# Effect of Larval Competition on Extrinsic Incubation Period and Vectorial Capacity of *Aedes albopictus* for Dengue Virus

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## Abstract

Despite the growing awareness that larval competition can influence adult mosquito life history traits including susceptibility to pathogens, the net effect of larval competition on human risk of exposure to mosquito-borne pathogens remains poorly understood. We examined how intraspecific larval competition affects dengue-2 virus (DENV-2) extrinsic incubation period and vectorial capacity of its natural vector *Aedes albopictus*. Adult *Ae. albopictus* from low and high-larval density conditions were orally challenged with DENV-2 and then assayed for virus infection and dissemination rates following a 6, 9, or 12-day incubation period using real-time quantitative reverse transcription PCR. We then modeled the effect of larval competition on vectorial capacity using parameter estimates obtained from peer-reviewed field and laboratory studies. Larval competition resulted in significantly longer development times, lower emergence rates, and smaller adults, but did not significantly affect the extrinsic incubation period of DENV-2 in *Ae. albopictus*. Our vectorial capacity models suggest that the effect of larval competition on adult mosquito longevity likely has a greater influence on vectorial capacity relative to any competition-induced changes in vector competence. Furthermore, we found that large increases in the viral dissemination rate may be necessary to compensate for small competition-induced reductions in daily survivorship. Our results indicate that mosquito populations that experience stress from larval competition are likely to have a reduced vectorial capacity, even when susceptibility to pathogens is enhanced.

## Introduction

Dengue fever is a rapidly emerging arthropod-borne virus (arbovirus) primarily transmitted to humans by two container-dwelling mosquito species, *Aedes aegypti* and *Ae. albopictus* [1], [2]. Annually, an estimated 100–390 million people become infected with one of the four closely related dengue virus serotypes (DENV 1–4) resulting in approximately 500,000 hospitalizations and 25,000 deaths [1, 3, 4]. With no immediate prospects for an effective vaccine or antiviral treatment, current dengue prevention efforts depend on vector control strategies which

integrate the use of insecticides with natural enemies, elimination of breeding sites, and physical barriers such as house screening [5, 6]. Despite these efforts, reducing DENV incidence through mosquito control continues to be a significant challenge due in part to our limited understanding of the ecological factors that regulate DENV transmission and their relative importance in disease prevention and management.

For a mosquito to transmit DENV, it must acquire a blood meal from an infected host, support viral replication and dissemination to the salivary glands (vector competence), survive the extrinsic incubation period (EIP, the duration between acquisition of an infectious agent and the potential to transmit that agent), and take a subsequent blood meal from a susceptible host. The length of the EIP has a significant effect on the temporal dynamics of DENV transmission and may be affected by a variety of environmental factors including the fitness of the infecting genotype, viral titer of the infectious blood meal, and temperature [7–10]. Under natural conditions, the EIP of many mosquito-borne pathogens is long relative to mosquito lifespan, and less than 10% of female mosquitoes are estimated to survive the EIP to become potential vectors [11]. The EIP of DENV is estimated to be between 8–12 days [4, 9, 12] while the mean lifespan of *Ae. albopictus* females is estimated to be between 3–11 days [13–16]. Therefore, slight changes in either EIP or mosquito longevity can have significant consequences on the ability of *Ae. albopictus* to transmit DENV [11, 17].

Vectorial capacity describes the capacity of a vector population to transmit a pathogen and takes into account tripartite interactions between host, virus, and vector [17–21]. An accurate estimate of vectorial capacity is necessary to evaluate vector control strategies and predict the risk of pathogen transmission [19, 21]. Vectorial capacity (*C*) is expressed as:

$$C = \frac{ma^2bp^n}{-\ln(p)}$$

Where *m* is the ratio of mosquitoes to humans, *a* is the human biting rate, *b* is vector competence, *p* is the daily survival rate of the vector, and *n* is the EIP.

The parameters of vectorial capacity vary spatially and temporally depending on biotic and abiotic environmental fluctuations, and an individual environmental factor can affect multiple vectorial capacity parameters in complex or antagonistic ways [22]. For example, elevated temperatures may enhance vector competence (*b*) and decrease EIP (*n*) while concomitantly reducing adult longevity (*p*) [10,23,24]. Competition for limited resources may also reduce adult mosquito lifespan (*p*) while enhancing vector competence (*b*) [25, 26]. Understanding how environmental factors affect the relationship between the components of vectorial capacity, especially *p* and *n*, is therefore essential for accurate estimation of disease transmission risk.

A growing body of knowledge has shown that adult mosquito life history traits are largely influenced by environmental conditions experienced during larval development [23–33]. These studies have stimulated interest in understanding the contribution of the larval environment to the risk of mosquito-borne pathogen transmission [28–31, 34]. However, our understanding of how these interactions affect the vectorial capacity of mosquito populations is limited, due in part to the complex ways in which the larval environment affects the ability of adults to transmit pathogens.

Larval competition is an ubiquitous ecological stressor that has the potential to affect the vectorial capacity of mosquito populations [25, 26, 28, 34]. *Ae. albopictus* larvae develop in a variety of artificial water holding containers (e.g. water tanks, discarded tires, trash cans, flower vases) [14, 35] that derive most of their energy through decomposition of allochthonous detrital inputs, primarily leaf litter [15, 34, 36]. Larvae in these habitats often attain very high densities and populations are usually regulated by intra- and interspecific resource competition [28,

34, 37]. When larval competition is severe enough to affect larval survivorship, it can reduce adult emergence rates and in turn vector density ($m$). Further, surviving mosquitoes that reach adulthood may have altered behavior, physiology, or life history traits that could affect their potential to vector pathogens. For example, stress from larval competition often results in the production of smaller adults, which is correlated with reduced longevity ($p$) [27, 38–41], altered biting rates ($a$) [40, 42, 43], and higher vector competence ($b$) [25, 28–30].

Larval competition can also influence viral virulence because it often yields adult mosquitoes with a shorter lifespan, thereby reducing future transmission opportunities. Consequently, competition could select for viral strains with rapid replication, shorter EIPs ($n$) and higher virulence [44]. Alternatively, if larval competition results in enhanced viral susceptibility and diminished physiological barriers to dissemination and transmission, it could reduce viral virulence by reducing the force of selection.

In this study, we evaluated the effect of intraspecific larval competition on the EIP of DENV-2 in *Ae. albopictus*. We then used the cumulative vectorial capacity equation (cVC) developed by Christofferson and Mores [20] to assess the net effect of larval competition on vectorial capacity of *Ae. albopictus* for DENV. We tested the hypotheses that: 1) larval competition would enhance *Ae. albopictus* susceptibility to DENV-2 and shorten the EIP for this virus, and 2) the effects of larval competition on adult mosquito longevity will outweigh its effects on vector competence, and ultimately have an overall negative effect on vectorial capacity.

