 60-70% relative humidity (RH) on a 12:12 hr light/dark cycle. 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 de-fibrinated 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 [51, 52]. These sgRNAs were generated by *in vitro* transcription following Bassett et al.[53]. 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 TCGTACACCGAGGGCCACTACAAGC in exon 5 (AAEL002049-RD) was selected for further injection. An injection solution was prepared by combining 100ng/μL of sgRNA and 200ng/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 14000 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 de-fibrinated calf blood and maintained in the insectary without access to egg paper. After four days, ~30 adult female *Ae. aegypti* were placed into a 50mL 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 (1mm x 0.5mm x 10cm) using the P-1000 Pipette Puller and beveled using the BV-10 Micropipette Beveller (Sutter Instrument). Injections were performed using a Pneumatic Picopump (World Precision Instruments) and visualized with the Dino-Lite Edge Digital Microscope (Model 7115MZTL) at 150x magnification and a laptop computer running 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 conducted 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 *Cyc*^{+/+} 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 hours of darkness (DD) after entrainment to LD cycles (Table S1). mRNA was extracted from 10 female heads per replicate using the Dynabeads® mRNA DIRECT™ 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*, *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 were calculated relative to ribosomal protein S6, which has previously shown to have constitutive expression across the light/dark cycles in *Ae. aegypti* [5, 8].

Luciferase transcriptional assay

The pGL-*dpPer4Ep* and pAC5.1V5/HisA plasmids were provided by Zhu et al., [54] and pAC-*Renilla*-Luc control plasmid was provided by McDonald et al. [55]. pAC plasmids containing wildtype *AeClk*, *AeCyc*^{WT}, 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 *AeClk* was subcloned between *KpnI* and *XhoI*, wildtype *AeCyc* was subcloned between *EcoRI* and *XbaI*, and the mutant *AeCyc* was subcloned between *EcoRI* and *XhoI*. 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 described [17, 56] 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 5ng/well of pAC plasmids expressing wildtype *AeClk*, wildtype *AeCyc* and/or the mutant *AeCyc*. For luciferase assay, cells were lysed with 50µl of 1X Passive lysis buffer (Promega). Firefly and renilla luciferase activities were quantified with a Dual-Luciferase reporter assay system (Promega) using 5µl 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 fabric wick that enters the glass vial. Glass vials were placed in the Locomotor Activity Monitor 25 (Trikinetics). 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 was quantified by infrared beam breaks in the activity monitor which was collected by the accompanying DAMSystem3 software. The first 24 hours of activity monitor data were discarded. Data was collected for an additional eight days. On the first two days, mosquitoes were exposed to a 12:12 light-dark cycle, followed by three days of darkness, and three subsequent days of 12:12 light/dark cycle.

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 30x30x30 cm cages and allowed to mate for 1h, 24h, 48h, and 72h. The 1h 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 three hours.

The dual choice olfactometer measures 6ft x 2.5ft x 2.5ft. Warm humidified air was released into the olfactometer through two odor ports at a speed of ~0.5m/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 the olfactometer from a position in between the two odor ports. Experiments conducted during the dark phase were conducted at ~5 lux.

After opening the release cage, experiments were run for 20 min. At that point the number of mosquitoes that left the holding chamber were counted. These were classified as “active”. Additionally, the number of mosquitoes entering one of the two odor ports were counted and classified as “responsive”. Experiments were run at three different times: 6 hours into the light phase (Zeitgeber time 6; ZT6), 12 hours into the light phase (ZT12), and 6 hours into the dark phase (ZT18).

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 comparing *AeClk:AeCyc*^{-/-} vs. *AeClk:AeCyc*^{WT}. 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 statistical analyses were performed using R software (version 3.6.3).

