Males of *Aedes aegypti* show different clock gene expression profiles in the presence of conspecific females

Jéssica Rodrigues Assunção Bezerra1, Rafaela Vieira Bruno1,2 and Luciana Ordunha Araripe1*

**Abstract**

**Background:** The study of behavioral and physiological traits in mosquitoes has been mainly focused on females since males are not hematophagous and thus do not transfer the parasites that cause diseases in human populations. However, the performance of male mosquitoes is key for the expansion of populations and the perpetuation of mosquito species. Pre-copulatory communication between males and females is the initial and essential step for the success of copulation and studying the male facet of this interaction provides fertile ground for the improvement of vector control strategies. Like in most animals, reproduction, feeding, and oviposition are closely associated with locomotor activity in mosquitoes. Rhythmic cycles of locomotor activity have been previously described in *Aedes aegypti*, and in females, they are known to be altered by blood-feeding and arbovirus infection. In previous work, we found that males in the presence of females significantly change their locomotor activity profiles, with a shift in the phase of the activity peak. Here, we investigated whether this shift is associated with changes in the expression level of three central circadian clock genes.

**Methods:** Real-time PCR reactions were performed for the gene *period*, *cycle*, and *cryptochrome 2* in samples of heads, antennae, and abdominal tips of solitary males and males in the presence of females. Assays with antennae-ablated males were also performed, asking whether this is an essential organ mediating the communication and the variation in activity profiles.

**Results:** The gene *period* showed a conserved expression pattern in all tissues and conditions, while the other two genes varied according to the male condition. A remarking pattern was observed in *cry2*, where the difference between the amplitude of expression at the beginning of photophase and the expression peak in the scotophase was greater when males were in the presence of females. Antennae ablation in males did not have a significant effect on the expression profiles, suggesting that female recognition may involve other senses besides hearing and olfaction.

**Conclusion:** Our results suggest that the expression of gene *cryptochrome 2* varies in association with the interaction between males and females.

**Keywords:** *Aedes aegypti*, Social interaction, Reproductive behavior, Clock genes, Circadian gene expression, *cryptochrome 2*
traits and environmental factors contribute to the maintenance and expansion of *Ae. aegypti* populations, favoring the risk of epidemic diseases. Therefore, it is important to study the behavioral and physiological traits that account for the rise in mosquito populations to mitigate these risks. One particularity of mosquito biology is that only females can blood seek and bite, given that a blood meal is necessary for completing ovogenesis but not required for survival [2]. Thus, most research focuses on females, the actual pathogen vectors, and studies focusing on males are still incipient [3–6]. However, the expansion of mosquito populations depends on reproductive success, and understanding male reproductive behavior and physiology is crucial for the framework of any program of vector control. Pre-copulatory communication is an important initial step to recognition and courtship among the events contributing to reproductive success. Thus, a failure in the recognition between males and females can restrain the growth of mosquito populations.

Behaviors such as reproduction, foraging, feeding, and oviposition are closely associated with the locomotor activity. Like most organisms, mosquitoes exhibit rhythmic cycles of locomotor activity, which are regulated by an endogenous pacemaker called the circadian clock [7]. In the model species *Drosophila melanogaster*, the circadian clock works through genes that interact in negative feedback loops and determines that physiological and behavioral processes occur in approximately 24 h. This pattern of regulation has been found in a variety of other organisms, from microbes to mammals [8, 9], being considered a conserved characteristic of the endogenous clock. Environmental factors such as light and temperature synchronize the circadian clock in cycles of exact 24 h [10].

In *D. melanogaster*, the central clock is located in the brain and is composed of about 150 neurons that express clock genes [11]. In addition, peripheral clocks in other organs also express the clock genes and may exhibit rhythmic patterns. These peripheral clocks can function independently of the central clock, regulating specific functions of each organ [11–14], and were described in various tissues in *Drosophila* and other organisms, such as in reproductive organs, antennae, Malpighian tubules, and others [15–20]. In a critical study, using transgenic *Drosophila* expressing the construct *per-luc* (the clock gene *period* fused to the luciferase gene—*luc*) or *per-driven* GFP (*period*-driven green fluorescent protein), the authors analyzed the circadian oscillations in different tissues [16]. Through experiments where different organs were dissected from transgenic flies and subsequently incubated, it was found that heads, thoraxes, abdomens, and also small organs like antennae and proboscis exhibited rhythmic oscillations. The results suggest that individual cells can keep their circadian oscillations even in the absence of a brain [16].

