DOI: 10.1534/genetics.110.122390

Dynamics of the "Popcorn" Wolbachia Infection in Outbred *Aedes aegypti* Informs Prospects for Mosquito Vector Control

H. L. Yeap,*,[1] P. Mee,[†] T. Walker,[†] A. R. Weeks,* S. L. O'Neill,[†] P. Johnson,[‡] S. A. Ritchie,[‡] K. M. Richardson,* C. Doig,* N. M. Endersby* and A. A. Hoffmann*

**Bio21 Institute, Department of Genetics, University of Melbourne, Victoria 3010, Australia, [†]School of Biological Sciences, University of Queensland, Brisbane, Queensland 4072, Australia and [‡]School of Public Health and Tropical Medicine, James Cook University, Townsville, Queensland 4811, Australia*

Manuscript received August 18, 2010
Accepted for publication November 18, 2010

ABSTRACT

Forty percent of the world's population is at risk of contracting dengue virus, which produces dengue fever with a potentially fatal hemorrhagic form. The *w*MelPop Wolbachia infection of *Drosophila melanogaster* reduces life span and interferes with viral transmission when introduced into the mosquito *Aedes aegypti*, the primary vector of dengue virus. Wolbachia has been proposed as an agent for preventing transmission of dengue virus. Population invasion by Wolbachia depends on levels of cytoplasmic incompatibility, fitness effects, and maternal transmission. Here we characterized these traits in an outbred genetic background of a potential target population of *Ae. aegypti* using two crossing schemes. Cytoplasmic incompatibility was strong in this background, and the maternal transmission rate of Wolbachia was high. The infection substantially reduced longevity of infected adult females, regardless of whether adults came from larvae cultured under high or low levels of nutrition or density. The infection reduced the viability of diapausing and nondiapausing eggs. Viability was particularly low when eggs were laid by older females and when diapausing eggs had been stored for a few weeks. The infection affected mosquito larval development time and adult body size under different larval nutrition levels and densities. The results were used to assess the potential for *w*MelPop-CLA to invade natural populations of *Ae. aegypti* and to develop recommendations for the maintenance of fitness in infected mosquitoes that need to compete against field insects.

AEDES aegypti is the primary mosquito vector of dengue viruses, the leading causes of arboviral infection, which affect up to 1 in 100 people globally (Kyle and Harris 2008). According to the World Health Organization, 2.5 billion people (40% of the world population) are at risk of contracting dengue. *Ae. aegypti* is anthropophilic and has established in densely populated urban and semiurban areas in the tropics and subtropics (Wilder-Smith and Gubler 2008). International travel increases likelihood of the multiple dengue serotypes crossing borders, leading to secondary infections that can manifest into debilitating dengue hemorrhagic fever and cause 1–20% mortality, mostly in young children (Wilder-Smith and Gubler 2008). With no licensed vaccine available (Wilder-Smith *et al.* 2010), vector control targeting *Ae. aegypti* is the only effective way of limiting dengue transmission and epidemics.

The Wolbachia bacterium strain *w*MelPop-CLA, isolated from a laboratory strain of *Drosophila melanogaster* (Min and Benzer 1997; McMeniman *et al.* 2008), has been introduced into *Ae. aegypti*, where it shortens life span while maintaining strong cytoplasmic incompatibility and a high level of maternal transmission (McMeniman *et al.* 2009). Although *w*MelPop-CLA affects the fitness of *Ae. aegypti* by changing factors such as activity levels and decreasing biting behavior (Evans *et al.* 2009; Moreira *et al.* 2009b; Turley *et al.* 2009), it may nevertheless be able to invade populations and prevent the spread of dengue by older females (McMeniman *et al.* 2009). The *w*MelPop-CLA infection may have other beneficial side effects, including direct suppression of dengue virus and other pathogens such as Chikungunya virus, Plasmodium, and filarial nematodes inside mosquitoes (Kambris *et al.* 2009; Moreira *et al.* 2009a; Bian *et al.* 2010).

The spread of *w*MelPop-CLA into natural populations is aided by strong cytoplasmic incompatibility and high rates of maternal transmission of the infection (Turelli 2010). To predict whether Wolbachia will spread across multiple host generations, these parameters and others should ideally be estimated on the genetic background of the host population, particularly as the spread of Wolbachia requires multiple generations (Turelli and Hoffmann 1995), during which time the introduced genetic background becomes intermingled with the

Supporting information is available online at http://www.genetics.org/cgi/content/full/genetics.110.122390/DC1.

[1]*Corresponding author:* Bio21 Institute, Department of Genetics, University of Melbourne, Victoria 3010, Australia.
E-mail: h.yeap@pgrad.unimelb.edu.au

local target population background. Genetic background can influence host fitness effects mediated by Wolbachia (Dean 2006), as well as patterns of cytoplasmic incompatibility and bacterial density (Kondo *et al.* 2005; Sinkins *et al.* 2005). With respect to the *w*MelPop-CLA infection, nuclear genetic background influences expression of longevity effects after introduction into *D. simulans* (McGraw *et al.* 2002; Carrington *et al.* 2010) and may also attenuate infection effects of Wolbachia in *D. melanogaster* (Carrington *et al.* 2009).

When moving from laboratory experiments to large-scale releases, it is essential to generate stocks with high competitive ability to increase the likelihood of infections spreading into natural populations, an issue well recognized in insect sterile release programs (Alphey 2002). *Ae. aegypti* populations show isolation by distance, reflecting relatively high levels of genetic isolation in populations (Failloux *et al.* 2002; Huber *et al.* 2002; Endersby *et al.* 2009) likely to generate local adaptation. Competitive strains that capture a locally adapted background can be generated through backcrossing strains to the genetic background of the target population.

Here we undertake a comprehensive evaluation of *w*MelPop-CLA effects in *Ae. aegypti* following backcrossing to a target background from Cairns, Australia. We characterize outcrossing effects on cytoplasmic incompatibility when females are aged from 1 to 3 weeks, on maternal transmission and on egg viability when eggs are tested immediately after being laid or after they have entered diapause (Juliano *et al.* 2002). We also consider *w*MelPop-CLA infection effects on larval development time, adult body size, and longevity following rearing under different conditions. The results are interpreted within the context of models that predict conditions required for the spread of *w*MelPop-CLA in natural populations.

