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Research article

Vector/Pathogen/Host Interaction

Effects of Larval Nutrition on *Wolbachia*-Based Dengue Virus Interference in *Aedes aegypti* (Diptera: Culicidae)

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Abstract

In order to assess the broad-scale applicability of field releases of *Wolbachia* for the biological control of insect-transmitted diseases, we determined the relationship between the larval diet of *Aedes aegypti* L. mosquitoes infected with *Wolbachia* strains and their susceptibility to dengue virus (DENV) infection via intrathoracic injection and oral inoculation. Larvae were reared on diets that varied in the quantity of food which had the effect of modifying development time and adult body size. *Wolbachia w*Mel infection was associated with highly significant reductions in dengue serotype 2 (DENV-2) infection rates of between 80 and 97.5% following intrathoracic injection of adults emerging from three diet levels. Reductions were 100% in two diet level treatments following oral inoculation. Similarly, *w*MelPop infection was associated with highly significant reductions in DENV-2 infection rates of between 95 and 100% for intrathoracic injection and 97.5 and 100% for oral inoculation across diet level treatments. Larval diet level had no significant effect on DENV-2 infection rates in the presence of *Wolbachia* infection in mosquitoes that were intrathoracically injected with the virus. This indicates that the effectiveness of *Wolbachia* on vector competence disruption within *Ae. aegypti* is unlikely to be compromised by variable larval nutrition in field settings.

Key words: *Wolbachia*, dengue virus, *Aedes aegypti*, larval nutrition, virus interference

Dengue fever, a vector-borne, human febrile disease caused by four closely related dengue virus (DENV) serotypes, DENV-1–4 (Gubler 1998, Pandey et al. 2008), has expanded its geographical distribution and severity in the last century (Stephenson 2005, Gibbons 2010). Development of a universal vaccine could provide long-lasting immune protection from all four DENV serotypes (Webster et al. 2009, Wilder-Smith et al. 2010). However, recent phase three efficacy trials of the Sanofi Pasteur tetravalent dengue vaccine have shown incomplete protection, with efficacy varying according to DENV serotype and seroprevalence of children with overall efficacy of 56.5% in Asia (Capeding et al. 2014) and 60.8% in Latin America (Villar et al. 2015). Until a vaccine is available, vector control will remain the mainstay of strategies to reduce dengue morbidity and mortality (Wilder-Smith et al. 2010).

A rapidly developing mosquito-borne disease control initiative is based around the bacterium *Wolbachia pipientis* (Iturbe-Ormaetxe et al. 2011). *Wolbachia* is an obligate intracellular bacterium infecting up to 76% of insect species (Jeyaprakash and Hoy 2000, Hilgenboecker et al. 2008). The infections are maternally transmitted and induce phenotypes in the insect host that have potential for use in strategies against insect-borne disease (Werren et al. 2008). A major breakthrough for the development of a dengue control strategy has been the generation of stable *Wolbachia* infections in the primary dengue vector, *Aedes aegypti* L. (McMeniman et al. 2009).

A second breakthrough came with discoveries that *Wolbachia* infections may inhibit infection of the host insect by a diverse range of pathogens (Hedges et al. 2008, Teixeira et al. 2008, Moreira et al. 2009, Hughes et al. 2011). *w*MelPop and *w*Mel infections in *Ae. aegypti* have induced complete and near-complete inhibition of infection of the mosquito by dengue serotype 2 (DENV-2), respectively (Moreira et al. 2009, Walker et al. 2011). The capacity for establishment and spread of the *w*Mel infection within wild *Ae. aegypti* populations has been demonstrated in north Queensland, Australia (Hoffmann et al. 2011). Thus, *Wolbachia*-based strategies have raised hopes for the control of dengue and potentially of other mosquito-borne diseases. However, the underlying mechanisms behind the *Wolbachia*-induced phenotypes are not well understood.

One of the leading hypotheses for the mechanism of virus interference is that the host insect immune system is "primed" against subsequent infection with pathogens. In support of this hypothesis, there have been numerous examples of a heightened activation of the innate immune response in *Wolbachia*-infected mosquitoes (Kambris et al. 2009, 2010; Moreira et al. 2009; Bian et al. 2010; Pan et al. 2012). However, the insect host immune system may be

suppressed following *Wolbachia* transinfection allowing higher pathogen infection rates (Douglas et al. 2010, Hughes et al. 2011). Further, *Wolbachia* was found to be providing protection against pathogens in insect cell lines that lack whole-organism or tissue-specific immunity (Osborne et al. 2009, Frentiu et al. 2010), and an increased immune response is not always associated with the protective effect of *Wolbachia* against virus pathogens (Wong et al. 2011, Rancès et al. 2012). An alternate hypothesis for the inhibition of dengue virus infection in *Wolbachia*-infected mosquitoes is that the observed interference is due to competition for host cellular resources (Moreira et al. 2009), as shown for the inhibition of viral pathogens by *Wolbachia* endosymbionts in *Drosophila melanogaster* Meigen (Caragata et al. 2013).