Previous efforts have been made to elucidate the molecular bases of the circadian clock of *Ae. aegypti* mosquitoes. Our group described the RNA expression profiles of the main circadian clock genes in females of this species [21]. The results revealed remarkable contrast with *D. melanogaster* profiles: for instance, unlike the arrhythmic pattern in the fly [22], the gene *cry* showed rhythmic expression in *Ae. aegypti* females [21]. Furthermore, while *D. melanogaster*’s genome has one *cryptochrome* gene with photoreceptor function, two *cryptochrome* genes were found and analyzed in *Ae. aegypti*: *cry1*, orthologous to *Drosophila*’s *cry*, and *cry2*, a plausibly transcriptional repressor also found in butterflies, beetles, and bees [21, 23, 24]. Only *cry2* showed a rhythmic expression profile, exhibiting a bimodal pattern with two expression peaks: one at the early hours of the photophase and the second peak in the scotophase [21].

Besides varying among organisms, rhythmic patterns of locomotor activity are not steady within a given species. Factors that influence the physiological state of organisms may lead to alterations in locomotor activity profiles, as described in female *Ae. aegypti* after blood-feeding [25] and after arboviruses infection [26, 27]. Intraspecific interactions may also bring in locomotor activity variation, as shown in laboratory experiments with male *Ae. aegypti* in the presence of females [28]. In this previous study, we analyzed the locomotor/flight activity over 24 h of solitary males and males exposed to females at a perceptible distance. Males exposed to females had their locomotor/flight activity patterns significantly altered compared to solitary males: at dusk, their second activity peak was significantly greater than the second peak of solitary males. Also, this second activity peak overlaps with the single peak of females, suggesting that males match their activity peak with the phase in which females are most active. We conducted experiments using individuals with ablated antennae or ablated wings to understand which signals could change males’ activity patterns when exposed to females. The results suggested that males perceive females even when females are wingless and do not produce the wing-beat sounds, indicating that signals other than wing-beat sounds may be involved in recognition between males and females, possibly chemical and/or visual cues [28]. Evidence of sexual pheromones, although still uncertain for *Ae. aegypti*, was seen in other dipterans like *Lutzomyia longipalpis* [29, 30].

Whether alterations in rhythmic behavior are backed by changes in the cycling pattern of clock genes’
expression is a relevant question, especially in the frame of reproductive behavior and its significance for the expansion of Ae. aegypti populations. Here, we describe the cycling of three of the main clock genes in tissues of male Ae. aegypti, in and out of female presence, and associate the gene expression profiles with the previous work's locomotor/flight activity profiles. Furthermore, we investigate the role of the male antennae, the main hearing and olfaction organ in mosquitoes, in perceiving the female conspecific and mediating the variation in gene expression cycling.

The gene **period** was the first clock gene described and has been shown to carry several polymorphisms associated with reproductive traits, for instance the mating activity [31] and copulation duration in Drosophila [32], and to be an important molecular marker for taxonomy and evolutionary studies of sympatric species in Drosophila [33] and in the dipteran Lutzomyia longipalpis [34–36]. The gene **cycle**, while constitutively expressed in Drosophila [22], shows rhythmic expression in Ae. aegypti [21]. Also, it is known that **CYC** has an important role in the regulation of output genes [37]. The gene cry2, not found in Drosophila, has been cited as a gene with variable expression in previous literature on mosquitoes and other organisms [21] and has been indicated to be a transcriptional repressor acting on the regulation of reproductive behavior in a species of moth [38].

**Methods**

**Mosquitoes**

The experiments were carried out with Ae. aegypti mosquitoes (Rockefeller strain) provided by the Laboratory of Physiology and Control of Arthropod Vectors (Fiocruz, Brazil). Eggs were hatched in plastic containers with 1.5 l Milli-RO water and 1 g yeast (Vitalab®, Brazil) as a food source. The larvae were redistributed to 300 larvae per container at the first instar. The water was changed, and 1 g of yeast was added every 2 days until the pupation stage [39]. The pupae were counted, collected, and separated in cages with 10% sucrose solution ad libitum. After emergence, the virgin females and males were collected twice a day and placed in different cages until further use in experiments. Individuals from larval to adult stages were synchronized to 12 h of light and 12 h of darkness (LD12:12) under a constant temperature of 25 °C. All individuals entered the experimental tubes at 3–5 days of age.

