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Temperature and Dengue Virus Infection in Mosquitoes: Independent Effects on the Immature and Adult Stages

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Abstract. Temperature is one of the most important environmental factors affecting biological processes of mosquitoes, including their interactions with viruses. In these studies, we show independent effects of rearing temperature on the immature aquatic stages and holding temperature on the adult terrestrial stage in terms of alterations in adult survival and progression of dengue-1 virus infection in the Asian tiger mosquito *Aedes* (*Stegomyia*) *albopictus*. Our studies show that adult survival was determined by adult-holding temperature, regardless of rearing conditions of the immature stages. In contrast, spread of virus throughout the body of the mosquito, a pre-requisite for transmission, was reduced when the immature stages were reared in cool conditions. These results show that immature-rearing temperature selectively modified mosquito traits that influence competency for viruses, and they further our understanding of the nature of temperature effects on interactions between mosquitoes and virus pathogens and risk of disease transmission.

INTRODUCTION

Biological invasions pose a major threat to ecological communities. Improving our understanding about underlying processes affecting populations of introduced species and changes to communities after introduction will assist in minimizing negative effects on native species. Invasive vector species such as mosquitoes not only have the potential to disrupt communities but also represent an epidemiological concern. Human-aided shipments of used tires have facilitated the spread of Asian tiger mosquito *Aedes* (*Stegomyia*) *albopictus*, an invasive species capable of transmitting numerous arthropod-borne (arbo) viruses.[1] Originally found in Southeast Asia, the geographic range of *Ae. albopictus* has greatly expanded. In recent years, the public health concern of *Ae. albopictus* has become a reality as witnessed by its incrimination as the vector responsible for outbreaks of dengue in Hawaii and chikungunya virus in India, La Reunion, Italy, and South Asian countries.[2–6] *Ae. albopictus* is considered a maintenance vector of dengue viruses, is occasionally involved in epidemics in Asia, and found to be naturally infected with dengue virus in the Americas.[7,8] However, the role of *Ae. albopictus* in dengue virus transmission is likely to be less than the role of the primary mosquito vector *Ae.* (*Stegomyia*) *aegypti*.[9] In North America, *Ae. albopictus* has been found to be infected with West Nile virus, LaCrosse virus, and Eastern equine encephalitis virus in nature.[10–12] However, this species does not seem to be the predominant vector of these arboviruses in North America.

Expanded range of *Ae. albopictus* directly increases risk for vector-borne diseases (e.g., dengue and chikungunya viruses) but also, may indirectly alter risk of disease transmission as mediated through interactions with other mosquito species (e.g., competitive displacement of other vector species; Yellow fever mosquito *Ae. aegypti*).[13–17] Temperature likely plays an important role in the distribution pattern of *Ae. albopictus*, which is by similar Northern isotherm limits of distribution in its native range in Asia and invaded range in North America.[18]

Temperature is regarded as one of the most important abiotic environmental factors affecting biological processes of mosquitoes, including interactions with arboviruses. Seasonal and geographic differences in temperature and anticipated climate change undoubtedly influence mosquito population dynamics, individuals' traits related to vector biology (lifespan and vector competence for arboviruses), and disease transmission patterns. Increases in adult-holding temperatures have usually been associated with enhanced vector competence.[19–27] However, some studies have identified reduced vector competence and activity in nature associated with increases in temperature (western equine encephalitis virus [WEEV] and St. Louis encephalitis virus [SLEV]).[28–31] Additionally, cool rearing temperature of the immature stages may be associated with reduced virus infection and dissemination in the adult stage.[32,33] It has long been recognized that increases in temperature reduce the extrinsic incubation period (the time from initial acquisition of pathogens until transmission is possible).[19,20] However, environmental temperature may also influence the expression of modulation, limiting virus replication and dissemination.[28] Along the same lines, increases in temperature reduce the adult lifespan of mosquitoes and may impinge transmission. Temperature effects may drastically alter risk of disease transmission, especially under conditions where the extrinsic incubation period approaches the lifespan of the mosquito. For dengue viruses, several studies have identified the role of temperature in infection and transmission by *Ae. albopictus* and *Ae. aegypti* mosquitoes and its role on the incidence of dengue in nature.[34–37] Despite the long history of relating temperature to vector biology, relatively few studies have evaluated the net effect of temperature on multiple traits (adult lifespan and vector competences for viruses) as it relates to risk of disease transmission.

Ae. albopictus as well as other holometabolous insect vectors of pathogens occupy entirely different environmental niches during the immature and adult stages. Because the immature stages of container mosquitoes are confined to aquatic habitats, the microclimate experienced by the immature stages of *Ae. albopictus* is largely determined by the placement of containers in nature and whether containers are exposed to direct sunlight for part of the day or mostly shade. In contrast, the adult stage is mobile, and diel temperature regimen is associated with mosquito activity, including movement between habitats.[38–41] Thus, shifts in environmental

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temperature between the immature and adult stages may be common in nature. Environmental temperature acting on the immature stages shapes the adult phenotype (e.g., nutritional reserves)[42–44] and therefore, may also modify traits of adults associated with ability to transmit arboviruses (e.g., lifespan and susceptibility to virus infection). Temperature-associated changes in morphology and physiology may translate to altered permissibility of midgut and salivary gland barriers that must be overcome for virus transmission to subsequent hosts.

