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Larval stress alters dengue virus susceptibility in *Aedes aegypti* (L.) adult females

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Abstract

In addition to genetic history, environmental conditions during larval stages are critical to the development, success and phenotypic fate of the *Aedes aegypti* mosquito. In particular, previous studies have shown a strong genotype-by-environment component to adult mosquito body size in response to optimal vs stressed larval conditions. Here, we expand upon those results by investigating the effects of larval-stage crowding and nutritional limitation on the susceptibility of a recent field isolate of *Aedes aegypti* to dengue virus serotype-2. Interestingly, female mosquitoes from larvae subjected to a stressed regime exhibited significantly reduced susceptibility to disseminated dengue infection 14 days post infection compared to those subjected to optimal regimes. Short term survivorship post-infected blood feeding was not significantly different. As with body size, dengue virus susceptibility of a mosquito population is determined by a combination of genetic and environmental factors and is likely maintained by balancing selection. Here, we provide evidence that under different environmental conditions, the innate immune response of field-reared mosquitoes exhibits a large range of phenotypic variability with regard to dengue virus susceptibility. Further, as with body size, our results suggest that mosquitoes reared under optimal laboratory conditions, as employed in all mosquito-pathogen studies to date, may not always be realistic proxies for natural populations.

Graphical abstract

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Keywords

Aedes aegypti; dengue virus; susceptibility; larval stress; vector competence

1. Introduction

The control of the mosquito *Aedes aegypti* (Diptera: Culicidae), the main global vector of the dengue virus (DENV), presents a significant challenge to public health organizations. Approximately 390 million people a year are infected with DENV, and about 96 million of these exhibit clinical symptoms (Bhatt et al., 2013). Effective vaccine development has been disappointing and no drug treatments exist, so only palliative care is available presently. Further, the potential side effects of DENV vaccines and their efficacy is largely dependent on factors such as preexisting flavivirus immunity, age group and local transmission intensity, raising concerns about their use (Ferguson et al., 2016). At present, mosquito vector control based approaches remain the primary mechanisms for preventing DENV transmission and limiting outbreaks (Bhatt et al., 2013; Morens and Fauci, 2008).

Genotype-by-genotype ($G \times G$) interactions between specific combinations of mosquito and virus strains play significant roles in determining vector competence and, as such, are essential to considerations of the innate immune response of the mosquito to pathogen infection (Lambrechts, 2011). Genetically distinct strains of *A. aegypti*, as well as individuals within the same populations, exhibit variable levels of DENV susceptibility and vector competence (Tardieux et al., 1990). Midgut infection and subsequent dissemination of DENV in *A. aegypti* are quantitative genetic traits (Bosio et al., 1998) and differences in DENV susceptibility have been shown to be conditioned by multiple quantitative trait loci (QTL) (Bennett et al., 2005; Bosio et al., 2000). However, the role of genotype-by-

environment ($G \times E$) interactions, and specifically the effects of variable environmental conditions on a given genotype, have been insufficiently investigated.

A. aegypti often lay eggs at the edges of standing water in man-made containers (Christophers, 1960). This semi-opportunistic habitat selection introduces intense competition during the aquatic larval stages of mosquito development and is affected by seasonal drops in precipitation where habitats become rare and crowded. Further, nutrient availability and daily temperature regimes can vary significantly among active breeding containers distributed in the same household area (Hemme et al., 2009). Despite shared genetic ancestry, larval stress has proven a critical influence on adult phenotype, which in turn is strikingly correlated with a suite of life history traits including vector competence (De Jesus and Reiskind, 2016; Honek, 1993; Mourya et al., 2004; Ponlawat and Harrington, 2007; Price et al., 2015; Schluter et al., 1991; Vantaux et al., 2016). In a previous study, our laboratory demonstrated the $G \times E$ influence of larval stress on adult body size (Schneider et al., 2011). This variance is likely maintained in the wild through balancing selection introduced by periodic environmental stressors associated with heterogeneous environments (Schneider et al., 2011). Additionally, it has been demonstrated that body size and rearing density influence the field fitness of *Wolbachia*-infected *A. aegypti* destined for release (Hancock et al., 2016; Yeap et al., 2013). This emphasizes the importance of realistic environmental conditions during the conception of *A. aegypti* laboratory trials and any subsequent vector control strategies. Despite this, to date most previous studies of DENV susceptibility have been conducted under optimal laboratory conditions.