METHODS

Backcrossing: Wolbachia-popcorn (*w*MelPop-CLA)–infected *Ae. aegypti* strains were created by crossing two uninfected field strains with two *w*MelPop-CLA–infected laboratory strains. The uninfected strains (C1 and C2) were created at James Cook University (Cairns, Australia) from several hundred eggs collected from urban areas between Gordonvale and Machans Beach, Queensland, Australia, in 2008 using alfalfa-baited ovitraps (Ritchie 2001). A *w*MelPop-CLA strain was provided by the University of Queensland after infection via embryonic microinjection (line O tested at generations 34–42 after the line was established).

Four infected backcrossed lines were created by crossing the uninfected and *w*MelPop-CLA strains under two schemes. In scheme A (Figure 1), the infected line was crossed for three successive generations to each of the outbred uninfected lines (two generations removed from the field) to create lines AOC1 and AOC2. Because the lines being crossed were maintained under laboratory conditions for three generations, there was potential for laboratory adaptation, perhaps facilitating laboratory maintenance of the lines, but reducing their competitive ability when compared against field mosquitoes. In scheme B, eggs from the two parental uninfected lines were collected and stored, after which two successive generations of the four target lines were crossed back to this stored generation (to create lines BOC1 and BOC2), using eggs that had been dried (see below) to maintain a diapause state (Juliano *et al.* 2002). This scheme minimized laboratory adaptation and may help maintain the competitive ability of the backcrossed lines against field mosquitoes. All crosses involved at least 100 mosquitoes from each line.

Colony maintenance: All life stages of the backcrossed lines were maintained in a controlled 12:12 light:dark (L:D) laboratory environment at 26° and 75–85% humidity. Eggs were hatched in plastic trays (20 × 28.5 × 9 cm) containing 3 liters of reverse osmosis (RO) water, yeast (~0.09 mg), and one crushed tablet of TetraMin Tropical Fish Food tablets Rich Mix (hereafter TetraMin). Density was controlled to 200 individuals at the second instar stage and larvae were transferred to plastic trays (30 × 40 × 8 cm) containing 4 liters RO water and supplied with TetraMin in excess. Pupae were sexed by size; females are larger than males. Adults (100 males and 100 females) were housed in plastic containers with mesh sides (20 × 20 × 30 cm, covered in a plastic bag to maintain high humidity) and allowed constant access to 10% sucrose solution on a sponge. Females were given access to a human arm for blood feeding within 1 week of emergence and oviposited on a conical filter paper (Whatman qualitative circles, 150 mm diameter) inside a plastic cup containing 150 ml RO water. Filter papers were replaced daily for 1 week following blood feeding. Eggs were allowed to embryonate by leaving filter papers wet for 2 days after collection, partially drying them on paper towels for 30 sec, and leaving them moist for 1 more day. Filter papers were then dried until only traces of moisture by touch were apparent and stored in plastic containers with moist cotton wool to prevent desiccation.

Genetic variation: Genomic DNA was extracted from the thorax of females via a CTAB extraction method (Weeks *et al.* 2002) with 150 μl of 2% CTAB, 2 μl proteinase K (Roche Diagnostics), a single chloroform: isoamyl alcohol (24:1) wash, 200 μl absolute ethanol, 5 μl of 5 M sodium chloride, and a single wash of 200 μl 70% ethanol. DNA was resuspended in 60 μl sterile water and stored at −20°.

Two EPIC markers (Endersby *et al.* 2009) and six microsatellites (Chambers *et al.* 2007; Slotman *et al.* 2007; Endersby *et al.* 2009) were used to study variation in the backcrosses (see Endersby *et al.* 2009). PCR was performed as a 10-μl reaction per individual. This consisted of 2 μl of 4× diluted genomic DNA; 0.3 μl [γ^{33}P]ATP-end-labeled forward primer, 1 μM; 0.1 μl

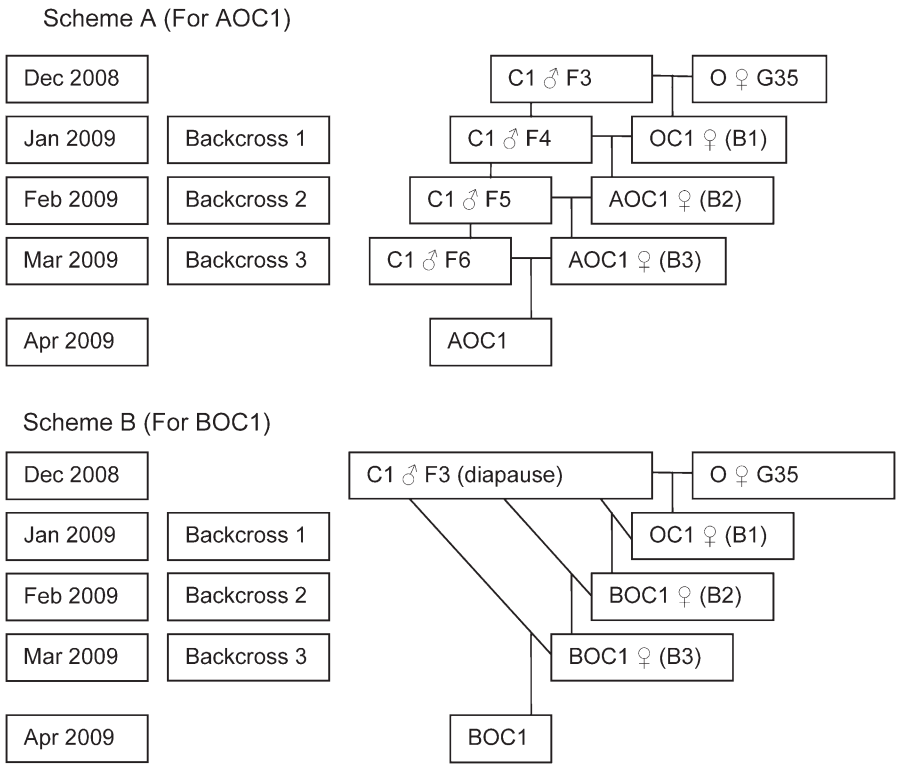

Figure 1.—Crossing schemes for developing outbred lines of *Aedes aegypti* carrying the *w*MelPop-CLA infection. The schemes are shown for the C1 mass-bred line (crossed in the third generation from the field) and equivalent crosses were carried out for the C2 mass-bred line to generate four outcrossed lines (AOC1, AOC2, BOC1, and BOC2). In Scheme A, the mass-bred line was crossed to the infected O line (35 generations after introduction of *w*MelPop-CLA) and then backcrossed to the mass-bred line maintained in culture. In Scheme B, the mass-bred line was put in diapause and resurrected for each generation of backcrossing to minimize laboratory adaptation.

