

Investigations of dengue-2 susceptibility and body size among *Aedes aegypti* populations

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Abstract. The mosquito *Aedes aegypti* (L.) (Diptera: Culicidae) is the primary global vector for dengue virus (DENV), yet considerable genetic variation exists among populations in terms of its competence to vector DENV. Variability in adult body size has also been observed among various mosquito populations and several studies have reported a relationship between body size and arbovirus dissemination, although most of these relied on artificially derived variation in body size. Here we examine the relationship between body size and disseminated DENV infection among 10 *Ae. aegypti* populations reared under optimum laboratory conditions. Body size variability was inferred from wing length measurements and DENV competence was evaluated as the proportion of individuals with disseminated infections following exposure to the dengue-2 JAM1409 strain. There were significant differences in mean wing lengths among populations (ANOVA, $F_{9,22} = 7.10$, $P < 0.0001$), ranging from 2.16 mm (Bangkok population) to 2.79 mm (MOYO-S [susceptible] population). We also observed significant differences among some populations in mean DENV infection rates (Waller–Duncan K-ratio *t*-test), ranging from 19.54% (MOYO-R [refractory] population) to 56.60% (MOYO-S population). However, we did not observe evidence for significant interactions between body size and DENV dissemination. We suggest that either the two traits are genetically independent or that our ability to detect interactions between them was limited by their respective inheritances as quantitative traits.

Key words. *Aedes aegypti*, body size, dengue virus, population variation, quantitative trait, vector competence.

Introduction

Dengue fever (DF) is the world's most important arboviral disease. Its symptoms range from general malaise to the potentially lethal dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). Over 2.5 billion people live in high-risk areas and millions of cases of DF occur each year (Centers for Disease Control, 2005). *Aedes aegypti* (L.), the primary vector for dengue virus (DENV), exists in most subtropical and tropical regions worldwide. The elucidation of factors affecting susceptibility or resistance to infection in the mosquito vector could help identify potential novel disease intervention strategies. This is particularly relevant to *Ae. aegypti* and DENV, as the

traditional tools used for disease prevention and control have largely failed. Lack of success in vaccine development, coupled with rapid emergence of insecticide resistance and the general failure of organized mosquito control programmes emphasize the need for efforts to develop new tools. Additionally, although the global disease burden of DF and DHF/DSS falls most heavily on developing tropical countries, the rise in global travel and trade increases the risk for exposure to this potentially lethal disease in other regions. The ease with which West Nile virus took hold in the U.S.A. clearly shows how quickly an arbovirus can spread throughout a naïve population (Bledsoe, 2004).

Considerable variation exists in the ability of *Ae. aegypti* populations to vector DF (Gubler *et al.*, 1979; Tardieux *et al.*,

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1990; Bennett *et al.*, 2002; Failloux *et al.*, 2002; Ocampo & Wesson, 2004). Populations which are highly susceptible to one of the four serotypes may be much less so to the other serotypes (Gubler *et al.*, 1979), and seasonal variability in susceptibility is evident (Paupy *et al.*, 2003; Ocampo & Wesson, 2004). Results from efforts to explore the genetics of population substructure and the relationship between substructure and dengue susceptibility have been inconsistent (Bennett *et al.*, 2002; Gorrochotegui-Escalante *et al.*, 2005). Consequently, although population sub-structure is clearly important for understanding dengue vector competence, the complexities of population-level susceptibility have not been thoroughly explained.

Variability in adult body size among individuals in mosquito populations has been observed, and several studies have shown a relationship between body size and arbovirus dissemination in mosquitoes; however, these studies have relied upon environmentally induced variation in body size (Takahashi, 1976; Baqar *et al.*, 1980; Grimstad & Haramis, 1984; Klowden *et al.*, 1988; Grimstad & Walker, 1991; Nasci & Mitchell, 1994; Sumanochitrapon *et al.*, 1998). Consequently, these studies have been limited to demonstrating how environmental conditions affect body size variation and the potential impact on pathogen dissemination. Bosio *et al.* (1998) found no significant correlation between genetic control of body size and DENV susceptibility within two *Ae. aegypti* strains showing high and low DENV susceptibility. However, the high DENV susceptibility strain was significantly larger than the low DENV susceptibility strain. It is clear that low food availability and/or crowding can lead to the development of smaller mosquitoes (Chadee & Beier, 1997). However, the genetic control of body size and subsequent interactions with environmental parameters have not been thoroughly investigated, although an understanding of these effects is critical to the elucidation of factors affecting vector competence.