Here, we addressed the independent effects of temperature on immature and adult stages in relation to life history traits and interactions with dengue-1 virus. Specifically, we hypothesize that cool rearing temperature of immature stages (1) buffers against life-shortening effects of warm holding conditions of adults and (2) reduces rates of dengue-1 virus infection and dissemination of adults.

MATERIALS AND METHODS

Field-collected larvae from discarded tires were reared to adulthood in enamel pans with ~1.0 L tap water and 0.2 g equal mixture of brewers yeast and lactalbumin as food resources. Food resources were renewed on a weekly basis. Adults were maintained in 0.32-m^3 cages with access to a 20% sucrose solution and weekly bloodmeals from guinea pigs and chickens. Eggs were collected on paper towels that lined cups with water placed inside the cages. Mosquitoes were kept in climate-controlled rooms at ~24°C and a 14:10-hour light:dark cycle. Subsequent generations of mosquitoes were maintained using the same methodologies.

Experiment 1: Life history traits. *Ae. albopictus* used were progeny of mosquitoes collected in Florida (F_6–F_7). Experimental units consisted of 400 mL Tri-Pour polypropylene beakers with 1.0 g *Quercus virginiana* live oak leaves (dried for at least 24 hours at 60°C), 0.25 g *Setaria faberi* giant foxtail grass, 350 mL deionized water, and 10 mL live oak leaf infusion water[45]; 60 newly hatched larvae less than 24 hours old were added to experimental units 5 days after setup. We manipulated temperature treatments during the immature and adult stages as 20–20°C, 20–25°C, 20–30°C, 25–25°C, 30–20°C, 30–25°C, and 30–30°C using environmental chambers where the first number refers to rearing temperature of immature stages and the second number refers to holding temperature of adults. Supplemental food resources (1.0 g oak leaves + 0.25 g foxtail grass) were added on days 7 and 14 after addition of larvae. The original water volume was maintained throughout the experiment by weekly additions of deionized water to experimental units. Each treatment was replicated eight times for a total of 56 experimental units. Pupae were transferred from experimental units to plastic vials with a cotton seal to capture adults.

On emergence, adults were recorded as male or female, and date of development to adulthood was recorded. Adult females were transferred to 16-oz cylindrical paperboard cages with screen tops and given cotton soaked in water. Mosquitoes were held in groups according to the treatment experimental units. Adult females were monitored for survival at 12-hour intervals. Dead adults were recorded and stored at −20°C. Dead females were dried at 60°C for at least 24 hours, and their dry weights were determined to the nearest milligram. For each experimental unit, we measured response variables survivorship to adulthood, development time (male and female), female weight, and female lifespan.

Experiment 2: Vector competence for dengue-1 virus. *Experimental setup and bloodfeeding adult females. Ae. albopictus* used were progeny of mosquitoes collected in Florida (F_3). Experimental units consisted of plastic containers with lids, 2.0 L water, and 0.1 g equal mixture of brewers yeast and lactalbumin as food resources; 200 newly hatched larvae less than 24 hours old were added to experimental units on the same day of the experimental setup. Additions of 0.1 g supplemental food resources were added on days 2, 7, and 12 after addition of larvae. To achieve sufficient sample sizes to estimate infection and dissemination rates, we needed to increase the scale of environmental conditions (larval resources and initial number of mosquitoes) from experiment 1. We manipulated temperature treatments during the immature and adult stages as 20–20°C, 20–30°C, 25–25°C, 30–20°C, and 30–30°C using environmental chambers. Treatment temperatures were chosen based on the range of environmental temperature in tropical and subtropical dengue-endemic regions where *Ae. albopictus* occurs.[46] Each treatment was replicated seven times for a total of 35 experimental units. Pupae were transferred from experimental units to plastic vials with a cotton seal to capture adults.

On emergence, adults were recorded as male or female; date of development to adulthood was recorded, and they were placed in cages together to facilitate mating. Mosquitoes were held together according to the treatment experimental units. Females were deprived of sucrose but not water 48 hours before bloodfeeding trials. Ages of adult females were between 7 and 10 days at the time of feeding. *Ae. albopictus* females were provided with blood infected with dengue-1 virus (strain BOL-KW010) isolated from a human infected in Key West, Florida in 2010 (Florida Department of Health). The virus was passaged three times in African green monkey kidney (Vero) cells. Mosquitoes were allowed to imbibe blood for 60 minutes from an artificial membrane feeding system (Hemotek, Discovery Workshops, Accrington, UK). During feeding trials, all females were kept at 30°C to maximize feeding rates. Immediately after the feeding trials, females were returned to their respective holding temperature treatments.

After bloodfeeding trials, *Ae. albopictus* were cold-anesthetized, and fully engorged females were transferred to 0.5-L cardboard cages with mesh screening along with a 30-mL plastic cup attached to the bottom of the cage for an oviposition site and a cotton ball soaked in 20% sucrose solution. Oviposition cups were kept moist during the time that mosquitoes underwent oviposition. Mosquitoes were then placed at the appropriate holding temperature treatment along with ~70–80% humidity and a 14:10-hour light:dark cycle. Incubator temperatures (mean ± SD) were monitored (HOBO data logger; Onset, Bourne, MA) throughout the experiment. After 14 days of incubation, mosquitoes were killed and stored at −80°C; they were later tested for presence of dengue-1 virus RNA.

