The Effect of Temperature on *Wolbachia*-Mediated Dengue Virus Blocking in *Aedes aegypti*

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Abstract. Dengue fever, caused by dengue virus (DENV), is endemic in more than 100 countries. The lack of effective treatment of patients and the suboptimal efficacies of the tetravalent vaccine in trials highlight the urgent need to develop alternative strategies to lessen the burden of dengue fever. *Wolbachia pipientis*, an obligate intracellular bacterium, is being developed as a biocontrol strategy against dengue because it limits the replication of the DENV in the mosquito vector, *Aedes aegypti*. However, several recent studies have demonstrated the sensitivity of pathogens, vectors, and their symbionts to temperature. To understand how the tripartite interactions between the mosquito, DENV, and *Wolbachia* may change under different temperature regimes, we assessed the vector competence and transmission potential of DENV-infected mosquitoes reared at a common laboratory setting of a constant 25°C and at two diurnal temperature settings with mean of 25°C and 28°C and a fluctuating range of 8°C (±4°C). Temperature significantly affected DENV infection rate in the mosquitoes. Furthermore, temperature significantly influenced the proportion of mosquitoes that achieved transmission potential as measured by the presence of virus in the saliva. Regardless of the temperature regimes, *Wolbachia* significantly and efficiently reduced the proportion of mosquitoes achieving infection and transmission potential across all the temperature regimes studied. This work reinforces the robustness of the *Wolbachia* biocontrol strategy to field conditions in Cairns, Australia, and suggests that similar studies are required for local mosquito genotypes and field relevant temperatures for emerging field release sites globally.

INTRODUCTION

An estimated 390 million people throughout tropical and subtropical regions of the globe contract dengue fever each year.[1] The symptoms of clinically apparent cases of human infection range from mild to severe fever with some cases leading to fatal dengue shock syndrome.[2–5] The ribonucleic acid (RNA) virus that causes the disease is vectored between humans mainly by the mosquito, *Aedes aegypti* and *Aedes albopictus*. Viruses such as dengue, West Nile, and yellow fever and other members of the *Flavivirus* genus have collectively become the most widespread arthropod-borne viruses affecting humans today.[6] The lack of specific medical treatment of patients, the suboptimal performance of dengue vaccines in recent phase IIb and phase III trials, and rising rates of insecticide resistance in mosquito populations has highlighted the pressing need for alternative strategies to lessen the dengue burden.[7–9] One of the most promising strategies in development is the use of the obligate endosymbiont *Wolbachia pipientis* to render *Ae. aegypti*, which does not naturally carry *Wolbachia*, incapable or less efficient at carrying the virus.[8]

Wolbachia is a maternally inherited intracellular bacterium that infects upwards of ~40% of insect species.[10] *Wolbachia* manipulates its host reproduction for its own benefit and this serves as a driver for invading host populations.[11] Additionally, the presence of *Wolbachia* reduces the replication of viruses in the host[12,13] by competing for host resources critical for viruses[14] and manipulating the host viral defense pathways such as the microRNA pathway.[15] This antiviral property is the cornerstone of an initiative to use *Wolbachia* as a biocontrol agent against dengue virus (DENV) in the mosquito, *Ae. aegypti*.[16–19] In controlled field releases in Cairns, Australia, the *w*Mel strain of *Wolbachia* has successfully invaded natural populations of *Ae. aegypti* and infection frequencies have reached near-fixation a few months after the cessation of releases.[20] More than 2 years later, the *Wolbachia* infection rate in the mosquito population remains high[21] and *Wolbachia*-infected mosquitoes from these field populations continue to demonstrate reduced susceptibility to DENV under laboratory conditions.[22] To assess the capacity of *w*Mel-infected (*w*Mel.F) mosquitoes to lower the transmission of the virus in a dengue endemic region, field-release trials are currently been carried out in Vietnam and Indonesia.[8]