**Experimental design**

To recreate the same conditions used in our previous work, where individual males' locomotor/flight activity was monitored in or out of females’ presence [28], a confinement system set with pairs of transparent plastic tubes was created. The confinement system consisted of 15-ml Falcon® tubes, whose caps were cut through in their center for the adaptation of a tulle net. Males and females were placed individually inside the tubes, and pairs of tubes were tape-attached, with their caps facing each other. While the tulle net allows males and females to perceive each other's presence, it prevents copulation. A set of tubes was left empty and paired with tubes with males. Inside the tubes, a piece of cotton soaked in 10% sucrose solution was provided as a food source ad libitum. The experiments were performed in four conditions: “solitary males” and “males with females” with 50 pairs of tubes for each collection point, and “antennae-ablated solitary males” and “antennae-ablated males with females” with 20 pairs of tubes for each collection points.

Male mosquitoes with ablated antennae were used as an experiment condition because antennae are the main communication organs between males and females, acting in hearing and olfaction. Since the sound of wing beating during the flight is perceived by specific structures in the antennae [40, 41], the ablation of males' antennae can interfere with the communication required for the encounter and copulation. For antennae ablation, males were anesthetized on ice, and the organs were mechanically removed with a pair of tweezers. Males were placed in the experimental tubes before waking up and were observed for several minutes after waking up to assure they were alive and moving.

**Experimental conditions and samples**

To study the expression of clock genes over 24 h, we maintained the mosquitoes in the paired tubes under LD12:12 and 25 °C over 4 days. The study of circadian gene expression requires a sampling schedule at regular intervals over 24 h. The collection and immediate freezing of mosquitoes every 4 h allow a reliable assembly of the expression profiles of circadian clock genes [42]. The samples were collected on the 4th day at six different Zeitgeber times (ZTs) (ZT1, ZT5, ZT9, ZT13, ZT17, and ZT21). The ZTs stand for the number of hours passed after lights turn on. Tubes were immersed in liquid nitrogen for males' instant freeze. Males were then transferred to the surface of an ice-frozen metal plate and dissected under a stereoscopic microscope. The tissues of interest were heads, antennae, and abdominal tips (containing testes and accessory glands), which were separately stored in microcentrifuge tubes with 100 µl of TRIzol and maintained at − 80 °C for posterior RNA extraction. For each biological replicate, 50 pairs of antennae, 20 heads, and 20 abdominal tips were dissected from the males of each condition and ZT.
RNA extraction, cDNA synthesis, and qPCR

The plumose antennae of male mosquitoes are composed of different structures that show varying degrees of sclerotization [43]. However, the tissue is mostly stiff, and standard RNA extraction protocols yield insignificant mass, even with an increased number of individuals sampled. After several trials, we developed a hybrid RNA extraction protocol that showed successful results, providing enough mass for all the replicates in every quantitative real-time PCR (qRT-PCR) experiment.

The hybrid protocol merges the TRIzol RNA extraction protocol (Invitrogen, Carlsbad, CA, USA) and the ReliaPrep™ RNA Tissue Miniprep System (Promega Corp., Madison, WI, USA). Briefly, the samples were homogenized in 100 µl TRIzol, and the volume was completed to 1000 µl with the same reagent. After that, 200 µl chloroform was added, the samples were centrifuged, and the aqueous phase was transferred to new microcentrifuge tubes. From this stage on, starting with the addition of 340 µl of isopropanol (Sigma-Aldrich), the ReliaPrep protocol was followed. The total RNA was quantified by Qubit Fluorometer Quantification (Invitrogen, Carlsbad, CA, USA), using the Qubit RNA HS Assay Kit, according to the manufacturer’s protocol. After quantification, the samples were diluted to 5 ng/µl. The cDNA was synthesized with TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA), as described by the manufacturer’s protocol, and diluted to 1 ng/µl. The relative mRNA abundances of each condition, tissue, and collection times were determined by qRT-PCR using the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in a StepOnePlus™ Real-Time PCR System (Applied Biosystems). The analyses were performed for the clock genes period, cycle, and cryptochrome 2, using gene rp49 as an endogenous control. The oligonucleotides of all genes used here were designed previously by Gentile et al. [21, 44] and fit the amplification efficiency criteria required for the analysis. We used the $2^{-\Delta\Delta CT}$ method to analyze the data from three technical replicates for each sample [45].