Preparation of infectious bloodmeals. Propagation of dengue virus (DENV) for bloodmeals was accomplished by inoculating tissue culture flasks (175 cm^2) with confluent monolayers of Vero cells with 250 μL virus stock (multiplicity of infection approximated at 0.0004 plaque-forming units [pfu] per cell). Dengue virus inoculum was allowed to incubate for 1 hour at 35°C with a 5% carbon dioxide atmosphere. After incubation, 25 mL media (199 media, 10% fetal bovine serum, 0.2% antimycotic, and 2% penicillin-streptomycin) were added

to each flask. Infectious bloodmeals were prepared using freshly harvested media and virus from tissue culture flasks inoculated 7 days previously and combined with defibrinated bovine blood (Hemostat, Dixon, CA) in a 1:1 ratio of media/virus:blood. A total of 12 bloodmeals were provided to cohorts of mosquitoes. Aliquots of infected bloodmeals were stored at −80°C for later determination of virus titer.

Mosquito processing. Mosquitoes stored at −80°C were dissected to remove their legs and one wing from the remainder of the body. Separate assays of body and legs were used to determine infection and dissemination rates of DENV as well as viral titer. Wing length measurements were used as an indicator of mosquito size. Samples were triturated separately in microcentrifuge tubes with two copper clad beads and 0.9 mL BA-1 at 25 Hz for 3 minutes (TissueLyser; Qiagen, Inc., Valencia, CA) and subsequently, were subject to centrifugation at 4°C. Nucleic acid was extracted from a 250-μL sample and eluted in 50 μL buffer using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, Indianapolis, IN). The amount of viral RNA present in samples was determined using the Superscript III One-Step Quantitative RT-PCR System (qRT-PCR; Invitrogen, Carlsbad, CA) with a Light Cycler 480 system (Roche, Mannheim, Germany) using methods described elsewhere.[47] A standard curve method was used to relate the amount of DENV RNA present in samples to 10-fold serial dilutions of virus stock with known concentrations expressed in pfu per milliliter.[48,49] Mosquitoes were categorized based on status of infection: disseminated infections, positively infected bodies and legs; non-disseminated infections, infected bodies but the absence of virus in legs; and uninfected, absence of virus in the body. The infection rate was the percentage of all mosquitoes tested having infected bodies. The dissemination rate was the percentage of mosquitoes with infected bodies that also had infected legs. A total of 633 individuals was assayed for dengue virus infection and dissemination. Viral titers were obtained for the same individual mosquitoes.

Statistical analyses. Treatment effects on life history traits were analyzed using analysis of variance (ANOVA) using container microcosm as the experimental unit. We lacked the facilities to independently replicate temperature independently for each container and cage. Rather, temperature treatment was manipulated for the entire environmental chamber, and therefore, we assume that between-environmental chamber variation (other than temperature) is negligible. When significant effects were detected, we used pair-wise contrasts of means adjusted for an experiment-wise α of 0.05 (Tukey–Kramer adjustment for multiple comparisons; PROC GLM; SAS 9.22). Treatment effects on age-specific survival of adults using lifespan of adult females were compared using non-parametric survival analysis (PROC LIFETEST; SAS 9.22). When significant effects were detected, we used log-rank test statistics to compare pair-wise estimates of survival adjusting for multiple comparisons using the Sidak method. Correlation analyses were used to relate relationships between adult female lifespan and dry weight measured after death for temperature treatments. Treatment effects on vector competence (virus infection, dissemination, and titer) were analyzed similarly using ANOVA and pair-wise contrasts of means adjusting level of significance for multiple comparisons.

RESULTS

Experiment 1: Life history traits. ANOVA showed no temperature treatment effects on survivorship to adulthood or dry weight of adult females (Figure 1A and B and Table 1). There was a significant effect of treatment on development time to adulthood (Table 1). For both females and males, significantly shorter development times were observed at warmer larval-rearing conditions relative to cooler conditions for all three larval-rearing temperature treatments (Figure 1C). There was a significant effect of treatment on female survival of adults (Table 1). Mosquitoes held at 20°C during the adult stage had significantly greater survival than other temperatures, regardless of rearing temperature of immature stages (Figure 1D). Mosquitoes held at 25°C during the adult stage had significantly greater survival, regardless of rearing temperature of immature stages, than individuals held at 30°C during the adult stages (Figure 1D). Adult female survival was not modified by immature-rearing temperature treatments (Figure 1D).

Correlation analyses showed significant positive relationships between adult female lifespan and dry weight measured after death for temperature treatments 20–20°C ($r = 0.33$, $N = 98$, $P = 0.0004$), 30–20°C ($r = 0.37$, $N = 82$, $P = 0.0007$), and 20–30°C ($r = 0.43$, $N = 100$, $P < 0.0001$). All remaining comparisons were not significant after correcting P values for multiple comparisons.