Vectorial capacity is a measure of a vector-borne disease's transmission potential among humans. It is determined by a range of parameters including the contact rate between vector and human, the vector biting rate, daily survival of the vector, vector competence (VC), and the extrinsic incubation period (EIP).[23,24] VC or the ability of the mosquito to carry a parasite is known to be influenced by mosquito genetics,[25] virus genotype,[26] mosquito by virus interactions,[27] and mosquito gut microbiota.[28] Furthermore, additional factors such as larval competition,[29] nutrition,[30] and temperature[31] can influence the outcome of infection. Temperature in particular can influence how efficiently the host becomes infected with a parasite and how readily the parasite is transmitted by the host.[32] For example, changes in temperature affect the length of the EIP[33] or the time lag between when a mosquito consumes an infectious blood meal to when it is capable of secreting DENV in its saliva.[34] EIP, relative to the other components of vectorial capacity, has the largest impact on rates of virus transmission as the earlier virus arrives in the saliva, the more humans the mosquito can potentially infect.[24] This is particularly the case for *Ae. aegypti* that may seek blood meals every few days.[35] Lastly, the diurnal temperature range (DTR), which emulates the degree or amplitude of daily cyclic temperature, was found to significantly influence the outcome of infection and survival of the mosquitoes, but not the EIP of DENV in *Ae. aegypti* as compared with a constant temperature.[36,37] This relationship may be broadly applicable for other vector–virus interactions, with evidence of a similar effect on *Plasmodium* in *Anopheles*.[38]

The effects of changing temperature are not restricted to the vector. *Wolbachia* densities can also be affected by temperature,[39] and DENV undergoes conformational changes

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under different temperatures.[40] Such sensitivity to environmental temperature raises the possibility that the *Wolbachia*-mediated DENV-blocking phenotype is likely to be influenced by temperature.[41] Indeed, various constant temperature regimes have been shown to affect the strength of *Plasmodium* inhibition in somatically *Wolbachia* transinfected *Anopheles* mosquitoes.[42] However, previous knowledge gained on *Wolbachia*-mediated DENV blocking at constant temperatures, especially the most commonly used laboratory setting of 25°C, reveals very little on how the DENV blocking phenotype may vary in a natural environment. For example, mean daily temperature in Cairns, where dengue outbreaks occur in Australia,[43] can range from 21°C to 28°C throughout the year with a DTR reaching 8°C (±4°C).[44]

To understand how the interactions between mosquitoes, DENV and *Wolbachia* may change in terms of different temperature regimes, we assessed the effects of both constant and diurnal temperature around a mean of 25°C on *Wolbachia*-mediated DENV blocking in *w*Mel.F mosquitoes recaptured from the *Wolbachia* field release sites in Cairns. To simulate the warmer weather during the dengue outbreak seasons in this region, we further studied the effects of a DTR with a mean of 28°C. We demonstrated that temperature not only changes VC of the mosquitoes and DENV blocking, it also alters *Wolbachia* density in mosquitoes. *Wolbachia*-infected mosquitoes continued to strongly limit the replication of DENV, however, significantly reducing the proportion of mosquitoes achieving infection and transmission potential. This reinforces the likely robustness of *Wolbachia*-based biocontrol strategy to field condition variability temperatures in Cairns and affirms that experimental work carried out at 25°C may be taken as representative.

MATERIALS AND METHODS

Ethical considerations. Blood feeding of mosquitoes with human volunteers was performed in accordance to Human Research Ethics Committee of Monash University (permit CF11/0766-2011000387). The volunteers gave written informed consent prior to taking part in the study.

Temperature regimes. Three temperature regimes were used in this study: a constant temperature of 25°C (25°C ± 0°C), a fluctuating range with a mean of 25°C and a DTR of 8°C (25°C ± 4°C), and a fluctuating range with a mean of 28°C with a DTR of 8°C (28°C ± 4°C). Fluctuating temperature regimes followed a sinusoidal progression during the day and a negative exponential decrease at night. The maximum and minimum temperatures were reached at 14:00 and 2:00, respectively. After oral infection of DENV, experimental mosquitoes were housed in MLR-352H-PE incubators (Panasonic Australia, New South Wales, Australia) that maintained the respective temperature regimes at ~70% relative humidity, with 12:12 hours light : dark cycle. One fluorescent light was scheduled to turn on at 08:00 and off at 20:00. Temperature and humidity data loggers (EL-USB-2; Lascar Electronics, Salisbury, United Kingdom) recorded temperatures on half an hourly basis in the incubators. Actual air temperatures within the incubators matched the programmed temperature profile.