## Materials and Methods

### Competition study

We combined a laboratory competition and infection experiment with mathematical models to investigate the effect of intraspecific larval competition on EIP and vectorial capacity of *Ae. albopictus* for DENV-2. The competition experiment was conducted using $F_{15}$ and $F_{16}$ generations of *Ae. albopictus* from field collections in Florida. First-instar larvae ($< 24$ hr old) were added to 1.6 liters of filtered oak infusion held in 5-liter plastic containers at an initial larval density of 150 or 300 per container. The containers were maintained at 25°C. Each container was supplemented with 0.2 g of larval food (1:1 albumin: yeast) on day 1 and 0.05 g on days 7 and 10 of the experiment. Each container was examined daily until all larvae had either pupated or died. Pupae from each replicate were removed daily and placed into plastic vials with water until eclosion. Newly emerged adults (both males and females) from each container (replicate) were housed in paperboard cages (11 cm high x 9.5 cm diameter) by date of emergence. There were 4 biological replicates for each larval density-EIP combination for a total of 24 containers.

### Vector competence study

*Aedes albopictus* epithelial cells (C6/36) (ATCC # CRL-1660) were maintained at 35°C in Leibovitz L-15 media (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS) (Atlanta Biological, Norcross GA) and 10 µg/ml of penicillin and streptomycin (Invitrogen, Carlsbad, CA). We used DENV-2 strain 16803 (Southeast Asian genotype) in this study. This strain was isolated from a patient in Thailand in 1974 and was inoculated into *Toxorhynchites amboinensis* once, passaged three times in primary African green monkey kidney (PGMK) cells, twice in C6/36 cells and four times in Vero cells before we obtained this strain from Walter Reed Army Institute of Research. Confluent monolayers of C6/36 cells in 25-cm$^2$ flasks were inoculated with DENV-2 at a multiplicity of infection of 0.1 and incubated at 35°C for 90 minutes with occasional rocking to allow for viral attachment and entry. Six milliliters of L-15 media was then added to each flask for a final volume of 6.3 ml/flask and incubated for 6

days. Freshly harvested C6/36 cell monolayers and media were used to prepare the infectious blood meals consisting of a mixture of citrated bovine blood (BB*100, HemoStat Laboratories, Dixon, CA) and media in a 3:2 ratio for a final DENV-2 titer of log $10^{6.32}$–$10^{6.49}$ focus forming units (ffu) /ml.

Four to 7-day-old female *Ae. albopicus* that had been sugar-starved for 48 hours were provided 40 min access to the infectious blood meal via the Hemotek membrane feeding system (Lancashire, UK). Blood-fed females were transferred into paperboard cages and maintained on 10% sucrose at 28°C and 70% relative humidity for an incubation period of 6, 9, or 12 days. Following the incubation period, individual mosquitoes were killed and their legs and wings detached from their bodies. The wings were used for body size determination while the bodies and legs were preserved separately at -80°C and later assayed for DENV-2.

**Quantification of DENV in infectious blood meal.** Vero cells (ATCC # CCL-81) were propagated in L-15 medium (Invitrogen), supplemented with 10% FBS and 10 µg/ml of penicillin and streptomycin (Invitrogen) and maintained at 37°C and 5% $CO_2$. Ten-fold serial dilutions of DENV-2 in L-15 media were inoculated in triplicate on confluent Vero cell monolayers in 24-well plates and incubated at 37°C for 90 minutes with periodic rocking to facilitate virus adsorption. Subsequently, wells were overlaid with 1 ml of 0.8% methylcellulose (Sigma-Aldrich, St. Louis, MO) diluted in warm L-15 media supplemented with 5% FBS, and 10 µg/ml of penicillin and streptomycin (Invitrogen), then incubated for 5 days at 37°C and virus plaques were visualized by immunoperoxidase staining as described by Durbin et al.[45].

**DENV quantification in mosquitoes by real-time PCR.** Bodies and legs of individual mosquitoes were separately homogenized in 1 ml of media prior to RNA extraction. Total RNA was extracted using Qiamp virus Biorobot 9604 kit according to manufacturer's protocol (Qiagen, Valencia, CA). A one-step Taqman probe qRT-PCR was used to determine the infection status of body and leg samples as well as the body titer of infected mosquitoes. qRT-PCR was conducted in 20 µL reactions containing 10 µL of one-step Sensifast RT-PCR master mix (Bioline, Tauton, MA), 0.2 µL RNAse inhibitor, 0.8 µL of each 10 µM forward and reverse primer stock, 0.4 µL of 10 µM Taqman probe, 2.8 µL nuclease-free water (Integrated DNA Technologies, Coralville IA) and 5 µL template RNA. Primers and probe used for this study targeted the nonstructural protein 1 portion of DENV genome; forward primer (5′ –CATGGCCCTKGTGGC–3′ ), reverse primer (5′ –CCCCATCTYTTCAGTATCCCT –3′ ), and probe (5′ FAM–TCCTTCGTTTCCTAACAATCC–BHQ1–3′ ). qRT-PCR reactions were conducted in a 7300 real time PCR system (Applied Biosystems, Foster City, CA). Thermocycling conditions were: 48°C for 30 min, 95°C for 2 min followed by 45 cycles of 95°C for 15 s, 60°C for 1 min, and 72°C for 1 min. To determine DENV titer within mosquito bodies, total RNA was standardized to 40 ng/ml and qRT-PCR was conducted as described above. Threshold cycle (Ct) values were compared to a DENV-2 standard curve with Ct values generated from DENV-2 with a known titer (ffu/ml) in cell culture. *Ae. albopictus* with DENV-2 RNA in their bodies and legs were considered to have a disseminated infection, while those with an infected body and uninfected legs represent a non-disseminated infection. The body infection rate was calculated as the number of females with a non-disseminated infection divided by the number of mosquitoes exposed. The disseminated infection rate was calculated as the number of mosquitoes with DENV-2 RNA in their legs divided by the number of mosquitoes exposed.

## Vectorial capacity models

We used the cumulative vectorial capacity equation developed by Christofferson and Mores [17] to investigate the effect of larval competition on vectorial capacity. The cumulative vectorial capacity equation calculates effective vector competence (EVC), which is the rate of

pathogen dissemination over time, instead of a single discrete value using the equation:

$$b_i(N) = \beta_{li}N + \beta_{0i}$$

Where $b_i(N)$ is the function for larval density i; $\beta_1$ is the determined change in $\boldsymbol{b}$ per unit change in N; N is day post exposure; and $\beta_0$ is the y-intercept. This line represents the rate of change in dissemination rate over time. Effective vector competence ($\varphi$), is defined as:

$$EVC = \varphi = \int_a^z p^N(\beta_{li}N + \beta_{0i})dN$$

The interval over which this line is constructed has a lower limit of time $a$ and an upper limit of time $z$. The values of $a$ and $z$ in our study are 6 and 12 days, respectively. The EIP of DENV is believed to be between 8–12 days [4, 9, 11, 12], therefore our three time points measure early, middle, and late viral dissemination, respectively. Effective vector competence is then inserted into the cumulative vectorial capacity (cVC) defined as:

$$cVC = \frac{ma^2\varphi}{-\ln(p)}$$

To address our second hypothesis, we generated three cVC models, based on parameter estimates derived from peer reviewed laboratory and field studies (Table 1). Small adult body size in mosquitoes is often correlated with shorter lifespan [39–41]. In our vectorial capacity models, we assumed that small mosquitoes from the high-density treatment would have a 0–6% decrease in daily survivorship based on values observed in field and laboratory studies (Table 2). Overall, there was a lack of peer-reviewed studies on the human biting rate ($\boldsymbol{a}$) and mosquito density ($\boldsymbol{m}$) for *Ae. albopictus*. Therefore we based our estimates of $\boldsymbol{m}$ and $\boldsymbol{a}$ from field studies on *Ae. aegypti*. For both parameters $\boldsymbol{m}$ and $\boldsymbol{a}$, there was a range of values reported in the literature and we based our parameter estimates on the approximate median of these values (Table 1). The vectorial capacity models assume a naïve end-host population with no significant vertical transmission [20]. In our first model we used our disseminated infection rate data to assess the effect of larval competition on cVC assuming equal daily survivorship (0.85) between the high and low density treatments, or a 5% higher daily survivorship (0.90) for the low density treatment, holding human-biting rate ($\boldsymbol{a}$ = 2) and mosquito density constant ($\boldsymbol{m}$ = 1.5). Second, we modeled the effect of larval competition on cVC using our disseminated infection rate data across a range of human-biting rates, which varied from 0–3, while holding mosquito density constant ($\boldsymbol{m}$ = 1.5) and assuming equal survivorship (0.90 or 0.85), or a 1% (0.85 vs 0.84) difference in survivorship between the low and high density treatments, respectively. Lastly, we estimated the change in the viral dissemination rate that is necessary to compensate for a 3% or 6% decrease in the daily survivorship rate assuming $\boldsymbol{a}$ = 2 and $\boldsymbol{m}$ = 1.5.

**Table 1. Vectorial Capacity model parameters.**

| Parameter | Value | References |
|---|---|---|
| Probability of Daily Survival (p) | 0.85–0.90 | [10–13, 46–49] |
| Man Biting Rate (a) | 0–3 | [50–52] |
| Mosquito Density (m) | 1.5 | [53, 54] |
| Extrinsic Incubation Period (n) | 6–12 days | [4, 9, 12] |
| Vector competence[a] (b) | - | - |

[a] obtained from experimental data

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**Table 2. Estimated reduction in daily survivorship rate in small mosquitoes.**

| Species | Environmental stressor | Reduction in female daily survivorship rate | Study type | Reference |
|---|---|---|---|---|
| *Culex tarsalis* | intraspecific competition | 0–3%$^\$$ | laboratory | [53] |
| *Aedes albopictus* | intraspecific competition | 2–4%* | laboratory | [41] |
| *Aedes aegypti* | diet modification | 3–4% | field | [39] |
| *Aedes albopictus* | intraspecific competition | 0–1%# | laboratory | [38] |
| *Aedes aegypti* | intraspecific competition | 1–2%# | laboratory | [38] |

$^\$$ based on larval density per container

\* derived from mean $LT_{50}$ and $LT_{90}$ values from water + blood treatment

\# estimated from mean survivorship of low density and high density treatments at 85% humidity

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## Statistical analysis

Statistical analyses were conducted using SAS 9.3 (SAS Corporation, Cary, NC). Data were checked for normality (Kolmogorov-Smirnov test) and homogeneity of variances (examination of residuals) and multivariate analysis of variance (MANOVA) was used to determine the effect of larval density on emergence rate, time to emergence, and female wing length. Standardized canonical coefficients were used to describe the relative contribution of larval density to the significant multivariate effect. Separate analysis of variance (ANOVAs) were used to determine the effect of larval density, incubation period, and their 2-way interaction on infection rate and viral titer respectively. A separate ANOVA was used to determine the effect of larval density on cVC assuming equal survivorship (0.85) for the high and low density treatments, or assuming a 5% higher daily survivorship rate (0.90) for the low density treatment. Separate logistic regressions were conducted by larval density, to test the relationship between female body size and body and disseminated infection rate. The probability of DENV infection (infected = 1, uninfected = 0) and infection status (disseminated infection = 1, non-disseminated infection = 0) was tested against the continuous variable wing length. For all analyses, significant main effects and interactions were followed by pairwise contrasts of least-square means using the Tukey-Kramer adjustment for multiple comparisons.

## Results

### Effect of larval density on adult life history traits

MANOVA revealed a significant effect of larval density on emergence rate, female time to emergence and wing length (S1 Table). Wing length contributed most to variation followed by emergence rate (Table 3). Emergence rates were significantly higher in the low-density treatment relative to the high-density treatment (ANOVA; F = 23.99; df = 1,19; P < 0.001) (Fig 1A). In addition, females from low-density treatment were significantly larger (ANOVA F = 34.37; df = 1,19; P < 0.001), and had a shorter time to emergence (ANOVA F = 31.17; df = 1,19; P<0.001) relative to females from the high-density treatment (Fig 1B).

**Table 3. Multivariate ANOVA for the effect of larval density on emergence rate, female time to emergence, and female body size.**

| Variable | df | Pillai's trace | P | Standardized canonical coefficients (SCC) | | |
|---|---|---|---|---|---|---|
| | | | | Emergence rate | Time to emergence | Wing length |
| Larval Density | 3,17 | 0.8605 | <0.0001 | 1.229 | -0.332 | 1.545 |

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### *Ae. albopictus* susceptibility to DENV

A total of 496 adult *Ae. albopictus* females were assayed for DENV-2 infection and dissemination. The proportion of *Ae. albopictus* with a body infection was not significantly affected by larval density, incubation period or their interaction (Table 4; Fig 2A–2C). The proportion of *Ae. albopictus* with a disseminated infection was significantly affected by incubation period (IP) but not larval density (Fig 2D and 2E). DENV-2 dissemination rates were significantly higher at the 12-day IP compared to either the 6- ($P = 0.002$) or 9-day IP ($P = 0.01$; Fig 2E). The larval density by incubation period interaction was not significant but depicted a trend of higher DENV dissemination rates at higher compared to lower larval density at the 6-day IP but not at 9 or 12-day IP (Table 4, Fig 2F). DENV-2 titer in infected *Ae. albopictus* females was not significantly influenced by larval density (Fig 3A) or the incubation period by larval density interaction (Table 4). However, the length of the incubation period had a significant effect on viral titer (Fig 3B). DENV-2 titer in mosquitoes was significantly higher at 12-day IP relative to either 6-day ($P = 0.001$) or 9-day IP dpe ($P = 0.01$).

**Relationship between body size and vector competence.** At both high and low-larval density treatments, the likelihood of a body infection was not influenced by female body size. However, for the high-density treatment, the probability of a disseminated infection was inversely related to body size (Table 5).