unlabeled forward primer, 10 μM; 0.4 μl reverse primer, 10 μM; 2 μl dNTPs (Bioline), 1 mM; 0.5 μl bovine serum albumin (New England Biolabs, Ipswich, MA), 1%; 1 μl 10× PCR buffer with 2.0 mM Mg^{2+}; 0.4 unit of *Taq* polymerase (New England Biolabs); and 3.62 μl sterile water. PCR specifications are based on previous studies (Chambers *et al.* 2007; Slotman *et al.* 2007; Endersby *et al.* 2009). Thirty females per line were genotyped.

Maternal transmission: The rate of maternal transmission of *w*MelPop-CLA was tested by screening mothers and their offspring for Wolbachia by PCR with *ftsZ* primers (Weeks and Breeuwer 2001). Maternal transmission in the BOC2 backcrossed line (generation 4) and the O-infected line (42 generations after establishment) was tested. Females were blood fed 7, 14, and 20 days after emergence. After blood feeding at day 7 and day 20, 20 females per line were isolated individually in 50-ml glass vials with a foam stopper. Conical filter paper (5.5 mm) wicked in water at the base of the vial provided an oviposition surface. Filter papers with eggs were collected daily for the first 1–2 days of egg laying after which adult females were stored in ethanol and frozen at −80°. Within 1 week of embryonation (see *Colony maintenance* above), eggs were hatched in plastic trays containing 400 ml RO water, one grain of yeast, and one-eighth of a crushed tablet of TetraMin. Female progeny were preserved in absolute ethanol at −80°. Only female parents producing at least 10 progeny were included in the analysis and ∼10 offspring per female were tested for Wolbachia infection. This resulted in 123 offspring from the BOC2 line (and 125 from the O line) being tested for the 7-day-old females and 36 (38 from the O line) for the 20-day-old females.

Longevity in groups: To investigate life-shortening effects when infected mosquitoes were held in groups, three replicates of 50 pupae per backcrossed line (1:1 sex ratio) were immersed in a small plastic cup containing 170 ml RO water inside a plastic container with mesh sides (3 liters capacity). The longevity of eclosed adults from infected outcrossed lines from scheme A and scheme B was compared to that of adults from C1 and C2 lines (which were at the F_7 generation). Containers were housed in a temperature-controlled cabinet at 29° with a photoperiod of 12:12 (L:D). Females were blood fed within 1 week of eclosion and at weekly intervals for the duration of their life. Adults were given constant access to a 10% sucrose solution as well as to filter paper oviposition sites as described above. Dead individuals were counted and removed three times weekly.

Egg viability after drying: Relative humidity (RH) inside environmental chambers (30 × 20 × 8 cm) was controlled at 20–25%, 55–60%, 75–80%, and 95–100% using saturated solutions of potassium acetate, sodium bromide, sodium chloride, and water, respectively (Juliano *et al.* 2002). Solutions were placed in cups housed in plastic environmental chambers sealed with petroleum jelly. Incubators were set to 24°, 26°, or 30° with a 12:12 (L:D) cycle. One week prior to eggs being introduced, RH was monitored in chambers using a hygrochron (1-wire; iButton.com).

Eggs on filter papers were collected from C1 and C2 (controls), AOC1 and AOC2 (infected outbred), and O

(infected inbred) lines. Papers were replaced daily over 10 days to acquire at least 3000 eggs per cross. After eggs embryonated, filter papers were cut into batches of at least 25 eggs, which were counted and placed in plastic containers. Eggs were considered unviable if their chorion collapsed, if they hatched precociously, or if they appeared partially or completely unmelanized. Five egg batches from each cross were placed in containers (17 × 11 × 3.5 cm) inside each environmental chamber. Chambers were resealed with petroleum jelly and then placed into incubators. After 3 days (day 3), one egg batch was removed from each cross and submerged in a cup (height 8 cm, radius 2.5–3.5 cm) filled with 170 ml RO water. One tablet of TetraMin was distributed across 60 cups. After 6 days, the numbers of larvae (dead or alive) and pupae were counted. This was repeated after 10, 17, 24, and 31 days.

Cytoplasmic incompatibility and egg viability: Cytoplasmic incompatibility induced by *w*MelPop was tested in the C1 backcrossed lines (AOC1 and BOC1) by reciprocally crossing the backcrossed lines (one generation after backcrossing was completed) with the uninfected C1 line that was at F_7. Hatch rates in the compatible (with C1 males) and incompatible (with C1 females) crosses were compared. Compatible crosses within infected (C1) and uninfected (O line 39 generations postestablishment) parental lines were also carried out.

For the crosses, 14 pupae per replicate (1:1 sex ratio) were transferred to plastic cups containing RO water (170 ml) housed inside plastic containers with mesh sides (1.4 liter capacity). Adults were given 10% sucrose solution (see *Colony maintenance*) and one blood feed. Oviposition sites were provided after feeding and eggs were collected daily over 3–5 days (depending on fecundity), counted, and conditioned. Eggs were hatched within 1 week of collection in plastic containers (6.5 cm height and 9.2–11.5 cm radii) with 500 ml RO water, one grain of yeast (~0.01 mg), and one-quarter of a crushed tablet of TetraMin. Filter papers were removed 3–4 days postimmersion, dried briefly, and reimmersed to ensure hatching. Larvae were counted 1 week after egg immersion. Three replicates were carried out for each cross. To determine if cytoplasmic incompatibility as well as hatch rate in compatible crosses changed over time, this process was repeated three more times (including the blood feed) at weekly intervals after the first blood meal.

