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Reduction of *Aedes aegypti* vector competence for dengue virus under large temperature fluctuations

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2 1 LRH: CARRINGTON AND OTHERS

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4 2 RRH: LARGE TEMPERATURE FLUCTUATIONS REDUCE DENV TRANSMISSION

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9 4 *Aedes aegypti* vector competence for dengue virus is reduced under large temperature fluctuations

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13 6 Lauren B. Carrington, Stephanie N. Seifert, M. Veronica Armijos, Louis Lambrechts
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29
30 13 ABSTRACT

31
32 14 Diurnal temperature fluctuations can fundamentally alter mosquito biology and mosquito-
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34 15 virus interactions in ways that impact pathogen transmission. We investigated the effect of two
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36 16 daily fluctuating temperature profiles on *Aedes aegypti* vector competence for dengue virus
37
38 17 (DENV) serotype-1. A large diurnal temperature range (DTR) of 18.6°C around a 26°C mean,
39
40 18 corresponding with the low DENV transmission season in northwest Thailand, reduced midgut
41
42 19 infection rates and tended to extend the virus extrinsic incubation period (EIP). Dissemination was
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44 20 first observed at Day 7 under small fluctuations (7.6°C; corresponding with high DENV
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46 21 transmission) and constant control temperature, but not until Day 11 for the large DTR. Results
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48 22 indicate that female *Ae. aegypti* in northwest Thailand are less likely to transmit DENV during the
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50 23 low than high transmission season because of reduced DENV susceptibility and extended virus EIP.
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52 24 Better understanding of DENV transmission dynamics will come with improved knowledge of
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54 25 temperature effects on mosquito-virus interactions.
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INTRODUCTION

In Mae Sot, northwestern Thailand, dengue virus (DENV) transmission is predictably seasonal, although the mean temperature across seasons varies minimally, remaining at approximately 26°C. The low transmission season in Thailand typically occurs from November to April and the high transmission season from June to October.¹ Temperatures during February (low transmission) are associated with relatively large fluctuations in temperature (15--20°C), while during July/August (high transmission) there is a smaller daily temperature swing (5--10°C).

The interaction between DENV and its principal mosquito vector, *Aedes aegypti* (Linnaeus), has been studied in detail in the laboratory under constant temperatures. Experimental transmission was observed at a range of constant temperatures from 13°C² to 35°C.³ Increasing constant temperature reduced the extrinsic incubation period (EIP), increased mosquito mortality,^{3,4} and resulted in a higher proportion of infected mosquitoes.

There are relatively few studies that explored vector-virus interactions under variable, more environmentally realistic temperature regimes. Bates and Roca-Garcia in 1946 and Chamberlain and Sudia in 1955 examined the vector competence of *Haemagogus* sp. and *Ae. triseriatus* for yellow fever virus and eastern equine encephalitis virus, respectively, but found little difference between the constant and alternating temperature regimes.^{5,6} At low temperatures, a diurnal temperature range (DTR) of 14°C (10°C--24°C) around a mean of 17°C did not influence *Ae. taeniorhynchus*⁷ or *Culex pipiens*⁸ susceptibility to infection with Ockelbo virus.

More recently, Lambrechts et al.⁹ examined the vector competence of *Ae. aegypti* for DENV under fluctuating temperatures, using small and large sinusoidal temperature fluctuations like the ones noted above for Mae Sot. Three temperature regimes were considered, all with a mean temperature of 26°C; i.e., a constant temperature, a DTR of 10°C (representing the high transmission season), and a DTR of 20°C (low transmission season). They found that mosquitoes

1
2 51 exposed to DENV-1 and DENV-2 had reduced longevity and lower midgut infection rates when
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4 52 exposed to the large DTR, compared to small DTR or constant 26°C. Dissemination rates did not
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6 53 vary across temperature regimes, but as expected did increase with the duration of extrinsic
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8 54 incubation. Results from that study support the notion that large temperature fluctuations can
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10 55 contribute to a reduction in DENV transmission intensity during the low DENV transmission
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12 56 season in northwestern Thailand.
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16 57 There are, however, additional details in the DENV-*Ae. aegypti* interaction that were not
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18 58 addressed in the Lambrechts et al.⁹ study. First, as acknowledged by the authors, a symmetrical,
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20 59 sinusoidal temperature profile was used in their experiments, as opposed to a more realistic
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22 60 asymmetrical profile. Parton and Logan¹⁰ describe air temperatures that follow a truncated
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24 61 sinusoidal progression during the day with an exponential decrease at night. The symmetrical
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26 62 profile has increases in temperature above the mean that are directly offset by reciprocal decreases
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28 63 below, whereas that is not necessarily the case for the asymmetrical profile. Second, during larval
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30 64 development, all mosquitoes were held at a constant 26°C. They were not exposed to the same
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32 65 fluctuating temperatures as adults until 3 d before adult females were exposed to virus. Third,
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34 66 infection status of females used to estimate the survival curves was unknown (i.e., the survival of
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36 67 virus-exposed females was compared regardless of whether infection was successfully established
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38 68 or not). Results could be confounded by DENV infection status, which can alter female survival.¹¹,
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40 69 ¹² Fourth, Lambrechts et al.⁹ did not evaluate adult female size, which has been associated with
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42 70 *Aedes* spp. vector competence. Other investigators reported conflicting results regarding vector
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44 71 competence and mosquito size.^{13, 14, 15} Results from one study using DENV-2 indicated that smaller
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46 72 *Ae. aegypti* are more susceptible to the infection than larger ones.¹³ Rearing effects may have
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48 73 confounded the outcome of some previous experiments.
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56 74 The Lambrechts et al.⁹ study also modeled the thermodynamics of virus infection and
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58 75 transmission by mosquitoes, under both symmetric and asymmetric temperature profiles. Their
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2 76 model was based on empirical data from three flaviviruses (West Nile virus, St Louis Encephalitis
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4 77 virus and Murray Valley encephalitis virus) and lead to the following predictions. Temperature
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6 78 fluctuations were expected to alter the direction of the response, dependent upon the mean
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8 79 temperature. At a low mean temperature ($< 18^{\circ}\text{C}$), fluctuations were predicted to increase the
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10 80 probabilities of infection and dissemination, and at higher mean temperatures ($> 18^{\circ}\text{C}$) fluctuations
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12 81 reduce these probabilities. In addition, under an asymmetric temperature profile a larger DTR was
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14 82 predicted to shorten EIP. The expected EIP at a constant 26°C is 11--12 d, and 10 d or less with a
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16 83 DTR of 20°C .
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21 84 Carrington et al.¹⁶ recently assessed the effect of DTR on *Ae. aegypti* immature and
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23 85 reproductive traits. Using the same asymmetrical temperature regimes as those used in this study,
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25 86 they reported that a large DTR reduced immature survival and extended development time. A small
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27 87 DTR, however, slightly increased reproduction.
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30 88 In this study we extended the Lambrechts et al.⁹ experiments by examining *Ae. aegypti*
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32 89 vector competence for DENV (midgut infection and dissemination of the virus to head tissue) and
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34 90 survival under asymmetrical temperature fluctuations (sinusoidal progression + exponential
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36 91 decrease; the same temperature profiles as those used in Carrington et al.¹⁶) that represent the high
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38 92 and low DENV transmission seasons in Mae Sot. Our aim was to include variables not examined in
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40 93 previous studies and to investigate the relationship between mosquito body size and vector
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42 94 competence using DENV-1 and *Ae. aegypti*. We hypothesized that females in the large DTR
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44 95 treatment are less susceptible to DENV-1 infection,⁹ and that smaller females are more likely to
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46 96 become infected with DENV-1 than larger conspecifics.¹³ As part of our analysis, we measured the
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48 97 proportion of mosquitoes with a midgut (body) and disseminated (head) infection, and the length of
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50 98 the EIP compared to predictions from the Lambrechts et al.⁹ thermodynamic model.
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METHODS