## Vectorial Capacity models

Assuming an equal survivorship of 0.85 for adult females from both high and low-density treatments, there was a marginally significant effect of larval competition on vectorial capacity at the 6-day IP ($F = 5.65$, df = 1,6; $P = 0.055$) but not at the 9 ($F = 0.94$, df = 1,6; $P = 0.37$) or 12-day IP ($F = 0.36$, df = 1,6; $P = 0.56$) or cVC ($F = 0.20$, df = 1,7; $P = 0.66$) (Fig 4A and 4B). However, when we assumed that larger adult mosquitoes from low-density treatment had a 0.90 daily survival rate compared to 0.85 for smaller mosquitoes from high larval density, there was a significant effect of larval competition on vectorial capacity at the 9-day IP ($F = 6.58$, df = 1,6; $P = 0.042$), 12-day IP ($F = 37.11$, df = 1,6; $P = 0.0009$) as well as on cVC ($F = 21.07$, df = 1,7; $P = 0.0013$). Mosquitoes from low-density treatment had significantly higher vectorial capacity at the 9 and 12-day IP as well as higher cVC (Fig 4A and 4B). As shown in our second model, a 5% decrease in daily survivorship has a significant effect on cVC reducing it by a magnitude of at least 2.5 fold from 50 to 20 for low density treatment and 45 to 17 for high density treatment (Fig 5A and 5B). Secondly, differences in cVC become more prominent as the biting rate increases. At biting rates below 1 the cVC of the high and low-density populations are equivalent (Fig 5A and 5B). Overall, the high-density treatment had a higher cVC when equal survivorships of 0.85 and 0.90 were assumed (Fig 5A and 5B). When a 1% difference in survivorship was assumed for mosquitoes from low (0.85) and high (0.84) density treatments an equal cVC between the two populations was observed (Fig 5C). In our third model, we calculated the fold change in viral dissemination necessary to compensate for a 3% or 6% decrease in survivorship, assuming a 90% daily survivorship. To compensate for a 3% decrease in daily survivorship, the viral dissemination rate would need to increase by 1.6 to 2.1 fold across a 6–14 day incubation period, while a 2.5 to 4.3 fold increase is necessary to compensate for a 6% decrease in survivorship across a 6–14 day incubation period (Fig 6).

## Discussion

In this study we tested the hypotheses that: 1) larval competition would enhance *Ae. albopictus* susceptibility to DENV-2 and shorten its EIP, and 2) the effects of larval competition on longevity will outweigh its effects on vector competence, and ultimately have an overall negative effect on vectorial capacity. Our results weakly supported our first hypothesis and strongly
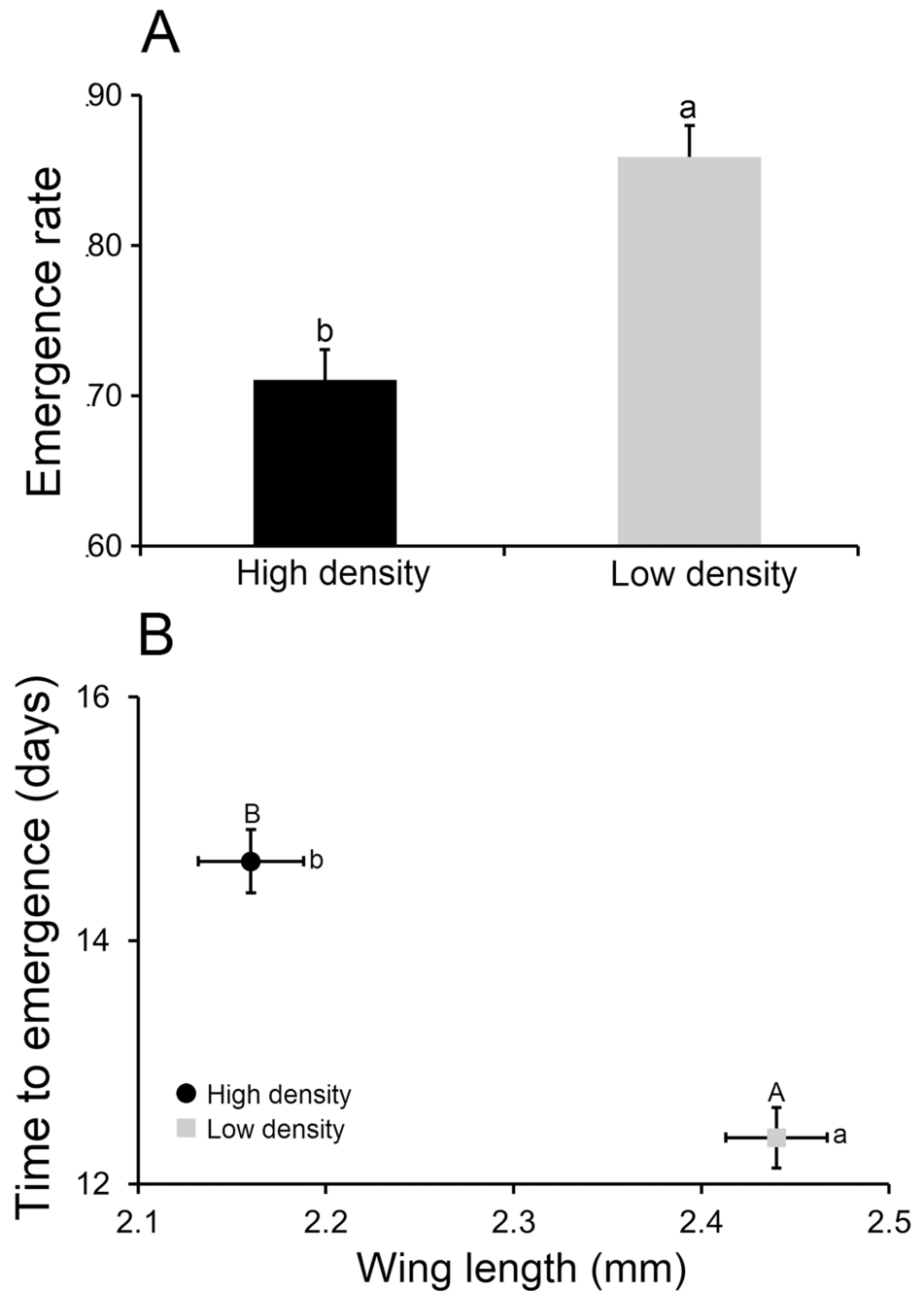
**Fig 1. Effect of larval competition on *Aedes albopictus* life history traits.** Panel A displays the effect of larval density on emergence rate. Panel B presents a bivariate plot of female time to emergence and female wing length. Error bars represent the standard errors of the mean. Tests of significance were corrected for multiple comparisons using the Tukey-Kramer adjustment. Bivariate means followed by different lower—and uppercase letters indicate significant differences in wing length and time to emergence respectively.

supported our second hypothesis providing evidence that larval competition may have a significant effect on the vectorial capacity of mosquito populations.

