She’s a femme fatale: low-density larval development produces good disease vectors

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Two hypotheses for how conditions for larval mosquitoes affect vectorial capacity make opposite predictions about the relationship of adult size and frequency of infection with vector-borne pathogens. Competition among larvae produces small adult females. The competition-susceptibility hypothesis postulates that small females are more susceptible to infection and predicts frequency of infection should decrease with size. The competition-longevity hypothesis postulates that small females have lower longevity and lower probability of becoming competent to transmit the pathogen and thus predicts frequency of infection should increase with size. We tested these hypotheses for Aedes aegypti in Rio de Janeiro, Brazil, during a dengue outbreak. In the laboratory, longevity increases with size, then decreases at the largest sizes. For field-collected females, generalised linear mixed model comparisons showed that a model with a linear increase of frequency of dengue with size produced the best Akaike’s information criterion with a correction for small sample sizes (AICc). Consensus prediction of three competing models indicated that frequency of infection increases monotonically with female size, consistent with the competition-longevity hypothesis. Site frequency of infection was not significantly related to site mean size of females. Thus, our data indicate that uncrowded, low competition conditions for larvae produce the females that are most likely to be important vectors of dengue. More generally, ecological conditions, particularly crowding and intraspecific competition among larvae, are likely to affect vector-borne pathogen transmission in nature, in this case via effects on longevity of resulting adults. Heterogeneity among individual vectors in likelihood of infection is a generally important outcome of ecological conditions impacting vectors as larvae.

Key words: dengue - Aedes aegypti - competition - adult size - longevity - trans-stadial effects
Harris 2008, Bhatt et al. 2013). Larval competition in laboratory microcosms significantly increased the susceptibility of the vector *Aedes albopictus* to experimental infection with dengue virus (DENV) and potential transmission (Alto et al. 2008a). In both *Aedes aegypti* and *Ae. albopictus*, adult female body size, which covaries with both larval rearing temperature and competition intensity, was negatively correlated with dengue infection rates in laboratory studies (Alto et al. 2008b). Independent investigations also show that adult longevity in *Ae. aegypti* is positively correlated with body size (Steinwascher 1982, Reiskind & Lounibos 2009) and negatively correlated with intraspecific larval competition (Reiskind & Lounibos 2009). Thus, there are potentially complex and interacting effects of larval density and competition on an adult female’s ability to act as a vector. This leads to two hypotheses for how competition among larvae affects production of likely dengue vectors and ultimately dengue transmission. On the one hand, greater competition among larvae produces small adult females that are more susceptible to acquiring dengue infection (competition-susceptibility hypothesis). On the other hand, greater competition among larvae produces small adult females with shorter adult lifespans, rendering them less likely to live long enough both to acquire the infection and to become competent to pass the infection on during subsequent blood meals (competition-longevity hypothesis). Though these hypotheses are not mutually exclusive, we can ask the question: which postulated effect of competition among larvae is actually most important in natural transmission of DENV?

In many dengue-endemic areas of the tropics, temperatures experienced by larvae do not change substantially during the transmission season (e.g., Aldstadt et al. 2011), so natural variation in body size of adult vectors is likely to be primarily related to crowding, resource limitation and competition among larvae in the aquatic environment (e.g., Strickman & Kittayapong 2003). The two hypotheses for effects of competition on dengue transmission thus yield two different predicted relationships between frequency of dengue infection and female body size: the competition-susceptibility hypothesis predicts that frequency of infection should decrease as body size increases; in contrast, the competition-longevity hypothesis predicts that frequency of infection should increase as body size increases, assuming larger females have greater lifespan. In this report, we tested the predictions of these two alternative hypotheses during a dengue epidemic in Rio de Janeiro, Brazil. This study has two parts. Part one is a laboratory investigation of the effects of dengue infection and female size on adult longevity under laboratory conditions. This portion of the project is a reanalysis, with new methods, of data presented by Sylvestre et al. (2013), and tests the assumption that longevity is related to size. Part two is a test of the relationship of frequency of dengue infection to female size among females collected in neighbourhoods of Rio de Janeiro during the 2008 dengue outbreak, controlling for both time and location of collection.