To date, investigations into the effects of *Wolbachia* on DENV inhibition were performed on mosquitoes reared under standard laboratory conditions that produce larger and more nourished mosquitoes in comparison with wild mosquitoes originating from larval habitats with a diversity of nutrient levels. Laboratory-reared *Ae. aegypti* can be quite dissimilar to wild-type *Ae. aegypti*, as indicated by measurements of wing length and sugar, glycogen, and protein content under different diet regimens (Knox et al. 2010).

In this study, we investigated the influence of larval diet (quantity of food provided) on *Wolbachia*-mediated inhibition of DENV infection. By varying the diet, we produced *Ae. aegypti* adults of three different size classes: from small, nutritionally stressed mosquitoes to large adults. We challenged these mosquitoes with DENV-2 via two inoculation routes—oral feeding and intrathoracic microinjection. Testing *Wolbachia*-mediated dengue virus inhibition over the broad range of nutritional backgrounds will indicate the robustness of *Wolbachia*-based biological control of insect-transmitted diseases under operational conditions.

Materials and Methods

Human Research Ethics Statement

Approval for allowing colonized (DENV-free) mosquitoes to feed on volunteers was obtained from the QIMR Berghofer Medical Research Institute Human Research Ethics Committee approval P361. Written informed consent was provided by study participants.

Mosquito Rearing

Aedes aegypti strains used in these experiments were the *w*Mel2 strain (outbred *Wolbachia w*Mel-infected *Ae. aegypti* strain generated by backcrossing for three generations to F1 eggs from wild *Ae. aegypti* collected in Cairns, Australia; Walker et al. 2011), the PGYP1.out strain (outbred *Wolbachia w*MelPop-CLA-infected *Ae. aegypti* generated by backcrossing for five generations to F1 eggs from wild *Ae. aegypti* collected from Cairns; Moreira et al. 2009) and their corresponding tetracycline-treated uninfected strains (*w*Mel2.tet and *w*MelPop-CLA.tet) of matching genetic background. We refer to these strains as *w*Mel and *w*Mel.tet, and to *w*MelPop and *w*MelPop.tet throughout this paper. Lines were maintained in a climate-controlled, biosecurity level two insectary, at a temperature of 27°C, relative humidity of 70%, and a photoperiod of 12:12 (L:D) h with 30-min dawn and dusk periods. For colony maintenance, larvae were fed ground fish food pellets (TetraMin Tropical Tablets, The Rich Mix, Tetra, Germany) ad libatum, and adults were blood fed on human volunteers for egg production. Eggs from the above lines were vacuum-hatched, and larvae were reared under one of three diet regimens that differed in the daily quantity of food provided to larvae (0.10, 0.25, and 1.00 mg/larva/d of ground fish food pellets in an aqueous suspension). Larvae were reared at the three diet levels to produce adult mosquitoes for intrathoracic injection with DENV-2 (Experiment 1). A second cohort of mosquitoes was fed at 0.10 and 1.00 mg/larva/d diet treatments to produce adult mosquitoes for oral inoculation with DENV-2 (Experiment 2). Adult mosquitoes were maintained in cages (30 by 20 by 20 cm^3) under the insectary conditions described above and were provided with 10% sucrose solution on cotton pads and slices of apple.

Entomological Parameters

Larval development time was determined from the mean interval between hatching and the date of pupation for individuals of a cohort. For the analysis of nutritional status, the wing length of 4- to 5-d-old females was measured from the axillary incision to the apical margin, excluding the fringe of scales (Nasci 1986).

Dengue Virus Preparation

DENV-2 isolate 92T (Knox et al. 2003) was obtained from an existing collection of the Queensland Health Scientific Services, Coopers Plains, Brisbane. The anonymized sample was originally collected in 1992 from a patient in Townsville showing clinical signs of dengue infection. Work was conducted under safety and risk management practices described by QIMR Berghofer Medical Research Institute project P980. The virus stock had been passaged six times in *Aedes albopictus* (Skuse) (C6/36) cells in RPMI 1640 media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin or streptomycin antibiotics, and 1% glutamax (Invitrogen, Australia) and stored at −80°C. The virus stock had a titer of $10^{7.8}$ $CCID_{50}$ (50% cell culture infectious dose) per milliliter, which was then diluted to $10^{3.8}$ $CCID_{50}$/ml, a predetermined dosage in RPMI 1640 medium for intrathoracic inoculation, and in sheep blood to $10^{4.3}$ $CCID_{50}$/mosquito as a bloodmeal for oral infection.

Experiment 1: Intrathoracic Injection of Mosquitoes With DENV-2

Female mosquitoes that were previously reared on food regimens of 0.10, 0.25, and 1.00 mg/larva/d were briefly anesthetized with CO_2 and placed on a Petri dish over ice. Mosquitoes were held in position using forceps under a dissecting microscope. A handheld Nanoject II microinjector (Drummond Scientific Scientific, Broomall, PA) with a pulled glass capillary needle was used to inject 69 µl of diluted DENV-2 stock into the thorax of each mosquito. Injected mosquitoes were maintained in plastic containers with gauze lids (11 cm in diameter by 15 cm in height) for 10 d under the insectary conditions described above and provided with 10% sugar solution and slices of apple.