Mosquitoes. The Wildtype (WT) mosquito colony (not infected with *Wolbachia*) was established from eggs routinely collected from ovitraps from neighboring suburbs outside of the *Wolbachia* field-release populations in Cairns, Australia. The *w*Mel.F population was founded by mosquito eggs collected once from within the *Wolbachia* release areas in Cairns, Australia. *Ae. aegypti* species identification and *Wolbachia* screening at the point of collection was as previously described.[20] All experimental work was carried out within four generations of field collection for the WT colony to limit the effects of inbreeding on genetic diversity. To prevent genetic drift between the two lines while maintaining *Wolbachia* infection, 20% of the males in the *w*Mel.F line were replaced with WT males each generation. After hatching, mosquito larvae were reared at a constant 25°C, ~70% relative humidity, with 12:12 hours light : dark cycle, at a density of 150 individuals/3 L of distilled water in plastic trays (30 × 40 × 8 cm) and fed fish food (Tetramin Tropical Tablets; Tetra, Melle, Germany) until pupation. Pupae were transferred to 30 × 30 × 30 cm cages to allow adult emergence at a density of approximately 400 individuals per cage. Adults were fed a 10% sucrose diet *ad libitum*.[45]

Virus. A dengue virus serotype 3 (DENV-3) strain was used that was originally isolated from a patient diagnosed with dengue in the 2008/2009 outbreak in Cairns. This strain caused one of the largest outbreaks in recorded history (>900 cases) in far north Queensland, Australia.[46] Virus was passaged, grown, and collected fresh from cell culture as previously described.[47] The freezing and thawing of viruses reduces infectivity[48] and therefore we used freshly propagated virus to achieve the greatest possible infection rate in the mosquitoes.

DENV infection. Five- to 8-day-old female *Ae. aegypti* were starved for 24 hours and then provided defibrinated sheep blood containing live DENV to a final concentration of 3×10^6 plaque-forming units/mL as determined by plaque assay.[45] The virus blood meal was held over a water-jacketed membrane-feeding apparatus by a piece of desalted pig intestine. The feeding apparatus was maintained at 37°C, and mosquitoes were allowed to feed for 3 hours. Previous research by our group demonstrated the stability of virus over this period.[45] Unfed mosquitoes were removed from populations 24 hours later under carbon dioxide (CO_2). Fed mosquitoes were then used for VC, transmission, and survival assays under their respective temperature regimes.

VC and *Wolbachia* density. The head and carcass (body) of the mosquito 7 days postinfection (DPI) were used to measure the rate of DENV dissemination and infection, respectively. Head and carcass samples were homogenized using 2-mm-diameter glass beads (Merck KGaA, Darmstadt, Germany) in a mini-Beadbeater (BioSpec Products, Bartlesville, OK). Multiplex quantitative polymerase chain reaction assay was performed on a LightCycler 480 II machine using 4×TaqMan Fast Virus 1-Step Master Mix (Life Technologies Australia, New South Wales, Australia) according to the manufacturer's instructions. DENV concentrations in the samples were extrapolated from a standard curve of known DENV copy number. The data were expressed as concentration of virus per tissue by back calculating to the initial concentration of RNA.[47,49] Quantification of *Wolbachia* density used *Ae. aegypti* ribosomal protein S17 as the reference gene. *Wolbachia* strain *w*Mel was detected with primers/probes specific to the *WD0513* gene as previously published.[50]

Transmission potential. To estimate the transmission potential of the mosquitoes, saliva was collected from individual mosquitoes as modified from a previously established method.[45]

In brief, individual mosquitoes were lightly anesthetized with CO_2 and placed inside a 70-mL polypropylene cup (Sarstedt, Nümbrecht, Germany) capped by a piece of 100% polyester mesh (Spotlight Pty Ltd., Victoria, Australia). The lid of a 2-mL polypropylene screw-cap tube (Sarstedt) was affixed to the bottom of the inside of the cup using adhesive plasticine (Bostik, Thomastown, Vic, Australia). The lid was then filled with 200 μL of 10% sucrose. The sucrose solution provided was the only source of food and fluid for the mosquito. The sucrose solution was collected every 2 days from 4 to 20 DPI to test for the presence of virus expectorated during feeding. Any mosquito death was recorded before each saliva collection. During each collection, the mosquito was lightly anesthetized using CO_2 so that the bottom half of the 2-mL tube could be screwed onto the lid containing the sucrose solution and removed. A new lid was then provided containing fresh sucrose solution. DENV was detected in the collection tube as previously described.[45]

Data analysis. Binary phenotypes (infection, dissemination, and infectivity) were analyzed using nominal logistic regression fitted with a binomial error distribution.

For the continuous phenotype (DENV titer in head and carcass), the dependent variable was log-transformed and analyzed using a linear regression model fitted with a normal error distribution. EIP and *Wolbachia* density data were analyzed using a general linear model fitted with an identity link function and normally distributed errors. The number of days DENV was detectable in mosquito saliva was analyzed using Mann–Whitney U tests due to its deviation from normality. Survival data were analyzed using log-rank statistics. Statistical analyses were performed using the software Statistica 8.0 (StatSoft, Inc., Tulsa, OK).

