ABSTRACT: The extrinsic incubation period is a critical component in the assessment of arboviral transmission potential. It defines the time it takes for a mosquito to become infectious following exposure to an arbovirus. Since this is a temporal process, the lifespan of a mosquito is intimately tied to the extrinsic incubation period and thus transmission potential of these viruses. Temperature is a known effector of both vector competence (the ability of a vector to transmit a pathogen) and mosquito mortality, but the interaction among temperature, vector competence, and mosquito mortality is not well characterized. Herein, we investigate this interaction for dengue virus, serotype 2, and its primary vector Aedes aegypti where we found that at 30 °C, infection and/or dissemination shortened the average lifespan of the mosquito and that when considering only mosquitoes with a disseminated infection, those incubated at 26 °C lived significantly longer.

KEYWORDS: A. aegypti, dengue, extrinsic incubation period, mortality, temperature, survival

Introduction

Global expansion of dengue virus (DENV) has lead to endemicity in several regions, including Central and South America.¹,² Each year, returning travelers from these and other locations introduce the virus into a largely naive population in the United States.³ With the exception of self-limited chains of local transmission in Florida, there has yet to be a sustained outbreak in the US.⁴-⁹ However, the threat remains as global travel, trade, and transport increase.¹ To establish local transmission when virus is introduced, competent mosquito vectors need to be present. The primary vector, Aedes aegypti, is present year-round throughout the southern US, including seasonally in subtropical and more temperate areas.⁹

Vector competence is the ability of a mosquito population to become infected with and ultimately transmit an arbovirus. The vector competence of DENV in A. aegypti is known to be affected by both intrinsic and extrinsic factors, such as viral strain and temperature/humidity, respectively.¹⁰⁻¹⁵ The influence of temperature on vector competence is multifaceted and influences the rate of dissemination through the mosquito to the salivary glands, as well as the lifespan of the mosquito.¹⁴

The extrinsic incubation period (EIP) is an important epidemiological measure and is defined as the time it takes for a virus to disseminate through a mosquito from the midgut where virus enters the mosquito after blood feeding to the salivary glands where it is then expectorated upon subsequent feeding. The EIP, while often reported as an average, discrete value, is a continuous process and is therefore tied to the mortality rate of a mosquito. If, for example, a specific strain of virus takes seven days (on average) to disseminate through a mosquito vector, the lifespan of that mosquito will determine how many days of infectiousness results from exposure (Fig. 1). In a mosquito that is known to have multiple blood meals per gonotrophic cycle, such as A. aegypti, this is especially relevant as it results in a much higher potential for transmission through an increased rate of human contact. Temperature, as a driver of vector competence, EIP, and mortality, is a critical environmental factor when considering transmission dynamics of arboviruses. In addition, previous studies have observed altered protein expression in salivary glands of mosquitoes with disseminated DENV2 infections, including metabolic proteins,¹⁵ which may indicate that there is some alteration in fitness to mosquitoes with a disseminated infection. To that end, we explored the interaction among temperature, infection/dissemination status, and mosquito mortality. Specifically, we investigated whether mosquitoes had different likelihoods of survival depending on their infection and/or dissemination status and the temperature to which they were exposed during the EIP.
Materials and Methods

A. aegypti mosquitoes (Rockefeller strain) from the Louisiana State University (LSU) colony were reared at a constant temperature of 28 °C with approximately 85% humidity and a 16:8 light/dark cycle. Pupae were separated into batches of 100 per carton and allowed to emerge. Three to five days postemergence, cartons of adult mosquitoes were exposed to a blood meal containing a 1:2 mixture of either uninfected cell culture supernatant and blood or infected cell culture and blood. Titters of virus were matched prior to feeding (10^5 pfu/mL). The virus used was DENV2, strain 1232 was originally isolated from a patient in Indonesia.

