

Larval Temperature–Food Effects on Adult Mosquito Infection and Vertical Transmission of Dengue-1 Virus

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Abstract

Temperature–food interactions in the larval environment can affect life history and population growth of container mosquitoes *Aedes aegypti* (L.) and *Aedes albopictus* Skuse, the primary vectors of chikungunya and dengue viruses. We used *Ae. aegypti*, *Ae. albopictus*, and dengue-1 virus (DENV-1) from Florida to investigate whether larval rearing temperature can alter the effects of larval food levels on *Ae. aegypti* and *Ae. albopictus* life history and DENV-1 infection and vertical transmission. Although we found no effect of larval treatments on survivorship to adulthood, DENV-1 titer, or DENV-1 vertical transmission, rates of vertical transmission up to 16–24% were observed in *Ae. albopictus* and *Ae. aegypti*, which may contribute to maintenance of this virus in nature. Larval treatments had no effect on number of progeny and DENV-1 infection in *Ae. aegypti*, but the interaction between temperature and food affected number of progeny and DENV-1 infection of the female *Ae. albopictus* parent. The cooler temperature (24°C) yielded the most progeny and this effect was accentuated by high food relative to the other conditions. Low and high food led to the highest (~90%) and lowest (~65%) parental infection at the cooler temperature, respectively, whereas intermediate infection rates (~75–80%) were observed for all food conditions at the elevated temperature. These results suggest that temperature and food availability have minimal influence on rate of vertical transmission and a stronger influence on adults of *Ae. albopictus* than of *Ae. aegypti*, which could have consequences for dengue virus epidemiology.

Key words: larval ecology, infection, transmission, dengue

Dengue viruses are responsible for up to 390 million infections per year (Kroeger et al. 2004, WHO 2012). Transmission to humans occurs by widespread invasive mosquitoes *Aedes aegypti* and *Aedes albopictus* (WHO 2012). Horizontal (human–mosquito–human) transmission is the best known mechanism of DENV transmission. However, vertical transmission, the transfer of virions from an infected female mosquito to her progeny has also been documented in natural populations of *Ae. aegypti* and *Ae. albopictus* (Angel and Joshi 2008, Martins et al. 2012, Espinosa et al. 2014). Additionally, DENV vertical transmission has been demonstrated in both mosquito species in the laboratory (Rosen et al. 1983, Shroyer 1990, Bosio et al. 1992, Castro et al. 2004, Buckner et al. 2013). Though the occurrence of DENV vertical transmission is clear, its role in the DENV transmission cycle is not well-understood. A frequently cited hypothesis is that vertical transmission serves as a mechanism that allows DENV to persist in nature during inter-epidemic periods (Rosen et al. 1983, Hull et al. 1984, Joshi et al. 2002, Guo et al. 2007, Angel and Joshi 2008).

Mosquitoes possess a complex life cycle in which the immature and adult stages occupy distinctly different environments. An expansive literature demonstrates that the environmental factors

experienced by an adult female mosquito can affect her vector competence, the innate ability of the vector to support replication and transmission of a pathogen (Turell 1993, Richards et al. 2007). Additionally, growing evidence suggests that effects of the larval environment can continue through the adult stage to also influence vector competence, because innate immunity, infection barriers, and escape barriers develop during larval stages (Grimstad and Walker 1991; Reiskind and Lounibos 2009; Muturi et al. 2011a, 2012; Alto and Lounibos 2013). In particular, the interaction between larval temperature and nutrient availability may affect mosquito biology and vector competence, especially for container mosquitoes (Padmanabha et al. 2011). In urban and suburban areas in DENV endemic climates (≈20–30°C), *Ae. aegypti* and *Ae. albopictus* larvae primarily inhabit water-filled artificial containers like water storage containers, plant vases, and tires. Container habitats often are associated with episodic and irregular inputs of basal nutrients and crowded larval conditions causing larvae to experience food limitation (Southwood et al. 1972, Subra and Mouchet 1984, Arrivillaga and Barrera 2004, Juliano 2007, Padmanabha et al. 2010), which may affect adult nutrient reserves (Briegel 1990) and so may influence adult morphology (size) and physiology (immune functions;

Ahmed et al. 2002, Schwartz and Koella 2004, Telang et al. 2012, Kim and Muturi et al. 2013). Temperature in these container habitats may also affect mosquito larval development and survival within food-limited conditions by modifying feeding rate, efficiency of food conversion into biomass, or ability to survive starvation (Rashed and Mulla 1989, Padmanabha et al. 2011). Therefore, by affecting adult morphology, physiology, and survival, temperature–food interactions in the larval environment could have consequences for *Ae. aegypti* and *Ae. albopictus* biology and DENV vector competence. If observed larval environment-induced changes in infection and horizontal transmission also affect changes in vertical infection (Turell 1993, Alto and Bettinardi 2013), then the parental environment may contribute to infection and transmission by the progeny. Further, we may expect that the relative role of vertical transmission in dengue epidemiology is dynamic and changes with environmental conditions. Based on findings investigating single treatment effects, we predict that nutrient stress and elevated temperature may reduce barriers to dengue virus vertical transmission (Zhang et al. 1993, Alto and Bettinardi 2013, Alto and Lounibos 2013).