Increasing larval density two-fold resulted in a significantly longer time to emergence, lower emergence rates, and smaller females. These findings are consistent with previous observations

**Table 4. Univariate ANOVA for the effect of larval density, incubation period, and larval density by incubation period on midgut infection rate, disseminated infection rate and viral titer.**

| Model term | Source | numDF | denDF | F-value | P-value | Significance |
|---|---|---|---|---|---|---|
| **Body infection rate** | | | | | | |
| Main effect | Larval density (LD) | 1 | 18 | 0.06 | 0.81 | N.S. |
| Main effect | EIP | 2 | 18 | 1.03 | 0.37 | N.S. |
| Interaction | EIP*LD | 2 | 6 | 0.18 | 0.832 | N.S. |
| *Disseminated infection rate* | | | | | | |
| **Model term** | **Source** | **numDF** | **denDF** | **F-value** | **P-value** | **Significance** |
| Main effect | Larval density (LD) | 1 | 18 | 0.21 | 0.65 | N.S. |
| Main effect | EIP | 2 | 18 | 8.64 | **0.0023** | ** |
| Interaction | EIP*LD | 2 | 6 | 2.84 | 0.08 | N.S. |
| *Viral titer (body)* | | | | | | |
| **Model term** | **Source** | **numDF** | **denDF** | **F-value** | **P-value** | **Significance** |
| Main effect | Larval density (LD) | 1 | 18 | 2.32 | 0.145 | N.S. |
| Main effect | EIP | 2 | 18 | 10.2 | **0.001** | *** |
| Interaction | EIP*LD | 2 | 6 | 1.21 | 0.322 | N.S. |

N.S. $p > 0.05$;

* $p < 0.05$;

** $0.001 < p < 0.01$;

*** $p < 0.001$

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that intraspecific larval competition can negatively impact adult mosquito fitness both at individual [41, 55, 56], and population level [29, 50, 57]. How larval competition impacts vectorial capacity is primarily driven by its effects on daily survivorship, the most important determinant of a mosquito's ability to transmit pathogens [19, 58]. We found that slight reductions in longevity that were not statistically significant may still have important biological consequences. Additionally, the fitness cost of DENV infection may be greater in small females, which have lower nutrient reserves relative to large females [27], further contributing to differences in longevity between large and small mosquitoes.

Larval competition had no significant effect on the susceptibility of *Ae. albopictus* to DENV-2. The effect of larval competition on the susceptibility of mosquitoes to arboviruses remains unclear as there is evidence to suggest that larval competition decreases [59, 60] has no effect [61] or increases mosquito susceptibility to arboviruses [28–30]. We hypothesized that smaller mosquitoes from the high-density treatment would have enhanced susceptibility to DENV based on previous observations that small mosquitoes may have reduced immune function [56] and reduced physiological barriers to viral dissemination [62]. However, our results did not support our hypothesis. This could be attributed in some measure to the differences in experimental design as all previous studies utilized a single time point to assess vector competence. Though our results did not support our hypothesis, they do suggest that larval competition may alter mosquito-virus interactions as we found a significant correlation between body size and viral dissemination among mosquitoes in the high-density treatment.

Our findings partially supported our hypothesis that larval competition would shorten the DENV-2 EIP in *Ae. albopictus*. We observed a trend of earlier viral dissemination in mosquitoes from the high density treatment at the 6-day IP that resulted in a 4.5 fold increase in vectorial capacity relative to the low-density treatment. Although this result narrowly failed statistical significance ($P = 0.055$) under the conditions of our experiment, it could have significant
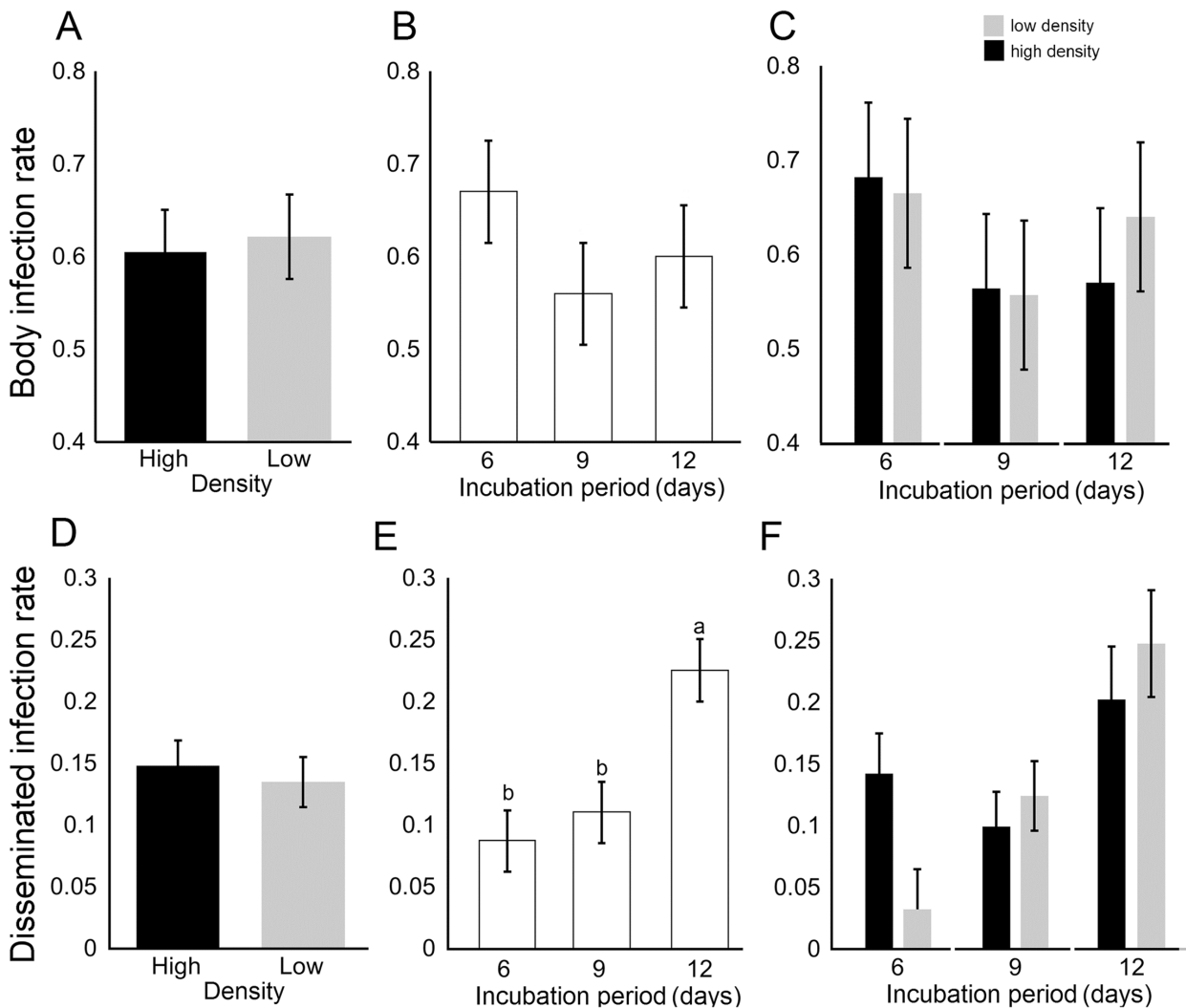
**Fig 2. *Ae. albopictus* body and disseminated leg infection rates.** Panel A-C shows the body infection rate by larval density, incubation period, and larval density by incubation period respectively. Panel D-F shows the disseminated infection rate by larval density, incubation period and larval density by incubation period. Bars above and below the means represent the standard errors of the mean. For each panel, estimates with a different letter are significantly different at *P* = 0.05, after controlling for multiple comparisons using the Tukey-Kramer adjustment.