Here, we characterize and compare differences in susceptibility in a recent *A. aegypti* field isolate from Trinidad to dengue virus (DENV2 JAM1409). We examine effects of optimized laboratory regimes and conditions simulating realistic, stress-inducing field conditions on individuals sharing the same underlying genetic architecture. We hypothesized that stress introduced by larval crowding and nutrient deprivation should be directly correlated with a mosquito's vectorial capacity: their ability to host the virus and subsequently transmit to a naïve human host. We present infection rates of each mosquito treatment to DENV in female *A. aegypti* mosquitoes 14 days after infection, as well as differences in several associated life history traits, including body size and longevity post-infection. The implications of gene-by-environment interactions on innate immunity are then further discussed.

2. Materials and methods

2.1. Mosquito rearing

Experiments employed F_3 generation mosquitoes from a colony of *A. aegypti* established at the University of Notre Dame originating from ovitrap collections in Curepe, Trinidad in November of 2015. Mosquitoes were reared and maintained in a 26°C environmental chamber kept at 85% relative humidity, with a 16 hour light:8 hour dark (L:D) cycle and a 30 minute crepuscular period following our standard lab protocol (Clemons et al., 2010). First instar larvae were subjected to either optimal or stressed rearing conditions as described by Ponlawat and Harrington (2007). Briefly, 75 first instar F_3 Trinidad larvae were placed in a container with one liter of autoclaved water and provided 75 mg of bovine liver powder (MP Biomedicals, LLC) on day one after hatching, 0 mg on day two, 38 mg on day

three, 75 mg on day four, 113 mg on day five, and 150 mg on day six. The rearing conditions of stressed individuals were identical to optimal, except 750 individual larvae were added to the container. Pupae were transferred to fresh water in 500 mL plastic cups and placed in 20×20×30 cm mesh cages for adult eclosion, where they were maintained on 10% sucrose-soaked cotton balls. Six days after adult eclosion, females were transferred to 500 mL paper cups and starved for 24 hours before infectious blood feeding.

2.2. Dengue infection

Aedes albopictus C6/36 cells were cultured using a standard laboratory protocol with minor modifications (Gaines et al., 1996; Schneider et al., 2007). Cells were grown with 10% fetal bovine serum (FBS) without CO₂ at 28°C to near confluence (75–80%) in 75 cm³ flasks. Cells were next inoculated with DENV2 (strain JAM1409) at a multiplicity of infection (MOI) of 0.1 and incubated for 7 days with 2% FBS infused L-15 media without CO₂ at 28°C (Deubel et al., 1986). After the 7 days, the cells were centrifuged for 10 minutes at 2500 RPM before collection of supernatant.

A standard infectious blood feeding protocol was performed with slight modification (Schneider et al., 2007). Frozen DENV2 supernatant at a TCID₅₀ of 10^{6.5} was thawed and mixed with defibrinated sheep blood (Colorado Serum Company) in a 1:1 ratio. A shaved rat skin was utilized as an artificial membrane on a glass membrane feeder loaded with the infectious blood mixture and kept warm with water circulating at 37°C. Animals use was in accordance with the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health. The animal use protocol was approved by the University of Notre Dame Institutional Animal Care and Use Committee (Study #11–036). Females were allowed to feed for 10 minutes. Mosquitoes were then briefly anesthetized with CO₂ and engorged females were transferred to a new 475 mL container where they were maintained on a 10% sucrose solution at 26°C and 85% relative humidity until 14 days post infection.

2.3. RT-PCR

Standard RT-PCR was employed on the mosquito heads to determine the frequency of disseminated infection in DENV2 challenged females (Lanciotti et al., 1992). The remaining carcasses were stored at –80°C for later use. Total RNA was removed from the head via standard TRIzol (Thermo Fisher Scientific) extraction as described by the manufacturer. An aliquot (50 ng) of RNA was reverse transcribed and amplified utilizing a Superscript III One-Step RT-PCR kit (Invitrogen) per manufacturer's instructions. A multiplex of primers specific for DENV2 (DENV511 F: 5'-TCAATATGCTGAAACGCGCGAGAAACCG-3'; DENV511 R: 5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3') (Lanciotti et al., 1992) and a ribosomal protein S7 as an endogenous control (RPS7 F: 5'-CCCAACAAGCAGAAGCGTCCACG-3'; RPS7 R: 5'-TCGAACGTAACGTCACGTCCGGTC-3'), designed from previously published sequences (Geiser et al., 2006), were used to detect virus. This procedure was then repeated on the abdomens of individual mosquitoes testing negative for disseminated head infection to check for presence of DENV2 in the midgut.

