Abstract

Background: *Aedes aegypti* is the main vector of the virus causing Dengue fever, a disease that has increased dramatically in importance in recent decades, affecting many tropical and sub-tropical areas of the globe. It is known that viruses and other parasites can potentially alter vector behavior. We investigated whether infection with Dengue virus modifies the behavior of *Aedes aegypti* females with respect to their activity level.

Methods/Principal Findings: We carried out intrathoracic Dengue 2 virus (DENV-2) infections in *Aedes aegypti* females and recorded their locomotor activity behavior. We observed an increase of up to ~50% in the activity of infected mosquitoes compared to the uninfected controls.

Conclusions: Dengue infection alters mosquito locomotor activity behavior. We speculate that the higher levels of activity observed in infected *Aedes aegypti* females might involve the circadian clock. Further studies are needed to assess whether this behavioral change could have implications for the dynamics of Dengue virus transmission.

Introduction

Over the last decades, Dengue outbreaks have been a major public health concern in many parts of the World, where Dengue epidemics have been registered with a significant number of deaths [1]. There are four antigenically distinct RNA viruses that can cause the disease, and in Brazil, three Dengue serotypes (DENV-1; DENV-2 and DENV-3) have co-circulated in several areas and caused some severe Dengue epidemics [2].

*Aedes aegypti* (Diptera: Culicidae) is the urban vector of Yellow Fever and Dengue viruses. This diurnal mosquito is very anthropophilic and endophilic, being commonly found in urban and suburban areas, especially where house and human densities are high and where it seems to live longer and disperse to short distances (e.g. [3]–[5]).

It is known that parasites can alter vector behavior (reviewed by [6]–[8]) and a number of studies have reported behavioral changes in *Ae. aegypti* infected with pathogens and symbionts. For example, Rossignol et al [9] observed that *Ae. aegypti* females experimentally infected with an avian malaria parasite, *Plasmodium gallinaceum*, take more time to locate blood in guinea pigs than the uninfected ones. Rowland and Lindsay [10] studied the flight activity of females of this species infected with the filarial parasite *Brugia pahangi* and observed a decrease in flight capacity in heavily-infected mosquitoes under laboratory conditions. Recently, it has been shown that the infection by the symbiotic bacterium *Wolbachia* can also drastically alter this mosquito’s behavior and physiology [11]–[14].

In the current study, we investigated whether infection with Dengue virus causes changes in the locomotor activity behavior of *Ae. aegypti* females under laboratory conditions.

Methods

Infection of Mosquitoes with the Dengue virus

The Paea strain of *Ae. aegypti* was used in all infection experiments. This laboratory strain is known to be highly susceptible to Dengue virus serotype 2 (DENV-2) infection [15]. Mosquito colony was reared according to procedures described in [16]. Males and females remained together and were fed with 15% sucrose solution since emergence.

Five-day-old female mosquitoes were individually infected by intrathoracic inoculation with 0.21 µl of L-15 (Lelovitz) Medium containing Dengue virus (DENV-2 strain FIOCRUZ-66985 [17]) in a concentration of 10⁷ PFU using a Nanoject microinjector (Drummond Scientific). Control mosquitoes were
inoculated with 0.21 μl of only L-15 (Leibovitz) Medium.

To verify the success of the experimental infections, the heads of a number of mosquitoes that were inoculated with virus and that were alive by the end of the locomotor activity experiments (around 8–10 days after inoculation), plus negative controls, were tested for Dengue infection via RT-PCR, as described below. The results indicated that over 95% (70/73) of the mosquitoes inoculated with the Dengue virus were infected (data not shown).

**Detection of Dengue virus in mosquitoes**

Mosquito heads were separated from bodies on a metal plate placed on dry ice and viral RNA was extracted from each head using the QiAmp Viral Mini Kit (Qiagen) according to the manufacturer’s protocol. RT-PCR for detecting DENV2 was conducted in a PCR System 9700 GeneAmp (Applied Biosystems) following the manufacturer’s protocol. RT-PCR for detecting DENV2 was conducted in a PCR System 9700 GeneAmp (Applied Biosystems) according to the manufacturer’s protocol. RT-PCR for detecting DENV2 was conducted in a PCR System 9700 GeneAmp (Applied Biosystems) using SuperScript™ One-Step RT-PCR with Platinum® Taq (Invitrogen) and Dengue virus consensus primers D1 and D2 [18], followed by a semi-nested PCR on the resulting product using GoTaq Green Master Mix (Promega) and primers D1 and TS2 [18]. PCR products were electrophoresed on 2.5% agarose gels. A band of 119 pb corresponding to DENV-2 could be seen under UV light in the infected mosquitoes.