REFERENCES

1. Deborah Bell-Pedersen, Vincent M. Cassone DJE, Susan S. Golden*, Paul E. Hardin§ TLT and MJZ. Circadian rhythms from multiple oscillators: lessons from diverse organisms. *Nature Reviews Drug Discovery*. 2005;4:121–30.
2. Taylor B, Jones MD. The circadian rhythm of flight activity in the mosquito *Aedes aegypti* (L.). The phase-setting effects of light-on and light-off. *Journal of Experimental Biology*. 1969;51:59–70.
3. Jones MDR. The programming of circadian flight-activity in relation to mating and the gonotrophic cycle in the mosquito,. 1981;:307–13.
4. Lee H, Yang Y, Liu Y, Teng H, Sauman I. Circadian control of permethrin-resistance in the mosquito *Aedes aegypti*. 2010;56:1219–23.
5. Ptitsyn AA, Reyes-solis G, Saavedra-rodriguez K, Betz J, Suchman EL, Carlson JO, et al. Rhythms and synchronization patterns in gene expression in the *Aedes aegypti* mosquito. 2011.
6. Rund SSC, Hou TY, Ward SM, Collins FH, Duf GE. Genome-wide pro fi ling of diel and circadian gene expression in the malaria vector *Anopheles gambiae*. *Annu Rev Genet*. 2011;108:419–444.
7. Rund SSC, Gentile JE, Duffield GE. Extensive circadian and light regulation of the transcriptome in the malaria mosquito *Anopheles gambiae*. *BMC Genomics*. 2013;14:218.
8. Leming MT, Rund SSC, Behura SK, Duffield GE, O’Tousa JE. A database of circadian and diel rhythmic gene expression in the yellow fever mosquito *Aedes aegypti*. *BMC Genomics*. 2014;15:1–9.
9. Faria N. R., Quick J., Claro I. M., Thézé J., de Jesus J. G., Giovanetti M., Kraemer M. U. G., Hill S. C., Black A., da Costa A. C., Franco L. C., Silva S. P., Wu C.-H., Raghvani J., Cauchemez S., du Plessis L., Verotti M. P., de Oliveira W. K., Carmo E. POG. Establishment and cryptic transmission of Zika virus in Brazil and the Americas. *Nature*. 2017;546:406–410. doi:10.1038/nature22401.
10. Araujo M da S, Guo F, Rosbash M. Video Recording Can Conveniently Assay Mosquito Locomotor Activity. *Scientific Reports*. 2020;10:1–9. doi:10.1038/s41598-020-61733-5.
11. Lima-Camara TN, Bruno R v., Luz PM, Castro MG, Lourenço-de-Oliveira R, Sorgine MHF, et al. Dengue infection increases the locomotor activity of *Aedes aegypti* females. *PLoS ONE*. 2011;6:1–5.
12. Das S, Dimopoulos G. Molecular analysis of photic inhibition of blood-feeding in *Anopheles gambiae*. 2008;19:1–19.

13. Gentile C, Bueno G, Rivas S, Lima JBP, Bruno RV, Peixoto AA. Circadian clock of *Aedes aegypti* : effects of blood-feeding , insemination and RNA interference. 2013;108:80–7.
14. Meireles-filho ACA, Kyriacou CP. Circadian rhythms in insect disease vectors. *Mem Inst Oswaldo Cruz*. 2013;108 September:48–58.
15. Yuan Q, Metterville D, Briscoe AD, Reppert SM. Insect cryptochromes: Gene duplication and loss define diverse ways to construct insect circadian clocks. *Molecular Biology and Evolution*. 2007;24:948–55.
16. Gentile C, Rivas GBS, Meireles-Filho ACA, Lima JBP, Peixoto AA. Circadian expression of clock genes in two mosquito disease vectors: *Cry2* is different. *Journal of Biological Rhythms*. 2009;24:444–51.
17. Zhang Y, Markert MJ, Groves SC, Hardin PE, Merlin C. Vertebrate-like CRYPTOCHROME 2 from monarch regulates circadian transcription via independent repression of CLOCK and BMAL1 activity. *Proceedings of the National Academy of Sciences of the United States of America*. 2017;114:E7516–25.
18. Baylies MK, Bargiello TA, Jackson FR, Young MW. Changes in abundance or structure of the per gene product can alter periodicity of the *Drosophila* clock. 1987;48 December 1986:1986–8.
19. Sehgal A, Price JL, Man B, Young MW. Loss of circadian behavioral rhythms and per RNA oscillations in the *Drosophila* mutant timeless. *Science*. 1994;263:1603–6.
20. Allada R, White NE, So WV, Hall JC, Rosbash M. A mutant *Drosophila* homolog of mammalian clock disrupts circadian rhythms and transcription of period and timeless. *Cell*. 1998;93:791–804.
21. Rutila JE, Maltseva O, Rosbash M. The timSL Mutant Affects a Restricted Portion of the *Drosophila melanogaster* Circadian Cycle. *Journal of Biological Rhythms*. 1998;13:380–92.
22. Allada R, Chung BY. Circadian Organization of Behavior and Physiology in *Drosophila* . *Annual Review of Physiology*. 2010;72:605–24.
23. Xu K, Zheng X SA. Regulation of feeding and metabolism by neuronal and peripheral clocks in *Drosophila*. *Cell Metab*. 2008;8:289–300.
24. Tanoue, Shintaro; Krishnan, Parthasarathy; Krishnan, Balaji; Dryer, Stuart E; Hardin PE. Circadian Clocks in Antennal Neurons Are Necessary and Sufficient for Olfaction Rhythms in *Drosophila*. *Current Biology*,. 2004;14:638–49.
25. Rutila JE, Suri V, Le M, So WV, Rosbash M, Hall JC. Cycle is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of *Drosophila* period and timeless. *Cell*. 1998;93:805–14.
26. Krishnan B, Dryer SE, Hardin PE. Circadian rhythms in olfactory responses of *Drosophila melanogaster*. *Nature*. 1999;400:375–8.
27. Meireles-Filho ACA, Gustavo GB, Gesto JSM, Machado RC, Britto C, de Souza NA, et al. The biological clock of an hematophagous insect: Locomotor activity rhythms, circadian expression and downregulation after a blood meal. *FEBS Letters*. 2006;580:2–8.
28. Tallon AK, Hill SR, Ignell R. Sex and age modulate antennal chemosensory-related genes linked to the onset of host seeking in the yellow-fever mosquito , *Aedes aegypti*. 2019; August 2018:1–13.
29. Popp, Maximilian W. and Maquat LE. HHS Public Access. *Physiology & behavior*. 2016;176:139–48.
30. Hardin PE. Molecular genetic analysis of circadian timekeeping in *Drosophila*. 2011.
31. Tauber E, Roe H, Costa R, Hennessy JM, Kyriacou CP. Temporal mating isolation driven by a behavioral gene in *Drosophila*. *Current Biology*. 2003;13:140–5.