Larval nutrition, development time, and individual longevity: These experiments were undertaken with the AOC1 and C1 lines. A larval nutritional stress level was initially determined by feeding serially diluted slurries of TetraMin fish food in RO water with the following concentrations: 1, 0.75, 0.5, 0.25, 0.1, and 0.01 mg food per larva per day. Stress was defined as the level of nutrients that significantly increased larval development time and decreased adult size. Hatched first instar larvae were divided into groups of 50 with four replicate trays per treatment. Larvae were reared at a low-stress nutrient level of 0.25 mg/larva/day or a high nutritional stress level of 0.05 mg/larva/day under both low (1 larva/20 ml) and high (1 larva/ml) densities. Larval development time was measured as the time between first instar hatching and pupation.

Wing length was measured to assess nutritional effects on mosquito body size. Wings were removed and photographed. Seven digital landmarks were placed on the wing with the program tpsDIG2 (Rohlf 2008). Wing size was expressed as centroid size, the square root of the sum of squared distances between allocated landmarks and the central point of the wing (Rohlf 2009). Linear wing measurements were also taken using an ocular micrometer on a stereo dissecting microscope to measure the distance from the alular notch to the tip of the wing (Bock and Milby 1981).

Longevity of mosquitoes from the different treatments was tested by isolating individual pupae in plastic cups. Eclosed adult mosquitoes were provided with 10% sucrose and mortality was scored daily.

Analysis: *Genetic marker data:* Deviations from Hardy–Weinberg equilibrium were tested with Genepop web-version 4.0 (Rousset 1997). Markov-chain parameters were set to 100,000 dememorization, 100 batches, and 10,000 iterations per batch. Measures of genetic variation, including private alleles and expected (H_E) and observed heterozygosity (H_O), were obtained with Genetic Data Analysis (GDA) version 1.1 (Lewis and Zaykin 2001).

Null allele frequency (Brookfield 1996) was estimated with Micro-Checker version 2.2.3 (Oosterhout *et al.* 2004) and the number of alleles per locus calculated with FSTAT version 2.9.3.2 (Goudet 1995). The inbreeding coefficient (F_{IS}) (Weir and Cockerham 1984) and pairwise F_{ST} among populations were also computed with FSTAT. Significance was determined through permutation (100,000) and table-wide *P*-values were adjusted for multiple comparisons. Overall genetic population structure was assessed via analysis of molecular variance (AMOVA) in Arlequin version 3.11 (Excoffier *et al.* 2005) with 10,000 permutations.

Quantitative data: To assess the rate of maternal transmission in the BOC2 and O lines, the number of uninfected mosquitoes produced from infected mothers and binomial confidence intervals were computed.

For egg viability after drying, data from day 0 were not analyzed because only a single egg mass was available for each line. For the remaining data, ANOVA was performed with SPSS for Windows (version 13) on hatch proportions after angular transformation to improve normality and decrease heteroscedasticity. Humidity, temperature, and infection status (infected or uninfected) were treated as fixed factors with four, three, and two levels, respectively. Population was treated as a random factor nested within infection status while "days of treatment" was treated as a linear variable. Equality of variances and normality of data were tested prior to analysis via *q-q* normal plots and plots of standardized residuals. Further ANOVAs were performed on data

partitioned into separate days of treatment, *i.e.*, 3, 10, 17, 24, or 31 days.

To assess cytoplasmic incompatibility in the C1 female by AOC1/BOC1 male crosses, we computed the proportion of eggs in a group (containing at least 10 eggs) that failed to hatch. For cytoplasmic incompatibility, there was an obvious effect on hatch rate not requiring analysis, but we did compare by ANOVA the effect of female age and cross type on hatch rate in the compatible crosses (which included O × O and C1 × C1 crosses), using angular transformed hatch rates.

To compare survival curves in the group longevity assay that involved all outbred lines, the Cox regression procedure was applied to produce a survival function that predicts the probability that the event of interest has occurred at a given time, *t*, for a predictor variable. We initially compared survival curves of the replicate lines; because these did not differ significantly within treatments, we then combined data from replicates when comparing treatments.

For the larval nutrition data collected on the AOC1 and C1 lines, the effects of line, nutrition, and density on development time and wing size were investigated through ANOVAs. Because of the very large effects of nutrition on these traits, we also ran ANOVAs separately on the two nutrition levels. Development time was square-root transformed for normality, but an association between means and variances could not be entirely removed through transformation in the ANOVA on all data. Within the nutrition treatments, data were normally distributed after square-root transformation (Kolmogorov–Smirnov tests). Wing length was not transformed, but nutrition levels were treated separately because of a correlation between means and variances across the nutrition levels. Tray effects were included in the wing length analyses although these tended to be nonsignificant.

Estimating unstable equilibrium frequency: Estimates were based on an equation (Turelli 2010) that assumes age-independent survival as well as fecundity, perfect maternal transmission, and random mating,

$$\hat{p} = \frac{(v_U - v_I)\lambda_I^{\tau_U} + (F_U - F_I\lambda_I^{\tau_U - \tau_I})}{F_U s_h}, \qquad (1)$$

where $v_I(v_U)$ is the daily survival rate of infected (uninfected) reproductive adults and λ_I is the asymptotic geometric growth rate of a pure infected population. This is estimated by solving

$$\lambda_I^{\tau_I+1} = v_I\lambda_I^{\tau_I} + F_I, \qquad (2)$$

where λ_I is the largest positive solution of this characteristic equation. F_U is the expected number of future reproductive uninfected females produced by an uninfected reproductive female each day in the absence of cytoplasmic incompatibility (CI). F_I is the expected number of future reproductive infected females produced by an infected reproductive female each day, while τ_I (τ_U) is the length of the prereproductive period for an infected (uninfected) female in days beginning with fertilization. Reproduction for an infected (uninfected) female starts on day $\tau_I + 1$ ($\tau_U + 1$) and s_h is the intensity of CI, where $s_h = 1$ equates to perfect CI while $s_h = 0$ means fully compatible.