Experiment 2: Oral Inoculation of Mosquitoes With DENV-2

Four-day-old female *Ae. aegypti* reared on 0.1 and 1.0 mg/larvae/d regimens were starved for 24 h before being transferred to separate plastic containers with gauze lids for blood feeding. Female mosquitoes were allowed to feed on prepared blood or virus suspension for ~1 h via glass membrane feeders, maintained at 37°C using a recirculating water pump (Rutledge et al. 1964, Moreira et al. 2009). After feeding, mosquitoes were anesthetized using CO_2 and sorted on a chill table. Only fully engorged mosquitoes were retained, and

these were maintained in the plastic containers for 10 d as described for Experiment 1.

Sample Testing for DENV-2

DENV-2 was detected and titrated in mosquito samples using cell culture enzyme-linked immunosorbent assay (CCELISA) based on the methods of Oliviera et al. (Oliviera et al. 1995). Forty mosquito samples from each group were dissected to isolate the body (thorax and abdomen), which was homogenized in 0.5 ml of grinding medium (RPMI 1640 medium supplemented with 10% FBS, 1% penicillin and streptomycin [100 µg/ml], 1% Glutamax [Invitrogen], and 2% Fungizone amphotericin B [Apothecon, Princeton, NJ]), before centrifuging at 11,200 × *g* for 10 min at 4°C to collect the supernatant. C6/36 cell monolayers were grown to 80% confluency in 96-well plates and inoculated with 50 µl/well of the supernatant from each mosquito grind. Plates were then incubated at 28°C with 5% CO_2 for 5 d. Cell monolayers were fixed by adding 100 µl/well of fixative buffer (80% v/v phosphate buffered saline (PBS), 20% v/v acetone, 0.2% v/v bovine serum albumin). Fixative buffer was removed, and plates were left to dry for an hour within a biosafety cabinet. The fixed C6/36 cell monolayer was then blocked by incubating cells in 100 µl/well of blocking buffer (6.05% w/v Tris, 0.372%w/v ethylenediaminetetra-acetic acid, 8.77% w/v NaCl, 0.05% w/v Tween 20, 0.2% w/v casein) for 1 h at 28°C and then removing the blocking buffer. Each well contained 50 µl of a monoclonal antibody (mAb) cocktail comprising 1:40 dilutions of 4G2 and 4G4 mAb hybridoma cell culture supernatant (Arbovirus Laboratory, University of Queensland, Brisbane, Australia) in blocking buffer. The plates were then incubated for 1 h at 28°C before the mAb cocktail was removed. Cell monolayers were then washed four times in wash buffer (0.05% Tween 20 in PBS), and 50 µl per well of goat antimouse horse radish peroxidise conjugated secondary antibody diluted 1/2000 in blocking buffer was added. The plates were incubated at 28°C for one h before six washes in wash buffer. One hundred microliters per well of 0.02% v/v 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) and 0.02% v/v H_2O_2 in substrate buffer (1.52% w/v Na_2HPO_4, 1.05% w/v citrate) was then added and the plates incubated at 28°C for 1 h in the dark. Absorbance was measured at 405 nm using a Multiskan EX microplate reader (Thermo Labsystems Oy, Helsinki, Finland). Presence of virus was scored when a test well containing viable cells produced an optical density greater than twice the mean of uninfected control wells of the same plate.

Mosquito grinds from adult females that tested positive for DENV-2 from each diet group were titrated 10-fold in growth media before performing CCELISA, and the $CCID_{50}$ estimates were then calculated (Reed and Muench 1938).

Statistical Analysis

The effect of *Wolbachia* infection and larval diet on larval development (time to pupation), including synergistic effects, was determined using unrestricted permutation tests (Manly 2007). Tests were performed separately for *w*Mel/*w*Mel.tet and *w*MelPop/*w*MelPop.tet strain pairs and for injection and oral infection datasets. The effect of larval diet and *Wolbachia* strain on adult mosquito wing length was determined using analogous permutation tests with both strain and diet as fixed effects for the *Wolbachia* strain pairs. Diet level groups were then compared by post hoc tests with Bonferroni adjustment. The effect of *Wolbachia* infection on DENV-2 infection rates was determined for each diet level or strain combination by Fisher's exact tests with two-tailed probability. Logistic regression analysis was performed on the DENV-2 infection status (infected or noninfected) for the *Wolbachia* strain pairs to quantify the diet effect in the presence of *Wolbachia* infection and whether there was synergy between these factors.

Results

Larval Development Time and Adult Body Size

Larval development time was inversely proportional to the amount of larval food provided to a cohort of *Ae. aegypti* reared to examine the effect of larval diet on DENV-2 interference by *Wolbachia* following intrathoracic inoculation (Experiment 1, Fig 1a). The variances in larval development time were statistically different between strain or diet level groups ($P < 0.0001$). In particular, variances were disproportionately greater for mosquitoes fed at the lowest diet level (0.1 mg/larva/d). Therefore, the effect of *Wolbachia* strain and larval diet level on time to pupation was compared using unrestricted permutation tests that do not rely on equal-variance assumptions. Mean time to pupation of *w*Mel and *w*Mel.tet strains was highly significantly affected by larval diet level, mosquito strain (positive or negative for *Wolbachia* infection), and an interaction between mosquito strain and diet level (Table 1). There was a trend toward *Wolbachia*-infected mosquitoes developing faster than uninfected mosquitoes at lower diet levels (Fig 1a). For *w*MelPop and *w*MelPop.tet mosquitoes, there were highly significant effects of larval diet level and an interaction between diet level and mosquito strain on the time to pupation; however, the effect of strain alone was not significant (Table 1). Similar trends were observed to the effect of larval diet and strain on the development times of larvae for Experiment 2; however, larval diet was the only significant factor affecting development for *w*MelPop and *w*MelPop.tet strains (Table 1; Supp. Fig. 1 [online only]).