For each experimental group, an environmental chamber was programmed to a constant extrinsic incubation temperature (EIT) of 26 °C, 28 °C, or 30 °C with all other parameters as above. Six cartons each of unexposed mosquitoes and DENV2 exposed mosquitoes were placed in an environmental chamber and incubated at constant temperature for the duration of the EIP until the study concluded at 22 days postexposure. Total sample sizes per group are listed in Table 1.

Each day, mosquitoes that had died within the previous 24 hours were removed from the carton and the day postexposure was recorded. Each mosquito was processed for the detection of DENV2 RNA in the bodies and legs, as infected legs are an indication of a disseminated infection and has previously been used as a proxy for vector competence. Mosquitoes were grouped as follows: unexposed referring to mosquitoes receiving a blood meal with no virus present; infected referring to mosquitoes that were exposed to DENV2 and developed an infection in the abdomens at the time of death; and disseminated referring to mosquitoes that were exposed and developed a disseminated infection in the legs. At the end of the study (22 days postexposure), mosquitoes that were still alive were killed via flash freezing and processed as described in Ref. 20.

For the purposes of the survival analysis, these mosquitoes were coded in the dataset as right-censored observations, as they did not experience the event (death) during the study period.

A Kaplan–Meier nonparametric survival analysis was performed using PROC LIFETEST in SAS (version 9.4) to determine whether time to death among groups was significantly different. These survival analyses determine the significance among the entirety of the survival function. We set the type I error rate at \( \alpha = 0.05 \) for pairwise comparisons, and applied a Bonferroni adjustment to the type I error rate to account for multiple comparisons wherever appropriate. We first analyzed only the unexposed group to determine whether there was a significant effect of temperature on mosquito survival in the absence of infection. This required a Bonferroni adjustment to the significance level of \( \alpha/3 = 0.0167 \) to account for three pairwise comparisons across three temperatures. Next, we performed pairwise comparisons of unexposed mosquitoes to infected mosquitoes at each temperature, followed by pairwise comparisons of unexposed mosquitoes to mosquitoes that developed a disseminated infection at each temperature. Finally, we compared survival of infected mosquitoes at each temperature and that of disseminated mosquitoes at each temperature. These two tests also required the same Bonferroni adjustment to the significance level as described above.

Results

There was no significant effect of temperature when we compared mortality among mosquitoes in the unexposed group (\( P < 0.05 \)). Survival curves are depicted in Figure 2, and the average times to death for this and the other groups are given in Table 2. There was also no significant difference when time to death was compared between unexposed mosquitoes and either (1) infected mosquitoes or (2) disseminated mosquitoes at 26 °C and 28 °C. At 30 °C, there was a significant difference between the survival of unexposed mosquitoes and infected mosquitoes, with average times to death of 15.09 and 8.60 days (post exposure), respectively (Fig. 3A). When we then compared unexposed mosquitoes to mosquitoes that had developed a disseminated infection, we also observed a shorter lifespan in those mosquitoes with disseminated DENV2 infections at 30 °C by about half a day (Fig. 3B). Though these differences are small for both infected and disseminated comparisons with unexposed mosquitoes, they are significant (\( P \)-values = 0.0078 and 0.0219, respectively).

![Figure 1. Schematic demonstration of the impact of mosquito mortality on the cumulative transmission potential of an arbovirus.](image)
We then compared differences in survival for infected individuals only across the three temperatures and found a significant difference among the three ($P$-value = 0.002, adjusted $\alpha$ = 0.0167). Interestingly, the difference was observed between those kept at 26 °C and 30 °C (pairwise comparison $P$-value = 0.0004, adjusted $\alpha$ = 0.0167), but no difference was observed between the 28 °C and 30 °C or 26 °C and 28 °C (Fig. 4A). The same pattern was observed when we analyzed the survival of mosquitoes with disseminated infections across the three temperatures ($P$-value = 0.0029, adjusted $\alpha$ = 0.0167), with significance only between the 26 °C and 30 °C temperatures (pairwise $P$-value = 0.0014, adjusted $\alpha$ = 0.0167; Fig. 4B).