We further investigated age-dependent survival and fecundity but with perfect maternal transmission and random mating, by adapting equations

$$I_{A,t+1} = v_I I_{A,t} + F_I I_{A,t-\tau_I} \qquad (3)$$

$$U_{A,t+1} = v_U U_{A,t} + F_U(1 - s_h p_{t-\tau_U})U_{A,t-\tau_U} \qquad (4)$$

(Turelli 2010), where $I_{A,t}$ ($U_{A,t}$) is the number of infected (uninfected) reproductive females at time t, while p_t is the frequency of infection at time t, given by two life table matrices similar to those in Rasgon and Scott (2004):

$$\begin{pmatrix} m_0(I) & m_1(I) & \cdots & m_{k-2}(I) & m_{k-1}(I) & m_k(I) \\ \frac{l_1(I)}{l_0(I)} & 0 & \cdots & 0 & 0 & 0 \\ 0 & \frac{l_2(I)}{l_1(I)} & \cdots & 0 & 0 & 0 \\ \vdots & \vdots & \ddots & \vdots & \vdots & \vdots \\ 0 & 0 & \cdots & \frac{l_{k-1}(I)}{l_{k-2}(I)} & 0 & 0 \\ 0 & 0 & \cdots & 0 & \frac{l_k(I)}{l_{k-1}(I)} & 0 \end{pmatrix} \times \begin{pmatrix} I_{0,t} \\ I_{1,t} \\ I_{2,t} \\ \vdots \\ I_{k-1,t} \\ I_{k,t} \end{pmatrix} = \begin{pmatrix} I_{0,t+1} \\ I_{1,t+1} \\ I_{2,t+1} \\ \vdots \\ I_{k-1,t+1} \\ I_{k,t+1} \end{pmatrix} \qquad (5)$$

$$\begin{pmatrix} m_0(U) & m_1(U) & \cdots & m_{k-2}(U) & m_{k-1}(U) & m_k(U) \\ \frac{l_1(U)}{l_0(U)} & 0 & \cdots & 0 & 0 & 0 \\ 0 & \frac{l_2(U)}{l_1(U)} & \cdots & 0 & 0 & 0 \\ \vdots & \vdots & \ddots & \vdots & \vdots & \vdots \\ 0 & 0 & \cdots & \frac{l_{k-1}(U)}{l_{k-2}(U)} & 0 & 0 \\ 0 & 0 & \cdots & 0 & \frac{l_k(U)}{l_{k-1}(U)} & 0 \end{pmatrix} \times \begin{pmatrix} U_{0,t} \\ U_{1,t} \\ U_{2,t} \\ \vdots \\ U_{k-1,t} \\ U_{k,t} \end{pmatrix} = \begin{pmatrix} U_{0,t+1} \\ U_{1,t+1} \\ U_{2,t+1} \\ \vdots \\ U_{k-1,t+1} \\ U_{k,t+1} \end{pmatrix}. \qquad (6)$$

In these matrices, $I_{x,t}$ ($U_{x,t}$) is the number of infected (uninfected) mosquitoes aged x at time t, while l_x is the

number or proportion of mosquitoes surviving to age x where k is the oldest age class, and thus l_{x+1}/l_x is the proportion of mosquitoes surviving from age x to age $x + 1$; and m_x is the expected number of progeny from a mosquito at age x where $m_x(I) = 0$ at $x \leq \tau_I$ and $F_{I,x}$ at $x > \tau_I$, while $m_x(U) = 0$ at $x \leq \tau_U$ and $F_{U,x} \times [1 - s_h p]$ at $x > \tau_U$. Changes in $F_{I,x}$ and $F_{U,x}$ with age x were estimated on the basis of the CI experiment (below). We utilized Poptools version 3.2.3 (Hood 2010) to compute the eigenvalues λ_I and λ_U of the two matrices, which correspond to the asymptotic geometric growth rate of the infected and uninfected populations, respectively. Since λ_U is dependent on p, the unstable equilibrium frequency ($\hat{p}$) is achieved when

$$\lambda_U(\hat{p}) = \lambda_I(\text{equivalent growth rate}). \quad (7)$$

RESULTS

Genetic variation: Across all 811 individuals and eight molecular markers, 37 alleles were recorded. Three markers (*Rps20b*, *Rpl30a*, and *BbH08*) had 2 alleles while the most polymorphic marker (*BbB07*) had 11 alleles. Genotypic frequencies did not differ from Hardy–Weinberg equilibrium for any of the markers in populations after controlling for multiple comparisons. No significant null alleles were detected for any locus in a population.

There was more genetic variation in populations generated through both schemes A and B than in the parental O line (supporting information, Table S1). A total of 23 alleles were recorded in the O line, compared to 27/31 alleles in the two scheme A lines and 28/29 alleles in the two scheme B lines. This compares to 25/26 alleles in the control outbred lines. Backcrossed lines also had higher levels of observed heterozygosity regardless of crossing scheme. Initial heterozygosity in the O line was 0.41. This increased to an estimated 0.44/0.54 in the two A lines and 0.46 in the two B lines. Values for C1 and C2 lines (0.47/0.46) were similar to those in the backcrossed lines (Table S1).

In the AMOVA, the component among uninfected populations and backcrossed *w*MelPop-infected populations explained only 3.48% of the variation, that among populations within infected or uninfected subgroup explained only 2.38%, and that within population variance explained the remainder (Table S2). The backcrossed *w*MelPop-infected populations therefore have a genetic background similar to that of the uninfected populations. In the AMOVA on scheme A and scheme B lines, there was no significant difference between scheme A and B variance within control lines (*e.g.*, AOC1 *vs.* BOC1), but differences had developed between the subpopulations (*e.g.*, AOC1 *vs.* AOC2) presumably due to differences among control lines to which the infected populations had been crossed and additional drift effects during backcrossing (Table S2).

Pairwise F_{ST} values between scheme B lines tested during the backcross procedure and control lines decreased during backcrossing, while those between the scheme B lines and the original O line increased, although this difference leveled off (Figure S1). For the scheme A lines that were compared after only three generations of backcrossing, both the replicate lines differed significantly from their respective controls in pairwise comparisons (F_{ST} = 0.053/0.028 for C1/C2 comparisons) and they also differed from the O line with higher F_{ST} values (F_{ST} = 0.063/0.097 for AOC1/AOC2 comparisons).

Maternal transmission: For the 7-day females, all 125 O-line offspring were infected (maternal transmission rate 1, lower confidence interval of 0.98), while 121 of the 123 BOC2 offspring were infected (transmission rate 0.98, lower confidence interval 0.96). For the 20-day females, all 38 offspring from the O females were infected (transmission rate 1, lower confidence interval 0.93), as were the 36 offspring of the BOC2 mosquitoes (transmission 1, lower confidence interval 0.93).