Previously, for dengue-1 virus (DENV-1) isolated during the 2010 Key West, Florida outbreak, we demonstrated infection and dissemination rates of 93.3 and 80.2% for *Ae. aegypti* and 93.0 and 84.9% for *Ae. albopictus*, respectively, which had fed on infected blood with viremia levels observed in humans (Buckner et al. 2013). In this same study, we documented DENV-1 vertical transmission rates of 8.3% for *Ae. aegypti* and 11.1% for *Ae. albopictus* (Buckner et al. 2013). In the current work, we test the hypothesis that larval temperature alters the effects of larval food levels that continue through the adult stage and influence DENV-1 infection and transmission to their progeny (vertical transmission). We interpret this information in the context of measurements on mosquito life history: survivorship to adulthood, development time, and number of progeny of *Ae. aegypti* and *Ae. albopictus*.

Materials and Methods

Rearing of *Ae. aegypti* and *Ae. albopictus*

We manipulated temperature and food level during the immature stages of the parental (P_1) generation of *Ae. aegypti* and *Ae. albopictus* mosquitoes using a two-level full factorial experimental design with six replicates per treatment for 24 experimental units. Experiments were conducted independently for each species to increase the number of replicates per treatment. The P_1 generation used in the *Ae. aegypti* experiment was the F_6 progeny of larvae collected from Key West, FL. The P_1 generation used in the *Ae. albopictus* experiment was the F_2 progeny of larvae collected from the campus of the Florida Medical Entomology Laboratory in Vero Beach, FL. Experimental units consisted of 7.5-liter containers (height by diameter: 24 by 23.5 cm) with 5 liters of tap water and 500 first-instar larvae. We controlled larval temperature by maintaining experimental units in incubators at $24 \pm 0.5^\circ\text{C}$ and $30 \pm 0.5^\circ\text{C}$. These temperatures were chosen because they are within the range in which *Ae. aegypti* and *Ae. albopictus* develop and dengue virus may circulate. Larval food level was controlled by providing containers with either 250 mg or 500 mg of an equal mixture of brewer's yeast and lactalbumin on the day larvae were added. Supplemental food resources (250 mg or 500 mg depending on treatment) were added to containers on days 4 and 11 after addition of larvae.

The time until pupation and the number of mosquitoes surviving to pupation for each experimental unit were recorded, and pupae

were transferred to water-filled cups and placed in 0.3-m^3 adult cages labeled according to experimental unit and date. Only pupae collected for five consecutive days from the same experimental unit were placed in the same adult cage, which narrowed the ages of adult mosquitoes in each cage to a 5-d period to ensure that infectious bloodmeals would later be provided to similarly aged adult females to minimize the possibility for age-related variation in measurements (Buckner et al. 2013). After emergence, male and female mosquitoes were held together for approximately 7–12 d at $30 \pm 0.5^\circ\text{C}$ to allow mating and provided with 20% sucrose solution, except for 48 hours before infectious bloodmeals, when the sucrose solution was replaced by tap water. Adults were then aspirated from each cage, cold-anesthetized, and sorted on a chill table (BioQuip Products, Rancho Dominguez, CA). Males were separated from females and discarded. Females were transferred to 1-liter cylindrical cartons covered by screen, with approximately 75 adults per carton for the feeding trials with DENV-1-infected blood.

Preparation of Infectious Bloodmeal

Propagation of DENV-1 virus for bloodmeals followed previously established methods (Buckner et al. 2013). Briefly, confluent monolayers of African green monkey kidney (Vero) cells were inoculated with 250 μl of DENV-1 (GenBank accession no. JQ675358, originally isolated from a human infected in Key West, FL, in 2010 and passaged three times in Vero cells) at a multiplicity of infection of 0.3. After one hour of incubation at 35°C in a 5% CO_2 atmosphere, 25 ml of media (199 media, 10% fetal bovine serum, 0.2% antimycotic, and 2% penicillin-streptomycin) were added to each flask. After 7 d, media and virus were collected from flasks and combined with defibrinated bovine blood (Hemostat, Dixon, CA) in a 1:1 ratio of blood: media + virus. Aliquots of DENV-1-infected blood were taken just before feeding trials and stored at -80°C . Viral titers of infected blood were determined using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR; Buckner et al. 2013).

Oral Infection of Mosquitoes

Females were given the opportunity to feed on DENV-1-infected blood using an artificial membrane feeding system (Hemotek, Discovery Workshops, Accrington, Lancashire, UK) for approximately 45 min. After offering the infectious bloodmeal, females were cold-anesthetized and blood-fed were separated from unfed females based on abdominal color and distention under a $10\times$ stereo microscope. Fully engorged mosquitoes were housed together in cages with 10% sucrose solution, and females that did not initially feed were given subsequent opportunities to feed on infectious blood for each of the next 3 d. The six replicates per treatment were retained throughout the infection study.