2.4. Wing measurements

Wing length was used as a proxy for body size, a strong indicator of larval nutrition (Gleiser et al., 2000; Nasci and Mitchell, 1994; Schneider et al., 2007). The right wings of cohort females were removed 7 days post adult eclosion. Wings were attached to double-sided tape on glass slides and sealed with a coverslip before measurement under a dissection microscope. Measurements spanned the axillary margin to the apical notch, excluding fringe.

2.5. Statistical analysis

Statistical analyses were performed using STATISTICA 13.0 (StatSoft) or GraphPad Prism v. 7.0 (GraphPad Software). Independent two-sample t-tests were employed to ascertain significant differences in means across treatments. A χ^2 test of independence was employed to check for significant differences between categorical data.

3. Results and Discussion

The primary aim of this study was to further characterize differences in DENV susceptibility resulting from different rearing conditions among *A. aegypti* of common genetic ancestry. Additionally, we report and validate the impact of larval conditions on a suite of life history traits including body size, survival, blood feeding success and developmental time. In organisms with a complex life cycle, phenotypic responses in the pre-metamorphic phase can be carried over to the reproductive phase (Wilbur 1980; Nylin & Gotthard 1998). Specifically, the aquatic environmental conditions that mosquito larvae experience strongly influence their phenotypic characteristics as adults (Maciel-de-Freitas et al. 2007; Reiskind and Lounibos 2009; van Uitregt et al. 2012). These carry-over effects can have major consequences on the ability of vectors to transmit disease agents such as arboviruses. Finally, stress conditions encountered during the larval stages often lead to life history trade-offs in which energy should first be allocated to priority functions (e.g., survival and reproduction) at the expense of other fitness-related traits (e.g. immune defenses against disease agents; Zera & Harshman 2001).

Our results indicated that adult females reared under stress conditions exhibited significantly lower head dissemination rates compared to those reared under standard optimum laboratory conditions ($\chi^2 = 4.23$, $df = 1$, $P=0.04$; Fig. 1). The increased refractoriness of our stressed individuals may seem counterintuitive from an energetic approach, as one might assume that optimum-reared mosquitoes would be better prepared for the metabolic toll associated with combating infection, but the cost-benefit of warding off versus hosting an infection has yet to be definitively investigated. Although our study does not directly investigate innate immune factors, the significantly reduced susceptibilities of mosquitoes suggest that deficient larval rearing conditions may drive differences in immunity.

Two potential inhibitors of dengue infection in the mosquito are the midgut infection barrier (MIB), which prevents the infection of the epithelium, and the midgut escape barrier (MEB), which may prevent mature virus from escaping the midgut epithelial cells and entering the hemocoel for dissemination (Black et al., 2002; Bosio et al., 1998; Telang et al., 2012).

Here, females testing negative for head dissemination did not exhibit significantly different incidence of DENV2 midgut infection 14 days post infection ($\chi^2 = 0.05$, $df = 1$, $P=0.823$; Fig. 1). This suggests that while our larval stress conditions did result in an increased MIB among the adult females, they did not concomitantly increase the likelihood of a MEB. If the MIB is indeed responsible for our differences in susceptibility, it is likely that the critical innate immune responses occur very early after exposure, rather than preventing virus from escaping the midgut. This is supported by literature suggesting that DENV2 takes as little as 5–7 minutes to bind to the cell membrane of C6/36 *Aedes albopictus* cells in culture with endocytotic ingress occurring in as little as 30 minutes (Mosso et al., 2008; van der Schaar et al., 2008).

As both optimal and stressed treatment groups shared direct genetic ancestry, the differences between treatments are likely a result of a continuum of variation maintained within the gene pool by environmental shifts. Maintaining a range of phenotypic plasticity is important for a population to adapt to highly variable and sudden environmental shifts. Our results indicated individuals reared under optimal conditions had significantly higher larval survival rates ($\chi^2 = 982.28$, $df = 1$, $P<0.0001$) and blood feeding success ($\chi^2 = 8.6097$, $df=1$, $P<0.0033$) than those reared under stress conditions (Fig 2). However, we nonetheless observed similar levels of 14 day post-infection survivorship, well past their transmissive phase ($\chi^2 = 2.9559$, $df = 1$, $P 0.0856$). As expected based on previous literature, larvae reared under the optimal regime exhibited significantly faster development ($t = 104.44$, $df = 656$, $P < 0.0001$) and significantly larger adult body sizes ($t = 19.952$, $df = 58$, $P < 0.0001$). Here, we show that these traits reflected strong associations with DENV dissemination frequencies (Fig 3).