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6 101 **Experimental design.** We determined the effect of three temperature regimes on adult *Ae.*
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9 102 *aegypti* survival and vector competence for DENV; one constant temperature and two with daily
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11 103 temperature fluctuations (see Figure 1 of Carrington et al.¹⁶). Temperature profiles were intended to
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13 104 be representative of the high and low DENV transmission seasons in Mae Sot, Thailand. We used
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15 105 the average temperature of each hour of the day as measured over a three week period during the
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17 106 high and low DENV transmission seasons. Temperatures were recorded inside houses in villages
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19 107 near Mae Sot.¹⁷ The high transmission period was characterized by a 7.6°C swing around a mean of
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21 108 26.7°C (recorded over a 20 d period during July/August 2000). The low transmission season
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23 109 followed an 18.6°C swing around a mean of 26.1°C (recorded over 20 d during February 2000).
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25 110 Fluctuating temperature profiles followed a sinusoidal increase during the day, and a negative
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27 111 exponential decrease at night, with minimum and maximum temperatures reached at 6:00AM and
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29 112 2:00PM, respectively. Experimental climatic conditions were maintained for experimental
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31 113 mosquitoes in KBF115 incubators (Binder, Germany). Internal data loggers recorded temperatures
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33 114 in all incubators. Additionally, HOBO data loggers recorded temperatures on an hourly basis in the
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35 115 two incubators with fluctuations. Actual air temperatures that mosquitoes were exposed to during
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37 116 experiments were consistent ($\pm 0.3^\circ\text{C}$) with that programmed at each point during the day. The
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39 117 mean temperature for the small DTR treatment incubator was 26.71°C, while for the large DTR,
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41 118 temperature averaged at 26.34°C. Relative humidity was maintained between 70% and 80% across
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43 119 all treatments.
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51 120 **Mosquitoes.** *Aedes aegypti* used in our experiments were collected from Kamphaeng Phet
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53 121 Province, Thailand, which is less than 150 kmoutheast of Mae Sot and has a similar annual climate.
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55 122 Mosquitoes were collected as pupae during January 2011 and sent to UC Davis as F₁ eggs. After
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57 123 eggs were received, they were hatched and reared at a low density (1.3 larvae/10 mL) in 24 cm x 29
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2 124 cm x 5 cm containers with 1.5 L of deionised water. Colony maintenance was conducted under
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4 125 standard insectary conditions (constant $28^{\circ}\text{C} \pm 2^{\circ}\text{C}$, 70-80% RH) and a 12:12 hr light:dark cycle,
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6 126 with >500 females per generation. Mosquito rearing procedures for experiments are described
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8 127 below. Larvae were fed a 1:1 mix of bovine liver powder and puppy chow, with 0.1 g per 200
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10 128 larvae each day for the first four days, 0.2 g on the fifth day, 0.3 g on the sixth, and then 0.2 g on
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12 129 the remaining 2 d, at which time most larvae had pupated.
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16 130 **Experimental infections.** For each of the three treatments, F_3 generation mosquitoes were
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18 131 reared at the temperature at which their vector competence was to be tested. Due to space
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20 132 limitations, rearing density was increased (4--5 larvae/10 mL) and food was increased
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22 133 proportionately. To limit variation in adult emergence time, we staggered the hatching of eggs
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24 134 based on estimates from previous experiments of development time from egg hatch to adult
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26 135 emergence.¹⁶ Five to seven days after adult emergence, we exposed mosquitoes from each
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28 136 temperature regime to an infectious DENV-1 blood meal using an artificial feeding system.
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32 137 After having sugar removed for 30--36 hr, females were fed defibrinated sheep blood
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34 138 (QuadFive, Ryegate, MT), mixed with DENV-1 freshly grown in *Ae. albopictus* C6/36 cells prior
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36 139 to mosquito exposure. The virus supernatant was harvested after scraping and then separating all
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38 140 cells by centrifugation. Mosquitoes fed through a desalted porcine intestinal membrane stretched
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40 141 over the bottom of a warm water-filled jar to maintain a temperature of 37°C . The viral isolate
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42 142 used, SV2951 obtained from Ratchaburi, Thailand, had been passaged seven times in C6/36 cells
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44 143 prior to use in this study. Confluent cultures of C6/36 cells grown in 25-cm^2 flasks were inoculated
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46 144 at a virus multiplicity of infection of 0.01 and left to grow for 10 d at 28°C in 5% CO_2 . The
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48 145 infectious blood meal consisted of 45% viral supernatant harvested at Day 10, 50% defibrinated
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50 146 sheep blood (Quadfive, MT), and 5% sucrose solution (diluted 1:5 in water). An aliquot of the
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52 147 blood meal was taken at the end of the feed to determine the minimum viral titer of the blood that
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54 148 mosquitoes ingested.
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2 149 We prepared two blood meals, but fed each female only once (feeding mosquitoes in one of
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4 150 two feeding sessions), and limited the feeding time to 30 min, in an effort to minimize the effect of
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6 151 virus degradation in the infectious blood meal. The titers of the two blood meals were 4.16×10^5
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9 152 fluorescent focus units (FFU)/mL and 3.09×10^5 FFU/mL. After allowing 2--3 hr for mosquitoes to
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11 153 begin digestion, we sedated them using CO₂, and retained only fully engorged females. Thirty-six
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13 154 replicate 1-pint paper cartons (Science Supplies WLE, NJ) with mesh tops, each containing 20
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16 155 engorged females (12 cartons from each temperature regime), were returned to their respective
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18 156 incubators.