Larval diet was also the strongest determinant of adult wing length. Wing length was highly significantly affected by larval diet level for both *w*Mel/*w*Mel.tet and *w*MelPop/*w*MelPop strain pairs (Table 2). However, for the *w*MelPop and *w*MelPop.tet datasets, there was a significant interaction between diet and strain. There was a tendency for *w*MelPop-infected mosquitoes to have larger wings than uninfected mosquitoes at lower diet levels. Mean wing lengths were highly significantly different between larval diet level groups for all pairwise post hoc comparisons ($P < 0.001$). Therefore, the larval diet level treatments had produced three discrete size classes of mosquitoes that were used in experiments to examine the effect of larval diet level on virus interference by *Wolbachia* following intrathoracic DENV-2 injection. Highly significant effects of larval diet on adult wing length were also observed from mosquitoes reared at 0.1 and 1.0 mg/larva/d levels for Experiment 2 (Table 2). Significant effects of strain and an interaction between strain and diet indicated that *w*Mel mosquitoes had smaller wing lengths than their uninfected counterparts at the low diet level.

Experiment 1: DENV-2 Infection Following Intrathoracic Injection

DENV-2 infection rates of *w*Mel-infected females reared on 0.10, 0.25, and 1.0 mg/larva/d regimens 10 d postintrathoracic inoculation were 10.0, 2.5, and 5.0%, respectively (Fig 2a). In contrast, *w*Mel.tet females reared on the same diet regimens had much higher body infection rates of 97.5, 65.0, and 76.5%. The 90–96% lower DENV-2 infection rates for the *w*Mel infected groups were highly significantly different to the equivalent tetracycline-cured lines at each nutrition level ($P < 0.0001$). Similarly, DENV-2 infection rates