Longevity: Data were pooled for the replicate lines from scheme A (AOC1 and AOC2) and scheme B (BOC1 and BOC2) because these did not differ significantly from each other by Cox regression. The survival curves clearly differed among the line types ($\chi^2 = 130.2$, d.f. = 2, $P < 0.001$), with the controls dying much more slowly than the infected outcrossed lines, although the lines showed a similar rate of survival for the first 20 days (Figure 2). The survival curve for scheme A lines also differed from that for scheme B lines ($\chi^2 = 11.41$, d.f. = 1. $P = 0.001$), with scheme B mosquitoes showing a lower early mortality but higher later mortality (Figure 2). The average decrease in mean longevity between the outcrossed lines and controls was 43%.

Egg viability: For the eggs held in containers, there were significant two-way interactions between infection status (status) and days of treatment (days), between humidity and infection status and between temperature and days of treatment (Table 1). There was also a significant three-way interaction between temperature, infection status, and days of treatment. The interaction between infection status and days of treatment reflects a significant decrease in hatch proportion over days in the infected *vs.* uninfected populations (Figure 3). Humidity effects on viability were weak or not significant (Table 1).

Cytoplasmic incompatibility and hatch rate: The hatch rate in incompatible crosses was extremely low regardless of the age of the females (Figure 4). The hatch rate never exceeded 1% and averaged ~0.2% for each of the two crosses.

For the compatible crosses, we noted a marked difference in hatch rate between the control crosses and those involving females carrying the Wolbachia infection, particularly for older females (Figure 4). While control crosses averaged hatch rates of ≥0.8 even after females had been aged for 4 weeks, the crosses involving

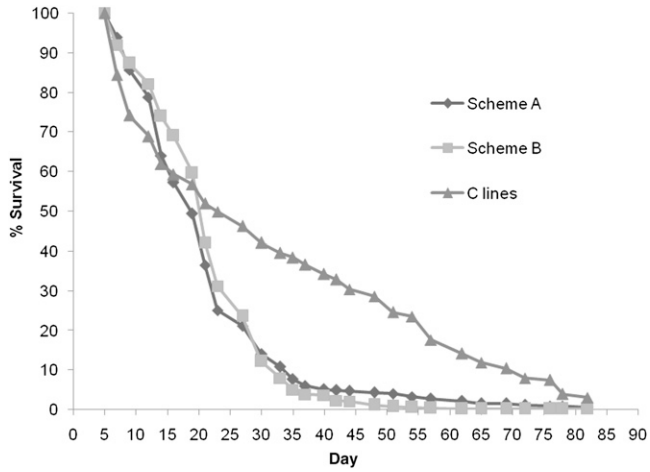

FIGURE 2.—Effect of Wolbachia infection on survival of *Aedes aegypti* females from the outbred lines when held in groups. Lines reflect the average survival of two replicates from each scheme.

infected lines showed a sharp decrease in hatch rates in older females. An ANOVA on hatch rates of the compatible crosses (angular transformed) indicated a significant effect of week [$F_{(3,17)} = 13.22$, $P < 0.001$], cross [$F_{(3,17)} = 5.77$, $P = 0.007$], and interaction between these terms [$F_{(9,17)} = 5.69$, $P = 0.001$]. This reflected the difference between the C1 × C1 crosses and those involving infected females, as there were no significant effects of cross on hatch rate (or interaction) when the three crosses with infected females were considered.

Larval fitness traits: Initial experiments were carried out to determine a larval nutritional stress level that resulted in changes in larval development time and adult body size, correlates of mosquito fitness (BEDHOMME *et al.* 2003; PONLAWAT and HARRINGTON 2009). A significant increase in larval development time, compared to control levels (1.0 mg food/larva/day), was first observed for larvae provided with 0.25 mg food/larva/day ($P < 0.01$, *t*-test). Development time was prolonged further at 0.1 mg/larva/day and 0.01 mg/larva/day (both $P < 0.001$). Wing size was also altered compared to controls, by a nutrient level of 0.25 mg/larva/day ($P < 0.002$). Further reductions in larval nutrition to 0.1 mg and 0.01 mg ($P < 0.001$) resulted in progressively smaller mosquitoes. An increase in development time at a constant temperature was therefore associated with smaller adult body size.

The effect of Wolbachia infection on mosquito fitness parameters was assessed under varying nutrient levels and densities. As shown in Table 2, both Wolbachia-infected (AOC1) and uninfected (C1) mosquitoes reared with high nutrient levels had a reduced larval development time at a higher density. In the ANOVA on the overall data, there were significant effects of nutrition [$F_{(1,1332)} = 5730.92$, $P < 0.001$] and density [$F_{(1,1332)} = 46.40$, $P < 0.001$], but not Wolbachia [$F_{(1,1332)} = 2.15$, $P > 0.05$]. However, there was a significant interaction between nutrition and Wolbachia [$F_{(1,1332)} = 14.11$, $P < 0.001$]. A high nutrient level and low density conditions extended the development time of Wolbachia-infected larvae relative to uninfected larvae, while a low nutrient level and high density conditions decreased it. When an ANOVA was carried out on development time under the low nutrition treatment, there was a significant effect of Wolbachia [$F_{(1,623)} = 6.42$, $P = 0.012$] and also density [$F_{(1,623)} = 20.26$, $P < 0.001$], but no interaction between these terms. Under the high nutrition treatment, there was a significant effect of Wolbachia [$F_{(1,709)} = 19.74$, $P < 0.001$] and also density [$F_{(1,709)} = 71.15$, $P < 0.001$], but again no interaction between these terms.