**MATERIALS AND METHODS**

*Dengue epidemiology in Rio de Janeiro in 2008 -* Dengue transmission in Rio de Janeiro is strongly associated with summer (December-April), when higher temperatures and intense rainfall are frequent (Honório et al. 2009). In 2008, the city of Rio de Janeiro suffered one of its most severe dengue outbreaks when DENV-2 and DENV-3 co-circulated. Between January-April 2008, more than 155,000 cases and 110 deaths were reported and an incidence of 2,453.7/100,000 inhabitants was observed (Teixeira et al. 2009). The epidemic reached its peak in March and April, with 86,036 and 90,945 human cases reported, respectively, and had ended by June (Malhão et al. 2013).

**Effect of dengue infection on *Ae. aegypti* survival in the laboratory -** To estimate the effect of dengue infection on *Ae. aegypti* longevity and how that effect may vary with mosquito size, we reanalysed data reported by Sylvestre et al. (2013), who gave a full description of experimental conditions and methods. Briefly, we orally challenged females with DENV-2 at a viral titre of 2 x 10⁸ TCID₅₀. We infected two *Ae. aegypti* populations: Paea (a population maintained as laboratory colony at Oswaldo Cruz Institute, Rio de Janeiro, for almost 13 years) (Sylvestre et al. 2013) and a field population from Rio de Janeiro. Sylvestre et al. (2013) reported that 93.3% of females challenged in this way had detectable dengue in the head, suggesting disseminated infection. After being orally infected, females were maintained individually in plastic vials (6.5 cm height, 2.5 cm diameter) with cotton soaked in a 10% sucrose solution *ad libitum* and an oviposition substrate at the bottom for egg laying. During the longevity study one blood meal on an anesthetised mouse [Oswaldo Cruz Foundation Ethical Committee for Animal Use (CEUA L-0007/09)] was offered to mosquitoes once a week. Adult female longevity was assessed daily. After death, wing length (axillary incision to wing tip, excluding fringe) (Alto et al. 2008b) was measured.

*Ae. aegypti* field collections - Mosquito collections started in March 2008 during the dengue outbreak and continued until June 2009. Adult *Ae. aegypti* were collected in 23 districts that encompassed three municipalities in metropolitan Region of Rio de Janeiro (Supplementary data). District selection was done together with the Health Municipal Secretary, which directed collectors to neighbourhoods in which dengue transmission had recently become intense. At each field-site, we randomly choose 10-20 houses per day for mosquito collection using backpack aspirators or BG-Sentinel traps (Clark et al. 1994, Maciel-de-Freitas et al. 2006). Mosquito collections began after explaining the objectives of this research project to householders and receiving their oral consent to use an aspirator or a mosquito trap to collect mosquitoes. Aspirating was performed for 15-20 min per house, depending on the premise area, and was also done in the peridomestic environment. BG-Sentinel traps were installed inside houses and remained in the same house for a 24-h trapping period.
Mosquito preparation - All mosquitoes collected in the field (1,586 males and 1,213 females) were brought to the laboratory for identification using taxonomic keys (Consoli & Lourenço-de-Oliveira 1994). Ae. aegypti females had their wing length measured from auxiliary incision to wing tip excluding the fringe (Alto et al. 2008b). Each adult was individually labelled with its sex, date and district of collection. Adult females from all 23 sites were individually macerated in 1 mL of Leibovitz L-15 medium (Sigma) plus antibiotics (penicillin-streptomycin, 10,000 units - Invitrogen) and centrifuged (6,000 rpm at 4°C for 30 min). Supernatant was transferred to an Eppendorf tube containing 100 mL of streptomycin/fungizone and penicillin, kept in an ice bath for 1 h and centrifuged (3,000 rpm at 4°C for 15 min). Supernatant was transferred to an Eppendorf tube containing 0.3 mL of foetal calf serum (FCS) (Invitrogen) and frozen (-70°C) (de Castro et al. 2012).