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21 157 **Vector competence.** At 7, 11, 15, 19, 23 and 27 days post-exposure (DPE) to the infectious
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23 158 blood meal (i.e., days of EIP), we randomly selected and removed two cartons from each incubator
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25 159 at each time point. For all surviving mosquitoes in each carton, we measured two components of
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28 160 vector competence, midgut infection and virus dissemination from the midgut, using a qualitative
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30 161 indirect fluorescence assay (Q-IFA).

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33 162 We separated and tested bodies (comprised of the thorax and abdomen) for midgut
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35 163 infection, and heads for disseminated infection, independently. Samples were placed into 1 mL
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37 164 viral transport medium (VTM; 77.2% low glucose DMEM, 18.5% heat-inactivated fetal bovine
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39 165 serum, 3.8% penicillin/streptomycin, and 0.15% gentamycin and nystatin) with approximately ten 2
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42 166 mm glass beads (Fisher Scientific, Pittsburg, PA) in a screw-top plastic vial. Following collection,
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44 167 all samples were frozen at -80°C for later analysis by IFA. We also collected the whole bodies
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46 168 (without separation of heads) of dead females daily and tested them for infection status. Results
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49 169 from analysis of dead mosquitoes were included in our survival analyses.

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51 170 **Wing lengths.** Female wings were removed at the same time as heads and bodies were
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53 171 separated, and mounted on microscope slides with double-sided tape. Photographs of the wings
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56 172 were taken and measurements of each wing were made, in millimeters, using AutoMontage
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59 173 (Syncroscopy, MD) software. Wings were measured from the distal end of the alula to the most
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2 174 posterior point of the R₃ vein, minus the fringe. Damaged wings or wings that were mounted
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4 175 imperfectly were excluded from analysis.

6 176 **Data analysis.** All data was analysed using JMP software, version 10 (SAS Institute Inc.,
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9 177 NC). Vector competence was analyzed by nominal logistic regression of the infection or
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11 178 dissemination status as a full-factorial function of temperature and DPE, and carton nested within
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13 179 temperature and DPE. To improve the statistical model, factors with a *p*-value above 0.5 were
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15 180 removed sequentially from the model, except for the nested carton effect, which was required to test
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17 181 the effects of temperature and DPE. Survival was analyzed by assessing the longevity of females
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19 182 exposed to an infectious blood meal using deaths throughout the duration of the experiment, as well
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21 183 as right-censored individuals that were sacrificed on the days we collected mosquitoes for testing
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23 184 midgut infection and dissemination (sampling days). We used a Kaplan-Meier (K-M) analysis to
24
25 185 test for differences in survival curves between different DTR and infection status of recently dead
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27 186 mosquitoes. Carton effects were not considered in survival analyses. We corrected for multiple
28
29 187 comparisons between treatment groups for logistic regression and Kaplan-Meier analyses using a
30
31 188 Bonferroni correction. Wing length was analyzed as a function of infection status and DTR.
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33 189 Replicate carton was nested within DTR, and we tested for an interaction between infection and
34
35 190 DTR.

42 191 **Fluorescent Focus Assay.** We used an infectious fluorescent focus assay (FFA) to titrate
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44 192 viral samples.¹⁸ One-day old confluent monolayers of Vero (green monkey kidney) cells in 8-well
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46 193 chamber slides (Nunc, Rochester, NY) were inoculated with serial 10-fold dilutions of virus and
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48 194 blood meal samples. Dilutions were prepared in 2% FBS maintenance media in duplicate and
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50 195 inoculum was allowed to infect the cells for 1 hr. A negative control (the maintenance media used
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52 196 for the dilutions) was included in all titrations. The overlay applied to the cells after the incubation
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54 197 was made of a 1:1 mix of 2% FBS maintenance media:carboxymethyl cellulose (CMC; 2% in
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56 198 PBS). We then allowed 2 d for virus to replicate in the monolayer, then the media was removed and
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2 199 the cells washed carefully. In each washing step, PBS was added to cells three times, allowed to
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4 200 rest for 3--5 min, before PBS was again removed. The cells were fixed with 3.7% formaldehyde
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6 201 and washed again before being stained with 75 μ L 1:250 dilution of primary mouse anti-DENV
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9 202 monoclonal antibody (MAB8705; Millipore, MA) at 37°C for 1 hr. The cells were again washed to
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11 203 minimize background fluorescence, and then stained with 75 μ L 1:100 dilution FITC-conjugated
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13 204 secondary goat anti-mouse antibody (AP124F; Millipore, MA) for 30 min, which was used to
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15 205 detect and count the number of fluorescent foci under a fluorescent microscope at 20x
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18 206 magnification.
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21 207 **Qualitative Indirect Fluorescence Assay (Q-IFA).** To test mosquito samples for DENV
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23 208 infection, we homogenized the tissue samples for 2 min in a Retsch Mixer Mill 400, at 30 Hz. We
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25 209 filtered 300 μ L of the sample through 0.22 μ m cellulose acetate centrifuge filters (Costar Spin-X,
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28 210 Corning, Japan) and 50 μ L of the filtered supernatant was inoculated in duplicate onto a 1 d old
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30 211 confluent monolayer of Vero cells, seeded at a density of 2.5×10^5 cells/well in a 96-well culture
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32 212 plate. The inoculum was allowed to infect the cells for one hour at 28°C, before a standard
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34 213 maintenance media containing 2% FBS overlay was applied to the cells in each well, and the plate
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37 214 was incubated 28°C for 4 d. Positive and negative controls were used in each plate. We then
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39 215 removed the overlay, washed and fixed the cells in 3.7% formaldehyde for 20 min. The washing
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41 216 and staining steps that followed were exactly the same as for the FFA, except that the volumes used
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44 217 for antibody staining were 50 μ L for each of the primary and secondary antibodies. We viewed
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46 218 cells under FITC-fitted FA microscope at 10x to screen for the presence or absence of green
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48 219 fluorescence, which was indicative of a sample being either infected or uninfected by DENV,
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51 220 respectively.
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RESULTS