For wing size measured under low nutrition conditions, Wolbachia did not have a significant effect overall

TABLE 1

ANOVA on *Aedes aegypti* egg hatch proportions (arcsine square-root transformed) testing the effects of temperature, humidity, and infection status (status) as factors; days of treatment (days) as a linear covariate; and population (pop) as a nested random effect within infection status

Source	d.f.	d.f. error	SS	*F*-value	*P*-value
Temperature	2	32.6	0.104	0.820	0.449
Humidity	3	59.1	0.423	2.387	0.078
Status	1	4.4	7.007	32.802	0.004**
Days	1	561	9.239	188.313	<0.001***
Pop (status)	3	7.7	2.097	4.806	0.036*
Temperature × status	2	32.6	0.280	2.201	0.127
Temperature × days	2	561	0.996	10.147	<0.001***
Pop (status) × temperature	6	561	0.640	2.175	0.044*
Humidity × status	3	9	1.471	5.588	0.019*
Humidity × days	3	561	0.021	0.142	0.935
Pop (status) × humidity × days	9	561	0.790	1.789	0.068
Status × days	1	561	0.776	15.810	<0.001***
Temperature × status × days	2	561	1.365	13.910	<0.001***

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

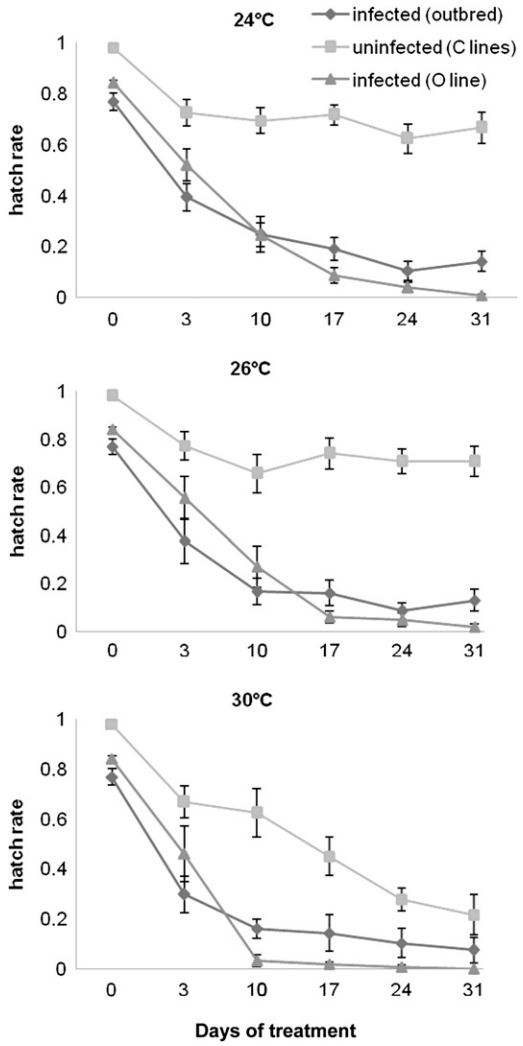

Figure 3.—Change in hatch rate of eggs of *Aedes aegypti* with number of days of treatment at three temperatures. Humidities were combined in these graphs because of the weak effects detected for humidity in the analyses (see Table 1). Error bars represent standard errors.

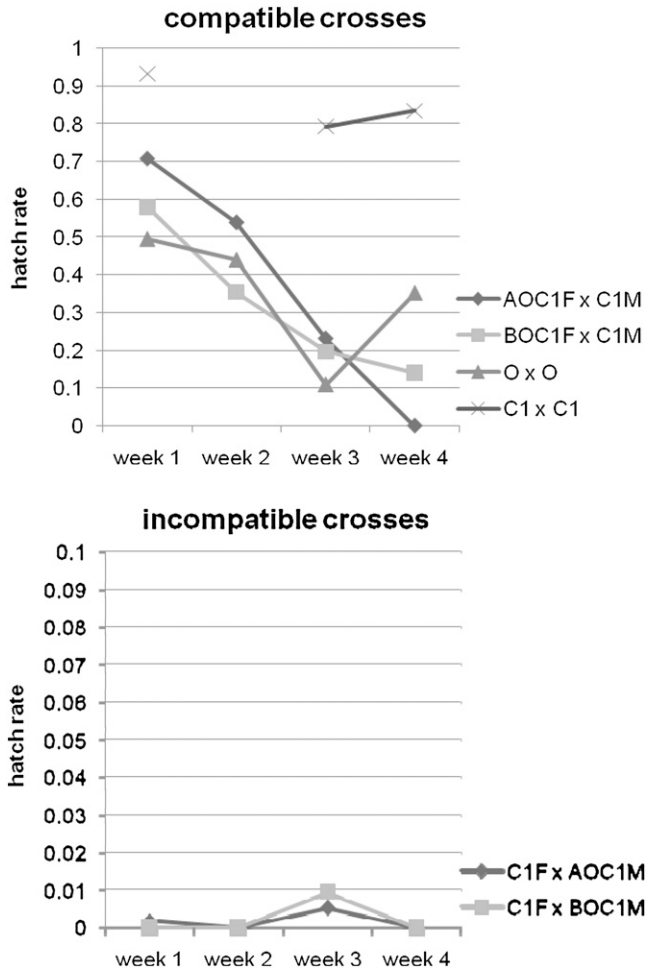

Figure 4.—Changes in hatch rate of *Aedes aegypti* in compatible and incompatible crosses in C1 lines with females of different ages.

[$F_{(1,14)} = 1.83$, $P = 0.197$], but there was an interaction between sex, density, and Wolbachia [$F_{(1,278)} = 6.84$, $P = 0.009$]. Wing size was generally greater in Wolbachia-infected males as shown in Table 3. At a high density, there was an overall effect of Wolbachia on wing size [$F_{(1,13.8)} = 17.7$, $P = 0.001$]. This is largely due to infected males being much larger than uninfected males at both high and low nutrient levels (Table 3). However, at a high density, infected females at low nutrient levels were relatively smaller than uninfected females.