Virus isolation - Virus isolation was performed by inoculation into monolayers of C6/36 Ae. albopictus cells (Igarashi 1978) in Leibovitz L-15 medium (Sigma) supplemented with 2% FCS (Invitrogen) and 0.2 mM of non-essential amino acids (Invitrogen). Cells were incubated at 28°C for five-seven days and observed for cytopathic effects (de Castro et al. 2012). Isolates were identified by indirect fluorescent antibody test (IFAT) using dengue serotype-specific monoclonal antibodies (Gubler et al. 1984) and infected supernatant was clarified by centrifugation and virus stocks stored in 1-mL aliquots at -70°C.

RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR) - Viral RNA was extracted using QIAamp Viral RNA Mini kit (Qiagen) following the manufacturer’s instructions. RT-PCR for detecting DENV was performed as described previously (Lanciotti et al. 1992).

Statistical analysis - Laboratory - For the laboratory longevity study, time to death was analysed by a proportional hazards model with classification effects of population (field, lab) and dengue infection (yes, no) and continuous effects of wing length (mm) and wing length² (mm²) (PROC PHREG) (SAS 2011). We initially tested a model with all interactions of the class variables and wing length and wing length². All interactions were far from significant (p > 0.15) and this full model yielded a poorer information theoretic index [Akaike’s information criterion with a correction for small sample sizes (AICc)] (Burnham et al. 2011) than a reduced model with only main effects. Similarly, effects of population on longevity were also far from significant (p > 0.15) and a model with population yielded a poorer AICc; hence our final analysis was a reduced model including only dengue infection, wing length and wing length². This same model also was the endpoint of using the STEPWISE option in PROC PHREG (SAS 2011).

Field - All dengue-positive female Ae. aegypti were collected from March to early June 2008, corresponding to epidemic period reported by the Ministry of Health (Malhão et al. 2013). We analysed wing lengths of female Ae. aegypti field collected during this epidemic period, testing effects of date of collection (expressed as days post 1 March 2008) and (date of collection)² and the random effect of site for 545 individuals from all 23 sites. We used a mixed model ANOVA (SAS PROC MIXED) (SAS 2011). Thirteen sites yielded some infected Ae. aegypti females and we analysed only these sites for frequency of dengue infection because we could not be sure dengue was active at a site unless we detected it. We analysed the relationship between frequency of dengue infection and wing length (mm) and (wing length)² across these 13 sites. All models tested included date of collection (days post 1 March 2008) (date of collection)² and the random effect of site for 492 individuals from these 13 positive sites. We used a mixed effects generalised linear model (PROC GLIMMIX) (SAS 2011), employing an information theoretic approach to model comparison (Anderson 2008) using AICc. A major advantage of this approach is quantification of weight of evidence (w) for different models (Anderson 2008, Burnham et al. 2011). We tested three models that included wing length as both linear and quadratic terms, wing length as a linear term only and no wing length term. We used a binary distribution of the dependent variable dengue (yes, no), with a logit link function (SAS 2011). We used multimodel estimation (Anderson 2008) to estimate weighted average consensus predicted probabilities of infection and their weighted standard errors (SE), as a function of wing length and date of collection based on all the models evaluated and weighting model contributions by w (Anderson 2008).

For all 23 sites, including those with no dengue-positive females, we determined mean female size (wing length) and proportion of females infected. We then tested for a relationship across sites of proportion dengue-infected and mean female size per site using Pearson’s correlation (PROC CORR) (SAS 2011).