Experiment 2: Vector competence for dengue-1 virus. *Life history traits.* ANOVA showed no temperature treatment effects on survivorship to adulthood (Figure 2A and Table 2). There was a significant effect of treatment on female wing length and development time to adulthood (Table 2). Significantly shorter development times and wing lengths were observed at warmer larval-rearing conditions relative to cooler conditions for all three larval-rearing temperature treatments (Figure 2B and C). These treatment effects on development time were similar for both females and males.

Susceptibility to dengue virus infection and dissemination. Bloodmeal titers estimated from an aliquot of the suspension were 7.09 ± 0.14 $\log_{10}$ pfu DENV/ml (mean ± SD). ANOVA showed no significant temperature treatment effect on susceptibility to dengue virus infection 14 days after imbibing dengue-infected blood (Table 2). There was a significant effect of treatment on virus dissemination 14 days after imbibing dengue-infected blood (Table 2). Mosquitoes held at 20°C during the adult stage had significantly lower dissemination rates than all other temperature treatments, regardless of rearing temperature of immature stages (Figure 3). Mosquitoes held at 25°C during the adult stage had significantly lower dissemination rates than individuals maintained at 30–30°C but not 20–30°C treatments (Figure 3). Individuals maintained at the 20–30°C treatment had significantly lower disseminations than individuals maintained at 30–30°C (Figure 3).

ANOVA on body titer of mosquitoes with non-disseminated infections showed that individuals in the 25–25°C treatment had significantly higher or similar body titers than individuals in the 20–20°C, 30–20°C, and 20–30°C treatments 14 days after imbibing dengue-infected blood (Figure 4 and Table 2). The body titers of mosquitoes from the 30–30°C treatments were significantly lower or similar than all other treatments. For mosquitoes with disseminated infections, ANOVA showed that body titers in the 20–30°C, 30–30°C, and 25–25°C treatments were significantly higher or equal to all other treatments (Figure 4 and Table 2). The lowest body titers were observed in 20–20°C and 30–20°C treatments, which were similar to each other (Figure 4). Leg titers were similar for all treatment groups except for the 30–20°C treatment, which was