There are two contrasting hypotheses regarding the effects of adult body size on vectorial capacity. First, smaller mosquitoes are more likely to become infected, as they will take smaller blood meals more frequently (Schneider et al., 2004; Xue et al., 1995). Second, larger mosquitoes take larger blood meals and consequently are more likely to become infected (Nasci, 1991; Paupy et al., 2003). While identifying individual mosquitoes that had successfully blood fed, we did not quantify the volume of each individual blood meal. As such, there remains a possibility that the higher rates of disseminated infection in mosquitoes raised under optimal conditions was due to imbibing larger blood meals, and consequently a higher total amount of virus.

In any realistic assessment of mosquito vectorial capacity, scientists should consider environmental stress as a variable. To date, the majority of experiments investigating mosquito-pathogen interactions employ laboratory reared mosquitoes under unrealistic, optimal conditions and fail to consider genotype-by-environment interactions. Further complicating matters, the landscape presented by the few studies that have included environmental stress is largely muddled by conflicting conclusions. One study suggests that among progeny originating from wild Thai mosquitoes, larger females present higher infection rates (10.7%) than smaller mosquitoes (5.7%). Additionally, the same study found that differences of geographic origin, as close as 100 km, strongly influenced the rate of DENV infection of mosquitoes (Sumanochitrapon et al., 1998). In contrast, a more recent investigation found a laboratory strain of *A. albopictus* (Lake Charles) exhibited lower susceptibility to DENV when crowded as larvae but that *A. aegypti* (Rockefeller), while

exhibiting a similar trend, did not exhibit significantly different infection rates (Alto et al., 2008).

The significantly higher susceptibility of our optimally-reared *A. aegypti* from a recent Trinidad field population seems to correlate well with the results from Thailand field populations (Sumanochitrapon et al., 1998), but is in contrast with generally opposite results reported for the Rockefeller strain (Alto et al., 2008). Given that the Rockefeller strain has been in lab culture since 1926 (Kuno, 2010) it is likely that the contemporary strain has limited remaining genetic variability that may be reflected in limited phenotypic plasticity in response to environmental conditions, including DENV vector competence. For example, our previous investigations on genetic variability in *A. aegypti* adult body size showed that laboratory strains showed highly reduced (Ghana strain) or essentially zero (MOYO-S strain) remaining genetic variability contributing to body size, in contrast with a field population collected in Trinidad (Schneider et al., 2011).

The lower susceptibility rates of adult females subjected to stress as larvae suggest that traditional laboratory conditions may overestimate the true potential of mosquitoes to transmit disease. In the estimation of vectorial capacity, type I experimental error is the more acceptable of the two errors, but it may still be more appropriate to report dengue susceptibilities in a range accounting for larval environmental stress. Here, we posit that rather than one of these factors possessing a causative influence on the other, both are correlated with the genetic architecture underlying environmental stress responses. Consequently, we suggest that stress via larval crowding and other environmental factors may severely influence dengue transmission by field populations.

5. Conclusions

Here we tested for and identified a strong correlation between larval stress, body size and DENV susceptibility in *A. aegypti*. We found that environmentally stressed mosquitoes present smaller bodies, longer developmental times with higher variance, and significantly less susceptibility to DENV2. Our study concludes that a genotype-by-genotype approach without consideration of environmental stress does not accurately reflect the potential DENV susceptibility in the field. We anticipate that further investigations of the genetic basis underlying these differences will improve existing models of vectorial capacity, as well as reveal mechanisms responsible for DENV resistance of stressed mosquitoes.

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Highlights

- Larval crowding and nutritional deprivation impact a suite of life history traits in adult *Aedes aegypti*.
- Stress-reared *A. aegypti* exhibit lower levels of susceptibility to dengue virus serotype-2, smaller bodies, and longer developmental times than cohorts reared under optimal conditions.
- Mosquitoes reared under optimal laboratory conditions may not always serve as realistic proxies for natural populations in studies of vector-pathogen interactions.

Fig 1. Comparisons of disseminated vs. midgut infections in optimum and stress reared females at 14 days post infection with DENV-2

Midgut infection assays were performed on individuals determined to be negative for disseminated infection by head tissue assays.

Fig 2. Effects of larval stress on survival from first instar larvae to pupation, adult blood feeding success at 7 days post eclosion, and adult survival 14 days after exposure to DENV2.

Fig 3. 3D plot showing associations between larval development time, adult body size, and disseminated DENV-2 infections among optimum and stress reared females
Error bars represent standard deviations.