32. Lin F-J, Song W, Meyer-Bernstein E, Naidoo N, Sehgal A. Photic Signaling by Cryptochrome in the *Drosophila* Circadian System. *Molecular and Cellular Biology*. 2001;21:7287–94.
33. Yadav P, Thandapani M, Sharma VK. Interaction of light regimes and circadian clocks modulate timing of pre-adult developmental events in *Drosophila*. *BMC Developmental Biology*. 2014;14:1–12.
34. JONES, M., REITER P. Entrainment of the pupation and adult activity rhythms during development in the mosquito *Anopheles gambiae*. *Nature*. 1968;254:242–244. <https://doi.org/10.1038/254242a0>.
35. Nayar JK. The Pupation Rhythm in *Aedes taeniorhynchus* (Diptera: Culicidae). II. Ontogenetic Timing, Rate of Development, and Endogenous Diurnal Rhythm of Pupation I. *Annals of the Entomological Society of America*. 1967;60:946–71. doi:10.1093/aesa/60.5.946.
36. Nijhout HF, Riddiford LM, Mirth C, Shingleton AW, Suzuki Y, Callier V. The developmental control of size in insects. *Wiley Interdisciplinary Reviews: Developmental Biology*. 2014;3:113–34.
37. Kaneko M, Hamblen MJ, Hall JC. Involvement of the period gene in developmental time-memory: Effect of the per(Short) mutation on phase shifts induced by light pulses delivered to *Drosophila* larvae. *Journal of Biological Rhythms*. 2000;15:13–30.
38. Srivastava M, James A, Varma V, Sharma VK, Sheeba V. Environmental cycles regulate development time via circadian clock mediated gating of adult emergence. *BMC Developmental Biology*. 2018;18:1–10.
39. Duffield GE, Best JD, Meurers BH, Bittner A, Loros JJ, Dunlap JC. Circadian programs of transcriptional activation, signaling, and protein turnover revealed by microarray analysis of mammalian cells. *Current Biology*. 2002;12:551–7.
40. Menon A, Varma V, Sharma VK. Rhythmic egg-laying behaviour in virgin females of fruit flies *Drosophila melanogaster*. *Chronobiology International*. 2014;31:433–41.
41. Kyriacou CP, Oldroyd M, Wood J, Sharp M, Hill M. Clock mutations alter developmental timing in *Drosophila*. *Heredity*. 1990;64:395–401.
42. Ravi Allada, Chung BY. Circadian Organization of Behavior and Physiology in *Drosophila* Ravi. *Annu Rev Physiol*. 2010;72:605–624.
43. Lima-Camara TN, Lima JBP, Bruno RV, Peixoto AA. Effects of insemination and blood-feeding on locomotor activity of *Aedes albopictus* and *Aedes aegypti* (Diptera: Culicidae) females under laboratory conditions. *Parasites and Vectors*. 2014;7:1–8.
44. Delventhal R, Pantalia M, O'Connor R, Ulgherait M, Kim H, Basturk M, et al. Dissection of central clock function in *Drosophila* through cell-specific CRISPR-mediated clock gene disruption. *bioRxiv*. 2019;:640011.
45. Nayar JK, Sauerman DM. The effect of light regimes on the circadian rhythm of flight activity in the mosquito *Aedes taeniorhynchus*. *Journal of Experimental Biology*. 1971;54:745–56.
46. Granados-Fuentes D, Tseng A, Herzog ED. A circadian clock in the olfactory bulb controls olfactory responsivity. *Journal of Neuroscience*. 2006;26:12219–25.
47. Eilerts DF, Vandergiesen M, Bose EA, Broxton K. Odor-Specific Daily Rhythms in the Olfactory Sensitivity and Behavior of *Aedes aegypti* Mosquitoes. 2018.
48. Cabrera M, Jaffe K. An aggregation pheromone modulates lekking behavior in the vector mosquito *Aedes aegypti* (Diptera: Culicidae). *Journal of the American Mosquito Control Association*. 2007;23:1–10.