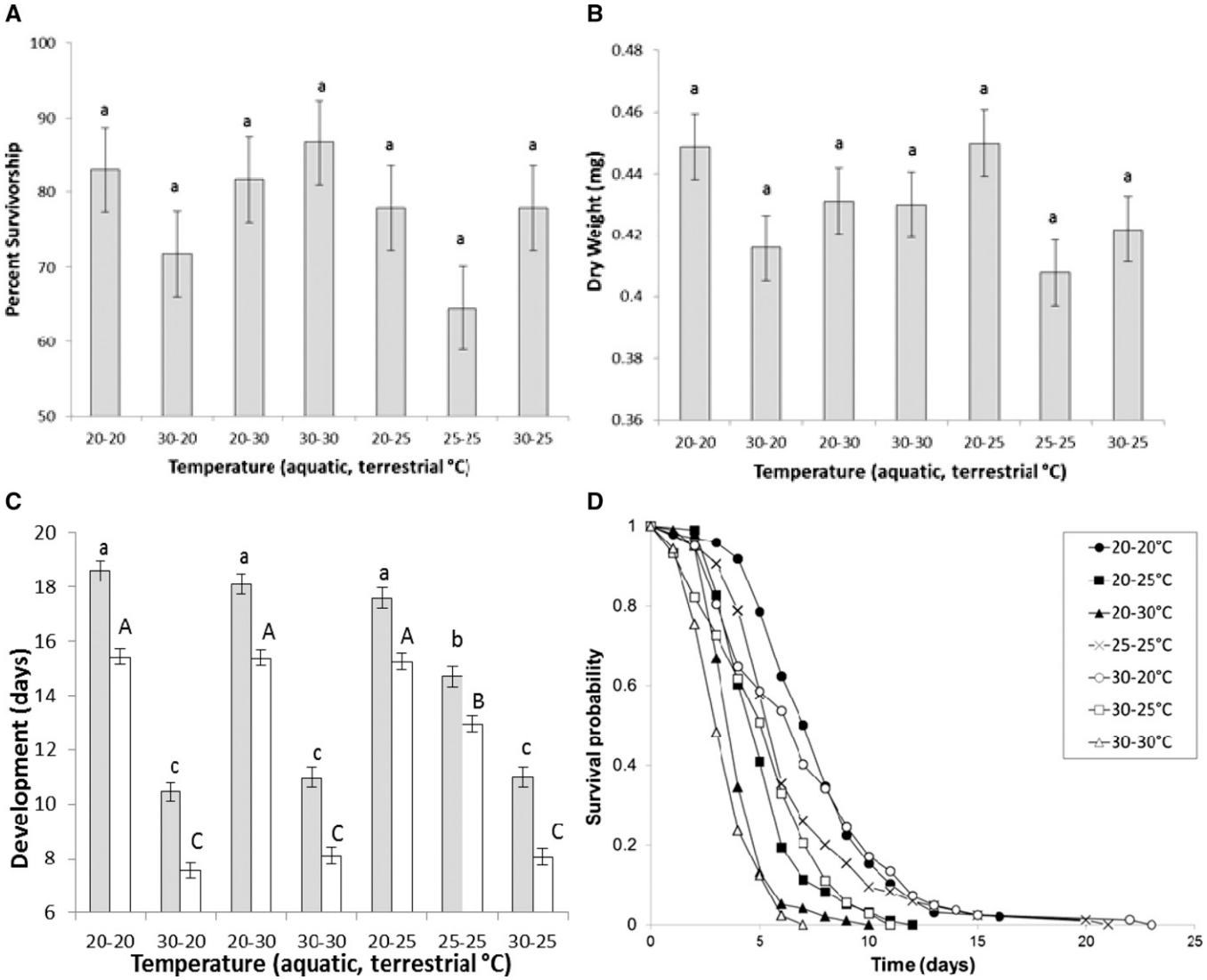

FIGURE 1. Adult lifespan and life history traits (experiment 1). Mean (± standard error) of (**A**) survivorship, (**B**) dry weight, (**C**) development (females and males shown by grey and white bars, respectively), and (**D**) lifespan of *Ae. albopictus* from temperature treatments. Means followed by different letters are significantly different from one another.

significantly lower than all other treatments (Figure 4 and Table 2).

DISCUSSION

Survivorship to adulthood was similar between the larval-rearing temperature range of 20°C and 30°C, suggesting that these temperatures did not impose substantial stress to induce mortality in *Ae. albopictus*. Similar results have been observed for temperatures in this range for *Ae. albopictus*,[32,33,50] although wider ranges of temperature have been shown to influence survivorship to adulthood.[51,52] Development time increased with decreasing temperatures for both males and females, with temperature-specific rates being similar for both experiments, despite several differences in experimental setup between the experiments (water volume, nutrients, and larval density). Development time to adulthood was shorter for males than females, likely attributable to lower nutritional thresholds for males of most mosquito species. Because development times were determined on emergence to adulthood, only environmental temperature of immature stages contributed to observed temperature effects on development.

Temperature-dependent differences were observed in wing lengths but not dry weight of adult females from the two experiments. The lack of an effect of temperature on dry weight is surprising given that other studies have identified differences in masses of adult *Ae. albopictus* reared within this range of environmental temperatures.[33] In experiment 1, mosquitoes were deprived access to adult nutrition and therefore, forced to

TABLE 1
Life history traits (experiment 1)

Life history trait	d.f.	F	χ^2	P
Percent survivorship to adulthood	6, 49	1.71	–	0.137
Female dry weight	6, 49	2.20	–	0.059
Female development	6, 49	95.62	–	< 0.0001
Male development	6, 49	149.27	–	< 0.0001
Female adult survival	6	17.36	149.26	< 0.0001

ANOVA for temperature effects on life history traits of *Ae. albopictus*. d.f. = degrees of freedom.

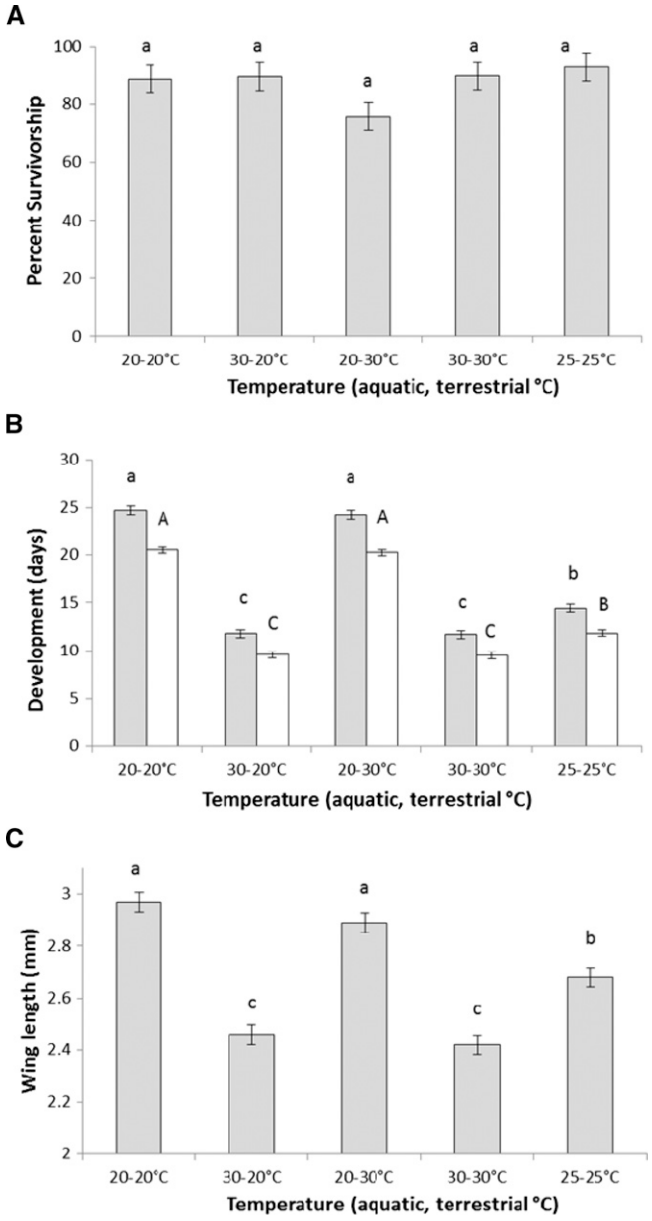

FIGURE 2. Adult life history traits for mosquitoes exposed to dengue-1 virus (experiment 2). Mean (± standard error) of (**A**) survivorship, (**B**) development (females and males shown by grey and white bars, respectively), and (**C**) wing length of *Ae. albopictus* from temperature treatments. Means followed by different letters are significantly different from one another.