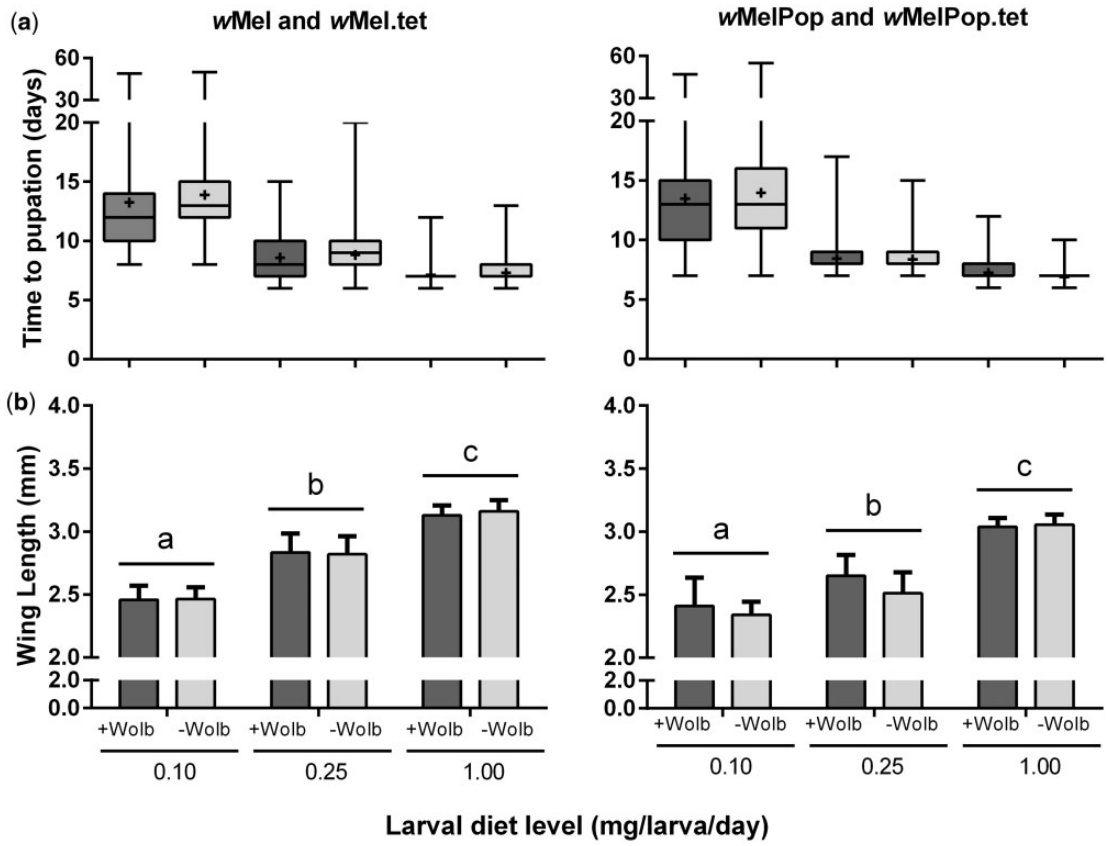

Fig. 1. Entomological traits of *Wolbachia*-infected and control mosquitoes reared under different diet regimens for DENV-2 intrathoracic injection challenge (experiments one). **(a)** Development time of mosquito larvae. Box and whisker plots (median, interquartile range, range) show variation in the time to pupation for *Wolbachia*-infected (dark shading) and control (light shading) strains fed food regimens that differed in the quantity of food provided. + symbols in columns indicate means. The results of unrestricted permutation analyses to determine the effect of *Wolbachia* infection and larval diet on development time are provided in Table 1. **(b)** Body size of emerging adults. Columns show mean (±SD) wing length for adult mosquitoes emerging from treatments shown in **a**. The results of unrestricted permutation analyses to determine the effect of *Wolbachia* infection and larval diet on adult wing length are provided in Table 2. The group means of columns represented by different letters are significantly different by post hoc comparison with Bonferroni adjustment.

Table 1. Comparison of the influence of *Wolbachia* infection and larval diet level on mosquito development time

	Diet	Strain	Diet × strain
Experiment 1 (for DENV-2 injection)			
*w*Mel and *w*Mel.tet	<0.001	<0.001	0.006
*w*MelPop and *w*MelPop.tet	<0.001	0.262	<0.001
Experiment 2 (for DENV-2 oral inoculation)			
*w*Mel and *w*Mel.tet	<0.001	<0.001	<0.001
*w*MelPop and *w*MelPop.tet	<0.001	0.332	0.077

Values are multiple-comparison adjusted *P* statistics from permutation tests on time to pupation with diet level and mosquito strain as factors.

Table 2. Comparison of the influence of *Wolbachia* infection and larval diet level on adult wing length

	Diet	Strain	Diet × strain
Experiment 1 (for DENV-2 injection)			
*w*Mel and *w*Mel.tet	<0.001	0.763	0.597
*w*MelPop and *w*MelPop.tet	<0.001	0.056	0.145
Experiment 2 (for DENV-2 oral inoculation)			
*w*Mel and *w*Mel.tet	<0.001	0.011	0.015
*w*MelPop and *w*MelPop.tet	<0.001	0.763	0.295

Values are multiple-comparison adjusted *P* statistics from permutation tests on adult wing length with diet level and mosquito strain as factors.

of *w*MelPop females reared on 0.10, 0.25, and 1.0 mg/larva/d regimens were 0, 5.0, and 2.5%, respectively (Fig. 2a), whereas the infection rates of the *w*MelPop.tet females were 65.0, 37.5, and 47.5%, respectively. The differences in DENV-2 infection rates between *w*MelPop-infected individuals and equivalent tetracycline-treated lines were highly significant at each diet level (Fig 2a).

By applying logistic regression analysis to analyze the effect of diet in the presence of mosquito strain and any synergies, we confirmed that there were highly statistically significant differences in DENV-2 infection rates between *w*Mel and *w*Mel.tet mosquitoes; however, larval diet did not have a statistically significant effect (Table 3). There was also no interaction between diet level and strain. The same outcome was observed for *w*MelPop and *w*MelPop.tet mosquitoes (Table 3).

DENV-2 titers within infected mosquitoes from the tetracycline-treated group reared at 0.25 and 1.0 mg/larva/d ranged between 4.8–5.3 log $CCID_{50}$/ml at 10 d postintrathoracic inoculation (Fig 2b). Titers within mosquitoes reared on 0.10 mg/larva/d were between 2.8–3.8 log $CCID_{50}$/ml. As DENV-2 was only detected in 10 *Wolbachia*-infected individuals (seven *w*Mel and three *w*MelPop), it was not possible to analyze differences in titer between *Wolbachia*-infected and uninfected mosquitoes.