49. Sakai T, Ishida N. Circadian rhythms of female mating activity governed by clock genes in *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America*. 2001;98:9221–5.
50. Petersen G, Hall JC, Rosbash M. The period gene of *Drosophila* carries species-specific behavioral instructions. *The EMBO journal*. 1988;7:3939–47.
51. Montague TG, Cruz JM, Gagnon JA, Church GM, Valen E. CHOPCHOP: A CRISPR/Cas9 and TALEN web tool for genome editing. *Nucleic Acids Research*. 2014;42:401–7.
52. Labun K, Montague TG, Gagnon JA, Thyme SB, Valen E. CHOPCHOP v2: a web tool for the next generation of CRISPR genome engineering. *Nucleic acids research*. 2016;44:W272–6.
53. Bassett AR, Tibbit C, Ponting CP, Liu JL. Highly Efficient Targeted Mutagenesis of *Drosophila* with the CRISPR/Cas9 System. *Cell Reports*. 2013;4:220–8. doi:10.1016/j.celrep.2013.06.020.
54. Zhu H, Yuan Q, Briscoe AD, Froy O, Casselman A, Reppert SM. The two CRYs of the butterfly (DOI:10.1016/j.cub.2005.11.030). *Current Biology*. 2005;15:730.
55. McDonald MJ, Rosbash M, Emery P. Wild-Type Circadian Rhythmicity Is Dependent on Closely Spaced E Boxes in the *Drosophila* timeless Promoter. *Molecular and Cellular Biology*. 2001;21:1207–17.
56. Chang DC, Reppert SM. A Novel C-Terminal Domain of *Drosophila* PERIOD Inhibits dCLOCK:CYCLE-Mediated Transcription. *Current Biology*. 2003;13 Ccid:654–8. doi:10.1016/S.

Acknowledgements

We wish to thank Drs Zachary Adelman and Kevin Myles for technical advice. We thank Christopher Janes, Alex Seua, Javier Pellacani and Xavier Tijerina for technical support. Figure 1 and figure 5 were created with Biorender.com under a license to Texas A&M University. This work was supported by an Insect Vectored Disease grant by Agrilife Research to MAS. Partial support was provided by NIH awards R21 AI115175 and R21 AI149100 to MAS, and NIH R01 GM124617 to CM.

Availability of supporting data

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

Completing interests

The authors declare that they have no competing interest.

Author contributions

VS designed and conducted experiments, analyzed data and co-wrote the manuscript. JIM co-designed the study, designed and conducted experiments, analyzed data and contributed to manuscript preparation. YZ conducted experiments. CM designed and supervised experiments and edited manuscript. MAS co-designed and supervised the study and co-wrote the manuscript.

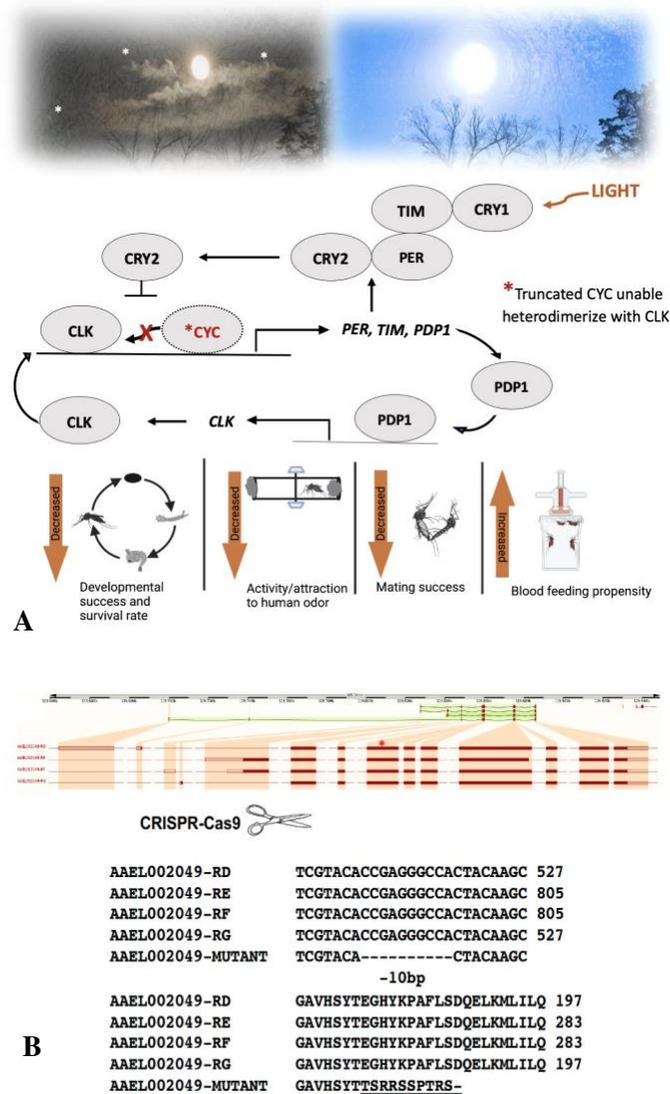


Figure 1: A.: Schematic illustration of the molecular mechanism of circadian rhythms at day/night cycle. *AeCYC* knockout is unable to bind with *AeCLK* and initiate the transcription of downstream genes in the pathway. *AeCyc*^{-/-} impacts on its developmental stages and other behaviors.

B.: CRISPR/Cas9-mediated deletion of 10 bp from the exon 5 of *cycle* gene in *Ae. aegypti*. AAEL002049-RD, RE, RF and RG are four alternative splice variants, and deletion of 10 bp from the exon 5 causing a frame shift, resulted in a premature stop codon in all the four gene splice variants.