RESULTS

Temperature and *Wolbachia* influence DENV infection rate in mosquitoes. We used DENV infection of the carcass (body) as a measure of infection and presence of DENV in the head as an indication of dissemination. Only mosquitoes with carcass infection were used to calculate rate of dissemination. Temperature had a significant effect on DENV infection rate in the mosquito carcass ($F = 6.8$, degrees of freedom [df] = 2, $P < 0.01$; Figure 1B and Table 1). Mosquitoes reared at a constant temperature of 25°C (25°C ± 0°C) had a higher dissemination rate ($F = 7.5$, df = 1, $P < 0.01$) as compared with those reared at a diurnal temperature around a mean temperature of 25°C (25°C ± 4°C). Raising the diurnal temperature mean from 25°C to 28°C significantly ($F = 11.4$, df = 1, $P < 0.01$) increased infection rate from 72% to 86% and from 5% to 23% in WT and *w*Mel.F, respectively. Of the mosquitoes that had a carcass infection, temperature had a significant effect on DENV dissemination rate to the mosquito head ($F = 4.3$, df = 2, $P < 0.05$; Figure 1 and Table 1). However, we did not find a significant change ($F = 0.35$, df = 1, $P = 0.56$) in dissemination rate when mosquitoes were reared at a constant temperature of 25°C (25°C ± 0°C) as compared with those reared at a diurnal temperature around a mean temperature of 25°C (25°C ± 4°C). Increasing the diurnal temperature mean from 25°C to 28°C also did not significantly ($F = 3.3$, df = 1, $P = 0.21$) change the dissemination rate of the mosquitoes.

Regardless of the temperature regimes, *Wolbachia* was able to significantly reduce the rate of DENV infection in the mosquitoes ($F = 242$, df = 1, $P < 0.0001$; Table 1) but did not significantly reduce the dissemination rate of mosquitoes that already had carcass infection ($F = 1.2$, df = 1, $P = 0.27$). Temperature did not have a significant effect on *Wolbachia*-mediated reduction in infection rate (temperature × *Wolbachia* effect, $F = 0.35$, df = 1, $P = 0.71$; Table 1) or dissemination rate ($F = 2.2$, df = 1, $P = 0.11$).

Of the mosquitoes that achieved dissemination, temperature had little effect on the titer of DENV in the head ($F = 2.2$, df = 2, $P = 0.11$; Figure 1C) or the carcass ($F = 0.79$, df = 2, $P = 0.45$; Figure 1D) of the mosquitoes. *Wolbachia* significantly lowered the DENV titer in the carcass ($F = 35$, df = 1, $P < 0.0001$) but not in the head ($F = 0.12$, df = 1, $P = 0.11$). Head DENV titer was significant influenced by temperature by *Wolbachia* interactions ($F = 6.1$, df = 1, $P < 0.01$; Table 1).

Temperature influences *Wolbachia* density. Temperature regimes significantly altered the *Wolbachia* density of the mosquitoes in both the head ($F = 10.1$, df = 2, $P < 0.0001$; Figure 1E) and the carcass ($F = 9.4$, df = 2, $P < 0.001$; Figure 1F). It was noted that *Wolbachia* density was highest when mosquitoes were reared on a diurnal temperature around the mean of 25°C, which coincided with the lowest DENV dissemination rate in *w*Mel.F mosquitoes. However, we observed no clear trend between the changes in *Wolbachia* density with DENV infection rate in the carcass.

We then tested for a correlation between *Wolbachia* density and DENV titer in mosquitoes. Due to the small number of *Wolbachia*-infected mosquitoes that were simultaneously infected with DENV, we plotted and analyzed these mosquitoes across all three temperature regimes. We observed no correlation between *Wolbachia* density and DENV titer in the head (Spearman's $r = 0.49$, $N = 14$, $P = 0.091$; Figure 1G) or the carcass (Spearman's $r = 0.09$, $N = 20$, $P = 0.71$; Figure 1H).