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.0.2 (GraphPad Software, San Diego, CA, USA). The $2^{-\Delta\Delta CT}$ values were compared among ZTs by ANOVA, followed by Tukey’s a posteriori test, where $P < 0.05$ was considered significant. The significance given by ANOVA indicates that gene expression varies among different collection points (ZTs). Graphic profiles of the gene expression variation were constructed with $2^{-\Delta\Delta CT}$ values. Next, the software CircWave (version 1.4, University of Groningen, The Netherlands) was run using the $2^{-\Delta\Delta CT}$ values. CircWave fits sinusoidal curves to the individual expression data and compares the fitting with a continuous line calculated through the data mean [46]. A significant difference between the fitted sinusoidal curve and the horizontal line, given by $P < 0.05$, was used to confirm the rhythmicity of expression.

Results

Results are presented in sections, each describing the expression profiles of a gene in the three tissues and four conditions studied. The $P$ values in parentheses stand for the significance of Tukey post hoc tests comparing high and low mRNA abundances (peaks and troughs) among ZTs. The average mRNA abundance values and standard errors for each collection point are presented in the Additional file 1: Table S1. Raw data sheets are provided as Additional files 2: Table S2, 3: Table S3, 4: Table S4, 5: Table S5, and 6: Table S6.

Period expression profile in tissues of Ae. aegypti males

The circadian expression profiles of per in heads, abdominal tips, and antennae are shown in Fig. 1. In all tissues and conditions, per showed expression varying significantly among ZTs (Table 1), with an expression peak at ZT1 (Tukey: $P < 0.001$), and rhythmicity was confirmed by the results of CircWave (Table 1). Within each tissue studied, the circadian profiles of the four conditions were akin.

Cycle expression profile in tissues of Ae. aegypti males

The circadian expression profiles of cyc in heads, abdominal tips, and antennae are shown in Fig. 2. In different tissues and conditions, the gene cyc showed an expression peak oscillating between ZT1 and ZT5 and a conserved trough at ZT13. In heads, cyc reached an expression peak at ZT1 in the conditions “solitary males” ($P < 0.001$) and “antennae-ablated males with females” ($P < 0.001$) (Fig. 2a). In the conditions “males with females” ($P < 0.001$) and “antennae-ablated solitary males” ($P < 0.001$), the expression peak was reached at ZT5 (Fig. 2a). In abdominal tips, the gene cyc showed an expression peak at ZT1 in the condition “solitary males” ($P < 0.05$) and at ZT5 in the conditions “males with females” ($P < 0.001$), “antennae-ablated solitary males” ($P < 0.001$) and “antennae-ablated males with females” ($P < 0.01$) (Fig. 2b). In antennae, cyc expression reached a peak at ZT1 in the condition “solitary males” ($P < 0.001$) and a peak at ZT5 in the condition “males with females” ($P < 0.001$) (Fig. 2c). ANOVA showed a significant difference among ZTs in all conditions, and CircWave confirmed the rhythmic profile of cyc (Table 2).
cryptochrome 2 expression profile in tissues of *Ae. aegypti* males

The circadian expression profiles of *cry2* in heads, abdominal tips, and antennae are shown in Fig. 3. For this gene, expression profiles were more heterogeneous among tissues and conditions. In heads, *cry2* showed two expression peaks (ZT1 and ZT17) in the conditions “solitary males” (*P* < 0.001) and “antennae-ablated solitary males” (*P* < 0.001) (Figs. 3a). In the conditions “males with females” and “antennae-ablated males with females” *cry2* showed a single peak at ZT17 (*P* < 0.001) (Fig. 3a). In all conditions, ANOVA showed significant expression variation among ZTs, and CircWave confirmed the rhythmic profile of *cry2* (Table 3). A remarking and replicable