*indicates the location of deletion in exon 5 (AAEL002049-RD).

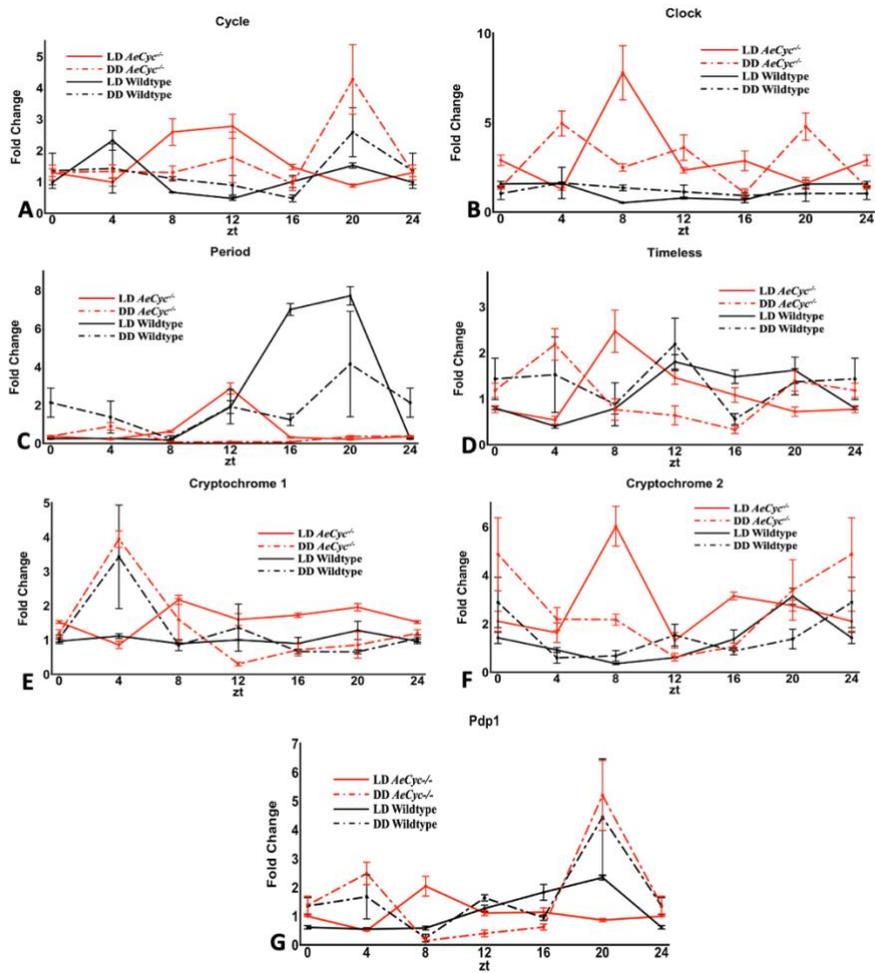


Figure 2: Gene expressions of A) *cycle* B) *clock* C) *per* D) *tim* E) *cry1* F) *cry2* G) *pdp1* in *AeCyc*^{-/-} and wildtype mosquitoes at different time intervals across LD and DD cycles. Relative mRNA expression measured by quantitative real-time PCR.

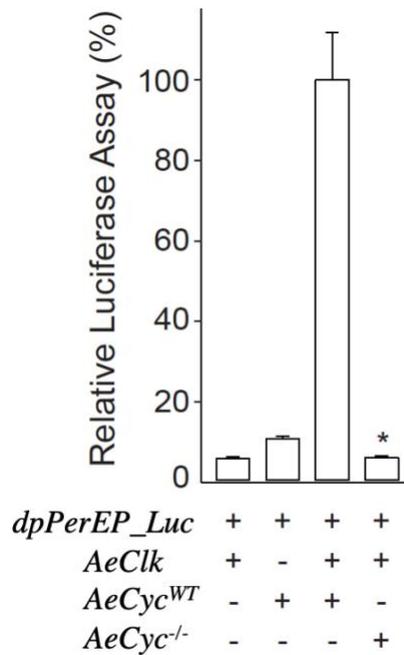


Figure 3: The mutant *AeCyc* lacking the PAS and transactivation domains is impaired in transcriptional activation in S2 cells. The monarch *per* E box luciferase reporter (*dpPerEp_Luc*; 10 ng) was used in presence (+) or absence (-) of wildtype *AeClk*, *AeCyc^{WT}*, *AeCyc^{-/-}* expression plasmids (5 ng each). Firefly luciferase activity was computed relative to renilla luciferase activity. Each value is mean \pm SEM of three replicates.

One-way ANOVA, *AeCLK:AeCYC^{-/-}* vs. *AeCLK:AeCYC^{WT}*, * $p < 0.002$.