RESULTS

Laboratory - Sizes of the 134 females used in the laboratory study ranged from 2.10-3.20 mm wing length, with a mean ± SE of 2.89 ± 0.01 mm. Infection with dengue significantly shortened the life of females ($\chi^2 = 7.71$, degrees of freedom (df) = 1, p = 0.0055), with a hazard ratio of control/infected of 0.560, indicating that controls had nearly half the hazard of death as infected individuals. Both wing length ($\chi^2 = 4.06$, df = 1, p = 0.0439) and (wing length)² ($\chi^2 = 4.81$, df = 1, p = 0.0283) were significantly related to the hazard of death. The linear term (± SE) was negative (-20.81 ± 10.30), indicating that hazard of death initially declines with adult size. The quadratic term was positive (4.09 ± 1.90), indicating that hazard of death increases as larger sizes are reached. Thus this analysis describes a hazard function of size that is significantly concave upward. Predicted survival curves for infected and uninfected females of wing lengths = 2.10, 2.70 and 3.30 mm (Fig. 1) indicated that longevity peaked at 2.70 mm and was actually least at 3.30 mm.

Field - Sizes of 545 field collected females ranged from 1.60-3.95 mm wing length, with a mean ± SE of 2.69 ± 0.01 mm. Wing length varied significantly among
sites ($z = 2.24$, $p = 0.0124$), but was unrelated to date of collection, as a model omitting date of collection, and only including site yielded the best AICc.

Frequency of dengue infection was best predicted by a model with wing length as a linear effect (Table I) and this linear effect was positive (slope $\pm$ SE $= 1.24 \pm 0.56$, $t_{476} = 2.20$, $p = 0.0285$). Evidence indicated that the model with linear + quadratic effects of wing length also had some support, given this data set, based on AICc [and, indeed, any of the other information theoretic indices reported by SAS Institute (2011)]. For the quadratic model, the linear effect of wing length was again positive (linear coefficient $\pm$ SE $= 9.33 \pm 7.33$, $t_{475} = 1.27$, $p = 0.2034$), whereas the quadratic effect was negative (quadratic coefficient $\pm$ SE $= -1.46 \pm 1.32$, $t_{475} = 1.11$, $p = 0.2688$). The model omitting wing length entirely was substantially less likely as a model generating these data (Table I). Predicted frequencies of dengue infection for these two most likely models, holding the random site effect constant at the value for the Valqueire collection (Supplementary data, Fig. 2A, B, Table II) show that in both cases predicted frequency of dengue infection increases from wing lengths of 2.0 mm to about 3.5 mm. At wing lengths from 3.5-4.0 mm the quadratic model predicts frequency of dengue infection declines, though never to the low levels observed for the smallest females (Fig. 2B). We expected frequency of dengue infection to be related to day of collection and the relationship proved to be quadratic, with frequency first increasing to a peak in the middle of the epidemic, then declining (Fig. 2A, B).

Multimodel consensus prediction of frequency of dengue infection, based on the weighted averages of the three models (Table II) indicated that frequency of dengue infection likely increased monotonically across the range of wing lengths from 2.0-4.0 mm. Multimodel predictions, particularly at the largest wing lengths, had high uncertainty (Table II) due to both inherent error of prediction and model selection error (Anderson 2008).

Site-wide frequency of dengue infection was not significantly correlated with mean wing length for a site (Fig. 3). The observed relationship was positive (Fig. 3), but highly variable and unlikely to be linear.

**DISCUSSION**

Our data support the competition-longevity hypothesis and suggest that the competition-susceptibility hypothesis does not account for patterns of dengue infection of these vectors in nature. However, a central point of this paper is that the frequency of dengue infection in the field may have a complex relationship to vector body size. Thus, it is important to determine that empirical

**TABLE I**

| Model effects          | AICc  | $\Delta$AICc | $\text{Exp}[-0.5(\Delta\text{AICc})]$ | $w_i$ |
|------------------------|-------|--------------|----------------------------------------|-------|
| Wing, day, day$^2$, site | 301.87| 0            | 1                                      | 0.5608|
| Wing, wing$^2$, day, day$^2$, site | 302.47| 0.60         | 0.7408                                 | 0.4155|
| Day, day$^2$, site     | 308.20| 6.33         | 0.0422                                 | 0.0237|
| Sum                    | -     | -            | 1.7830                                 | -     |