**Table 1** Statistical analysis of the gene period in tissues of *Ae. aegypti* males

| Conditions                  | Gene: tissue | period | Circwave | period | Circwave | period | Circwave |
|-----------------------------|--------------|--------|----------|--------|----------|--------|----------|
|                             | per—heads    |        |          | per—ab. tips |        | per— antennae |        |
|                             | ANOVA        | R²     |          | ANOVA  | R²       | ANOVA  | R²       |
| Solitary males              | F₅,₁₂ = 350.9 | 0.99167|          | F₅,₁₂ = 21.42 | 0.85767| F₅,₁₂ = 62.29 | 0.9584  |
|                            | *P* < 0.001  |        |          | *P* < 0.001 |        | *P* < 0.001 |        |
| Males w/females             | F₅,₁₂ = 138.5 | 0.96809|          | F₅,₁₂ = 28.36 | 0.89218| F₅,₁₂ = 26.46 | 0.89994 |
|                            | *P* < 0.001  |        |          | *P* < 0.001 |        | *P* < 0.001 |        |
| Antennae-ablated solitary males | F₅,₁₂ = 372.3 | 0.99312|          | F₅,₁₂ = 47.35 | 0.95106| F₅,₁₂ = 26.17 | 0.8784  |
|                            | *P* < 0.001  |        |          | *P* < 0.001 |        | *P* < 0.001 |        |
| Antennae-ablated males w/females | F₅,₁₂ = 277.5 | 0.99144|          | F₅,₁₂ = 26.17 | 0.8784 | F₅,₁₂ = 26.17 | 0.8784  |
|                            | *P* < 0.001  |        |          | *P* < 0.001 |        | *P* < 0.001 |        |
A difference in RNA abundances was found: in conditions where males were kept in the presence of females, the mRNA abundance of cry2 at ZT1 was lower than that observed for solitary males (Fig. 3a). The relative values at ZT1 were compared between solitary males and males with females with a Student t-test, which confirmed significant difference in heads of antennae-ablated males ($t = 4.360, P < 0.05$) and in antennae ($t = 3.074, P < 0.05$). In heads of non-ablated males the same trend was observed, though with no significant difference ($t = 2.157, P = 0.0972$).

In peripheral tissues, the patterns were not as consistent among conditions. In abdominal tips, the relative mRNA abundance of cry2 varied significantly for “solitary males” and the CircWave analysis confirmed rhythmicity (Table 3). In the condition “males with females” the patterns were not as consistent among conditions.
females” no significant differences between ZTs were found, and CircWave confirmed the arrhythmic profile (Table 3). Significant expression peaks at ZT1 ($P < 0.05$) and ZT13 ($P < 0.05$) were observed with ANOVA’s post-hoc Tukey in the condition “antennae-ablated solitary males” but CircWave revealed an arrhythmic profile (Table 3). In the condition “antennae-ablated males with females” cry2 presented a single expression peak at ZT17 ($P < 0.01$) (Fig. 3b), and CircWave showed a low value of $R^2$, though with significant rhythmicity (Table 3). In antennae, cry2 showed a significant expression variation among ZTs, with rhythmicity confirmed by CircWave (Table 3). A profile with one single peak
at ZT21 in the conditions “solitary males” ($P<0.001$) and “males with females” ($P<0.001$) was found (Fig. 3c).

Notably, as well as in heads, the RNA abundance of $cyc$ at ZT1 was relatively lower in “males with females” than in “solitary males” (Fig. 3c).

Discussion
In a precursor study, we measured the locomotor/flight activity of Ae. aegypti males’ mosquitoes in response to confined females of the same species. Diverse conditions were studied, among them males in the presence of females and males in the presence of wingless females. The results revealed that the males could notice the females even in the absence of wing-beat sounds and align their activity patterns with those of females [28]. Given that, we hypothesized that the changes in the activity patterns of males exposed to females could result from a difference in the RNA expression of circadian clock genes. Thus, we herein investigated the RNA circadian expression of genes period, involved in the first loop of the circadian clock described; cycle, whose protein forms the heterodimer CLK-CYC and activates the main clock genes’ expression; and cryptochrome 2, which was suggested to have a role in the temporal activity pattern that distinguishes nocturnal and diurnal mosquitoes [21].

Several species exhibit rhythmic fluctuations of a variety of behavioral and physiological processes. In the model species D. melanogaster, previous studies have demonstrated the influence of clock genes on reproductive behavior [47]. In one important example in the literature [31], the authors demonstrated that the circadian clock strongly controls the mating activity of this species, and experiments using individuals carrying per$^{01}$ mutation showed that their mating frequency was negatively affected [31]. In our results, the gene per showed a conserved expression profile in heads, antennae, and abdominal tips of Ae. aegypti males’ mosquitoes in all conditions studied, independently of females’ presence. We believe that, because per is involved in the central regulation of the circadian clock and showed consistent RNA expression profiles among tissues, its RNA expression may not be under the influence of social interaction represented by the proximity of a female, as studied here.