A potential association between genetic control of body size and vector competence in *Ae. aegypti* was serendipitously observed following bidirectional selection for subpopulations susceptible (MOYO-S) and refractory (MOYO-R) to the avian malaria parasite *Plasmodium gallinaceum* from the Moyo-In-Dry laboratory population (Thathy *et al.*, 1994). Individuals from the MOYO-R subpopulation were found to be highly refractory to *P. gallinaceum* infection, and signifi-

cantly smaller than those from the MOYO-S subpopulation (Yan *et al.*, 1997). Subsequent quantitative trait loci (QTL) mapping efforts involving MOYO-R as the refractory parent identified several QTL determining *P. gallinaceum* susceptibility, as well as multiple QTL determining body size (Meece, 2002). Of note, some body size QTL co-localized to the same, albeit broad, genome regions as those QTL identified for *P. gallinaceum* susceptibility, suggesting a possible genetic association between the two phenotypes. Another QTL study examined La Crosse virus (LACV) transmission and body size in *Aedes hendersoni* Cockerall (also known as *Ochlerotatus hendersoni*) and *Aedes triseriatus* Say (also known as *Ochlerotatus triseriatus*) interspecific hybrids, and again observed that some QTL determining LACV transmission were associated with genome regions containing body size QTL (Anderson *et al.*, 2005).

In this study, we investigated the relationship between body size and disseminated DENV infection in *Ae. aegypti* in order to determine whether body size was a significant predictor of vector competence. By rearing several *Ae. aegypti* populations of diverse geographic origins and genetic backgrounds under standard laboratory conditions (Table 1), we were able to address the questions of whether variation in body size exists under optimal rearing conditions, and how naturally occurring genetic variation in body size impacts dengue-2 (DEN-2) dissemination in *Ae. aegypti*.

Materials and methods

Mosquito rearing

Sources for *Ae. aegypti* laboratory populations used in this study are presented in Table 1. Rearing was conducted in an environmental chamber held at 26°C, 85% RH (relative humidity), with a light cycle of LD 16 : 8 h, which included a 1-h crepuscular period to simulate dawn and dusk. Similar numbers of eggs (~500) were hatched from each population in tepid water with a pinch of yeast. Larvae were reared in pans (27cm diameter × 9cm depth) filled with 2 L tepid water to prevent overcrowding, with an *ad libitum* solution of bovine liver powder (ICN Biomedicals, Inc, Irvine, CA). Pupae were moved into 500-mL plastic cups containing ~ 250 mL clean, tepid water.

Table 1. Sources for *Aedes aegypti* populations.

Strain	Origin or selected phenotype	Source or reference
Bangkok	Ovitraps samples, Thailand	U. Thavara, National Institute of Health, Thailand (1999)
DS3	Dengue susceptibility	Bennett <i>et al.</i> (2005)
Form	Flavivirus refractoriness, Nigeria	Miller & Mitchell (1991)
Ghana	Ovitraps samples, Ghana	K. O. Owusu-Daaku, Kwame Nkrumah University of Science and Technology, Ghana (2001)
Ibo11	Dengue refractoriness, Nigeria	Gomez-Machorro <i>et al.</i> (2004)
Mombasa	Ovitraps samples, Kenya	G. Yan, SUNY, Buffalo, NY (1998)
MOYO-R	<i>Plasmodium gallinaceum</i> refractoriness	Thathy <i>et al.</i> (1994)
MOYO-S	<i>Plasmodium gallinaceum</i> susceptibility	Thathy <i>et al.</i> (1994); G. Yan, State University of New York, Buffalo, NY (2003)
RED	Mutant marker stock	G. B. Craig, University of Notre Dame, Notre Dame, IN
Trinidad	Ovitraps samples, Trinidad	D. D. Chadee, Ministry of Health, St Joseph, Trinidad, 1998

Adults emerged into 20 × 20 × 30-cm mesh cages. All adults were maintained at 26°C and 85% RH and provided with 5% sucrose solution *ad libitum*. Females aged 3–7 days were transferred to 500-mL paper cups for infectious blood-feeding.