rely on nutrition acquired in the immature environment. Depletion of nutritional reserves and perhaps, associated biomass may obscure differences in dry weight between temperature treatments. In addition, recent studies have shown differences in temperature-dependent allometric relationship between body mass and wing length in *Ae. albopictus*,[53] accounting, in part, for discrepancies in temperature effects on size reported here.

Cooler holding temperatures of adult females were associated with greater survival relative to warmer temperatures, regardless of rearing temperature of the immature stages (20°C > 25°C > 30°C). Temperature during the immature stages did not impinge on adult survival. This result was somewhat surprising given that immature-rearing temperature

TABLE 2
Vector competence for dengue-1 virus (experiment 2)

Response variable	d.f.	*F*	*P*
Life history trait			
Survivorship	4, 30	1.97	0.123
Female wing length	4, 30	44.47	< 0.0001
Female development	4, 30	237.94	< 0.0001
Male development	4, 30	266.51	< 0.0001
Vector competence			
Virus infection	4, 30	1.29	0.2975
Virus dissemination	4, 30	72.89	< 0.0001
Body titer (non-disseminated infection)	4, 24	6.77	0.0008
Body titer (disseminated infection)	4, 24	7.15	0.0006
Leg titer (disseminated infection)	4, 24	6.45	0.0011

ANOVA for temperature effects on life history traits and vector competence of *Ae. albopictus* for dengue-1 virus. d.f. = degrees of freedom.

determines the phenotypic traits of adults (e.g., nutritional reserves[42–44]). These results suggest that cool larval-rearing temperature does not buffer against life-shortening effects of warm holding conditions of the adults. Rather, female adult survival is robust to temperature experienced during the immature stages. It is plausible that longer development times associated with cool immature-rearing conditions facilitate the production of large-sized adults because of greater nutrient uptake and energy reserves at emergence to adulthood,[42–44] perhaps extending survival of adults. However, we can exclude this explanation for the current study, because adult female size (dry weight) was similar across temperature treatments. Despite the lack of treatment effects on female weight, heavier adult female mosquitoes did experience greater survival in some treatments where either immature-rearing or adult-holding conditions included 20°C, suggesting that increased size has life-lengthening effects in some instances.[54–56]

We observed temperature treatment effects on *Ae. albopictus* vector competence for dengue-1 virus. Regardless of immature-rearing or adult-holding temperature, susceptibility to viral infection was similar for all temperature treatments. These results suggest that barriers to midgut infection were robust to a range of temperatures. We are unable to rule out the possibility that a common environmental temperature of 30°C during bloodfeeding trials may have contributed, in part, to similar infection rates. However, mosquitoes were only exposed to a common temperature of 30°C for a short period of time during bloodfeeding trials (60 minutes), after which time they were immediately returned to their respective temperature treatments.

In contrast to infection rates, midgut escape barriers preventing dissemination were strongly influences by both rearing temperature of immature stages and holding temperature of the adult stage. A holding temperature of 20°C during the adult stage resulted in the lowest rates of viral dissemination. Rates of dissemination were higher at 25°C and still higher at 30°C relative to cooler holding temperatures of adults. These results corroborate observations found for laboratory studies examining susceptibility to dengue virus infection and length of the extrinsic incubation period in *Ae. albopictus* and *Ae. aegypti*[37,57–59] as well as the relationship between temperature and the occurrence of dengue in nature.[34–37] The current experiment did not test mosquitoes at multiple time points post-exposure to dengue virus to identify the extrinsic incubation period (the time between initial acquisition of the pathogen and when transmission is possible).