222
223 **Vector competence.** Over the entire time course of the experiment, 28.1% of the
224 mosquitoes had a detectable DENV-1 midgut (body) infection, from a total of 712 mosquitoes that
225 imbibed the infectious blood meal. Of the 200 infected females, we tested 174 for a disseminated
226 infection, of which 69.5% (121) had positive head tissue samples. The 26 mosquitoes that were not
227 tested for dissemination died before the scheduled sampling period and were only assayed for
228 infection.

229 Using a nominal logistic regression to analyze samples collected from the six scheduled
230 sampling days of the experiment, we found a highly significant effect of DTR on DENV-1 infection
231 in mosquito bodies (Table 1). A small DTR resulted in the highest mean infection rates, while large
232 DTR had more than a 50% relative reduction in the proportion of infected females (Table 2). Pair-
233 wise analyses demonstrated that both the constant temperature and small DTR treatments had
234 significantly higher infection rates than the large DTR at two time points. After correcting for
235 multiple comparisons, we determined that the constant DTR had higher infection rates than the
236 large DTR at Day 27, while the small DTR produced significantly more infected females than the
237 large DTR at Days 15 and 27. Levels of infection did not differ at any time point between the
238 constant temperature and small DTR treatments (Table 3). A strong effect of DPE was also
239 observed. An increase in DPE was associated with higher infection rates. Replicate carton (nested
240 within DTR and DPE) did not significantly influence body infection rates. There was no detectable
241 effect of DTR x DPE, and this term was removed from the final statistical model.

242 For dissemination of the virus into head tissue (Table 1), no effect of DTR was observed,
243 but we found a strong effect of DPE. Dissemination into the head tissue was first observed at Day 7
244 for the constant 26°C treatment and small DTR, but not until Day 11 for the large DTR, although an
245 effect of DTR at Day 7 was statistically insignificant (sample sizes and pair-wise comparisons are
246 shown in Tables 4 and 5, respectively). Rates of dissemination increased sharply until Day 15, after

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2 247 which almost all infected females had a disseminated infection (Table 4). An interaction between
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4 248 DPE and DTR was observed (Table 1). Differences between treatments were observed at Days 11,
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6 249 and 27. There was no effect of carton within DTR and DPE. Pair-wise comparisons at each age
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8 250 (Table 5) demonstrated that the constant temperature regime had significantly lower dissemination
9
10 251 at Day 11 than both other treatments, but higher dissemination rate than the small DTR at Day 15.
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12 252 At Day 27, there was only a single mosquito tested from the large DTR (which was uninfected).
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14 253 We, therefore, did not consider this data point in the pair-wise analyses for that time point.
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18 254 **Survival.** A total of 186 females died during the experiment and 526 were sampled at
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20 255 scheduled time points and, therefore, were right-censored. We identified an overall significant
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22 256 effect of DTR on survival ($\chi^2 = 6.85$, $df = 2$, $p = 0.032$; Figure 1A). Lowest survival was for
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24 257 females held under large fluctuations, with a mean adult lifespan of 20 d, relative to females under a
25
26 258 constant temperature (23 d) and small fluctuations (21 d). We detected an overall effect of infection
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28 259 on survival ($\chi^2 = 25.17$, $df = 1$, $p < 0.0001$; Figure 1B), where females that were uninfected,
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30 260 although they had been exposed to the virus, had a lower survival than those that were infected.
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32 261 Uninfected females lived, on average, almost four days less (21 d) than females who had detectable
33
34 262 infections (25 d). We separated infected and uninfected females and analyzed the effect of DTR for
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36 263 each, but found no significant effect for either (infected females: $\chi^2 = 1.49$, $df = 2$, $p = 0.473$;
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38 264 exposed but uninfected females: $\chi^2 = 3.68$, $df = 2$, $p = 0.158$; Figure 2). We also assessed the effect
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40 265 of infection within each DTR. While there was a consistent reduction in the lifespan of females that
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42 266 had been exposed to the virus, but were uninfected (Figure 3), it was significant only for the small
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44 267 and large DTR regimes ($\chi^2 = 13.93$, $df = 1$, $p < 0.001$ and $\chi^2 = 6.59$, $df = 1$, $p = 0.010$ respectively).
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46 268 It should be noted that there were only three infected individuals from the large DTR treatment that
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48 269 died through the duration of the experiment. There was no statistical difference in the survival
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1
2 270 curves of infected and uninfected females from the constant temperature regime ($\chi^2 = 3.31$, $df = 1$,
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4 271 $p = 0.068$).

6 272 **Wing lengths.** Wing lengths were measured and analyzed for a total of 95 individuals of
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8
9 273 known infection status. There was no effect of carton nested within DTR ($F_{27, 62} = 1.383$, $p =$
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11 274 0.146), or a main effect of DTR ($F_{2, 62} = 2.548$, $p = 0.086$) on mosquito wing length. Mean wing
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13 275 lengths for mosquitoes reared under a constant temperature regime were 2.95 mm (± 0.020 mm SE;
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15 276 $n = 27$), a small DTR 2.90 mm (± 0.016 ; $n = 44$), and a large DTR 2.86 mm (± 0.021 ; $n = 35$). No
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17 277 difference was observed between the wing lengths of infected and uninfected individuals ($F_{1, 62} =$
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19 278 0.242 , $p = 0.624$). Mean wing length of individuals that became infected was 2.91 mm (± 0.018
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21 279 mm; $n = 39$) and 2.89 mm for uninfected mosquitoes (± 0.013 mm SE; $n = 63$). There was no
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23 280 interaction between infection status and DTR ($F_{2, 62} = 0.620$, $p = 0.541$).