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biological implications in nature. First, viral dissemination at earlier incubation periods greatly increases the number of females that may survive to become infectious. Second, early viral dissemination in mosquitoes can alter the temporal dynamics of pathogen transmission by reducing the latent period between vertebrate hosts. Third, by reducing mosquito longevity, larval competition would be expected to select for viral strains with the shortest EIP in mosquitoes [44]. This could have important implications for human health as DENV isolates with short EIPs in mosquitoes are associated with more severe clinical symptoms in humans [7].

One of the difficulties in evaluating the impact of altered vector competence on vectorial capacity is the degree to which other life history traits are affected. Reductions in adult body size, presumably due to a stressful larval environment are associated with lower biting rates, higher viral dissemination rates, and lower daily survivorship [30, 39–41]. These trade-offs are rarely evaluated properly when mosquitoes are tested for their susceptibility to an arbovirus, and it is often assumed that enhanced vector competence will increase the vectorial capacity of
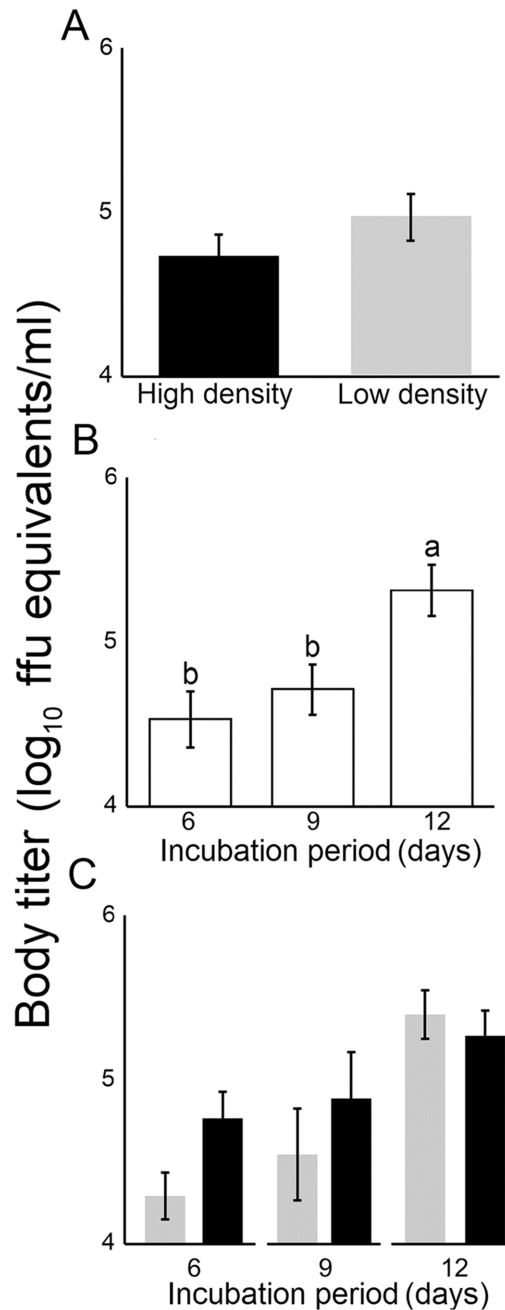
**Fig 3. Effect of larval density (A), incubation period (B) and larval density by incubation period (C) on DENV titer in infected mosquito bodies.** Bars above and below the means represent the standard errors of the mean. For each panel, estimates with a different letter are significantly different at $P = 0.05$, after controlling for multiple comparisons using the Tukey-Kramer adjustment.

mosquito populations. However, longevity and vector competence are not equivalent terms in the vectorial capacity equation; longevity is part of an exponential term, while vector competence is a linear term [17, 19]. To develop a better understanding of the relationship between longevity and vector competence, we estimated the change in the viral dissemination rate necessary to compensate for a 3% or 6% decrease in daily survivorship. We found that after a 14

**Table 5. Logistic regression assessing the relationship between DENV infection and dissemination status and female body size by larval density.**

| Infection status | Source | Density | df | Logit (SE) | t-value | P-value |
|---|---|---|---|---|---|---|
| **Body infection** | | High | | | | |
| | intercept | | 11 | 0.950 (1.906) | 0.50 | 0.627 |
| | size | | 266 | -0.115 (0.862) | -0.13 | 0.8933 |
| **Disseminated infection** | | High | | | | |
| | intercept | | 11 | 4.163 (2.472) | 1.68 | 0.1202 |
| | size | | 174 | -2.371 (1.131) | -2.10 | 0.0375 |
| **Body infection** | | Low | | | | |
| | intercept | | 11 | 2.337 (1.764) | 1.32 | 0.2121 |
| | size | | 126 | -0.648 (0.712) | -0.91 | 0.3641 |
| **Disseminated infection** | | Low | | | | |
| | intercept | | 11 | 1.466 (2.362) | 0.62 | 0.547 |
| | size | | 126 | -1.090 (0.965) | -1.13 | 0.26 |