During and after feeding on infectious blood, females were held within incubators at $30 \pm 0.5^\circ\text{C}$ on a daily photoperiod of 14:10 (L:D) h. After imbibing infectious blood, females were provided with 10% sucrose solution and held for 12 d, which approximates the extrinsic incubation period of dengue viruses at 30°C (Watts et al. 1987, Chan and Johansson 2012). During this time, females were allowed to lay their first gonotrophic cycle eggs. However, these eggs were not assayed for DENV-1 RNA because vertical transmission of DENV during the first gonotrophic cycle is rare (Mourya et al. 2001).

Provision of Noninfectious Bloodmeal

Twelve days after imbibing infectious blood, to stimulate a second gonotrophic cycle, females were given the opportunity to feed for

approximately 45 min on defibrinated bovine blood. After the non-infectious bloodmeal, mosquitoes were cold-anesthetized, and blood-fed and unfed females were separated using criteria described earlier. Fully engorged mosquitoes were placed individually in 37-ml plastic tubes (h by d: 8 by 3 cm), which were covered with screen and lined with moist germination paper for oviposition. Mosquitoes were given 5 d to lay their eggs.

Determination of Infection Status of P₁ and F₁ Generations

After the P₁ generation females laid eggs from their second gonotrophic cycle, they were anesthetized using CO₂ and stored in centrifuge tubes at -80°C. Hatching of F₁ eggs was stimulated by immersing germination papers with eggs in individual 0.47-liter water-filled containers containing 100 mg of larval food. Eggs from each female were hatched separately. The F₁ generation was reared to late larval instars, counted, and frozen in centrifuge tubes at -80°C. All progeny from each P₁ generation female were pooled into one tube to allow for determining vertical transmission rates for each larval treatment (the proportion of P₁ generation-infected females that infected at least one of their progeny). Whole-body P₁ generation females and their progeny samples were then homogenized using a TissueLyser (Qiagen, Valencia, CA) in centrifuge tubes containing 0.9 ml BA-1 diluent and two zinc-plated steel BBs.

The homogenized samples were clarified by centrifugation and total nucleic acids were extracted from 250 µl samples using a MagNA Pure Total Nucleic Acid kit (Roche Diagnostics, Chicago, IL) and a MagNA Pure LC robot (Roche Diagnostics, Chicago, IL). Following extraction of total nucleic acids, qRT-PCR was conducted on the samples using SuperScript III Platinum one step qRT-PCR (Invitrogen Company, Carlsbad, CA) and fluorogenic probe hydrolysis (TaqMan, Applied Biosystems, Foster City, CA) technology. Quantitative RT-PCR reactions were performed in 96-well reaction plates, with each containing: 0.4 µl SuperScript III RT/Platinum Taq mix, 10 µl 2x reaction mix, 1 µl DENV-1 specific forward primer (10 µmol/L), 1 µl DENV-1 specific reverse primer (10 µmol/L), 0.4 µl fluorogenic probe (10 µmol/L), 2.2 µl H₂O, and 5 µl test sample. Amplification and quantification of DENV-1 RNA in positive samples were performed by running the 96-well reaction plate in a LC480 thermocycler machine (Roche Diagnostics, Chicago, IL) programmed for the following: 60°C for 30 minutes, 2 minutes at 95°C, followed by 40 cycles of PCR (15 seconds at 95°C and 1 minute at 60°C), and 1 second at 45°C (Callahan et al. 2001). A negative control (water) and positive control standard (DENV-1 RNA 10⁻² dilution, GenBank accession no. JQ675358) were included on each 96-well reaction plate. The titer of each mosquito sample and blood from feeding trials was expressed in PFUeq/ml by a standard curve method that compares the known titer of the positive standard control to the titer of unknown samples (Buckner et al. 2013).

Calculations and Statistical Analysis

As a result of the separate experiments performed for *Ae. aegypti* and *Ae. albopictus*, data for the two species were analyzed separately. This allowed for qualitative but not quantitative comparisons of results between species. The following mosquito life history information was measured for each experimental unit for each species: the mean number of days to pupation, proportion of P₁ generation mosquitoes surviving to pupation, and mean number of F₁ progeny that hatched from second gonotrophic cycle eggs. Additionally, the mean DENV-1 titer of individual females was calculated for each experimental unit. P₁ generation infection rates of females were

calculated by dividing the number of females infected with DENV-1 by the total number of females that imbibed an infectious bloodmeal, expressed as a percent. Vertical transmission was calculated by dividing the number of P₁ generation females that had DENV-1-infected progeny by the total number of DENV-1-infected P₁ generation females, expressed as a percent. Here we calculate infection, viral titer, and vertical transmission only for mosquitoes that fed on both an infectious and a noninfectious bloodmeal.