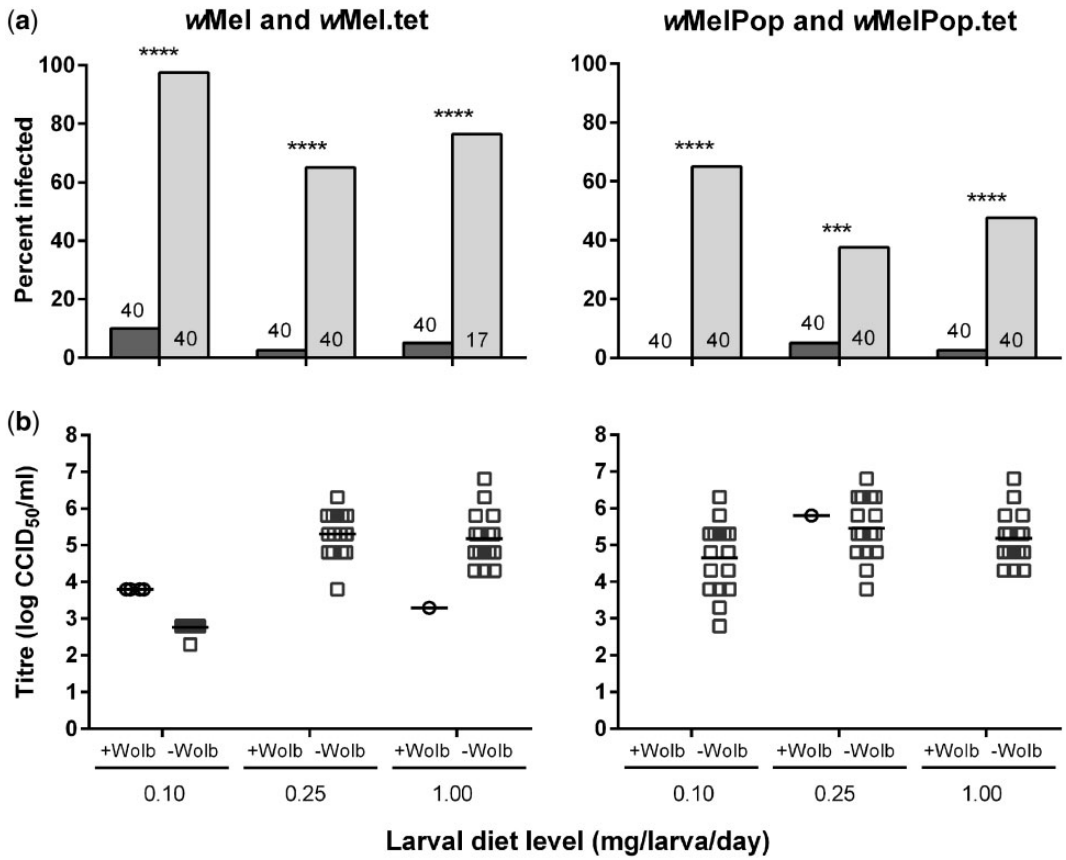

Fig. 2. Virus infection results for *Wolbachia*-infected and control mosquitoes reared under different diet regimens and inoculated with DENV-2 by intrathoracic microinjection (Experiment 1). **(a)** Virus infection rates (percentage of mosquitoes infected) 10 d postinoculation with DENV-2 for *Wolbachia*-infected (dark shading) and control (light shading) strains that were fed different larval food regimens. Numbers in columns show samples sizes. ***, $P < 0.001$. ****, $P < 0.0001$ (two-tailed Fisher's exact test). The results of logistic regression analyses to examine the effect of *Wolbachia* infection and larval diet level on DENV-2 infection are provided in Table 3. **(b)** DENV-2 titers (log CCID50/ml) from the bodies of *Wolbachia*-infected (circles) and uninfected (squares) mosquitoes fed on different diet regimens as larvae. Titers were only determined for DENV-2 positive individuals from a preceding CCELISA test. Absence of data points for *Wolbachia*-infected mosquitoes is due to an absence of DENV-2-infected samples as a result of strong virus interference.

Table 3. Logistic regression analysis of the effects of larval diet level and *Wolbachia* infection on the probability of infection of *Ae. aegypti* with DENV-2 following intrathoracic injection (Experiment 1)

	Estimate	SE	*P* value
***w*Mel and *w*Mel.tet**			
(intercept)	−2.564	0.564	<0.001
Diet	−0.524	1.066	0.623
Mosquito strain	4.063	0.682	<0.001
Diet × Strain	0.924	1.406	0.511
***w*MelPop and *w*MelPop.tet**			
(intercept)	−3.815	0.926	<0.001
Diet	0.320	1.441	0.824
Mosquito strain	4.028	0.967	<0.001
Diet × Strain	−0.719	1.514	0.635

Experiment 2: DENV-2 Infection Following Oral Feeding

*w*Mel- and *w*MelPop-infected *Ae. aegypti* lines and their equivalent tetracycline lines were orally inoculated with DENV-2 via artificial membrane feeding. DENV-2 infection rates for tetracycline-cured lines were lower than that achieved for the first cohort inoculated via intrathoracic injection with a maximum infection rate of 35.7% of inoculated mosquitoes (Fig 3a). However, DENV-2 infection rates were highly significantly reduced for *w*Mel and *w*MelPop infected groups compared with the equivalent tetracycline-treated mosquitoes for both the 0.1 and 1.0 mg/larva/d regimens ($P < 0.0001$).

Due to sparse infection data for *w*Mel and *w*MelPop mosquitoes, a logistic regression model that adjusted for diet was not estimable; therefore, we were unable to determine the strain-adjusted effect of larval diet level or synergies between larval diet and strain on oral infection rates for Experiment 2. Median DENV-2 titers for *Wolbachia*-infected and tetracycline-cured mosquitoes ranged between 3.8–4.3 log $CCID_{50}$/ml (Fig. 3b).