Both temperature and *Wolbachia* influence the proportion of mosquitoes achieving transmission potential. To determine the effect of temperature and *Wolbachia* on the transmission potential of the mosquitoes for DENV, we measured transmission traits including proportion of mosquitoes with detectable DENV in saliva at any collection time point from 4 to 20 DPI (infectivity), the earliest time point DENV can be detected in the saliva of mosquitoes (EIP), and the frequency (number of time points) that DENV can be detected in the saliva. Temperature significantly altered the proportion of mosquitoes that became infective ($F = 6.8$, df = 2, $P < 0.01$; Figure 2A). Consistent with the trend in dissemination rate (Figure 1A), mosquitoes reared at a constant temperature of 25°C are more likely to be infective ($F = 13.6$, df = 1, $P < 0.001$) compared with those reared at a diurnal regimen around a mean of 25°C (25°C ± 4°C). There was no significant temperature × *Wolbachia* interaction affecting whether a mosquito becomes infective ($F = 1.2$, df = 1, $P = 0.3$).

Neither temperature ($F = 0.3$, df = 2, $P = 0.71$) nor *Wolbachia* ($F = 2.3$, df = 1, $P = 0.13$; Figure 2B) significantly change the EIP of the mosquitoes. *Wolbachia* significantly reduced the frequency that DENV is detectable in the saliva ($F = 15.9$, df = 1, $P < 0.001$; Figure 2C). However, this frequency was not influenced by temperature ($F = 0.62$, df = 2, $P = 0.54$), nor was a temperature × *Wolbachia* interaction evident ($F = 0.07$, df = 1, $P = 0.93$). Lastly, we measured the
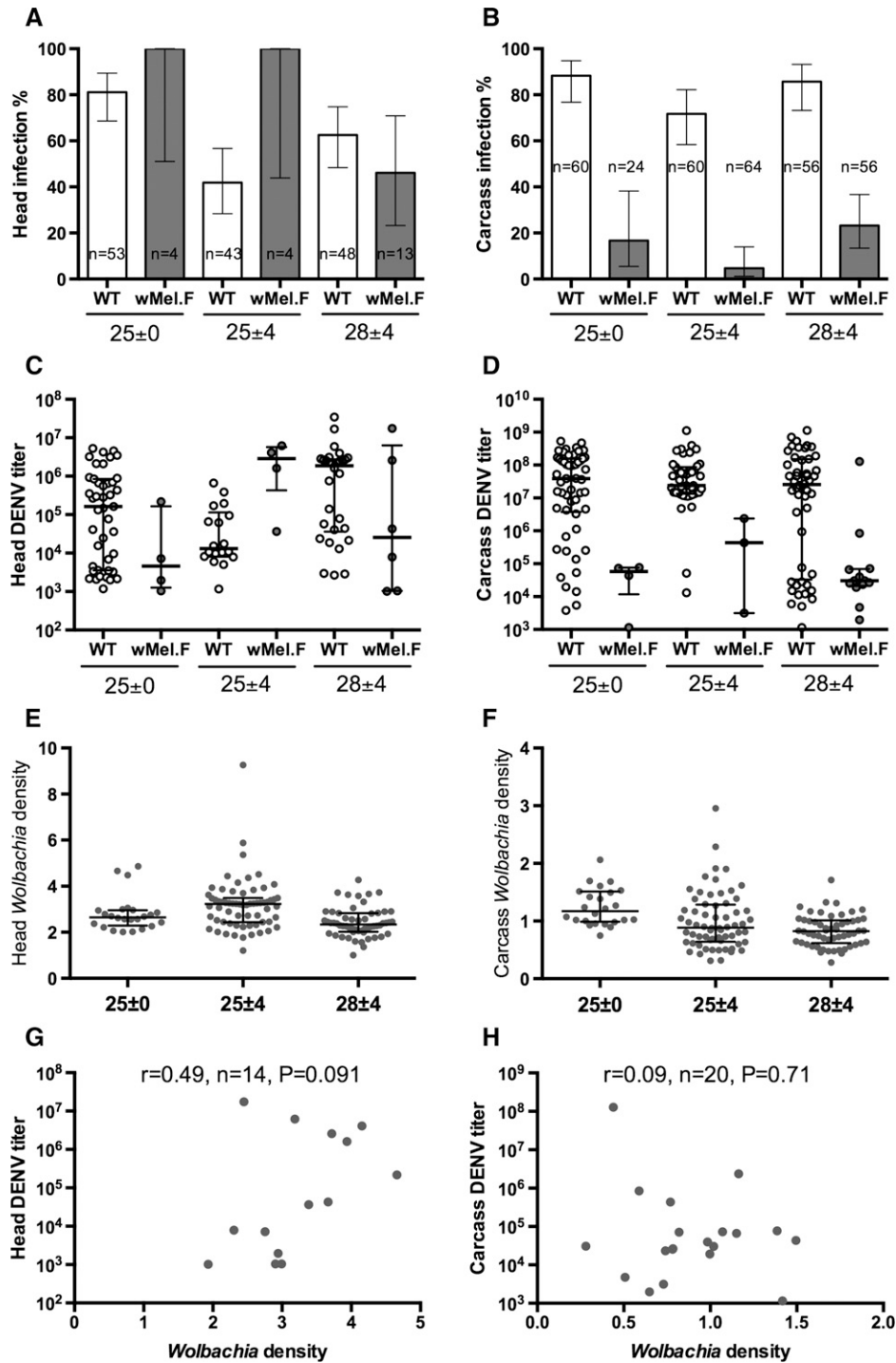
FIGURE 1. Infection rate ± 95% confidence intervals for (**A**) head and (**B**) carcass of WT (white) and *w*Mel-infected (*w*Mel.F) mosquito (gray) reared on three temperature regimes orally infected with dengue virus serotype 3 (DENV-3). To measure dissemination rate of virus to the head, only mosquitoes with carcass infection was used as denominator. Medians and interquartile ranges of viral titer for (**C**) head and (**D**) carcass of WT and *w*Mel.F mosquito reared on three temperature regimes. *Wolbachia* density of (**E**) head and (**F**) carcass of WT and *w*Mel.F mosquito reared on three temperature regimes following orally infected with DENV-3. Mean ± standard error was plotted. Each data point represents 24–64 females (mean = 53). *Wolbachia* density vs. (**G**) head and (**H**) carcass viral titer of *w*Mel.F mosquitoes across all three temperature regimes. Spearman's nonparametric *r* test is used to test for a correlation.