Infection of mosquitoes with dengue-2 virus

Cell culture and virus infections were carried out according to the protocol of Gaines *et al.* (1996), with minor modifications. Nearly confluent (~75–80% confluent) 75-cm³ flasks of C6/36 cells were grown at 28°C without CO₂ and infected with DEN-2 (strain JAM1409) at a multiplicity of infection (MOI) = 0.1. Maintenance media (E-MEM and supplements) with 10% fetal bovine serum (FBS) was added to the flasks after inoculation with virus. Media in the flasks was changed to 2% FBS on day 7 post-infection (p.i.) and the cells were harvested on day 14 p.i. by scraping the cells from the flask with a cell scraper. Equal parts of infectious cell suspension and warmed defibrinated sheep blood (Colorado Serum Company, Denver, CO, U.S.A.) were mixed for the infectious bloodmeal. Standard techniques for artificial infectious bloodmeals were followed (Rutledge *et al.*, 1964). Sausage casings were used to cover the artificial membrane feeder and the blood/virus mixture was kept warm throughout the mosquito feeding by circulating 37°C water across the feeder. Females were given ≤15 min to feed. Females that did not feed were removed from the cages. Females that fed to repletion were provided with a 5% sucrose solution via a soaked cotton ball *ad libitum* and maintained at 28°C and ~80% RH. On day 14 p.i. all surviving females were frozen at –80°C.

Determination of disseminated infection

Head squashes and indirect immunofluorescence assay (IFA) were used to determine presence of a disseminated infection (Romero-Vivas *et al.*, 1998). Heads were removed using forceps and a razor blade and immediately transferred to a 12-well slide and squashed under a coverslip using a pencil eraser. Head squashes were fixed in cold acetone (–20°C) for 10 min and air dried.

Primary antibody (mouse-ascites derived monoclonal antibody 4G2) was added to each head squash and incubated at 37°C for 40 min. Slides were washed three times in phosphate-buffered saline (PBS) for 2 min per wash and allowed to air dry. Secondary antibody (biotinylated sheep anti-mouse IgG) was added to each head squash and incubated at 37°C for 40 min. Slides were again washed three times in PBS for 2 min per wash and allowed to air dry. Streptavidin-fluorescein was then spotted to each head and allowed to incubate in the dark for 10 min. Slides were then washed three times in PBS for 2 min per wash and allowed to air dry. Finally, slides were covered using nine parts glycerol to one part PBS as the mounting media for the coverslips. Heads were scored visually as positive or negative using a Zeiss immunofluorescence microscope (Zeiss, Thornwood, NY). The ACCESS RT-PCR (reverse transcription-polymerase chain reaction) kit (Promega Corporation, Madison,

WI, U.S.A.), following the manufacturer's protocol, and DEN-2 specific primers (Mustafa *et al.*, 2001) were used to accurately identify ambiguous head squashes and to spot-check the IFA results of others.

Determination of body size

Wing length was used as a proxy for body size as wing length has been shown to be a valid estimate of overall body size (Van Handel & Day, 1989). One wing, either the left or the right, from each female was removed at the time of the head squash. Both wings on an individual mosquito have been shown to be of statistically similar size and, consequently, either can be used without compromising the data (Gleiser *et al.*, 2000). Wings were adhered to a glass slide by a piece of double-sided tape and overlaid with a coverslip. Wings were measured with an ocular micrometer from the apical notch to the axillary margin, excluding the wing fringe.

Statistical analysis

Statistical analyses were performed using SAS[®] 9.1 (SAS Institute, 2004). PROC MEANS was used to determine the distribution of body size for the combined populations and within populations. We measured dengue infection incidence per population as the proportion of mosquitoes infected, divided by the total number of mosquitoes exposed. PROC FREQ was used to determine the prevalence of infection among the overall population and within populations. The proportion of individuals infected was arcsine square root-transformed because the proportions ranged outside the interval of 0.3–0.7. PROC GLM was used to analyse the mean wing length and transformed dengue infection incidence of populations and PROC REG was used to examine the general predictive value of wing length mean per population on dengue infection incidence. A Waller–Duncan K-ratio *t*-test was used to detect differences among any subset of populations.

Results

To investigate the relationship between adult female body size and disseminated dengue infection, we reared 10 *Ae. aegypti* populations under optimum conditions, exposed females from these populations to a DEN-2 JAM1409 infective bloodmeal, and then scored individuals for disseminated infection by IFA and determined wing length (as a proxy for body size). For each population, we examined three or four independent replicates. A total of 845 mosquitoes from 10 strains survived the ~2-week extrinsic incubation period (EIP) (Watts *et al.*, 1987) and were scored for both wing length and disseminated dengue infection. The continuous distribution of body size within individual populations and the differences in wing length means among populations (Fig. 1) suggest that wing length (e.g. body size) is inherited as a quantitative trait. In the 845 individuals measured, wing lengths ranged from 1.8 mm to 3.1 mm, reflecting a difference of 72%.