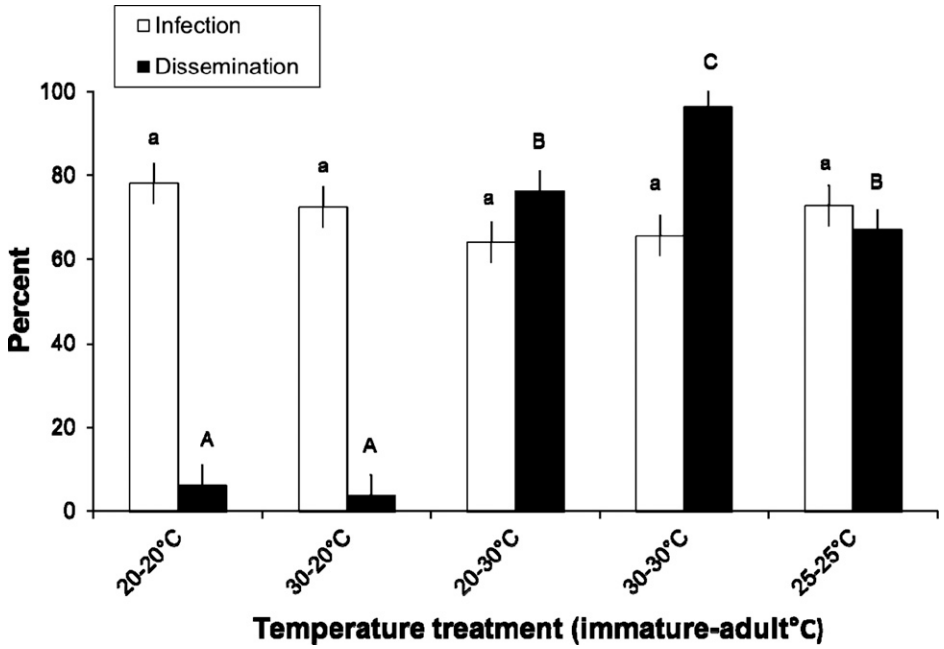

FIGURE 3. Vector competence for mosquitoes exposed to dengue-1 virus-infected blood (experiment 2). Mean (± standard error) susceptibility to dengue virus infection and dissemination of *Ae. albopictus* from temperature treatments. The numbers of mosquitoes assayed to determine infection and dissemination rates were 141 (20–20°C), 144 (30–20°C), 85 (20–30°C), 125 (30–30°C), and 138 (25–25°C). Means followed by different letters are significantly different from one another.

The extrinsic incubation period of dengue virus depends on temperature, and therefore, it is unclear whether sampling at later than 14 days post-exposure to dengue virus may have resulted in higher rates of dissemination, especially at cooler temperatures known to slow the progression of virus infection in the mosquito.[37] We also identified that the temperature during the immature stages influences rates of dengue-1 virus dissemination independent of the adult-holding temperature.

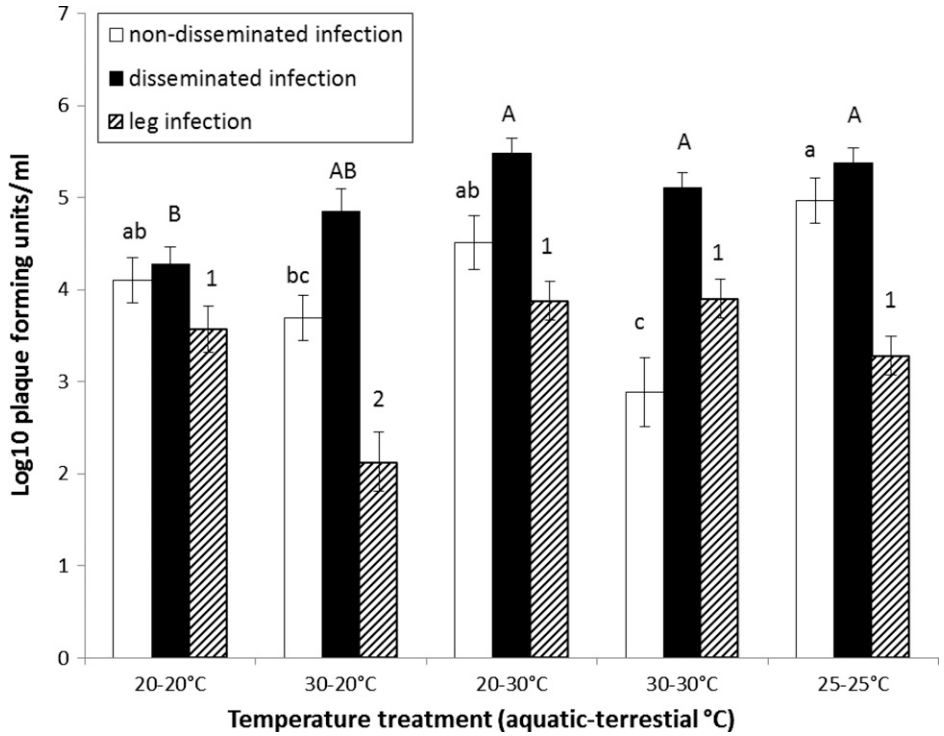

FIGURE 4. Viral titers in mosquitoes with non-disseminated and disseminated dengue-1 virus infection (experiment 2). Mean (± standard error) viral titers of individual adult female *Ae. albopictus* from temperature treatments. Means followed by different lower and upper case letters denote significant differences of body titer for non-disseminated and disseminated infections, respectively. Means followed by different numbers show significant differences of leg titers. No comparisons are made between body titers of individuals with non-disseminated and disseminated infections or leg titers.