survival of DENV-3 fed WT and *w*Mel.F mosquitoes from the point of DENV feed to 20 DPI. We found that change in temperature regimes had little effect on the mosquito survival of either WT or *w*Mel.F mosquitoes (Figure 2D and Table 2).

DISCUSSION

Wolbachia replication and density in its host is known to be sensitive to temperature changes. In *Ae. albopictus*, an increase of constant temperature from 25°C to 37°C reduced

Summary of ANOVA on the effect of temperature regimes on DENV-3 infection (carcass) and dissemination (head) in *Aedes aegypti* and *Wolbachia* density

	All temperature regimes	25°C ± 0°C vs. 25°C ± 4°C	25°C ± 4°C vs. 28°C ± 4°C
	P value	Adjusted *P* value	Adjusted *P* value
Head dissemination rate			
Temperature	*	0.56	0.21
Wolbachia	0.27	0.23	0.47
Temperature × *Wolbachia*	0.11	0.56	0.14
Carcass infection rate			
Temperature	**	**	**
Wolbachia	****	****	****
Temperature × *Wolbachia*	0.71	0.65	0.64
Head DENV titer			
Temperature	0.11	0.86	0.11
Wolbachia	0.73	0.56	0.60
Temperature × *Wolbachia*	**	**	**
Carcass DENV titer			
Temperature	0.45	0.34	0.34
Wolbachia	****	****	****
Temperature × *Wolbachia*	0.65	0.74	0.74
Head *Wolbachia* density	****	0.15	***
Carcass *Wolbachia* density	***	*	*

ANOVA = analysis of variance; DENV = dengue virus; DENV-3 = dengue virus serotype 3. Only mosquitoes with carcass infection were used to calculated dissemination rate. *P* values for post hoc pairwise comparison statistics (between two temperature regimes) were corrected using the Benjamini and Hochberg method.[51] *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

Wolbachia density.[52] Changes in temperature can also affect a wide range of *Wolbachia*-induced phenotypes in its host such as maternal transmission, cytoplasmic incompatibility, and host fitness including longevity.[53,54] Effects of temperature on *Wolbachia*-mediated pathogen blocking have only been reported in somatically infected *Anopheles*.[42] To our knowledge, this is the first report that compares constant and diurnal temperature regimes on *Wolbachia*-mediated