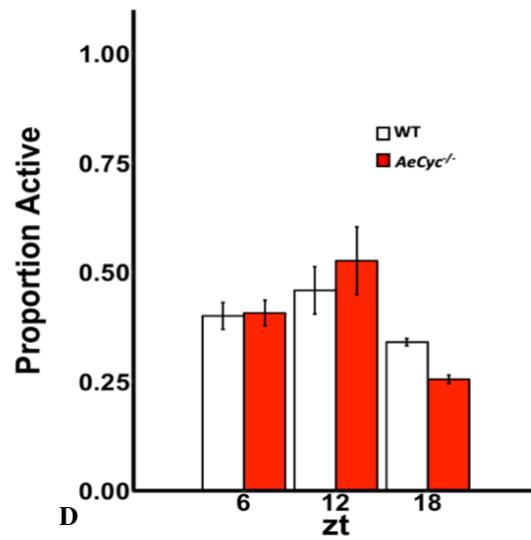
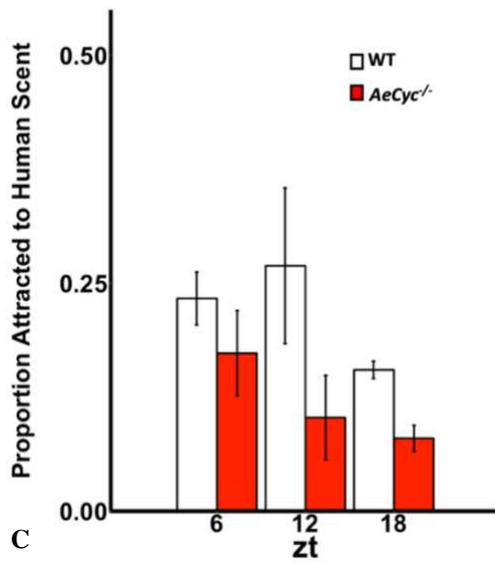
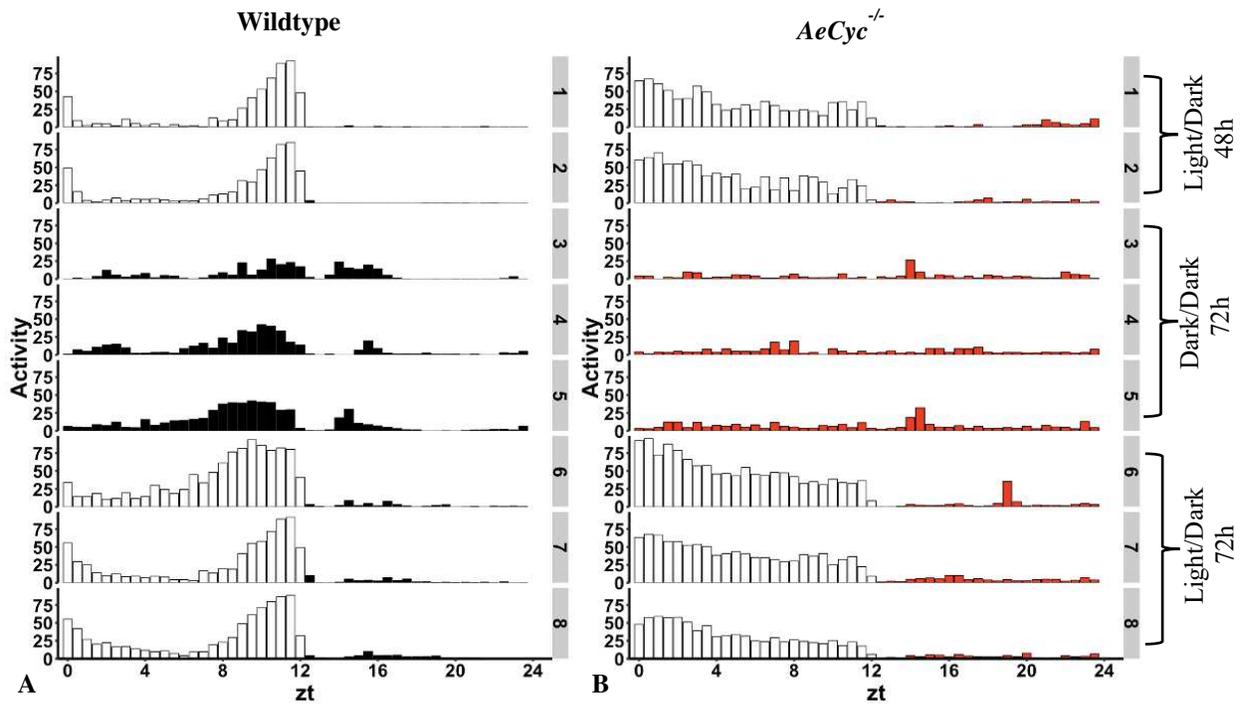


Figure 4A&B.: Circadian activity patterns in wildtype and *AeCyc*^{-/-} mutants in both both LD and DD conditions

C.: Attraction of wildtype and *AeCyc*^{-/-} females to human odor in olfactometer