The expression profile of cyc in females’ heads was described by our group as exhibiting a rhythmic pattern, with a peak of RNA expression at ZT3 and a trough at ZT13 in the LD regime [21]. In the current study, the expression peak of cyc varied in males’ tissues between ZT1 and ZT5 according to the condition studied. It is noticeable that when females are present with non-ablated males, cyc seemed to shift its expression peak to a later phase (from ZT1 to ZT5), as seen in Fig. 2. More specifically, in ZT5 the mRNA expression is higher in heads of males when females are present ($t=3.523, P<0.05$), but not in antennae-ablated males with females ($t=1.236, P=0.284$). The pattern of expression in antennae visually corroborates with the results in heads of non-ablated males (Fig. 2c), although the difference was not significant ($t=1.999, P=0.1162$). The similar profiles suggest that cycle’s expression may be influenced by the presence of females when antennae are present mediating the communication. In Gentile et al. [21], cycle expression was studied in female heads every 2 h, and they found the peak of expression at ZT3, which curiously is between the varying ZT1 and ZT5 we found. Interestingly, in every sample analyzed in our and Gentile’s works, the trough of expression was very conserved, at ZT13, which indicates that one gene’s circadian functionality may also be associated with the trough.

On the other hand, the gene cry2 had its expression modified in male tissues depending on the condition studied. cry2 is believed to function as a transcriptional repressor [23, 24] and has been implicated in the regulation of insect behavior, including reproductive behavior [38]. The RNA expression profiles of cry2 were described in female heads of Ae. aegypti and Culex quinquefasciatus by our group [21]. It was found that the pattern of expression differed among the species: while in Cx. quinquefasciatus cry2 showed a single expression peak in the scotophase in Ae. aegypti cry2 showed a bimodal profile, with an expression peak in the photophase and another peak in the scotophase. The authors argued that the difference between the two species might be related to their contrasting activity phases since Ae. aegypti has diurnal habits, and Cx quinquefasciatus is a nocturnal species [21].

Here, we found that cry2 showed a bimodal profile in heads of solitary males, with and without antennae, but when males were exposed to females, cry2 was much less expressed at ZT1, and the first peak collapsed (Fig. 3). Thus, males with females showed a single expression peak of cry2 in heads. This intriguing effect may suggest that the first expression peak of cry2 is influenced by the social interaction between males and females. In antennae, cry2 cycled with only one expression peak in both conditions, and the reason for this could be that antennae are peripheral organs and may be under the regulation of peripheral clocks, presenting their own rhythmic oscillations [16]. In abdominal tips, although the rhythmicity of cry2 expression was not statistically supported in all conditions, a graphical analysis suggests similar curves between rhythmic and arrhythmic profiles, which indicates a trend in daily patterns of expression variation.

Notably, the ablation of males’ antennae, which are the organs responsible for olfactory and acoustic perception [41, 48], did not seem to affect the gene expression...
of cry2 since the expression profiles were consistent between conditions that diverged solely by the presence of antennae (Fig. 3a). The most important factor associated with changes in cry2 expression profile seems to be the social interaction given by the presence of females (Fig. 3a, c). In a previous study, we observed that antennae ablation dramatically reduces the amplitude of locomotor/flight activity of males and makes their activity profiles with and without females even [28]. This result is somewhat paradoxical: the antennae are considered the main perception organ in mosquitoes, and, in fact, our results show that males without antennae do not perceive females’ presence in a way that provokes a shift in their behavior [28]. However, changes are seen at the regulatory levels when females are present, with significantly different profiles of cry2 expression. Therefore, in the absence of males’ antennae, the perception of confined females must be made possible by a sense other than hearing and olfaction, possibly through vision or touch (through the tulle net), without affecting locomotor/flight activity.

In addition, the fact that the main difference in cry2 expression is a variation in the relative amplitude of RNA abundance at ZT1 (beginning of the photophase) suggests that the first expression peak may be more influenced by external factors. In fact, in another study of our group, analyses of cry2 expression in Ae. aegypti female heads, submitted to different cycles of temperature and photoperiod, also showed variation in the amplitude of the morning peak of cry2 [49]. The authors also argued that the first expression peak of cry2 may have a function similar to cry1 expression in the mating isolation of two sibling species of tephritid fruit flies [49, 50]. At the phenotypic level, males exposed to females also showed a lower amplitude in the first evening peak activity than in the second evening peak [28]. However, the association of the observed difference in cry2 expression with the patterns observed in the locomotor/flight activity is still premature. A functional study of cry2, accompanied by observations of its effects on social interaction or female recognition, could help to elucidate whether the two phenomena are associated.