**Analysis of the locomotor activity behavior**

The activity of infected and uninfected control *Ae. aegypti* females was recorded using a larger version of the Drosophila Activity Monitor (TriKinetics) as described in [19]. After inoculation with Dengue virus or L-15 medium, females were individually placed in glass tubes (1 cm × 7 cm) with a cotton plug soaked in 15% sucrose solution and these tubes placed in the Activity Monitor inside a glass tubes (1 cm × 7 cm) with a cotton plug soaked in 15% sucrose solution and these tubes placed in the Activity Monitor inside a Precision Scientific Incubator Model 818 under a constant temperature of 25°C and a photoperiod of 12 hours of light and 12 hours of dark (LD 12:12). For each mosquito, the total locomotor activity of 1 hour-intervals was recorded for about seven days after inoculation. The data of individuals that died during the experiments were excluded, and the data analysis was carried out comparing the activity data of infected and uninfected mosquitoes from the second to sixth day after inoculation.

**Results**

Table 1 shows the mean hourly locomotor activity of control and Dengue virus infected females in four different experiments. We observed that infected females of *Ae. aegypti* showed more activity than controls in all experiments. The relative increase in activity ranged from ~10% to more than 50%. A two-way ANOVA indicated a significant difference in the activity between infected and uninfected control females (p<0.01). Although a significant difference in the overall activity levels was also observed between experiments (p<0.01), the interaction between experiments and infection was not significant (p = 0.82) indicating that the difference between infected and uninfected females was consistent.

Figure 1 shows the normalized locomotor activity patterns of infected and control females during a full LD 12:12 cycle (Fig. 1A) or during the photophase (Fig. 1B). As previously reported in the literature (reviewed in [20]), *Ae. aegypti* is a diurnal species showing higher activity levels towards the end of the photophase (“late afternoon”) and a characteristic startle response to the abrupt light-on/light-off transition [21]. The comparison of the normalized locomotor activity patterns of infected and uninfected females shows that Dengue infection causes an increase in activity throughout the 24 hour period. Although this effect is most dramatic during the light-on/light-off transition (Fig. 1A), an increase in activity is also seen throughout the day and night in infected females, especially during the “natural” activity peak occurring around ZT 9 and in the last hours of the photophase (Fig. 1B). In fact, this increase in activity associated with Dengue infection is still significant (p = 0.012) even when we exclude the light-on/light-off transition (Table 1). In summary, our results indicate that the locomotor activity of infected females is consistently increased when compared to that of uninfected females.

**Discussion**

Very little is known about the effects of viral infection on *Aedes* mosquitoes. Several authors have shown that Dengue virus exhibits

| Experiment | Status   | N  | Mean activity per hour* (± SEM) | Relative increase of activity in infected mosquitoes (%) |
|------------|----------|----|-------------------------------|-------------------------------------------------------|
| 1          | Control  | 17 | 5.06 ± 1.16 (4.73 ± 1.15)     | 52.6 (48.0)                                           |
|            | Infected | 23 | 7.72 ± 1.27 (7.00 ± 1.26)     |                                                        |
| 2          | Control  | 53 | 11.22 ± 1.45 (10.52 ± 1.49)   | 30.8 (30.5)                                           |
|            | Infected | 74 | 14.68 ± 1.50 (13.73 ± 1.49)   |                                                        |
| 3          | Control  | 45 | 9.84 ± 1.19 (8.97 ± 1.15)     | 43.2 (45.3)                                           |
|            | Infected | 66 | 14.09 ± 1.24 (13.03 ± 1.23)   | (13.03 ± 1.23)                                        |
| 4          | Control  | 70 | 11.88 ± 1.01 (10.95 ± 1.00)   | 13.3 (10.3)                                           |
|            | Infected | 83 | 13.46 ± 1.13 (12.39 ± 1.06)   |                                                        |

*The numbers in parenthesis refer to the activity excluding the light-on/light-off transition.

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a remarkable tropism for the mosquito nervous tissues. Linthicum et al [22] studied the tropism of DENV-3 in parenterally infected female *Aedes aegypti* mosquitoes using immunocytochemical methods and observed that the nervous tissues were among the first tissues to be infected. In fact, these authors suggested that the nervous system is the primary site of virus amplification in mosquitoes infected using this method [22]. Several years later, Salazar et al [23] corroborated these findings by showing that in mosquitoes orally infected with DENV-2, the nervous tissues are among the first to be infected, presenting detectable levels of viral antigens 5 days after an infective blood meal. Interestingly, these authors also showed that heads and salivary glands were the only tissues where viral antigens continued to accumulate throughout the 21 days observed in their study. All other mosquito infected tissues presented a decrease in the infection rate.

This remarkable tropism of Dengue virus for the insect nervous tissues led us to hypothesize that the infection might have some role in modulating the vector locomotor activity behavior, since it is known that activity rhythms in *Drosophila* and other Diptera are regulated by circadian clock neurons in the brain [reviewed in [24],[25]]. In fact, our results show that although the daily activity patterns of DENV-2 infected and uninfected mosquitoes are similar, the total level of activity is clearly increased upon infection. This increase is most evident in the light-on/light-off transition (Fig. 1A), an observation that is particularly interesting considering that the visual system is also highly infected [22],[23]. However, it is important to mention that this effect is also clearly detected in the “natural” activity peak occurring during the last hours of the photophase (Fig. 1B), which is under circadian control [20],[21], indicating that a similar effect is likely to occur in nature.
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