D.: Comparing activation of wildtype and *AeCyc*^{-/-} mutants in olfactometer

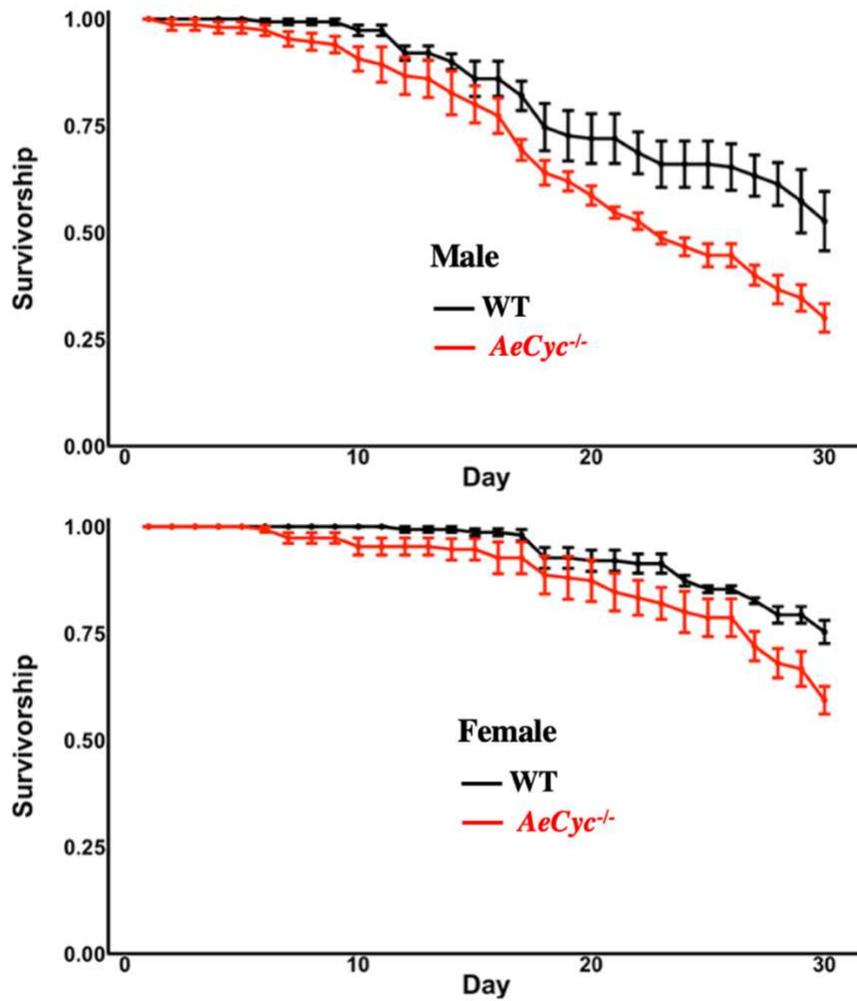


Figure 5: Survivorship of *AeCyc*^{-/-} and wildtype *Ae. aegypti* males and females