Conclusions
The current study advances the understanding of clock genes’ association with male mosquitoes’ sensory perception of mates, a field where studies are still incipient. In fact, to our knowledge, this is a pioneer study of clock genes’ expression in Ae. aegypti male tissues and in the context of reproductive behavior. Here, we explored the social interaction related to recognition for mating, given by the perception of females by males, and its effects on the RNA expression levels of three of the major circadian clock genes. In parallel, we compared the results obtained here with the locomotor/flight activity described for Ae. aegypti male mosquitoes. Our results suggest that at least one gene, cry2, may be a player involved in a regulatory pathway associated with pre-copulation social interaction. We believe that further functional studies of cry2, focusing on social interaction driven to reproduction, could help elucidate whether this gene has a role in Ae. aegypti’s reproductive success.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s13071-022-05529-8.

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Author contributions
JRAB and LOA: conceptualization and design of study. JRAB: performance. JRAB, LOA and RB: formal analysis, writing, revision, and editing. JRAB: writing of original draft. LOA and RB: supervision. LOA and RB: funding acquisition. All authors contributed to the article and approved the submitted version. All authors read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All authors are in accordance with the submission of the current manuscript for publication.

Competing interests
I declare that the authors have no competing interests as defined by BMC or other interests that might be perceived to influence the results and/or discussion reported in this paper.

Author details
1Laboratorio de Biología Molecular de Insetos, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil. 2Instituto Nacional de Ciência E Tecnologia Em Entomologia Molecular (INCT-EM)/CNpq, Rio de Janeiro, Brazil.

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References
1. Powell JR. Mosquitoes on the move. Science. 2016;354:971–2. https://doi.org/10.1126/science.aai7171.

2. Clements AN. The biology of mosquitoes: development nutrition and reproduction. London: Chapman and Hall; 1992.

3. Kloveden MJ. The check is in the male: male mosquitoes affect female physiology and behavior. J Am Mosq Control Assoc. 1999;15:213–20.

4. Ferguson H, John B, Ng'habi K, Knols B. Redressing the sex imbalance in knowledge of vector biology. Trends Ecol Evol. 2005;20:202–9. https://doi.org/10.1016/j.tree.2005.02.003.

5. Diabaté A, Tripet F. Targeting male mosquito mating behaviour for malaria control. Parasites Vectors. 2015;8:347. https://doi.org/10.1186/s13071-015-0961-8.

6. Vančíková L, Canale A, Benelli G. Sexual chemooecology of mosquitoes (Diptera, Culicidae): current knowledge and implications for vector control programs. Parasitol Int. 2016;66:190–5. https://doi.org/10.1016/j.parint.2016.09.010.

7. Taylor B, Jones MDR. The circadian rhythm of flight activity in the mosquito Aedes aegypti (L.): the phase-setting effects of lights-on and light-off. J Exp Biol. 1969;51:59–70.

8. Patke A, Young MW, Axelrod S. Molecular mechanisms and physiological level regulation of post-transcriptional processes by the circadian clock. https://doi.org/10.1073/rmm.004206.

9. Parnell AA, De Nobrega AK, Lyons LC. Translating around the clock: Multidisciplinary advances. Nature. 1997;386:664. https://doi.org/10.1038/386664a0.

10. Hansson S. The mammalian circadian system: a hierarchical multi-oscillator structure for generating circadian rhythm. J Physiol. 2018;668:207–19. https://doi.org/10.1101/s12576-018-0597-5.

11. Ito C, Tomioka K. Heterogeneity of the peripheral circadian systems in natural populations of Lutzomyia longipalpis (Diptera: Psychodidae: Phlebotominae). Parasit Vectors. 2013;6:429. https://doi.org/10.1186/1756-3305-7-304.

12. Padilha RP, Resck ME, Cirino OATD, Teles-de-Freitas SS, Campos MHF, Sorgine MH, et al. Dengue infection increases the locomotor activity of Aedes aegypti females. PLoS ONE. 2011;6:e17690. https://doi.org/10.1371/journal.pone.0017690.

13. Lima-Camara TN, Lima JB, Lumay P, Castro MG, Lourenço-de-Oliveira R, Sorgine MH, et al. Zika infection decreases Aedes aegypti locomotor activity but does not influence egg production or viability. Mem Inst Oswaldo Cruz. 2018;113:e180290. https://doi.org/10.1590/0074-02761180290.