All considered models included linear and quadratic effects of date of collection and the random effect of site. Weight of evidence ($w_i$) expresses $\text{exp}[-0.5(\Delta\text{AICc})]$ as a proportion of the sum of that column and indicates the evidence for the model (i.e., the probability that the model is the correct one). AICc: Akaike’s information criterion with a correction for small sample sizes.
relationship and to understand the ecological processes that cause heterogeneity in frequency of infection of vectors. Though our data refute the competition-susceptibility hypothesis, we do not reject the physiological mechanism underlying that hypothesis (i.e., physiological effects of competition and body size on probability of dengue infection given an infectious blood meal). That mechanism has been demonstrated in laboratory studies of infection of *Aedes* with dengue (Alto et al. 2008a, b) and other viruses (Alto et al. 2005, Muturi et al. 2011a, b). Our data indicate, however, that the demographic effects of competition (longevity of adults) are more important in nature than these physiological effects of competition (susceptibility to infection). In the epidemic in Rio de Janeiro, we find that the demographic effect overrides the physiological effect.

Our reanalysis of data on laboratory longevity of *Ae. aegypti* indicates that longevity is not linearly related to size, but rather shows a quadratic relationship, with maximal predicted longevity at intermediate sizes (wing lengths of 2.7 mm) and lower longevity at smaller or larger sizes. Typically mosquito ecologists assume, based on some data (e.g., Hawley 1985, Reiskind & Lounibos 2009), that adult *Aedes* longevity is positively related to body size. Some field studies have failed to find a relationship (e.g., Scott et al. 2000, Maciel-de-Freitas et al. 2007) and others find evidence for non-monotonic relationships (e.g., Hawley 1985). Our laboratory study of longevity had a relatively limited range of female wing lengths represented (2.1-3.2 mm) compared to our field collected females (1.6-4.0 mm); nevertheless this range was sufficient to show the nonlinear relationship of size to longevity.

### TABLE II

| Wing (mm) | Model          | Model (w) | Predicted frequency (dengue) | Conditional SE predicted | Model selection uncertainty | Average predicted frequency (dengue) | Unconditional SE predicted |
|-----------|----------------|-----------|------------------------------|--------------------------|---------------------------|--------------------------------------|---------------------------|
| 2.0       | Wing, wing²    | 0.4155    | 0.078                        | 0.062                    | 0.001                     | 0.112                                | 0.335                     |
|           | Wing           | 0.5608    | 0.145                        | 0.086                    | 0.011                     | 0.207                                | 0.547                     |
|           | -              | 0.0237    | 0.275                        | 0.116                    | 0.055                     | 0.022                                |                          |
| 3.0       | Wing, wing²    | 0.4155    | 0.399                        | 0.147                    | 0.074                     | 0.299                                | 0.547                     |
|           | Wing           | 0.5608    | 0.368                        | 0.141                    | 0.059                     | 0.082                                | 0.547                     |
|           | -              | 0.0237    | 0.275                        | 0.116                    | 0.022                     |                                       |                          |
| 4.0       | Wing, wing²    | 0.4155    | 0.220                        | 0.352                    | 0.004                     | 0.348                                | 0.590                     |
|           | Wing           | 0.5608    | 0.667                        | 0.208                    | 0.260                     | 0.014                                | 0.480                     |
|           | -              | 0.0237    | 0.275                        | 0.116                    | 0.014                     |                                       |                          |

All models include date and date². For prediction, the random effect of site was held constant at the value for Valqueire (Supplementary data), which had an intermediate frequency of dengue-positive females (0.1719) and date was held constant at 21 April (middle of the epidemic). Conditional SE includes only the uncertainty within the model. Unconditional SE includes that uncertainty plus model selection uncertainty weighted by evidence (w). For details see Anderson (2008).
In the field, frequency of dengue infection increases with female size and the linear model was the best description of our field data. Even if we accepted the quadratic model of frequency of dengue, it yields a peak predicted frequency of dengue for females between 3.0-3.5 mm wing length (relatively large females) (Fig. 2B). Wing lengths ≥ 3.5 mm represented less than 1% of all females collected for this study, which indicates that for the vast majority of females in the field in Rio de Janeiro during the epidemic, frequency of dengue infection increases with size for both the linear and quadratic models (Fig. 2A, B). If the decline in frequency of dengue among this largest 1% of females is real, its cause remains unknown. That decline may result from low crowding of larvae during development, yielding very large, less physiologically susceptible adults, as observed by Alto et al. (2008a, b). Alternatively, low crowding may yield very large, but short-lived adults, as observed in our laboratory analysis.