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DISCUSSION

Our results indicate that large DTRs can reduce DENV transmission probability by altering vector life history traits and vector-virus interactions. Depending on the location and climate (i.e., northwestern Thailand), our results may help explain seasonal changes in DENV transmission dynamics. During the high transmission season mosquitoes are more likely to become infected than during the low transmission season and a larger number of infected mosquitoes would be expected to have enhanced survival. Conversely during the low transmission season, relatively fewer mosquitoes become infected with DENV. Additionally, a large DTR around of mean of 26°C has other negative effects, including reduced fecundity, extended immature development time and decreased survival.¹⁶

Vector competence. We found that a large DTR reduces the proportion of females that become infected with DENV-1 compared to either a constant 26°C or a set of small fluctuations around the same mean, which is consistent with results reported by Lambrechts et al.⁹ Although the shape of the temperature profile was different between the two studies (symmetrical vs. asymmetrical fluctuations), results are similar across multiple collections of *Ae. aegypti* from two different locations in Thailand. We found no difference in overall detectable mosquito DENV-1 infection rates between the constant temperature and small fluctuation regimes; this is the same result that Lambrechts et al.⁹ reported for DENV-1 and DENV-2. Unlike the Lambrechts et al.⁹ study, however, we observed a slight increase in detectable infection rates over time, which may be partly explained by the lower infection rate in our study compared to the previous study. Because the overall detectable infection rate in the Lambrechts et al.⁹ study was close to 100%, a significant increase was not possible. Differences in infection rates between the two studies could be a result of the relatively low infectious titer of the blood meal used in the present experiment. Alternatively, viral- and/or mosquito-specific factors may have lead to variation in levels of infection.¹⁹ Despite this discrepancy, we are confident that the differences we detected are biologically meaningful,

1
2 307 because the infectious blood meal titers of this study fall within the range of observed viremia in
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4 308 humans.^{20, 21}

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6 309 Lambrechts et al.⁹ modeled the effect of fluctuating temperatures on infection and
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8 310 transmission of flaviviruses, using empirical data from West Nile, Murray Valley encephalitis, and
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10 311 St Louis encephalitis viruses. The model predicted that transmission probability under an
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12 312 asymmetric temperature profile with a mean of 26°C and large daily fluctuations (15--20°C) would
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14 313 be reduced from ~95% to ~50%. Although we found a reduction in rates of midgut (body) infection
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16 314 when compared to the constant temperature, we did not detect an empirical difference between
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18 315 DTR treatments in rates of dissemination (head infections). These results are consistent with the
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20 316 empirical data reported by Lambrechts et al.,⁹ in which a symmetrical temperature profile was used.
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22 317 It is likely that the discrepancy between the modeling outcome and the empirical data is a result of
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24 318 differences between vector-virus systems. It should be noted, however, that dissemination
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26 319 probability may not adequately represent transmission probability because a low titer of
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28 320 disseminated virus often does not correlate with successful transmission in laboratory assays.²²

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30 321 Previous studies have shown that the EIP is influenced by changes in mean temperature.^{2, 3,}
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32 322 ^{4, 23} In our experiment, the minimum EIP (i.e., the time when we first observed a disseminated
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34 323 infection in females), was not observed in any individual from the large DTR treatment until Day
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36 324 11, but was seen at Day 7 for both the constant and small DTR treatments. Again, the theoretical
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38 325 predictions made by Lambrechts et al.⁹ are contrary to this finding, although resolution of their
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40 326 model below 10 d is limited by available data, in some cases our sample sizes were modest. Further
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42 327 investigation is required to obtain greater resolution of dissemination potential at these early time
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44 328 points.

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46 329 Environmental temperatures experienced during the larval development phase may
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48 330 influence adult traits, such as vector competence.¹⁵ The present study was not designed to allow for
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50 331 us to discern between the effects of temperature on immature and adult mosquitoes, because it is

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2 332 highly unlikely that a mosquito would transition from one temperature regime to another between
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4 333 the aquatic and adult phases. For this reason, we consider that our experiment to be more
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6 334 representative of field conditions than if we had standardized immature development temperatures,
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9 335 as was the case in the Lambrechts et al.⁹ study.

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11 336 **Survival.** Survival curves differed between mosquitoes in our three DTR treatments. We
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13 337 tested mosquitoes up to 27 DPE because we suspect that this is the most important time period for
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15 338 transmission, rather than later in life when mortality rates have increased and we expect that most
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17 339 mosquitoes would have died^{24, 25} and external factors may skew the population age structure. As
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19 340 reported by Lambrechts et al.⁹ we detected a significant effect of DTR on mosquito survival; the
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21 341 magnitude of the observed response in our study, however, was not as strong at that previously
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23 342 reported. The data show that exposing *Ae. aegypti* females to a large DTR reduces survival relative
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25 343 to a constant temperature, under a smaller DTR the magnitude of this effect is reduced, but the
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27 344 biological significance of this is unclear. This difference in the magnitude of the effect could be due
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29 345 to the difference in the shape of temperature profile (symmetric vs. asymmetric) and perhaps the
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31 346 slight difference in magnitude of DTR between the two studies.

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33 347 Our inability to identify a consistent effect of DTR on survival when accounting for
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35 348 infection status suggests an interaction between DTR and survival. Although we observed an
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37 349 overall effect of DTR on mosquito survival, when we separated mosquitoes by infection status the
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39 350 effect was no longer detected in either group. This result can be most clearly seen by looking at the
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41 351 outcome from the large DTR treatment. Here, there was only a small number of infected females
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43 352 that died during the course of the experiment (n = 3), thus the overall survival curve for the large
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45 353 DTR was lowered more by the larger number of uninfected females that died. This is compared to
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47 354 constant temperatures where there was less of a discrepancy in the numbers between
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49 355 infected/uninfected females and the overall survival curve was weighted less by a single group of
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2 356 mosquitoes. We suspect, therefore, that the apparent low mortality rate of infected mosquitoes in
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4 357 the large DTR was due primarily to the small sample size in that category.

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6 358 Although the exact mechanisms for changes in survival and vector competence remain to be
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8 359 determined, a possible explanation is that for portions of the day temperatures exceed the optimal
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10 360 thermal range for adult mosquitoes resulting in the diversion of resources from maintenance to
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12 361 thermal stress responses; for example, the induction of cellular chaperone proteins.²⁶ Examining
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14 362 thermal tolerance at both high and low extremes of infected and uninfected mosquitoes from this
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16 363 population would test a possible physiological basis for this interaction, and indicate the tolerance
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18 364 of the mosquito and virus populations to variable habitats and environments. We are currently
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20 365 testing the optimal thermal range and critical thermal limits for vector competence of this
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22 366 Kamphaeng Phet *Ae. aegypti* population.