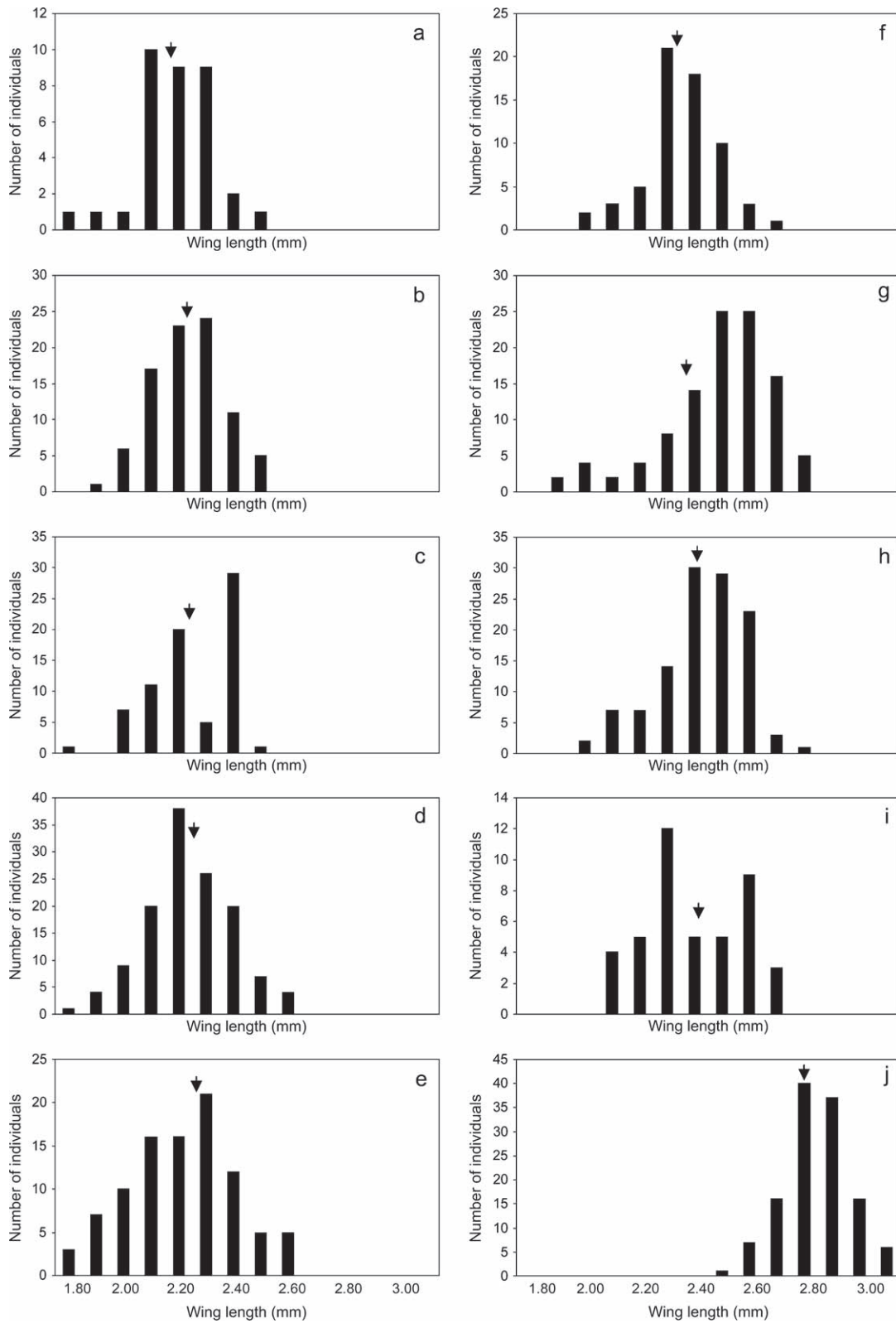


Fig. 1. Histogram showing the frequency distribution of wing length observed among all populations tested: (a) Bangkok; (b) MOYO-R; (c) DS3; (d) Ibo11; (e) Ghana; (f) Trinidad; (g) Form; (h) RED; (i) Mombasa; (j) MOYO-S. Arrows indicate individual population means.