14. Arakawa AS, Bezerra JRA, Lisboa NC, Sorgine MH, et al. High-temperature exposure increases the locomotor activity of Aedes aegypti females. Parasit Vectors. 2018;11:254. https://doi.org/10.1186/s13071-018-3655-9.

15. Spiegel CN, Santos dos Dias DB, Araki AS, et al. The Lutzomyia longipalpis complex: a brief natural history of aggregation-sex pheromone communication. Parasites Vectors. 2016. https://doi.org/10.1186/s13071-016-1866-x.

16. Araki AS, Vigoder FM, Bauzer LGSR, Ferreira GEM, Souza NA, et al. Zika infection decreases Aedes aegypti locomotor activity but does not influence egg production or viability. Mem Inst Oswaldo Cruz. 2018;113:e180290. https://doi.org/10.1590/0074-02761180290.
37. Rivas GB, Bauzer LG, Meireles-Filho AC. “The environment is everything that isn’t me”: molecular mechanisms and evolutionary dynamics of insect clocks in variable surroundings. Front Physiol. 2016;12:400. https://doi.org/10.3389/fphys.2015.00400.

38. Xu J, Gao B, Shi MR, Yu H, Huang LY, Chen P, et al. Copulation exerts significant effects on mRNA expression of Cryptochrome Genes in a Moth. J Insect Sci. 2019;19:1–8. https://doi.org/10.1093/jisesa/ezr016.

39. Farnesi LC, Martins AJ, Valee D, Rezende GL. Embryonic development of Aedes aegypti (Diptera: Culicidae): influence of different constant temperatures. Mem Inst Oswaldo Cruz. 2009;104:124–6. https://doi.org/10.1590/S0074-02762009000100020.

40. Göpfert MC, Briegel H, Robert D. Mosquito hearing: sound-induced antennal vibrations in male and female Aedes aegypti. J Exp Biol. 1999;202:2727–38.

41. Gibson G, Warren B, Russell IJ. Humming in tune: sex and species recognition by mosquitoes on the wing. JARO. 2010;11:527–40. https://doi.org/10.1007/s10162-010-0243-2.

42. Teles-de-Freitas R, Rivas GB, Peixoto AA, Bruno RV. The summer is coming: nocte and timeless genes are influenced by temperature cycles and may affect Aedes aegypti locomotor activity. Front Physiol. 2020;11:614722. https://doi.org/10.3389/fphys.2020.614722.

43. Saltin BD, Matsumura Y, Reid A, Windmill JF, Gorb SN, Jackson JC. Material stiffness variation in mosquito antennae. J R Soc. 2019;16:20190049. https://doi.org/10.1098/rsif.2019.0049.

44. Gentile C, Lima JBP, Peixoto AA. Isolation of a fragment homologous to the rp49 constitutive gene of Drosophila in the Neotropical malaria vector Anopheles aquasalis (Diptera: Culicidae). Mem Inst Oswaldo Cruz. 2005;1000:545–7. https://doi.org/10.1590/S0074-02762005000600008.

45. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25:402–8. https://doi.org/10.1006/meth.2001.1262.

46. van der Spek R, Fliers E, la Fleur SE, Kalsbeek A. Daily gene expression rhythms in rat white adipose tissue do not differ between subcutaneous and intra-abdominal depots. Front Endocrinol. 2018;9:206. https://doi.org/10.3389/fendo.2018.00206.

47. Franco DL, Frenkel L, Ceriani MF. The underlying genetics of drosophila circadian behaviors. Physiology (Bethesda). 2018;33:50–62. https://doi.org/10.1152/physiol.00020.2017.

48. Guidobaldi F, May-Concha IU, Guerenstein PG. Morphology and physiology of the olfactory system of blood-feeding insects. J Physiol Paris. 2014;108:96–111. https://doi.org/10.1016/j.jphysparis.2014.04.006.

49. Rivas GB, Teles-de-Freitas R, Pavan MG, Lima JBP, Peixoto AA, Bruno RV. Effects of light and temperature on daily activity and clock gene expression in two mosquito disease vectors. J Biol Rhythms. 2018;33:272–88. https://doi.org/10.1177/0748730418772175.

50. An X, Tebo M, Song S, Frommer M, Kathryn AR. The cryptochrome (cry) Gene and a Mating Isolation Mechanism in Tephritid fruit flies. Genetics. 2004;168:2025–36. https://doi.org/10.1534/genetics.104.028399.

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