DENV blocking. To simulate the daily cyclic temperature fluctuations, a DRT of 8°C (±4°C) was chosen to test if DENV blocking happens at the same efficiency in a diurnal temperature as compared with a constant temperature both with the same mean temperature. To further mimic the warmer months in summer in Cairns where dengue outbreaks occur,[43] a diurnal temperature around a mean temperature of 28°C was selected. We showed here that the
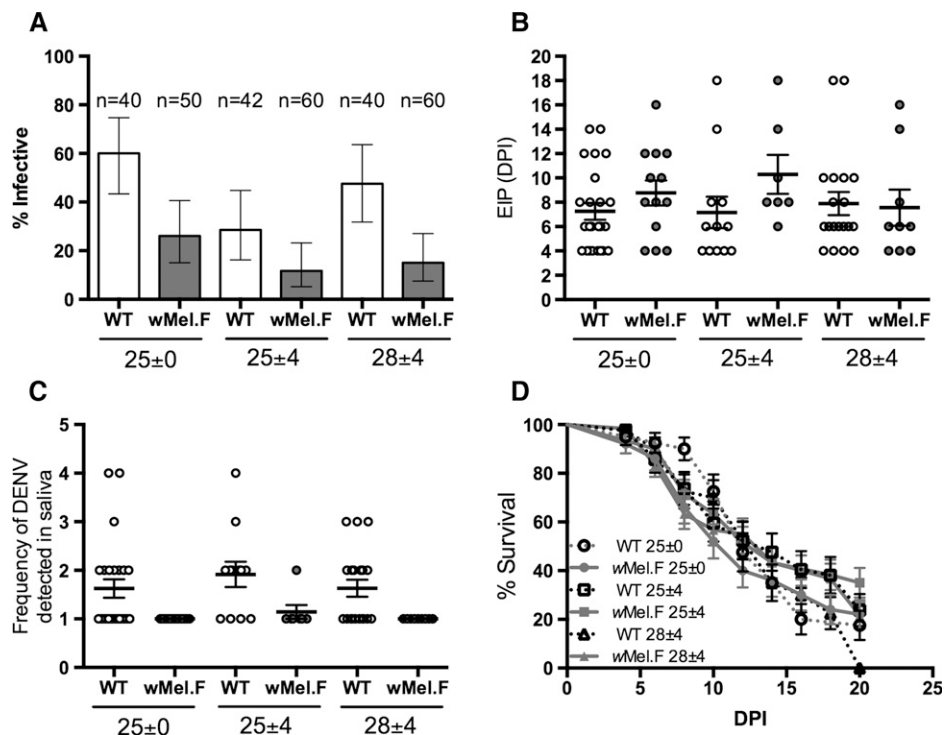

FIGURE 2. (**A**) Proportion of infective ± 95% confidence intervals, (**B**) extrinsic incubation period (EIP), (**C**) frequency of dengue virus (DENV) detected in saliva, (**D**) and survival of WT (white) and *w*Mel-infected (*w*Mel.F) mosquito (gray) reared on three temperature regimes orally infected with dengue virus serotype 3. Infective rate (A) of WT and *w*Mel.F mosquito. Each data point represents 40–60 females (mean = 49).

TABLE 2
Summary of ANOVA on the effect of temperature regimes on transmission potential of *Aedes aegypti* for DENV-3 and mosquito survival

	All temperature regimes	25°C ± 0°C vs. 25°C ± 4°C	25°C ± 4°C vs. 28°C ± 4°C
	P value	Adjusted *P* value	Adjusted *P* value
Proportion of infective mosquitoes			
Temperature	**	***	0.08
Wolbachia	****	***	***
Temperature × *Wolbachia*	0.3	0.18	0.18
EIP			
Temperature	0.71	0.52	0.46
Wolbachia	0.13	0.26	0.52
Temperature × *Wolbachia*	0.37	0.52	0.52
Frequency of DENV detected in saliva			
Temperature	0.54	0.49	0.49
Wolbachia	***	**	**
Temperature × *Wolbachia*	0.93	0.74	0.74
Survival			
WT	NA	0.57	0.24
*w*Mel.F	NA	0.24	0.32

ANOVA = analysis of variance; DENV = dengue virus; DENV-3 = dengue virus serotype 3; EIP = extrinsic incubation period; NA = not applicable; *w*Mel.F = *w*Mel-infected. *P* values for post hoc pairwise comparison statistics (between two temperature regimes) were corrected using the Benjamini and Hochberg method.[51] *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

rearing of mosquitoes at a constant temperature overestimates the body infection and the proportion of mosquitoes achieving transmission capability as compared with a diurnal of temperature around the same mean temperature of 25°C. We also found that raising the diurnal temperature regimes around the mean by 3°C enhances VC parameters and the transmissibly of DENV. *Wolbachia* efficiently reduced carcass infection rate and the proportion of mosquitoes achieving transmission potential across all temperature regimes. *Wolbachia* infection did not significantly change the dissemination rate of those mosquitoes that had already achieved infection in the body. It should be noted, however, that the small number of *w*Mel.F mosquitoes with carcass infection greatly limited the power to test the effect of temperature on dissemination rate.