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25 367 Across all temperature treatments, we observed higher mean survival estimates for females
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27 368 that were infected with DENV-1 compared to those that were uninfected. Our results do not support
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29 369 the expectation that there would be a relatively high cost of infection for *Ae. aegypti*, once exposed.
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31 370 Instead, they suggest there may be a cost in mounting a successful antiviral immune response. An
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33 371 equivalent result was observed in both *Ae. aegypti* infected with DENV-2¹¹ and *Cx. pipiens* infected
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35 372 with West Nile virus²⁷ where infected mosquitoes had higher survival than those that were exposed
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37 373 to the virus but remained uninfected. A cost of exposure to the virus could not be tested in our study
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39 374 because we did not include unexposed control mosquitoes. It is also possible that we did not detect
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41 375 infection in all dead mosquitoes. Dead females were collected every 24 hr and virus may have
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43 376 degraded below the limits of detection between death and collection.

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46 377 **Wing size.** A number of researchers have investigated the relationship between vector
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48 378 competence and female mosquito size and their results are contradictory. We measured variation in
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50 379 size within each temperature regime and could not attribute size as a factor influencing
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52 380 susceptibility to DENV infection. Alto et al.¹³ reported that smaller *Ae. aegypti* and *Ae. albopictus*

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2 381 were more likely to become infected with DENV-2, the effect being more prominent for *Ae.*
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4 382 *aegypti*. Westbrook et al.¹⁴ and Muturi and Alto¹⁵ reported that larger individuals were more likely
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6 383 to become infected with two alphaviruses (Chikungunya and Sindbis viruses). In the latter two
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8 384 studies, variation in the size of the adult females was produced through rearing larvae at different
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10 385 temperatures, which confounded size within developmental rearing conditions, rather than
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12 386 examining variation in size across different temperature treatments.
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16 387 The lack of consistency between our study and the one by Alto et al.¹³ may reflect our
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18 388 smaller sample size or the increased susceptibility of small females was associated with the DENV
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20 389 isolates used in the respective experiments (DENV-1 vs. DENV-2). Further work is required to
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22 390 resolve this discrepancy, including manipulation of immature rearing temperatures and adult
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24 391 exposure temperatures to determine whether smaller female size during the low DENV
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26 392 transmission season in Mae Sot is a contributing or confounding factor that alters mosquito
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28 393 susceptibility to DENV. Lambrechts et al.⁹ reared mosquitoes at the same temperature of 26°C (we
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30 394 would, therefore, expect wing size variation to be evenly distributed across temperature regimes)
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32 395 and then after feeding them virus infectious blood, exposed them to three different temperature
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34 396 treatments, and they still detected reduced infection rates under larger fluctuations. We detected
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36 397 some variation in mean wing length across different temperature regimes, but it was not statistically
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38 398 significantly different perhaps due to the small number of replicates within DTR treatments.
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44 399 **Comparison to other mosquito-pathogen interactions.** Recent work on interactions
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46 400 between mosquitoes and malaria parasites similarly indicates that DTR influences parasite
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48 401 transmission, although the mechanism(s) appears to be different from the *Ae. aegypti*-DENV
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50 402 system. Compared to constant temperatures, Paaijmans et al.²⁸ showed that under both a low and a
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52 403 high mean temperature regime, realistic magnitudes of fluctuations that would be observed in
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54 404 Africa altered the duration of the sporogony for *Plasmodium chabaudi* in *Anopheles stephensi*.
55
56 405 Dependent upon whether the mean temperature was low or high, fluctuations altered the direction
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2 406 of the change in the time estimate for transmission to be achieved. Around lower mean
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4 407 temperatures, a DTR of 12°C accelerated parasite development. At higher mean temperatures, the
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6 408 same DTR slowed development. This is consistent with what we measured for DENV at a
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8 409 relatively high mean temperature of 26°C, where increased DTR slows down the rate of virus
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11 410 dissemination in infected mosquitoes (i.e., a longer EIP).

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13
14 411 **Conclusions.** Our results support two important points raised by Lambrechts et al.⁹ First, it
15
16 412 is not always appropriate to assume in experimental studies that a constant mean temperature is
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18 413 adequate for representing the impact of natural fluctuating temperatures on mosquito-virus
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20 414 interactions. Second, the magnitude of DTR is important in determining the *Ae. aegypti*-DENV
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22 415 infection rate in mosquito bodies and this can have epidemiologically relevant implications for our
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24 416 understanding of transmission dynamics in natural populations. Additionally our results indicate
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26 417 that under a realistic temperature profile, a large DTR may extend the EIP of mosquitoes, and
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28 418 points out that diurnal temperature fluctuations can influence multiple components of vectorial
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30 419 capacity. These results are consistent with the hypothesis that under environmentally realistic
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32 420 temperature conditions female *Ae. aegypti* in northwestern Thailand would be less likely to transmit
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34 421 DENV-1 during the low than high transmission season because they have reduced susceptibility to
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36 422 DENV infection and an extended EIP.

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For Peer Review

1
2 520 **Table 1:** Test statistics of DTR and DPE effects on *Ae. aegypti* vector competence for DENV-1.

3
4 521 *Midgut (body) infection* refers to the proportion of females with infected bodies tested at the

5
6 522 sampling days. *Dissemination (head infection)* was tested for infected females only. NS means that

7
8 523 the factor was highly non-significant and therefore the factor was removed from the final statistical

9
10
11 524 model.

Factor	<i>Midgut (body) infection</i>			<i>Dissemination (head infection)</i>		
	df	χ^2	<i>p</i>	df	χ^2	<i>p</i>
DTR	2	26.316	<.0001	2	1.28x10 ⁻⁵	1.0000
DPE	5	16.291	0.0061	5	65.985	<.0001
DTR x DPE	--	NS	NS	10	23.278	0.0098
Carton (within DTR, DPE)	18	23.868	0.1594	17	14.693	0.6176

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526 **Table 2:** Overall *Ae. aegypti* DENV-1 infection rates in female bodies, separated by carton. For
 527 each carton, the percentage of infected females is given, along with the number of females tested in
 528 brackets.