All statistical analyses were performed using the SAS statistical package version 9.3 (SAS Institute, Cary, NC). Some of the measurements did not meet the assumptions of normality and homoscedasticity. Therefore, the effects of larval treatments on number surviving to pupation, time to pupation in days, number of F₁ progeny that hatched from second gonotrophic cycle eggs, and DENV-1 titer of infected females were analyzed using separate nonparametric Kruskal-Wallis tests. Post hoc comparisons used the least significant difference in mean ranks. The Bonferroni correction was used to adjust *P*-values for all post hoc comparisons. We tested for the interaction of temperature and food factors by reorganizing the data so that each temperature-food combination represented a single treatment, which yielded four individual treatments. Significant effects of treatment were further analyzed by determining the least significant difference in mean ranks. We tested for differences in the number of progeny from infected and uninfected parental mosquitoes using the Wilcoxon-Mann-Whitney test. The number of mosquitoes available for testing for DENV-1 infection was much lower than the number of mosquitoes surviving to adulthood, attributable to low blood feeding rates and mortality during the adult stage. The effects of larval treatments and their interaction on the number of *Ae. aegypti* and *Ae. albopictus* females that developed DENV-1 infections and vertically transmitted DENV-1 to their progeny were analyzed by maximum likelihood categorical analyses of contingency tables (PROC CATMOD, SAS Institute 2010, Agresti 1990). When significant interactions were detected, we reorganized the data in the manner described above and used pairwise contrasts to determine if effects of treatments were statistically different.

Results

Mosquito Life History

Temperature and food treatments did not affect *Ae. aegypti* or *Ae. albopictus* survival (*Ae. aegypti*, $H=4.80$, $df=3$, $P=0.19$, *Ae. albopictus*, $H=6.59$, $df=3$, $P=0.09$) but did affect days to pupation for both species (*Ae. aegypti*: $H=12.99$, $df=3$, $P=0.005$; *Ae. albopictus*: $H=18.33$, $df=3$, $P=0.0004$). Food, but not temperature, altered development time for *Ae. albopictus*. Specifically, more food resulted in significantly shorter times to pupation (Fig. 1). For *Ae. aegypti*, more food resulted in fewer days to pupation, but only at 30°C (Fig. 1). Larval factors did affect the number of *Ae. albopictus* progeny ($H=8.73$, $df=3$, $P<0.033$) but not *Ae. aegypti* progeny ($H=1.62$, $df=3$, $P=0.65$). *Ae. albopictus* females from the 24°C, 500 mg food larval treatment produced more progeny than cohorts from the 30°C, 250 mg larval food treatment (Fig. 1).

Dengue Virus Infection, Body Titer, and Vertical Transmission

The mean ± standard error viral titers of the infectious bloodmeals provided to *Ae. aegypti* and *Ae. albopictus* mosquitoes were determined to be 7.03 ± 0.06 and 6.12 ± 0.16 log₁₀ PFUeq DENV/mL, respectively, which is within the range of viremia levels observed in humans (Gubler et al. 1981, Vaughn et al. 2000, Stramer et al.