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day EIP, a 2-fold increase in the viral dissemination rate is necessary to compensate for a 3% decrease in daily survivorship. This increase in DENV susceptibility greatly exceeds the level of enhanced susceptibility reported in experimental studies [28–30]. We did not incorporate associated changes in the biting rate or mosquito density in this model in part due to the lack of
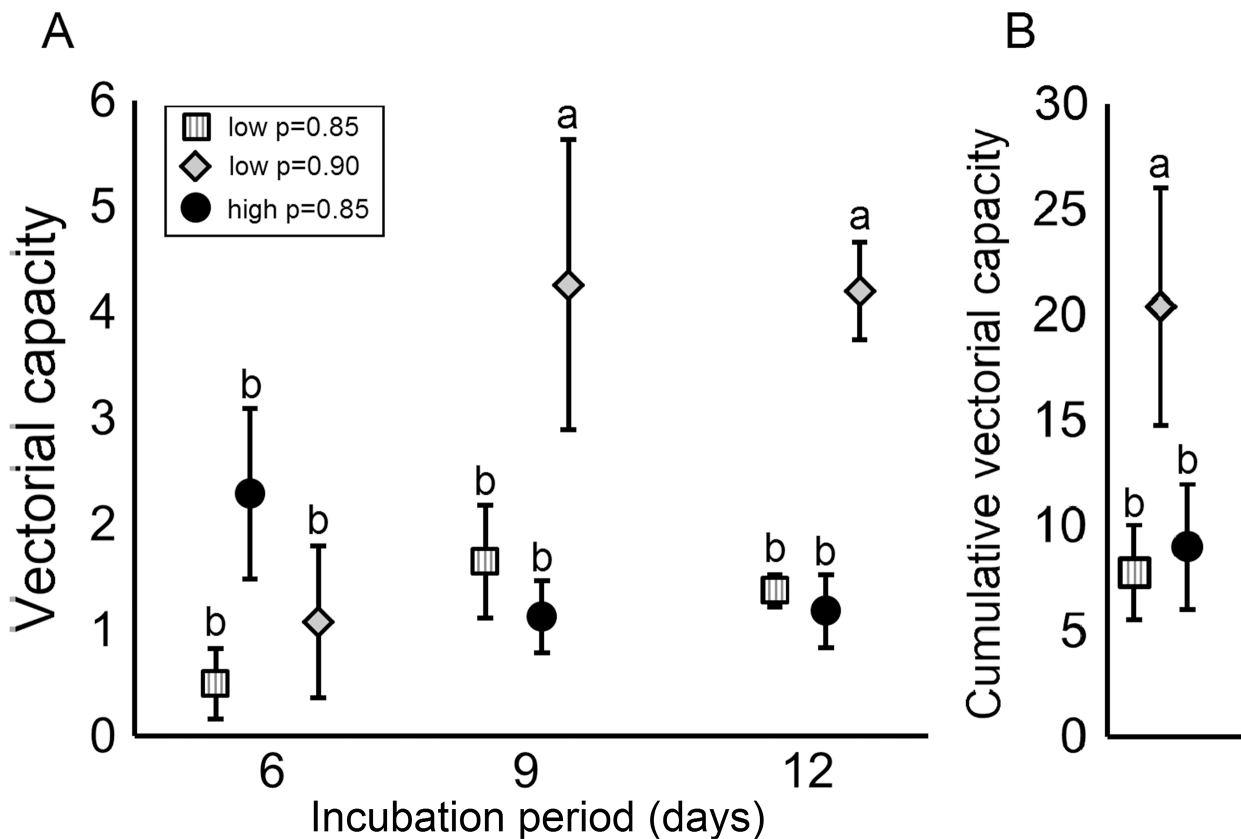


**Fig 4. Effect of larval competition on vectorial capacity.** Panel A displays the vectorial capacity values for high and low density treatments at the 6, 9, and 12-day incubation periods. Panel B displays the cumulative vectorial capacity for high and low-density treatments. Tests of significance were corrected for multiple comparisons using the Tukey-Kramer adjustment. Different letters indicate significant differences in means.
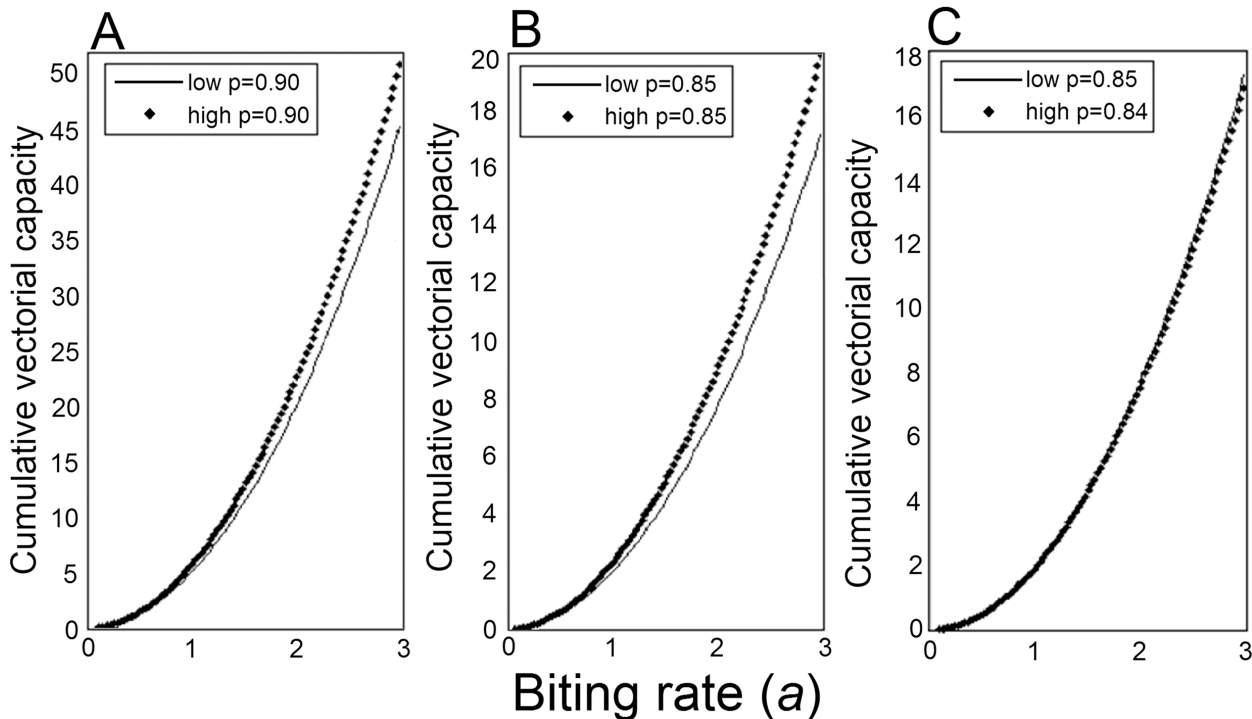
doi:10.1371/journal.pone.0126703.g004

**Fig 5. Cumulative vectorial capacity of high and low density treatments across a range of human biting rates.**

peer-reviewed studies on *Ae. albopictus*. Low larval densities yield higher adult emergence rates and larger *Ae. albopictus*, which may have a higher host attack rate relative to small mosquitoes [40]. Therefore by holding these two parameters constant, our model underestimates the negative effect of competition on vectorial capacity. Thus for most mosquito-borne pathogens, environmental factors that reduce female longevity are likely to decrease vectorial capacity, even when susceptibility is enhanced [40, 63]. These results are in agreement with recent studies by Araujo et al. [64], Moller-Jacobs et al. [65], Juliano et al. [66], and Breaux et al. [63] in indicating that stressful conditions within the larval environment yield adult mosquitoes with a reduced capacity to vector pathogens.

We also found that increases in viral dissemination rate over time often may not necessarily translate to increased vectorial capacity. Due to cumulative effect of adult mortality, increases in viral dissemination rate that occur at later incubation periods (> 9 days) become more unlikely to affect vectorial capacity. For example, although the viral dissemination rate of the high and low density treatments increased approximately two-fold from the 9 to 12-day IP, the vectorial capacity for both treatments actually decreased over this time period (S1 Fig). Similarly, Christofferson and Mores [20], found that DENV-2 strain with the highest disseminated infection rate in *Ae. aegypti* did not have the highest cVC, since the increase in viral dissemination rate for this strain primarily occurred after a 9-day IP. Alternatively, increases in the viral dissemination rate at early incubation periods (< 6 days) are more likely to affect vectorial capacity. This suggests that the biological relevance of vector competence studies is highly dependent on the use of relevant incubation periods for the vector-virus system.

Vector control remains the primary tool for reducing the incidence of vector-borne diseases.