Discussion

In nature, density-dependent competition by larval *Ae. aegypti* for varying amounts of food resources in different container types may vary seasonally (Tun-Lin et al. 2000, Lounibos et al. 2002) and this results in the production of different classes of adults, usually of low to moderate nutritional status (Knox et al. 2007). Here, we show that dengue virus inhibition by *Wolbachia* infection was not affected by the quantity of larval food provided to *Ae. aegypti* mosquitoes. Similar infection rates were achieved in mosquitoes reared under nutrient stress (indicated by prolonged larval development and low adult body size) to robust mosquitoes produced with an abundance of larval food. This has important implications for the *Wolbachia*-

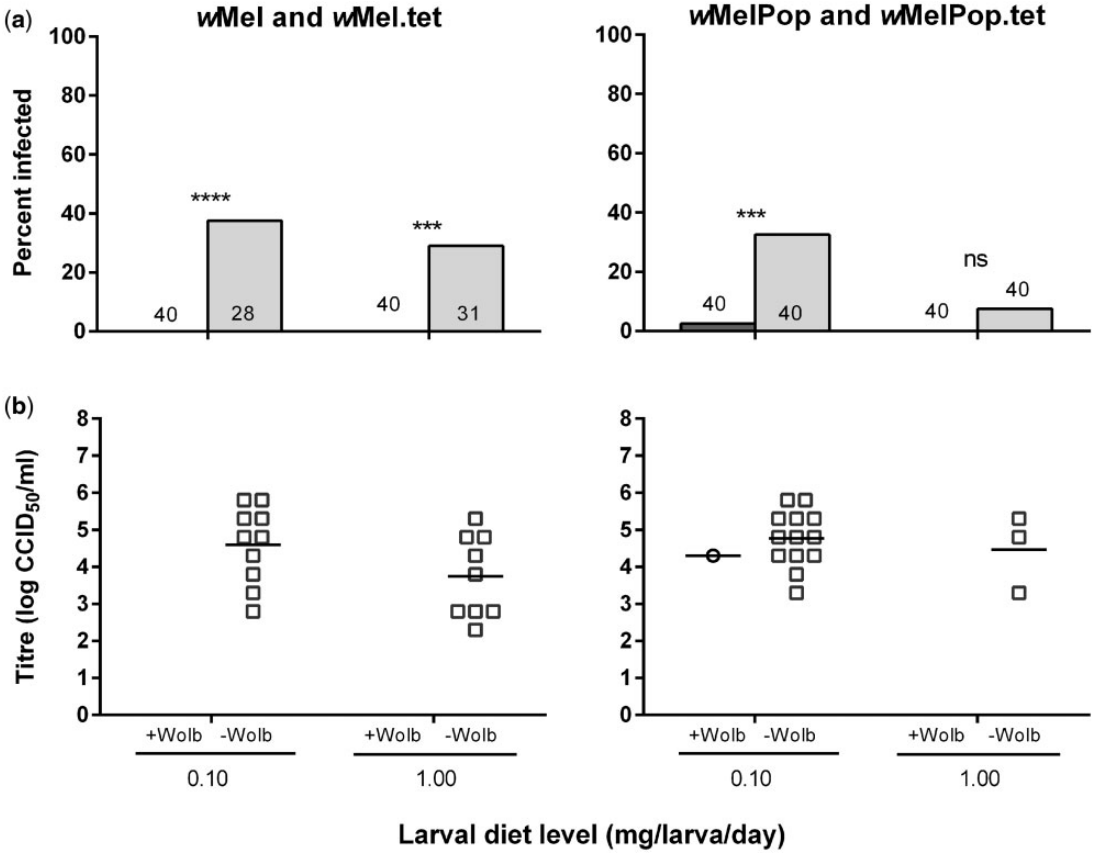

Fig. 3. Virus infection results for *Wolbachia*-infected and control mosquitoes reared under different diet regimens and orally inoculated with DENV-2 by artificial membrane feeding (Experiment 2). **(a)** Virus infection rates (percentage of mosquitoes infected) 10 d postinoculation with DENV-2 for *Wolbachia*-infected (dark shading) and control (light shading) strains that were fed different larval food regimens. Numbers in columns show samples sizes. ***, $P < 0.001$. ****, $P < 0.0001$. ns, not significant (two-tailed Fisher's exact test). **(b)** DENV-2 titers (log CCID50/ml) from the bodies of *Wolbachia*-infected (circles) and uninfected (squares) mosquitoes fed on different diet regimens as larvae. Titers were only determined for DENV-2 positive individuals from a preceding CCELISA test. Absence of data points for *Wolbachia*-infected mosquitoes is due to an absence of DENV-2-infected samples as a result of strong virus interference.

based viral interference strategy, as it is likely to apply to all nutrient classes. Highly significant dengue inhibition was achieved by both *w*Mel and *w*MelPop strains of *Wolbachia* following inoculation of mosquitoes with DENV by both intrathoracic microinjection and oral feeding. The reductions of DENV infection of up to 100% demonstrate the large potential of *Wolbachia* for blocking dengue virus transmission by mosquitoes, which is consistent with previous investigations (Moreira et al. 2009, Walker et al. 2011).