There were significant differences in mean wing length among populations (ANOVA, $F_{9,22} = 7.10$, $P < 0.0001$; Table 2). The MOYO-S population was significantly larger than all other populations, whereas the Bangkok population was significantly smaller than the Form, RED, Mombasa and MOYO-S populations (Waller–Duncan K-ratio t -test mean comparisons). The general linear model procedure did not reveal a strong relationship between mean dengue infection incidence and population (ANOVA, $F_{9,22} = 1.80$, $P = 0.126$; Table 2). However, the MOYO-R population was significantly less susceptible than the DS3, Form and MOYO-S populations (Waller–Duncan K-ratio t -test mean comparisons). The slope of the linear regression line for the predictor variable wing length and the response variable dengue infection incidence was positive and close to statistical significance ($R^2 = 0.11$, $F_{1,30} = 3.57$, $P = 0.068$). Because data for the MOYO-S population had a strong influence on the regression analysis (because of its extreme body size and high dengue infection incidence), the data were re-analysed without MOYO-S. We observed no predictive value of mean wing length for dengue infection incidence after removing the MOYO-S data ($R^2 = 0.02$, $F_{1,30} = 0.45$, $P = 0.51$).

Discussion

Vector competence is the ability of a vector to acquire a pathogen in an infected bloodmeal, develop a disseminated infection and infect a susceptible host during subsequent contact. In *Ae. aegypti*, the EIP for dengue viruses is ~ 14 days, by which time susceptible individuals will develop a high-titre infection in the salivary glands. If a mosquito does not develop a salivary gland infection, it will not be able to transmit virus during the next blood-feed. Once virus is present in the salivary glands, however, mosquitoes are able to pass virus during all probes for subsequent bloodmeals (Putnam & Scott, 1995). Many factors affect vector competence, including temperature, humidity,

infection and escape barriers within the mosquito, age of the mosquito and, potentially, body size (Hardy *et al.*, 1983).

Our results confirm that significant genetic variation exists among *Ae. aegypti* in both adult body size and dengue vector competence, but do not reveal evidence of significant interactions between the two phenotypes. One comparison of particular interest involves the MOYO-R and MOYO-S populations; as previously indicated, these populations were selected for low and high susceptibility to *P. gallinaceum*, respectively, and, unexpectedly, also attained significant differences in adult body size (Thathy *et al.*, 1994; Yan *et al.*, 1997). These two populations also expressed the extremes for dengue vector competence observed in the present study. However, most of the other population comparisons do not show strong associations between body size and dengue vector competence. We propose three hypotheses for these results. Firstly, there may be no genetic correlation between body size and dengue vector competence. Secondly, the power of our ability to detect the true relationship may have been negatively influenced by variability in the infectivity of the artificial bloodmeals. Although the observed variances in mean wing length between replicates was small, the proportion of individuals infected varied greatly among replicates within populations, thus reducing the statistical power of our analyses. High variability among replicates in oral DENV infections were also reported by Tardieux *et al.* (1990). It is important to note that although vector competence for DENV shows strong genetic components, considerable environmental influence is evident (Bosio *et al.*, 1998). Results from our studies and several others (Gubler *et al.*, 1979; Tardieux *et al.*, 1990; Ocampo & Wesson, 2004) reflect this phenomenon, as infection rates following exposure to infected bloodmeals were typically < 60% among even the most DENV-susceptible strains. Thirdly, although both traits clearly have strong complex genetic underpinnings, the suite of genes determining both phenotypes may reflect a combination of tightly linked or common genes (e.g. pleiotropy) and independent effect genes that would probably not be revealed by direct phenotypic comparisons, as employed in the present studies.

Although multiple studies involving a number of mosquito species and pathogens have shown significant relationships between body size and vector competence, little attention has been given to potential genetic interactions. Two independent studies have identified overlapping QTL regions influencing body size and *Ae. aegypti* susceptibility to *P. gallinaceum* (Meece, 2002), and LACV transmission in *Ae. triseriatus* (Anderson *et al.*, 2005), respectively. It is particularly relevant to the current study that the previous studies also showed that not all QTL influencing body size co-localized with those influencing vector competence (and vice versa). Thus, because both these phenotypes reflect quantitative traits with considerable genetic variance among populations, one might expect to find strong correlations between body size and vector competence only if the allelic configuration of genes within shared QTL were appropriately coupled with alleles at genes associated with independently segregating QTL. Conversely, one might find a general lack of correlation if some allelic configurations for at least some QTL were in repulsion. This might explain the apparently conflicting results between studies examining the

Table 2. Wing lengths and dengue infection status among populations.