	Carton	Constant		Small		Large	
		%	(n)	%	(n)	%	(n)
Day 7	1	15	(20)	43	(14)	25	(16)
	2	38	(16)	10	(10)	0	(20)
Day 11	1	30	(20)	47	(15)	37	(19)
	2	47	(15)	47	(19)	14	(14)
Day 15	1	50	(16)	65	(17)	37	(19)
	2	53	(15)	61	(18)	8	(13)
Day 19	1	11	(18)	33	(15)	24	(17)
	2	42	(19)	41	(17)	30	(10)
Day 23	1	20	(10)	38	(16)	21	(14)
	2	27	(15)	50	(14)	9	(11)
Day 27	1	54	(13)	38	(8)	13	(8)
	2	25	(12)	50	(14)	0	(6)

529

530 **Table 3:** Pair-wise comparisons between DTR treatments for body infection of DENV in *Ae.*
 531 *aegypti* at each time-point. Text in bold font indicates that the effect was significant ($p < 0.016$)
 532 after Bonferroni correction for multiple comparisons.

Source	df	Constant vs. Small		Constant vs. Large		Small vs. Large	
		χ^2	p	χ^2	p	χ^2	p
Day 7 DTR	1	0.044	0.833	5.043	0.025	3.104	0.078
Day 7 Carton [DTR]	2	5.511	0.064	9.280	0.010	10.473	0.005
Day 11 DTR	1	0.561	0.454	1.453	0.228	3.677	0.055
Day 11 Carton [DTR]	2	1.019	0.601	3.199	0.202	2.183	0.336
Day 15 DTR	1	1.422	0.233	5.639	0.018	12.513	<0.001
Day 15 Carton [DTR]	2	0.083	0.959	3.965	0.138	3.979	0.137
Day 19 DTR	1	1.098	0.295	0.028	0.867	0.719	0.397
Day 19 Carton [DTR]	2	5.043	0.080	4.969	0.083	0.345	0.841
Day 23 DTR	1	2.219	0.136	0.600	0.439	5.171	0.023
Day 23 Carton [DTR]	2	0.708	0.702	1.112	0.574	1.355	0.508
Day 27 DTR	1	0.137	0.711	5.856	0.016	7.146	0.008
Day 27 Carton [DTR]	2	1.586	0.453	2.439	0.295	1.500	0.472

533

534 **Table 4:** Overall *Ae. aegypti* DENV-1 dissemination rates, as assayed by testing heads of infected
 535 females, separated by carton. For each carton, the percentage of infected females is given, along
 536 with the number of females tested in brackets.

	Carton	Constant		Small		Large	
		%	(n)	%	(n)	%	(n)
Day 7	1	33	(3)	50	(2)	0	(4)
	2	0	(6)	0	(1)	--	(0)
Day 11	1	17	(6)	57	(7)	71	(7)
	2	0	(7)	44	(9)	50	(2)
Day 15	1	100	(8)	80	(10)	71	(7)
	2	100	(7)	73	(11)	100	(1)
Day 19	1	100	(2)	100	(5)	100	(4)
	2	63	(8)	100	(7)	100	(3)
Day 23	1	100	(2)	83	(6)	100	(3)
	2	100	(4)	100	(7)	0	(1)
Day 27	1	100	(6)	100	(3)	0	(1)
	2	100	(3)	100	(7)	--	(0)

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1
2 538 **Table 5:** Pair-wise comparisons between DTR treatments for DENV dissemination in *Aedes*
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4 539 *aegypti*. Dashes indicate that the test was not conducted because there was no difference in the
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6 540 response variables for these two DTR treatments (i.e., both cartons for both treatments had 100%
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8 541 infection). Asterisks indicate the test was not performed because there was only a single individual
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10 542 to test for the large DTR treatment. Text in bold font indicates that the effect was significant ($p <$
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12 543 0.016) after Bonferroni correction for multiple comparisons.
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Source	Constant vs. Small			Constant vs. Large			Small vs. Large		
	χ^2	df	p	χ^2	df	p	χ^2	df	p
Day 7 DTR	2.374	1	0.123	0.000	1	0.998	2.993	1	0.084
Carton [DTR]	2.634	2	0.268	2.460	1	0.292	0.174	1	0.917
Day 11 Temperature	7.855	1	0.005	6.600	1	0.010	0.194	1	0.660
Carton [DTR]	1.899	2	0.387	1.953	2	0.377	0.564	2	0.754
Day 15 DTR	5.804	1	0.016	0.000	1	0.999	0.370	1	0.543
Carton [DTR]	0.154	2	0.926	0.622	2	0.733	0.775	2	0.679
Day 19 DTR	0.000	1	0.998	0.000	1	0.998	--		
Carton [DTR]	1.632	2	0.442	1.632	2	0.442	--		
Day 23 DTR	0.000	1	0.999	0.000	1	0.996	0.000	1	0.998
Carton [DTR]	1.644	2	0.440	4.499	2	0.106	6.143	2	0.046
Day 27 DTR	--			*			*		
Carton [DTR]	--			*			*		