Infection and transmission of DENV in *Ae. aegypti* is influenced by the amplitude and pattern of daily temperature variation. This amplitude of change, which is represented by the DTR, affects both *Ae. aegypti* infected with DENV[36] and *Anopheles* carrying *Plasmodium*[38] in a nonlinear manner. DTR and mean temperature in combination dictates how fast *Ae. aegypti* can transmit DENV. With field release of *Wolbachia*-infected mosquitoes in dengue endemic region such as Vietnam and Indonesia, each site with its unique mean temperature and DTR, it will be important to examine how these two variables also impact the efficiency of *Wolbachia*-mediated DENV blocking.

Further, temperature is known to have an effect on insect immunity.[55] Though the fundamental mechanism involved in viral blocking, demonstrated in both *Drosophila* and *Aedes*, does not seem to be dependent on the upregulation of the immune effectors, it is likely that *Wolbachia*-associated immune priming in mosquitoes can enhance the virus blocking phenotype.[56,57] Furthermore, microbial gut flora of mosquito interacts with host immune system to determine the outcome of host–pathogen interactions,[28,58] and evidence is emerging on how temperature can affect the presence and quantity of symbionts in insect's gut to influence host fitness.[59] How different temperature regimes can affect DENV blocking by possibly changing host immunity and its gut microbiota also needs to be investigated.

Finally, VC parameters such as the proportion of mosquitoes with a body infection, rate of dissemination of virus, and virus titer in mosquito tissue are dependent on the combination of mosquito genotype and virus genotype.[27] To determine the role of environment on host and parasite interactions, a study of chikungunya viruses in *Ae. albopictus* populations extended the concept to include both interacting genotypes (virus and mosquito) and temperature. The study demonstrated that these sources of variation do not necessarily act independently and may interact with each other (mosquito genotype × virus genotype × environment) to determine the outcome of VC.[60] To accurately assess the impact and plasticity of *Wolbachia*-mediated DENV blocking, it is also necessary to take this G × G × E interaction into consideration.

A limitation of this study is the use of a single DENV strain. We selected this DENV-3 isolate as it achieves a high infection rate in WT mosquitoes. The efficiency of *Wolbachia*-mediated DENV blocking greatly limited our choice of virus strains in this study. For example, *w*Mel.F was found to completely limit some DENV strains from disseminating to the head of the mosquito.[18,22] To examine the generality of DENV blocking and effects on transmission parameters, a diversity of viral genotypes, including representatives of the other three serotypes, need be used in the future.

We have previously reported a lengthening of EIP in mosquitoes infected with *w*Mel.F as compared with uninfected ones. In that study, we used saliva pooled from a group of mosquitoes and at any one time point only ~5% of the *w*Mel.F cages/pools were DENV positive for saliva.[45] Here we used live DENV in individual mosquitoes to examine their EIP and have observed no significant delay in EIP due to the presence of *Wolbachia*. With only ~20% of the *w*Mel.F mosquitoes producing a valid EIP this greatly limits the power to tease apart the effects of temperature and *Wolbachia* on EIP. Challenging *Wolbachia*-infected mosquitoes with viremic blood from dengue patients or concentrating DENV using ultracentrifugation methods may help to increase the proportion of *w*Mel.F mosquitoes that achieve transmission potential to study the EIP trait at the individual mosquito level.

Lastly, recent studies showed that virus dissemination in the mosquito was reduced when the immature stages of

Ae. albopictus were spent in cooler conditions.[61] The interaction between temperature and food experienced during larval and pupal stages of the mosquitoes was also found to influence DENV-1 infection of adult *Ae. albopictus*.[62] Future experiments should extend to study the effect of rearing larvae and pupae under different temperature regimes on DENV infection and transmission.[62]

CONCLUSIONS

We showed that whether a mosquito becomes infected, reaches dissemination, and achieves transmission of dengue is heavily dependent on temperature. *Wolbachia* density is also sensitive to temperature changes, and temperature can interact with *Wolbachia* to determine the VC of the mosquito. However, we found that *Wolbachia* efficiently reduced the proportion of mosquitoes achieving infection and transmission potential across all temperature regimes tested. As field releases of *Wolbachia*-infected mosquitoes are currently underway in dengue-endemic regions each with its unique climate, it is important to take into consideration the mean temperature and extent of fluctuations in daily temperature to better access the impact of *Wolbachia* on DENV transmission. This work, however, shows a robustness of *Wolbachia*-based blocking to temperature variation that mimic the warmer months in summer in Cairns. Parallel studies should be carried out with mosquito genotypes and field temperatures relevant to emerging release sites globally.

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