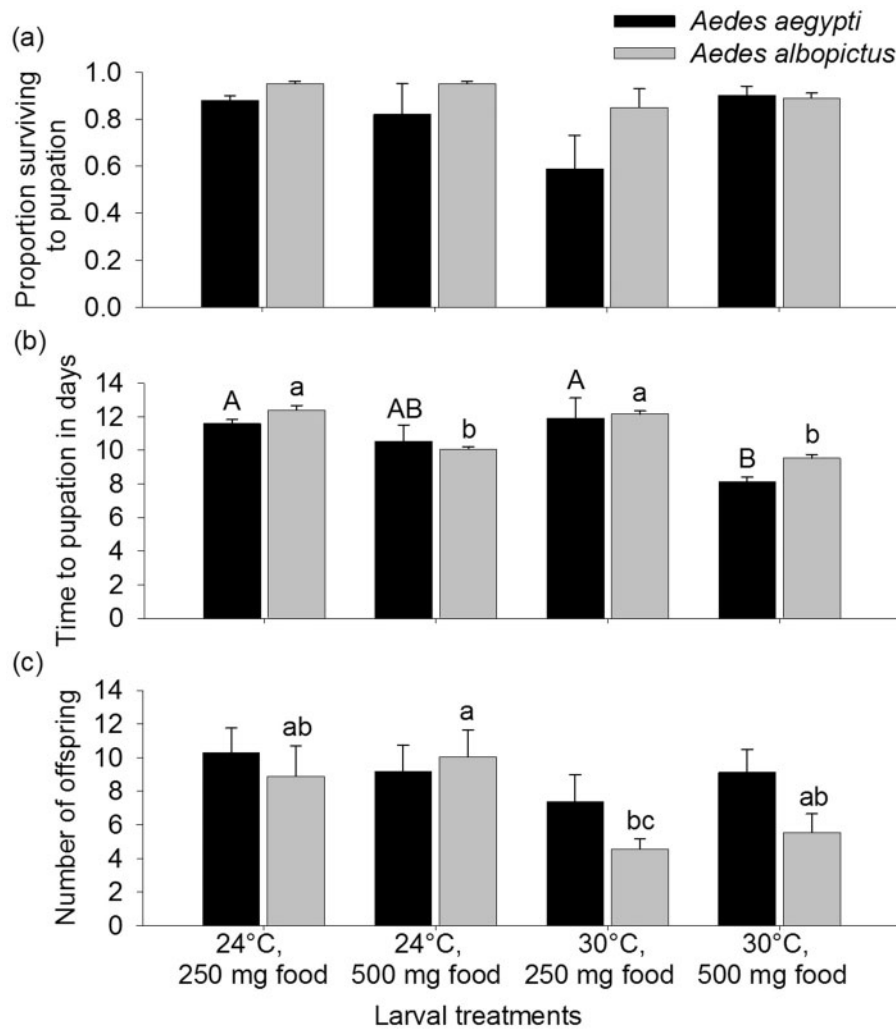


Fig. 1. Means (\pm SE) of proportion of *Aedes aegypti* and *Aedes albopictus* mosquitoes surviving to pupation (a), time to pupation in days (b), and number of 2nd gonotrophic cycle progeny (c) for larval treatments, which varied temperature (24°C or 30°C) and food levels (250 mg or 500 mg as initial amounts). Means followed by different letters (upper case for *Ae. aegypti* and lower case for *Ae. albopictus*) show significant differences in ranks. Post hoc comparisons used the least significant difference in mean ranks.

2012). Out of the 225 *Ae. aegypti* and 428 *Ae. albopictus* females that imbibed infected blood, 164 (72.9%) and 334 (78.0%) developed DENV-1 infections, respectively. Vertical transmission rates for *Ae. albopictus* ranged between 13–24% among treatments and 7–16% for *Ae. aegypti* (Fig. 2).

Food quantity and temperature, as well as their interaction, had no influence on the proportion of DENV-1-infected *Ae. aegypti*, the titer of *Ae. aegypti* and *Ae. albopictus* infected mosquitoes, and the proportion of *Ae. aegypti* and *Ae. albopictus* that vertically transmitted DENV-1 to their progeny ($P > 0.08$ in all instances; Figs. 2 and 3). However, food quantity and the interaction between food quantity and temperature did influence the proportion of DENV-1-infected *Ae. albopictus* (food: $\chi^2 = 15.01$, $df = 1$, $P = 0.0001$; food \times temperature: $\chi^2 = 9.94$, $df = 1$, $P = 0.002$), while temperature alone did not ($\chi^2 = 0.24$, $df = 1$, $P = 0.62$; Fig. 2). Infection rates were highest at low-temperature–low-nutrient treatment and lowest in low-temperature–high-nutrient treatment (Table 1; Fig. 2). Infection status of the female parent (uninfected vs infected) did not influence the number of progeny during the second gonotrophic cycle (Wilcoxon–Mann–Whitney test, *Ae. aegypti*, $z = -0.36$, $P = 0.72$; *Ae. albopictus*, $z = 0.08$, $P = 0.93$).

Discussion

We tested the hypothesis that larval temperature alters the effects of larval food level on *Ae. aegypti* and *Ae. albopictus* life history and DENV-1 infection and vertical transmission. Larval treatments were not limiting factors that influenced survivorship to adulthood, which supports results from experiments using similar larval rearing conditions (Lounibos et al. 2002; Muturi et al. 2011b, 2012; Yoshioka et al. 2012; Alto and Bettinardi 2013). However, we did observe species-specific patterns of treatments on larval development time. *Ae. albopictus* accelerated development with increased food quantity but was unaffected by larval rearing temperature. Larval diet has previously been shown to be a significant predictor of time to pupation for *Ae. albopictus*, but our finding of the lack of effect of larval temperature on *Ae. albopictus* development time contrasts with other studies that have documented higher larval temperature decreasing *Ae. albopictus* development time (Muturi et al. 2011c, Yoshioka et al. 2012, Alto and Bettinardi 2013). As predicted, more food led to a significantly shorter development time only at the elevated larval temperature in *Ae. aegypti*, demonstrating an interactive effect of larval temperature and food on *Ae. aegypti* development. Likewise, Padmanabha et al. (2011) documented an