Specifically, mosquitoes reared at a cool temperature during the immature stage but held at high temperature at the adult stage (20–30°C) had approximately 21% reduction in rates of viral dissemination relative to females maintained at warm temperatures during both the immature and adult stages (30–30°C). These results suggest long-lasting effects of the immature stage environment on phenotypes of adults related to progression of viral infection. In particular, the efficacy of the midgut escape barrier to dengue virus is substantially improved by cooler rearing conditions of the immature stages. Although the mechanism responsible for alterations in the midgut escape barrier is unclear, these results provide a direct association between immature-stage rearing temperature and progression of viral infection in adults. Previous studies have suggested that differences in the thickness of the basal lamina in response to larval conditions may influence the efficiency of arbovirus dissemination.[60] However, there are numerous other aspects of the mosquito biology beyond morphology that may affect the efficacy of the midgut escape barrier. The current experiment used nutrient-rich resources (yeast and lactalbumin) that do not mimic natural basal resources in container habitats (plant and invertebrate detritus). Future experiments will need to assess whether the observed temperature effects in this study translate to resources conditions found in nature.

Observations reported here are consistent with studies investigating the effects of heat shock and elevated temperature during immature stages and susceptibility to infection and dissemination of dengue and Sindbis viruses in *Ae. aegypti*.[32,33,61] In contrast, laboratory studies using other *Alphaviruses* and *Bunyaviruses* show enhanced viral infection or dissemination of mosquitoes reared in cool ambient temperatures (Chikungunya virus[52], Rift Valley Fever virus, and Venezuelan equine encephalitis virus[23]) relative to warmer conditions. Similarly, enhanced seasonal activity of *Alphavirus* Western equine encephalitis virus, but not *Flavivirus* St. Louis encephalitis virus, correlates to cool ambient temperature.[29] Taken together, these observations suggest that temperature effects on vector efficiency depend on the particular vector–virus system. Given these observations, it is tempting to draw the conclusions that warmer rearing conditions may enhance competence of mosquitoes for *Flaviviruses* but depress competence for other arboviruses (*Alphaviruses* and *Bunyaviruses*). However, caution is advised in interpreting potential fundamental differences in the influence of temperature on mosquito–virus interactions because of inconsistencies in observed temperature effects (e.g., warm larval-rearing conditions increased susceptibility to infection and dissemination for *Alphavirus* Sindbis[33,62]; cool larval-rearing conditions enhanced horizontal and vertical transmission of St. Louis encephalitis and Murray Valley encephalitis *Flaviviruses*[22,31]). The constant temperatures used here do not capture the daily temperature regimen in nature known to influence dengue virus[63] and malaria transmission,[64,65] an important consideration for predictive models and control efforts. Our experiment was designed as a general test of separating temperature effects acting on the immature and adult stages. An intriguing possibility for future work is an investigation to determine whether the drastic temperature shifts (up to 10°C) between the immature and adult stages have similar consequences on longevity and susceptibility to dengue virus infection in nature, where diel temperature is variable. Regardless, the current studies results may serve as a starting point in improving predictive models assessing the risk of dengue virus transmission and control efforts by incorporating stage dependency in the manner by which temperature influences transmission.[63,66,67]

For mosquitoes with disseminated infections, moderate to high holding temperatures as adults (25°C and 30°C) resulted in the highest viral titers, regardless of immature-rearing temperature. Mosquitoes held at low temperature as adults (20°C) had the lowest viral titers. These observed results are consistent with higher titers of dengue-2 virus in *Ae. aegypti* maintained at high temperatures[37] and the anticipated direct relationship between viral titer and duration of the extrinsic incubation period.[37,68] However, in the current study, this latter effect was modified by the immature temperature environment. Specifically, higher immature temperatures (30°C) resulted in viral titers similar to viral titers observed in mosquitoes held at 30°C as adults, despite being maintained at low adult-holding temperature (20°C). Thus, it seems that warm conditions experienced during the immature and adult stages may enhance viral replication in mosquitoes with disseminated infections as indicated by higher titers in these individuals. However, these effects do not translate to all mosquito tissues given that the opposite result was observed in the legs of individuals with disseminated infections for mosquitoes from the 30–20°C treatment. The explanation for these observed effects in body versus leg titers of mosquitoes with disseminated infections is not entirely clear, but it does suggest complex effects of temperature on virus replication and by extension, mosquito immune function[69] contingent on both immature- and adult-rearing environment. High viral loads and rates of dissemination associated with warm conditions may favor vertical transmission, allow dengue virus to persist during interepidemic periods,[70] and contribute to the epidemiology of dengue. In Thailand, where all four serotypes of dengue virus co-occur, a field study showed direct trends in increases in vertical transmission of dengue virus and temperature preceding increases of incidence of human infections.[71] However, in the current study, for mosquitoes with non-disseminated infections, individuals reared at 20°C and 25°C during their immature stages tended to have the highest body titers, whereas mosquitoes from elevated temperatures during the immature stages tended to have the lowest body titers. These results suggest complex relationships between temperature-dependent viral titers, which depend on progression of infection beyond the midgut of mosquitoes (non-disseminated versus disseminated infections).

The current study underscores the importance of the environmental temperature experienced by immature stages in shaping adult phenotypes. Environmental temperature of immature stages selectively modified traits of adult mosquitoes related to virus transmission, furthering our understanding of the nature of temperature effects on interactions between mosquitoes and virus pathogens and risk of disease transmission. We show that rearing temperature of immature stages affects adult mosquito interactions with dengue virus, most likely attributable to alterations in viral replication and efficacy of the midgut escape barrier.

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