Reduced longevity of small *Ae. aegypti* adults from crowded larval conditions was evident at adult body sizes at or below 2.6 mm mean wing length (Reiskind & Lounibos 2009), which suggests that the very small females < 2.5 mm wing length, which constituted 25% of our field sample, may be expected to have significantly reduced longevity relative to larger females. In contrast, based on logistic regressions, estimated physiological susceptibility to dengue infection and disseminated infection in the laboratory decline rapidly with increasing size for female *Ae. aegypti* across the range of wing lengths from 2.0-3.8 mm (Alto et al. 2008b). This wing length range encompasses about 99% of the field collected females in our study. As we observe increased frequency of dengue infection across virtually all of that size range, we conclude that the field patterns we observe are not consistent with the competition-susceptibility hypothesis and that the demographic mechanisms postulated in the competition-longevity hypothesis are most important in producing patterns of dengue infection with body size in the field.

*Female* *Ae. aegypti* clearly vary considerably in size *in nature* and this variation is related to probability of infection at the individual level. We found little evidence that inter-site variation in size, which was significant, was related to local frequency of dengue infection at the site level. Thus, individual variation does not obviously relate to larger-scale site-level patterns of risk of dengue. Whether spatial variation in dengue risk is associated with female body size and the ecological conditions that produce that size variation at smaller spatial scales (e.g., the urban block or the house level) remains to be tested. It is also well established that susceptibility to dengue infection has a genetic component (Black et al. 2002, Lourenço-de-Oliveira et al. 2004, Lambrechts & Failloux 2012) and that body size of *Ae. aegypti* varies due to environmental factors beyond larval density and competition [e.g., temperature (Muturi & Alto 2011)]. Thus, for genetically competent populations, we expect larval density and other environmental factors to foster heterogeneity in susceptibility to dengue infection among adult females. Whether genotypic variation in dengue susceptibility interacts with environmental effects remains to be investigated.

Beyond the present study, our results indicate that there are important patterns of frequency of dengue infection among *Ae. aegypti* that are evident during an outbreak of human cases of dengue. Larger females appear to be the most dangerous dengue vectors during the outbreak in Rio de Janeiro. This suggests that larval conditions that yield large, robust adult females are those that are most likely to produce the most dangerous individual vectors of dengue. Uncrowded aquatic habitats for larvae, with ample food, thus seem likely to be sources of vectors that pose the greatest threat. This indicates an important future direction for research in vector-borne disease: quantifying factors creating heterogeneity of disease vectors. Not all individual vectors are alike in their likelihood of infection and disease transmission and approaches to limit vector-borne disease need to consider the ecological conditions, particularly during larval development for vectors with complex life cycles that produce the most dangerous individuals. A similar concept is already enshrined in general ecology of host-pathogen interactions (Lloyd-Smith et al. 2005, Paull et al. 2012) where some individual hosts are considered “superspreaders” because of their disproportionately high tendency to spread the infection to other hosts. Similar effects are likely for insect vectors of human and animal disease, even though those effects may be more difficult to detect among the large numbers of individuals in vector populations. Even more important than understanding the lack of homogeneity of vectors (or hosts) for contributions to disease transmission is the identification of measurable traits, such as body size, that can be used to identify those individuals that are the most likely to transmit pathogens. This can enable us to identify the ecological conditions that are likely to produce those most dangerous vectors and thus may aid in limiting the transmission of vector-borne disease.
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