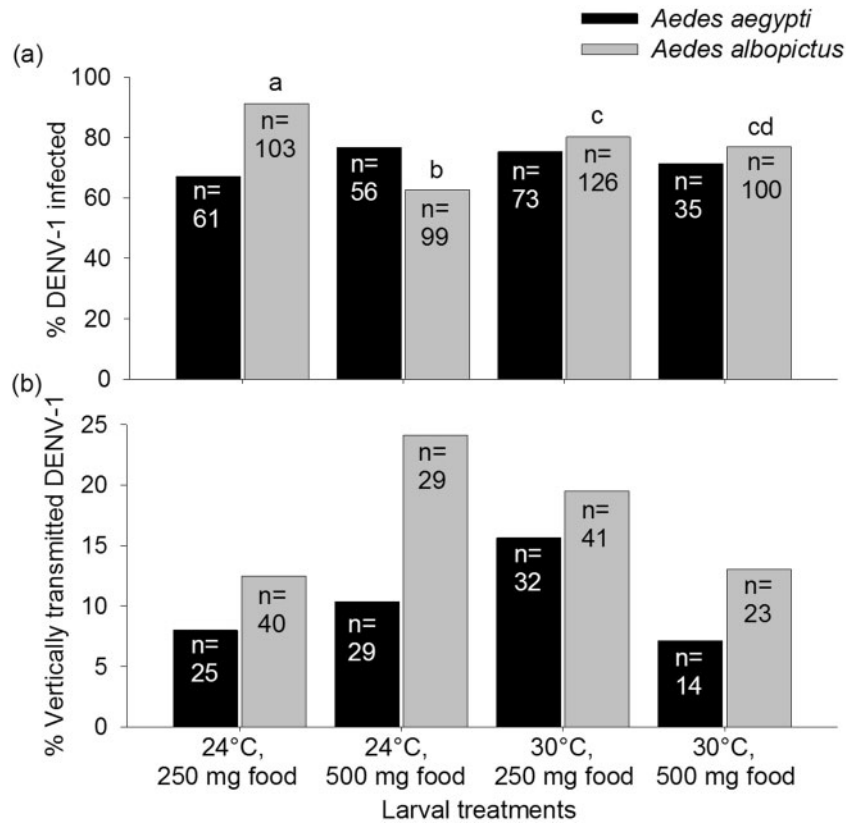


Fig. 2. Percentages of *Aedes aegypti* and *Aedes albopictus* mosquitoes infected with DENV-1 (a) and vertically transmitted DENV-1 to progeny (b) for larval treatments, which varied temperature and food levels. Numbers (n) associated with treatment groups indicate the total number of females provided with a DENV-1 bloodmeal (a) and total number of DENV-1-infected mothers (b) for each treatment. Percentages followed by different lowercase letters show significant differences determined by maximum likelihood follow-up contrasts (temperature – *Ae. albopictus*: $\chi^2 = 0.04$, $df = 1$, $P = 0.83$, *Ae. aegypti*: $\chi^2 = 0.06$, $df = 1$, $P = 0.81$; food – *Ae. albopictus*: $\chi^2 = 0.10$, $df = 1$, $P = 0.76$, *Ae. aegypti*: $\chi^2 = 0.16$, $df = 1$, $P = 0.69$; food \times temperature interaction – *Ae. albopictus*: $\chi^2 = 2.01$, $df = 1$, $P = 0.16$, *Ae. aegypti*: $\chi^2 = 0.61$, $df = 1$, $P = 0.44$). Post hoc comparisons used the least significant difference in mean ranks.

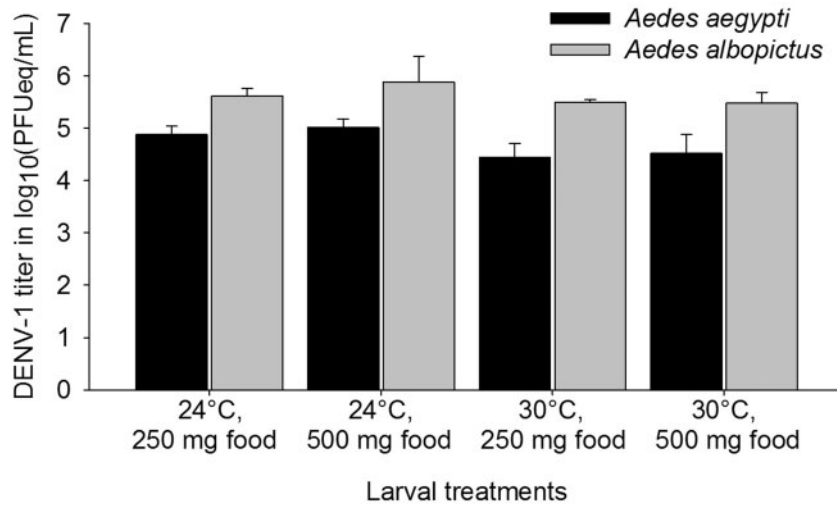


Fig. 3. Means (\pm SE) of DENV-1 titer in $\log_{10}(\text{PFUeq/mL})$ of infected *Aedes aegypti* and *Aedes albopictus* mosquitoes for larval treatments, which varied temperature (24°C or 30°C) and food levels (250 mg or 500 mg as initial amounts). Post hoc comparisons used the least significant difference in mean ranks.

interactive effect of larval temperature and food on wing cell size in *Ae. aegypti* mosquitoes, consistent with our findings on temperature modulation of the effects of larval food availability in that mosquito species.