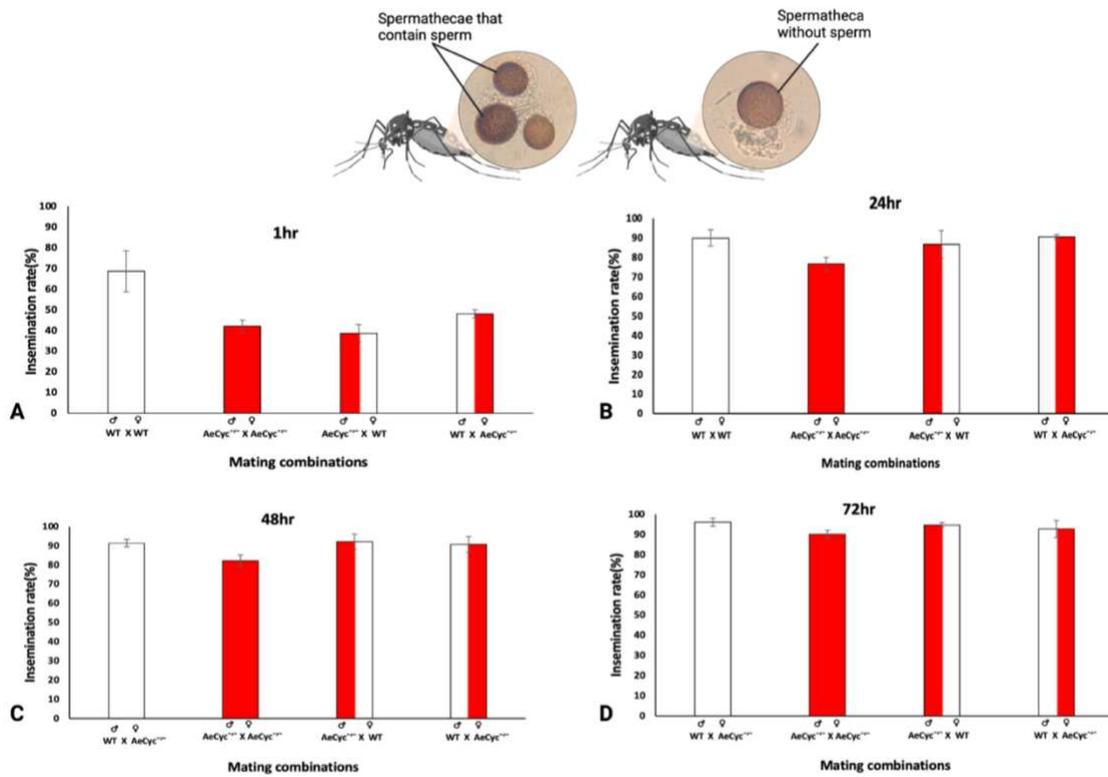


Figure 6: Insemination status of *AeCyc*^{-/-} (♂ & ♀) and wildtype (♂ & ♀) (Nonreciprocal) crossing, and reciprocal crossing groups *AeCyc*^{-/-}♂ and WT♀; *AeCyc*^{-/-}♀ and WT♂ mosquitoes after 1h(A), 24h(B), 48h(C) and 72h(D) of exposure to males for mating (copulation duration). Each value is mean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

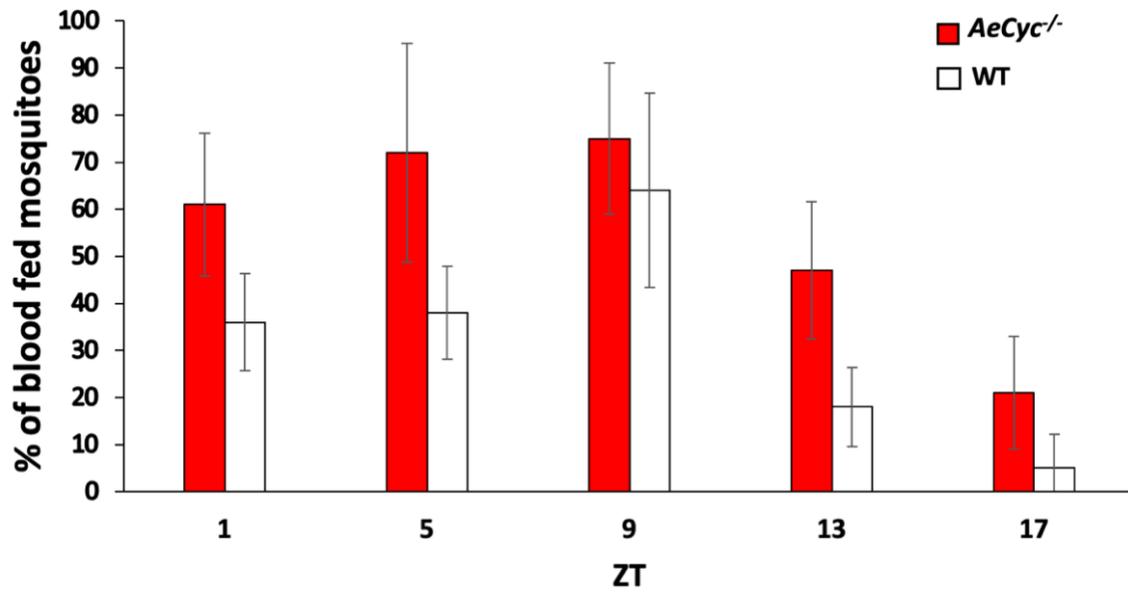


Figure 7: *AeCyc*^{-/-} and wildtype *Ae. aegypti* blood-feeding behavior.

Mosquitoes were allowed to feed for 20 min and the percentage of fed mosquitoes in each time point was scored and plotted. Error bars indicate the standard error.

Table 1: Comparison of life history traits between *AeCyc*^{-/-} and wildtype *Ae. aegypti* mosquitoes

	Genotype	Sex	Success Rate (Mean +/- SD)	Average Day of Success (Mean +/- SD)	Time to 50% Success (LT₅₀, 95% CI)
Egg to Larva	Wildtype		0.90 +/- 0.12	2.03 +/- 0.04	1.58 [1.21, 1.97]
	<i>AeCyc</i> ^{-/-}		0.55 +/- 0.18	7.12 +/- 4.51	3.72 [1.79, 5.23]
Larva to Pupa	Wildtype		0.99 +/- 0.01	5.23 +/- 0.28	4.73 [4.65, 4.81]
	<i>AeCyc</i> ^{-/-}		0.93 +/- 0.06	5.27 +/- 0.13	4.79 [4.71, 4.87]
Pupa to Adult	Wildtype	Male	1.00	1.36 +/- 0.09	0.95 [0.94, 0.97]
	<i>AeCyc</i> ^{-/-}		0.99 +/- 0.01	1.50 +/- 0.04	1.20 [1.08, 1.32]
	Wildtype	Female	0.99 +/- 0.01	1.66 +/- 0.08	0.94 [0.92, 0.96]
	<i>AeCyc</i> ^{-/-}		0.98 +/- 0.01	1.68 +/- 0.15	1.18 [1.07, 1.29]

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementarypages1.dotx](#)