**Discussion**

Vector competence is an important factor in the emergence, expansion, and persistence of arboviruses, but this static quantity does not often capture the whole picture.\(^{16}\) Quantification and assessment of relative differences in vector competence is incomplete without accounting for variation in temporality, which is in turn affected by factors such as temperature.\(^{21}\) As the global temperature trends toward warming, there will likely be two (very general) impacts on tropical mosquito vectors. First, indigenous species may experience prolonged exposure to higher temperatures as well as exposure to more extreme temperatures. Second, the geographical range of these vectors will expand into more temperate regions, albeit only seasonally in some areas.\(^{22}\)

Our results suggest that infection and dissemination may alter the long-term mortality rate of mosquitoes infected with DENV2 at higher temperatures, though others have shown – and our data do not dispute – that the short-term mortality of mosquitoes was not affected by infection and our result (Table 2).\(^{21}\) Our results indicate that there is a fitness cost associated with infection and dissemination at higher temperatures. The additional finding that, at a lower EIT, the lifespan of a mosquito is longer – coupled by the fact that viral dissemination is slower at lower temperatures – may also suggest a trade-off of viral efficiency and potential fitness cost (ie, differential mortality) for mosquitoes.

While temperature is a known effector of the rate of arboviral dissemination, our study demonstrates the nonlinearity of the relationship between mortality and virus replication.\(^{14,24–26}\) However, the differences observed in our study still do not offer a clear interpretation of this interaction as driven by changes in EIT. Thus, the interplay between these two processes should be further characterized, as our data suggest different likelihoods of survival depending on infection and/or dissemination of the vectors and the temperature to which they are exposed during EIT. This study offers

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**Table 2.** Estimates of the average time for unexposed mosquitoes, infected mosquitoes, and mosquitoes with a disseminated infection at each temperature.

| TEMP | GROUP       | TIME TO DEATH | STD. ERR OF MEAN |
|------|-------------|---------------|------------------|
| 26   | Unexposed   | 15.30         | 0.63             |
| 26   | Infected    | 15.73         | 1.71             |
| 26   | Disseminated| 18.60         | 0.076            |
| 28   | Unexposed   | 15.83         | 0.44             |
| 28   | Infected    | 11.64         | 1.86             |
| 28   | Disseminated| 16.39         | 0.7              |
| 30   | Unexposed   | 15.09         | 0.41             |
| 30   | Infected    | 8.60          | 1.94             |
| 30   | Disseminated| 14.51         | 0.51             |

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**Figure 2.** Survival curve of mosquitoes that were blood fed but not exposed to any virus and then kept at three difference temperatures (26 °C – red, 28 °C – green, 30 °C – blue) during the period correlating with the EiP of the treatment groups.
Figure 3. Survival curves for comparisons of (A) unexposed to infected mosquitoes at 30 °C and (B) unexposed to mosquitoes with a disseminated infection were significantly different.

Figure 4. Survival curves for comparisons of (A) infected mosquitoes across all three temperatures and (B) mosquitoes with a disseminated infection across all three temperatures. Significant differences were found only between 26 °C (red) and 30 °C (blue) in both cases.
insight into the combined processes of mortality and infection dynamics within the mosquito. Such information can not only offer insights into altered transmission patterns due to climate change and warming but also lead to more specific parameter development for mathematical models that look to predict expansion or emergence of future public health threats, such as dengue.

Author Contributions
Conceived and designed the experiments: RCC, CNM. Analyzed the data: RCC. Wrote the first draft of the manuscript: RCC. Contributed to the writing of the manuscript: RCC, CNM. Agree with manuscript results and conclusions: RCC, CNM. Jointly developed the structure and arguments for the paper: RCC, CNM. Made critical revisions and approved final version: RCC. Both authors reviewed and approved of the final manuscript.

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