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2 545 **Figure 1:** Survival curves for female *Ae. aegypti* exposed to DENV-1 from 0 to 27 d post exposure.
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4 546 (A) Effect of temperature fluctuations on the survival of *Aedes aegypti* females ($\chi^2 = 6.85$, df = 2, p
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7 547 = 0.032); (B) Effect of infection status on survival ($\chi^2 = 25.17$, df = 1, p < 0.0001).
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11 549 **Figure 2:** Effect of DTR on survival curves of female *Ae. aegypti*, separated by DENV-1 infection
12 550 status. (A) Exposed, infected with DENV-1 ($X^2 = 1.49$, df = 2, p = 0.473); (B) Exposed, uninfected
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15 551 ($X^2 = 3.68$, df = 2, p = 0.158)
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21 553 **Figure 3:** Effect of DENV-1 infection on female *Ae. aegypti* survival, for each of three temperature
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23 554 regime. (A) constant 26°C ($\chi^2 = 3.31$, p = 0.068); (B) small fluctuations ($\chi^2 = 13.93$, df = 1, p <
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26 555 0.001); and (C) large fluctuations ($\chi^2 = 6.59$, df = 1, p = 0.010).
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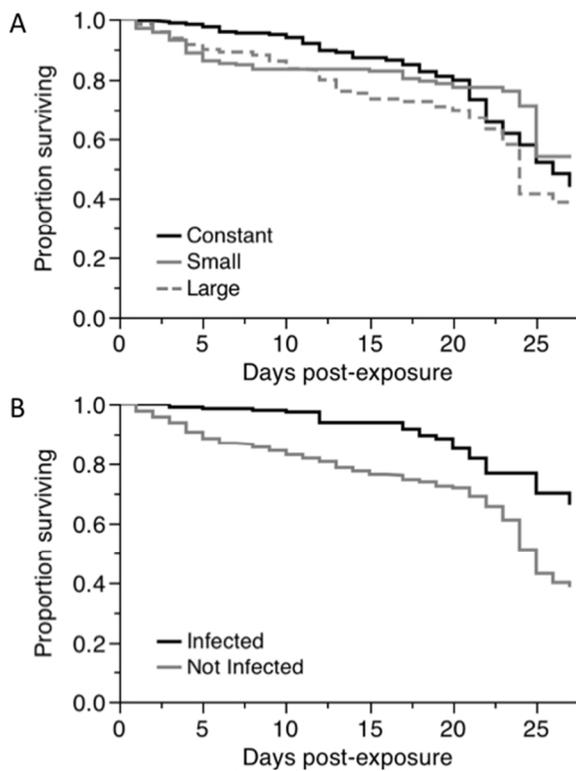


Figure 1: Survival curves for female *Ae. aegypti* exposed to DENV-1 from 0 to 27 d post exposure. (A) Effect of temperature fluctuations on the survival of *Aedes aegypti* females ($\chi^2 = 6.85$, $df = 2$, $p = 0.032$); (B) Effect of infection status on survival ($\chi^2 = 25.17$, $df = 1$, $p < 0.0001$).
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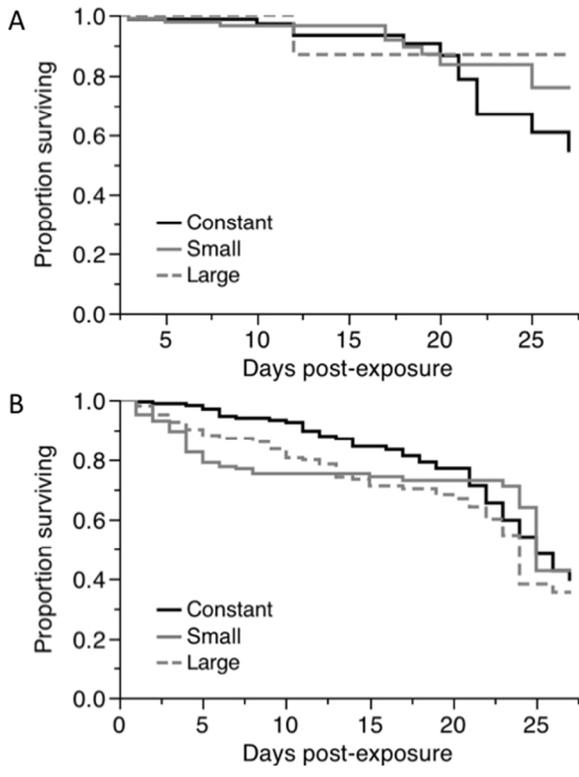


Figure 2: Effect of DTR on survival curves of female *Ae. aegypti*, separated by DENV-1 infection status. (A) Exposed, infected with DENV-1 ($\chi^2 = 1.49$, $df = 2$, $p = 0.473$); (B) Exposed, uninfected ($\chi^2 = 3.68$, $df = 2$, $p = 0.158$)
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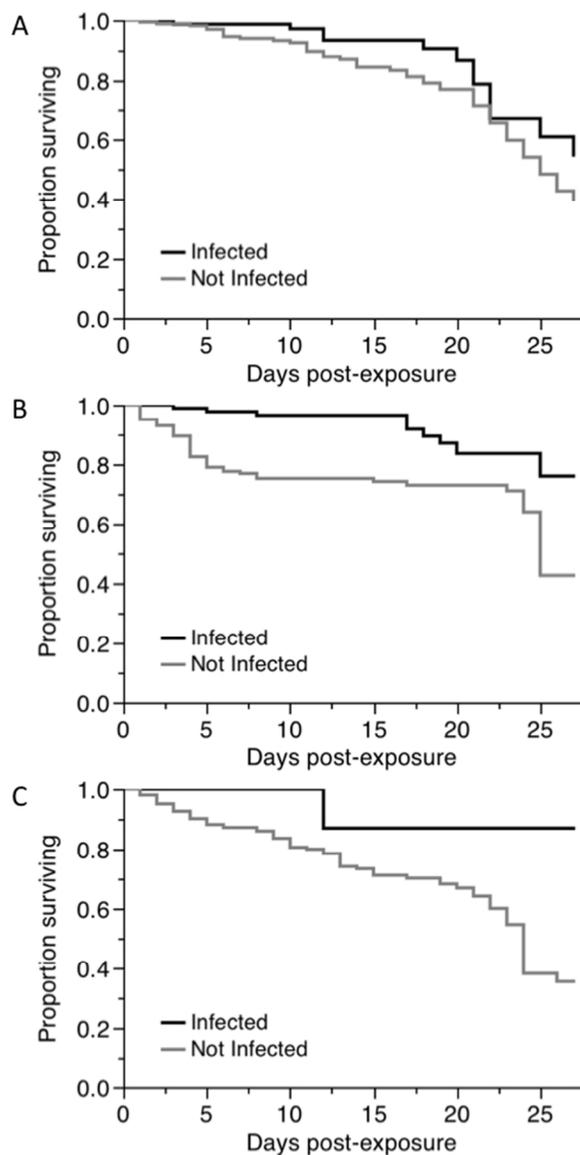


Figure 3: Effect of DENV-1 infection on female *Ae. aegypti* survival, for each of three temperature regime. (A) constant 26°C ($\chi^2 = 3.31$, $p = 0.068$); (B) small fluctuations ($\chi^2 = 13.93$, $df = 1$, $p < 0.001$); and (C) large fluctuations ($\chi^2 = 6.59$, $df = 1$, $p = 0.010$).
264x352mm (72 x 72 DPI)