Larval rearing conditions dictate nutrient reserves of teneral adults and so are likely to influence the biological processes of

female adults such as immune responses (Ahmed et al. 2002, Schwartz and Koella 2004) and reproduction (Briegel 1990). Specifically, infection with pathogens may decrease reproductive fitness (Sheldon and Verhulst 1996). We observed no relationship between the number of eggs laid by female mosquitoes and their infection rates, providing indirect support that reproductive output

Table 1. Maximum likelihood follow-up contrasts for proportions of *Aedes albopictus* females infected with DENV-1 from each treatment

Contrast	χ^2 value	df	P value
24°C, 250 mg vs 24°C, 500 mg	20.31	1	<0.0001*
24°C, 250 mg vs 30°C, 250 mg	5.26	1	0.02*
24°C, 250 mg vs 30°C, 500 mg	7.26	1	0.01*
24°C, 500 mg vs 30°C, 250 mg	8.32	1	0.004*
24°C, 500 mg vs 30°C, 500 mg	4.81	1	0.03*
30°C, 250 mg vs 30°C, 500 mg	0.33	1	0.62

An asterisk (*) denotes statistically significant differences between treatment groups. All P-values are adjusted using the Bonferroni approach.

was not compromised by different rates of DENV-1 infection. A caveat to this interpretation is that our experimental design did not include groups of mosquitoes that were unexposed to DENV-1, and so we cannot distinguish between individuals that mount an immune response associated with DENV-1 exposure and those that don't. The effects of larval treatment on number of progeny were only significant for *Ae. albopictus*. Females reared at the cooler temperature and provided with more food experienced a 44% increase in the number of progeny compared with females reared at the elevated temperature and provided with less food. These results suggest that elevated temperature and less nutrition may induce reproductive stress relative to females from other rearing conditions (Briegleb 1990, Telang and Wells 2004). While body sizes of mosquitoes were not determined in our study, larger *Ae. albopictus* females have previously been found to be more fecund than their smaller counterparts (Blackmore and Lord 2000). Body size, temperature, and food levels are known to have complex, population-dependent relationships in this species (Reiskind and Zarrabi 2012). Larger adult size has been shown to be associated with cooler larval conditions (e.g., <25°C) in mosquitoes, which may explain why females reared at the cooler larval temperature and provided with more food produced more progeny than those reared at the elevated larval temperature and provided with less food (Padmanabha et al. 2011).

Previous studies have demonstrated either larval nutrient or temperature-mediated changes in vector competence for arboviruses, but, to our knowledge, our study is one of the first to investigate the potential interaction between these factors and arbovirus infection and transmission. Cooler larval temperature and less food enhanced *Ae. albopictus* DENV-1 infection by approximately 33% relative to high food. The elevated larval temperature (30°C) resulted in similar rates of DENV-1 infection of adults from both low- and high-nutrient provisioned larvae. However, infection rates at the elevated temperature were intermediate and distinct from mosquitoes between low and high food at the cooler temperature, suggesting a complex interaction between these factors. These results provide partial support for our hypothesis that temperature alters the effects of larval food level on *Ae. albopictus* DENV-1 infection, although these effects do not appear to alter rates of vertical transmission, which indicate a unique reproductive barrier(s) to transmission. These results are consistent with Westbrook et al. (2010), who showed that *Ae. albopictus* reared at a cooler larval temperature (18°C) had higher chikungunya virus infection and dissemination rates compared with those reared at a warmer larval temperature (32°C). Zhang et al. (1993) observed small adult *Ae. albopictus* derived from a poor larval diet experienced enhanced DENV-2 dissemination relative to adults from nutrient-rich larval conditions. Additionally, these authors showed that field collections

of *Ae. albopictus* had sizes that more closely reflected those from poor larval diet, suggesting that the nutrient conditions used in the study were within the range encountered in nature. Along the same lines, nutrient-deprivation during the larval stages enhanced infection and transmission (horizontal and vertical) of LaCrosse encephalitis virus by *Aedes triseriatus* (Say; Grimstad and Haramis 1984, Patrican and DeFoliart 1985, Grimstad and Walker 1991). Together these observations are consistent with observations that temperature and nutrition may alter the expression of immunity-related genes and viral competence (Telang et al. 2012, Muturi et al. 2011c). Specifically, cooler rearing conditions may compromise the immune system in *Ae. albopictus* and lead to higher flavivirus and alphavirus infection, perhaps accentuated by nutrient deprivation (Adelman et al. 2013). *Ae. albopictus* adult mosquitoes that develop in areas of the world with cooler ambient temperatures (e.g., <25°C) and nutrient-poor conditions as larvae may be more likely infected with DENV compared with those exposed to nutrient-rich conditions as larvae. By contrast, other investigations have found little evidence relating larval nutrition to measurements of vector competence (e.g., *Culex annulirostris* Skuse and Murray Valley encephalitis virus, Kay et al. 1989; *Aedes vigilax* Skuse and Ross River virus, Jennings and Kay 1999; *Culex tarsalis* Coquillett and West Nile virus, Dodson et al. 2011). However, these studies used a single-temperature experimental design (range, 27–28°C), limiting the ability to identify possible nutrient-mediated changes in adult competence for arboviruses that may vary across a temperature gradient.