Population	No. individuals per replicate	Wing length*	% Infected*†
Bangkok	10/12/12	2.16 ^a (0.08)	32.22 ^{ab} (8.56)
MOYO-R	27/36/24	2.20 ^{ab} (0.02)	19.54 ^a (9.73)
DS3	11/10/52	2.22 ^{ab} (0.04)	45.95 ^b (17.76)
Ibo11	35/46/48	2.23 ^{ab} (0.12)	31.55 ^{ab} (2.44)
Ghana	37/36/12	2.25 ^{ab} (0.17)	27.44 ^{ab} (6.03)
Trinidad	24/15/24	2.32 ^{ab} (0.07)	34.92 ^{ab} (29.27)
Form	48/13/44	2.37 ^b (0.26)	48.42 ^b (6.68)
RED	32/15/35/10	2.38 ^b (0.06)	38.79 ^{ab} (14.17)
Mombasa	10/11/10/12	2.38 ^b (0.10)	30.23 ^{ab} (3.14)
MOYO-S	60/43/16	2.79 ^c (0.08)	53.60 ^b (14.16)

*Mean (standard deviation); means followed by the same letter in a column are not significantly different by Waller–Duncan K-ratio t -test at $P = 0.05$.

†Statistical comparisons were performed using the arcsine square root transformation of the proportion of individuals infected.

relationships between body size and vector competence within individual vector/pathogen systems. For example, *Ae. aegypti* vector competence for *P. gallinaceum* in one study showed that smaller individuals were highly immunocompetent (Yan *et al.*, 1997), whereas another study associated immunocompetence with larger individuals (Koella & Boëte, 2002). A parsimonious conclusion would be that both studies were correct and simply reflect the complexity of the quantitative inheritance of each trait.

As with many other important phenotypes, the development of body size has been studied more thoroughly in *Drosophila melanogaster* than in most other systems. Results from *Drosophila* are known to be applicable to a wide array of species, and it is expected that many of the findings concerning the regulation and allocation of resources for growth in *Drosophila* will be homologous to other dipterans, including *Ae. aegypti* (Oldham *et al.*, 2000). Work with *Drosophila* indicates that small insects are not just miniature versions of larger insects. Overall pattern and morphology are the same, but there may be fewer and/or smaller cells in smaller flies (Day & Lawrence, 2000). Body plan development, nutrient allocation and the attainment of size are regulated by a number of factors. It is thought that food availability and weight gain up to the second and early third instars dictate whether a larvae will achieve the minimum weight needed for pupation and the eventual final size of the adult insect (Prasad *et al.*, 2001). Although it is clear that nutrition plays a significant role in the development of body size in all species, individual genotypes dictate the biological processes of growth, including cell proliferation, differentiation and apoptosis. Therefore, genetics may lay the boundaries for growth, whereas nutrition may control how an individual can explore those boundaries.

Aedes aegypti genetics and genomics has reached the level wherein the factors determining DENV vector competence or body size can be elucidated by fine-scale genetic studies. Positional cloning of genes for individual QTL is nearly routine, even among organisms with highly complex and large genomes (Flaherty *et al.*, 2005; Keller *et al.*, 2005; Bortiri *et al.*, 2006). Several factors support the practicality of positional cloning in *Ae. aegypti*. Firstly, fine-scale evaluation of QTL in *Ae. aegypti* is facilitated by the ability to maintain genetic stocks that exhibit significant variation in both body size and DENV vector competence, and genetic and phenotypic information can be obtained for large populations segregating for these traits. Secondly, the completion of the annotated genome sequence for *Ae. aegypti* (Nene *et al.*, 2007) provides the opportunity for identification of genes within individual QTL. Finally, the ability to perform RNA interference-mediated (RNAi) knockdowns of candidate genes in *Ae. aegypti* (Attardo *et al.*, 2003; Sanchez-Vargas *et al.*, 2004) allows for the rapid evaluation of individual gene effects on a given phenotype. Thus, in principle, it should be possible to identify the genes that determine DENV vector competence and body size in *Ae. aegypti*.

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