In the face of global climate change, and expanding geographic ranges of mosquito species and the diseases they vector [6, 10, 67–69], improving quantitative assessment tools and
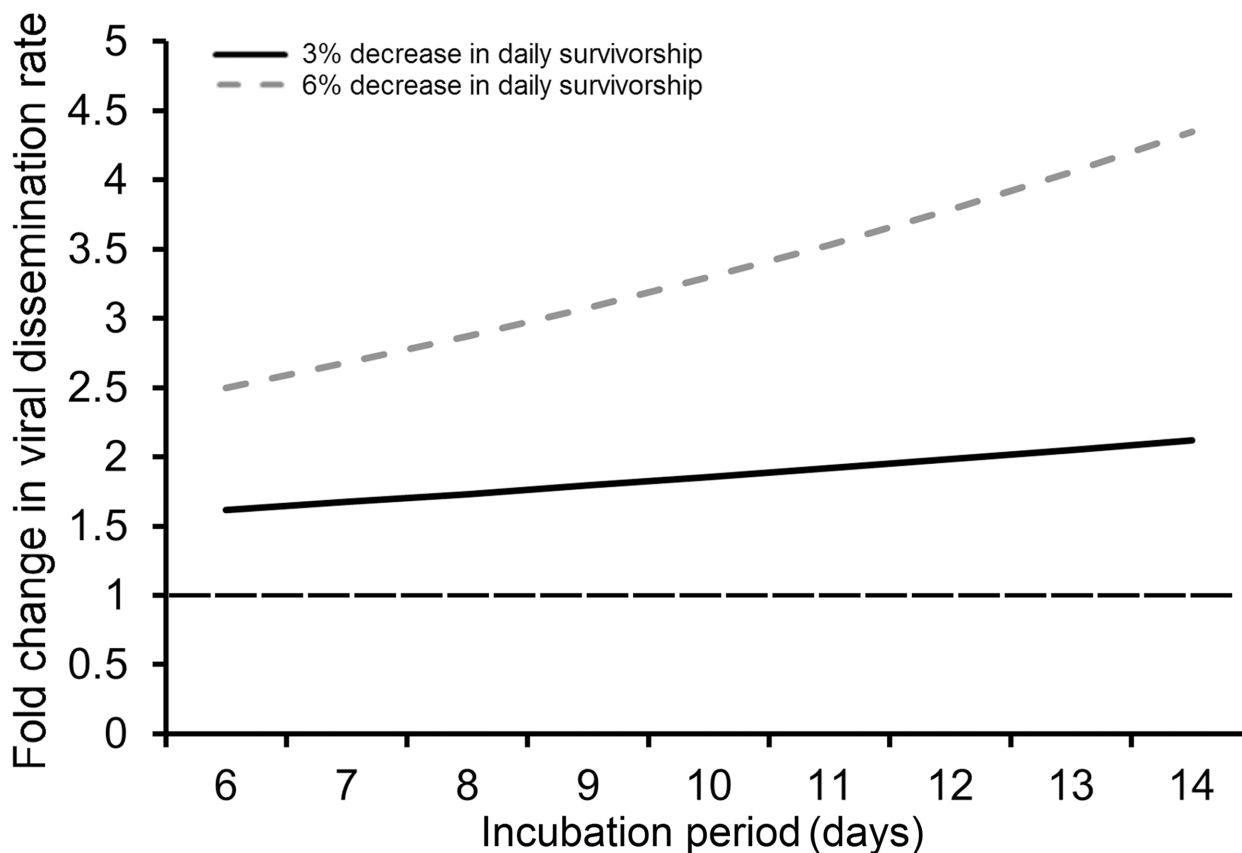
**Fig 6. Change in viral dissemination rate necessary to compensate for decrease in daily survivorship rate.** The figure above displays the change in viral dissemination rate necessary to compensate for a reduction in the daily survivorship rate by the specified amount from an estimated 90% survival rate.

mathematical models are necessary to accurately predict disease risks and improve vector control programs [21]. Determining the direction and magnitude of larval competition on vectorial capacity has important consequences for vector control programs. If competitive interactions have a negative effect on vectorial capacity, control measures (e.g. larvicides) that result in less than 100% mortality will release surviving larvae from competition, resulting in the production of larger adults with a higher vectorial capacity [70, 71]. Additionally, determining which artificial container habitats support *Ae. aegypti* and *Ae. albopictus* populations with the greatest capacity for DENV transmission can substantially improve the efficiency and cost effectiveness of mosquito control programs [6].

Some aspects of our study may influence how our results are translated to other systems. First, we used DENV RNA in mosquito legs as a proxy for the presence of DENV in the salivary glands, which marks the end of the EIP [9]. The presence of viral RNA in mosquito legs is highly correlated with viral dissemination into the head and salivary glands, but could potentially over estimate the number of infectious mosquitoes [72]. Second, given the lack of peer reviewed studies on the daily biting rates (a) and mosquito density (m) for *Ae. albopictus*, we used studies based on *Ae. aegypti* to estimate both parameters. While this approach provides insights into the potential consequences for larval competition on DENV vectorial capacity, further studies are necessary to generate the vectorial capacity parameters for *Ae. albopoctus*. Thirdly, as vector competence is influenced by genotype*genotype interactions, conclusions drawn from one vector-virus combination may not be representative of other vector-virus

systems under a variety of environmental conditions [73]. Lastly, this study was conducted using a laboratory strain of *Ae. albopictus* which may differ in susceptibility to DENV relative to natural populations [74]. However, our aim was to test for the effect of larval competition on DENV vectorial capacity and there is no evidence that laboratory colonization alters the effect of larval competition on vector susceptibility to DENV. For this reason, we believe our results provide an accurate indication of how larval competition may influence the vectorial capacity of this important vector. Additional studies using field collected populations of *Ae. albopictus* are needed to validate these findings.

In summary, our data suggest that larval competition has a weak effect on the EIP of DENV but can significantly alter the vectorial capacity of *Ae. albopictus*. In addition, our vectorial capacity models supported the hypothesis that stress-mediated decreases in the daily survivorship rate are likely to reduce the vectorial capacity of a mosquito population even if vector competence is enhanced. The effect of larval competition on adult fitness is context-dependent and may vary depending species interactions, detritus type or quantity, presence of anthropogenic chemicals (e.g. insecticides, pesticides) and temperature [24, 28, 75]. Therefore, additional studies investigating the effect of larval competition on arbovirus EIP and vector longevity under different environmental conditions are needed to understand how this ubiquitous ecological stressor affects transmission of mosquito-borne diseases.

## Supporting Information

**S1 Fig. The relationship between viral dissemination rate and vectorial capacity across the 6–12 day incubation period.**
(TIF)

**S1 Table. Mean life history traits, infection rates, and cumulative vectorial capacity values for the high and low-density treatments.**
(TIF)

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## Author Contributions

Conceived and designed the experiments: JB EJM. Performed the experiments: JB EJM ZR CEC. Analyzed the data: JB. Contributed reagents/materials/analysis tools: JB EJM. Wrote the paper: JB EJM. Minor revisions of manuscript: ZR CEC.

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