While treatment factors altered infection in *Ae. albopictus*, we did not observe effects on *Ae. aegypti* DENV-1 infection, suggesting that the stress of larval treatments used in our study may have been enough to influence *Ae. aegypti* development time to pupation but not infection barriers such as immune system function. Alto et al. (2008) also documented species-specific differences in effects of larval competition on DENV-2 infection rates in *Ae. aegypti* and *Ae. albopictus*. Specifically, they found that larval competition enhanced components of vector competence in *Ae. albopictus*, but not *Ae. aegypti*. While the reasons for differences between the two *Aedes* species in response to larval treatments are unknown, proposed explanations are species-specific differences in immune function and arbovirus infection barriers that may be differentially affected by the larval environment (Alto et al. 2008). Together the results indicate that larval environmental conditions, such as the interaction between temperature and food availability or competition, may have a stronger impact on *Ae. albopictus* compared with *Ae. aegypti* for dengue viruses (Alto et al. 2008).

To our knowledge, our study is the first to examine the effects of the larval environment on DENV vertical transmission in mosquitoes. Though there was no effect of larval treatments on DENV-1 titer and vertical transmission, we demonstrated that vertical transmission rates of up to 15.6 and 24.1% are possible in *Ae. aegypti* and *Ae. albopictus* mosquitoes, respectively. Using a mathematical modeling approach, Adams and Boots (2010) suggest that a DENV vertical infection rate of 5% can lead to local survival of the virus in between outbreaks for a matter of months, and they define vertical infection rate as vertical transmission rate (the proportion of infected females that infect at least one of their progeny) \times filial infection rate (the proportion of the progeny of an infected female that are infected given that vertical transmission occurred; Adams and Boots 2010). Additionally, these authors suggest that a DENV vertical infection rate of 10% may allow DENV to survive locally in the environment for up to two years in between outbreaks. In context of a vertical infection rate of 5% suggested by Adams and Boots (2010), if the filial infection rates exceed 25% in *Ae. albopictus*,

then the vertical transmission rate of 24.1% in *Ae. albopictus* documented in our study could lead to the persistence of DENV for months between outbreaks (Adams and Boots 2010). Also, if filial infection rates exceed 35% in *Ae. aegypti*, then the vertical transmission rate of 15.6% in *Ae. aegypti* documented in our study could lead to the persistence of DENV for months between outbreaks. However, because we pooled the progeny from each P₁ female and did not test each offspring separately for DENV, we cannot determine the actual filial infection rates for the progeny of each female. Filial infection rates have been found to be lower than rates suggested for the persistence of dengue viruses between outbreaks based on models by Adams and Boots (2010) (*Ae. albopictus* OAHU strain and DENV-1 Fiji strain, 0.4–5%, Shroyer 1990; DENV-1 Jamaican strain, 1.4–17.4%, Bosio et al. 1992; *Ae. aegypti* and DENV-2 16681 from Thailand, 1.4–3.7%, Wasinpiyamongkol et al. 2003), bringing into question the importance that vertical transmission plays in the persistence of DENV-1.

Though the separate larval manipulation experiments conducted for *Ae. aegypti* and *Ae. albopictus* in this study prevented a quantitative comparison between the DENV-1 vertical transmission rates of the *Aedes* species, it is worth noting that qualitatively higher vertical transmission rates were documented in *Ae. albopictus* (~13–24%) compared with *Ae. aegypti* (~7–16%), despite having fed on a lower dose of DENV-1-infected blood. Previously, we also documented a higher DENV-1 vertical transmission rate in *Ae. albopictus* (11%) compared with *Ae. aegypti* (8%; Buckner et al. 2013). Our observations are consistent with other comparative studies demonstrating higher rates of vertical transmission of dengue viruses by *Ae. albopictus* than *Ae. aegypti* (reviewed by Lambrechts et al. 2010). The higher DENV-1 vertical transmission rates documented in *Ae. albopictus* compared with *Ae. aegypti* potentially suggest that while *Ae. aegypti* may be the more efficient horizontal vector of DENV, *Ae. albopictus* could be the more efficient vertical vector of DENV. A meta-analysis of experimental laboratory studies revealed that overall *Ae. albopictus* is overall more susceptible to DENV midgut infection than *Ae. aegypti*, suggesting greater DENV adaptation to *Ae. albopictus* (Lambrechts et al. 2010). Therefore, the potential role of *Ae. albopictus* in the transmission cycle of DENV should not be overlooked.

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