Feeding larvae at various diet levels altered development times and had the necessary effect of producing adult mosquitoes in distinct body size classes. Well known relationships between larval food quantity and development time and adult body size were observed. Increasing larval food availability resulted in decreased time to pupation. There was indication of nutritional stress placed on 0.1 mg/larva/d larvae from the disproportionately increased variance in the time to pupation compared with the higher diet treatments. The body sizes of our mosquitoes were comparable with wild *Ae. aegypti* collected from Cairns, Australia (Yeap et al. 2014). Mosquitoes collected during January (Australian summer) had a median wing length of 2.94 mm, which falls between the mean wing lengths of mosquitoes fed on 0.25 and 1.0 mg/larva/d regimens in our experiments. The coefficient of variation of wing lengths was larger for these field collected specimens than laboratory-reared specimens in the previous study, probably because field-emerged mosquitoes develop in a range of habitats subject to a range of environmental conditions that affect size (water temperature, larval density, food availability). In our experiments, we created body size variation specifically by varying the quantity of larval food provided. The effect of *Wolbachia* infection on DENV vector competence may change depending on the constitution of the diet. Further studies are recommended to examine the influence of varying larval food quality, i.e., by feeding different food types, on *Wolbachia* density and DENV vector competence.

There was some evidence that *Wolbachia* infection increased the development rate of larvae compared with tetracycline-treated controls. This trend was observed for both cohorts for *w*Mel-infected larvae but was not consistently observed for *w*MelPop. A previous study found no effect of *w*MelPop infection on the rate of larval development (McMeniman and O'Neill 2010). The stimulation of development by *w*Mel infection has not been reported to date. It is possible that *w*Mel-infected *Ae. aegypti* larvae use nutrients more efficiently, thereby reducing development time. As a consequence, *w*Mel-infected mosquitoes emerge sooner, potentially conferring a fitness advantage.

Mosquito body size can significantly affect the susceptibility of mosquitoes to arbovirus infection and subsequent dissemination of the virus (Grimstad and Haramis 1984, Grimstad and Walker 1991, Alto et al. 2008). Smaller *Ae. aegypti* were more likely to become infected with DENV-2 and develop a disseminated infection than larger mosquitoes (Alto et al. 2008). However, the relationship can be complex and be modified by factors including geographic and genetic background (Sumanochitrapon et al. 1998, Schneider et al. 2007). In this study, a low infection rate following oral inoculation

was observed among large *w*Mel.tet mosquitoes that were reared on the 1.0 mg/larva/d diet (7.5%), and for all strains, the highest infection rates were obtained from the smallest mosquitoes (those reared on 0.1 mg/larva/d). Differences in infection rates between *w*Mel.tet and *w*MelPop.tet reared at the same diet level (Experiment 2) may be due to differences in the providences of the two strains. In a few instances, individuals from *Wolbachia* infection groups were infected with DENV-2 at titers higher than the means of tetracycline-treated groups. We hypothesize that this could be due to low *Wolbachia* densities in these samples.

Two factors may have limited the vector competence assessments of this study. First, the virus titers of the inocula were relatively low compared with similar challenge experiments that have shown *Wolbachia*-induced DENV inhibition following inoculation with titers above 10^7 plaque-forming units per ml (Walker et al. 2011). However, our inocula were within the range of titers obtained from patient sera during DENV outbreaks (Kuno et al. 1985). The lower titer inocula probably contributed to infection rates that were under 100% after intrathoracic microinjection in the tetracycline cured groups. Second, the assay of body infection may be more appropriate for *w*MelPop because of its broader tissue tropism compared with *w*Mel (Iturbe-Ormaetxe et al. 2011). Despite these limitations, the same methodology was applied to both *Wolbachia*-infected and uninfected controls allowing for successful examination of DENV-2 interference in mosquito bodies. As we measured the presence of virus from homogenates derived from whole mosquitoes, further work is needed to determine whether our results apply specifically to salivary gland infection and, therefore, transmission potential.

The relationship between host nutrition and *Wolbachia* infection dynamics is under scrutiny in order to define the mechanisms behind virus inhibition in insects. The endogenous level of cholesterol in *D. melanogaster* has been shown to influence virus inhibition by *Wolbachia* endosymbionts (Caragata et al. 2013). The normally protective effects of *Wolbachia* infection were reduced when flies were fed cholesterol-rich diets. Flies became more susceptible to *Drosophila* C virus-induced mortality. Dietary composition also modulates the effects of *Wolbachia* infection on fly fecundity and longevity (Ponton et al. 2015). *Wolbachia* infection decreased fly fecundity and longevity when either protein or carbohydrates was limiting, respectively. Infection of *Ae. aegypti* with either *w*MelPop or *w*Mel strains was associated with lower endogenous host cholesterol and cholesterol ester content (Caragata et al. 2014). *w*MelPop infection decreased mosquito fecundity and egg viability under conditions where particular amino acids were limited, traits that are important to the successful establishment of *Wolbachia* in biological control programs against DENV.

Based on the results of this study, *Wolbachia* prevents replication of dengue virus irrespective of the quantity of larval food provided. The inhibitory effect of *w*Mel and *w*MelPop against DENV-2 at all larval diet levels indicates that the nutritional background of the mosquitoes is unlikely to be important in field-based applications against dengue, which is a favorable outcome as wild *Ae. aegypti* emerge from artificial container habitats that vary widely in nutrition content. Our findings of independence from the nutritional status of the target insect (*Ae. aegypti)* and recent findings that *Wolbachia*-induced DENV inhibition are independent of the temperature regimen that adult mosquitoes are maintained under (Ye et al. 2016) highlight the robustness and enormous potential of the *Wolbachia* biological control strategy against dengue.

Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

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