Under low density/high nutrient conditions typically used in the laboratory, a 50% reduction in the life span of adult mosquitoes was observed for *w*MelPop-CLA–infected females (Figure 5) with a median longevity of 24 days, compared with 46 days for uninfected mosquitoes. The adult female life span was also significantly reduced in *w*MelPop-CLA–infected mosquitoes in the other treatments. No differences were observed in the mean or median longevity of Wolbachia-infected AOC1 females or males under different nutrient or density conditions (Figure 5). Differences between infected and uninfected lines were always significant by Cox regression analyses. These results suggest that larval nutrition and density did not affect the extent to which adult life span was reduced in Wolbachia-infected *Ae. aegypti.*

Unstable equilibrium frequency: Turelli (2010) used the parameters $v_U = 0.850$, $v_I = 0.752$, $F_U = 0.269$, $F_I = 0.255$, $s_h = 1$, and $\tau_I = \tau_U = 19$, which yields $\lambda_I = 1.001$ and $p = 0.425$. These are laboratory estimates based on McMeniman *et al.* (2009) scaled down to simulate field conditions. Turelli (2010) estimated the unstable point for invasion of a population with overlapping generations by *w*MelPop-CLA to be 0.425. Thus, to ensure invasion, *w*MelPop-CLA–infected mosquitoes need to be released in sufficient numbers such that their overall frequency in a population becomes >0.425. Here, we assume perfect CI ($s_h = 1$) and perfect maternal transmission, as well as daily survival rates (v_U, v_I) and fecundity (F_U, F_I) used by Turelli (2010).

TABLE 2

Mean larval development time (days) with standard errors for *w*MelPop-CLA–infected (AOC1) and uninfected (C1) populations of *Aedes aegypti* at two levels of nutrition and density

Nutrition	Density	Strain	Larval development time (days)
Low	Low	AOC1	28.77 ± 0.72
		C1	29.32 ± 0.55
	High	AOC1	24.89 ± 0.61
		C1	27.57 ± 0.63
High	Low	AOC1	8.77 ± 0.09
		C1	8.27 ± 0.08
	High	AOC1	7.94 ± 0.12
		C1	7.59 ± 0.08

Low nutrition, 0.05 mg food per larva per day; high nutrition, 0.25 mg food per larva per day; low density, 1 larva to every 20 ml water; high density, 1 larva to every 1 ml water.

TABLE 3

Mean wing centroid size (millimeters) with standard errors for *w*MelPop-CLA–infected (AOC1) and uninfected (C1) populations of *Aedes aegypti* at two levels of nutrition and density

			Wing centroid size (mm)	
Nutrition	Density	Strain	Males	Females
Low	Low	AOC1	1.443 ± 0.018	1.786 ± 0.030
		C1	1.419 ± 0.019	1.702 ± 0.036
	High	AOC1	1.499 ± 0.014	1.774 ± 0.025
		C1	1.416 ± 0.011	1.829 ± 0.025
High	Low	AOC1	1.765 ± 0.014	2.211 ± 0.020
		C1	1.711 ± 0.010	2.142 ± 0.025
	High	AOC1	1.755 ± 0.013	2.210 ± 0.019
		C1	1.705 ± 0.009	2.180 ± 0.027

Low nutrition, 0.05 mg per larva per day; high nutrition, 0.25 mg per larva per day; low density, 1 larva to every 20 ml water; high density, 1 larva to every 1 ml water.

Considering our data for suboptimal nutrient and high density conditions (Table 2), $\tau_I = 31$ and $\tau_U = 33$. This includes an additional 3 days for pupal development and 3 days for female refractoriness to insemination. During the wet season, with frequent rainfall, F_I remains the same at 0.255, so that $\lambda_I = 1.00079$ and $p = 0.425$, which is close to the estimate of Turelli (2010). However, in the dry season, with 17 days of no rainfall, F_I decreases to 0.045 and 0.06, while F_U decreases to 0.202 and 0.188 at 26° and 24°, respectively due to the decreasing hatch rate of diapause eggs (Figure 3), giving rise to $\lambda_I = 0.952885$ and 0.960601, and thus $p = 0.896$ and 0.844, respectively. If we assume $\tau_I = \tau_U = 19$ as in Turelli (2010), then for a dry season of 17 days of no rainfall, $\lambda_I = 0.923942$ and 0.934973, and thus $p = 0.898$ and 0.845 at 26° and 24°, respectively. Similar estimates are obtained at low nutrient and low density conditions.

On the basis of the age-dependent survival and fecundity model, at a low nutritional level and high density ($\tau_I = 31, \tau_U = 33$) and for a period with constant rainfall, $\lambda_I = 1.037172$ and $p = 0.466$, which is slightly higher than for the field-simulated age-independent model but still <0.5. Whereas for periods of 17 days without rainfall, $\lambda_I = 0.986141$ and 0.993308, with $p = 0.936$ and 0.895 at 26° and 24°, respectively. See File S1 to access the raw data.

DISCUSSION

We have trialed two backcrossing schemes for generating *w*MelPop-CLA–infected *Ae. aegypti* lines to increase their field fitness and reduce the effects of laboratory adaptation. The genetic markers indicate that both methods increased genetic diversity in the *w*MelPop-CLA line of *Ae. aegypti* and homogenized their genetic background with the two Cairns wild populations. While we did not directly test for laboratory adaptation, scheme B will potentially decrease laboratory adaptation more than scheme A, because of a reduced opportunity for selection in the laboratory.

Comparison with previously published *w*MelPop-CLA estimates in *Ae. aegypti*: Our parameter estimates are similar to those obtained in earlier work on laboratory strains. McMeniman *et al.* (2009) found that *w*MelPop-CLA decreased longevity by ~50% for females, which is similar to the difference between uninfected and infected mosquitoes recorded here when housed under different nutrition levels and densities. Our results also show that the effect of *w*MelPop-CLA on longevity appears to be robust regardless of experimental conditions. Even at low nutrition levels and high densities, the virulence of *w*MelPop-CLA is not compromised, which means that *w*MelPop-CLA modifies the age structure of *Ae. aegypti* regardless of environmental effects.

McMeniman *et al.* (2009) recorded strong CI (2 hatched eggs of 4400 tested or 0.05%) and we also found a hatch rate of <1% in incompatible crosses. Maternal transmission in an outbred strain was estimated at 99.4 and 99.7% in the earlier work, which compares with the >99% transmission rate estimated here. Wolbachia longevity effects, cytoplasmic incompatibility, and maternal transmission may therefore not be altered substantially through placing *w*MelPop-CLA in an outbred background. Transmission rates may also be high in the field, as demonstrated for Wolbachia infections in *Culex pipiens* (Rasgon and Scott 2003).

Other fitness effects: We found that the *w*MelPop-CLA infection caused host effects that may influence its spread in mosquito populations. First, the infected lines suffered from a sharp reduction in egg hatch rates as eggs were aged, whereas hatch rates of uninfected eggs

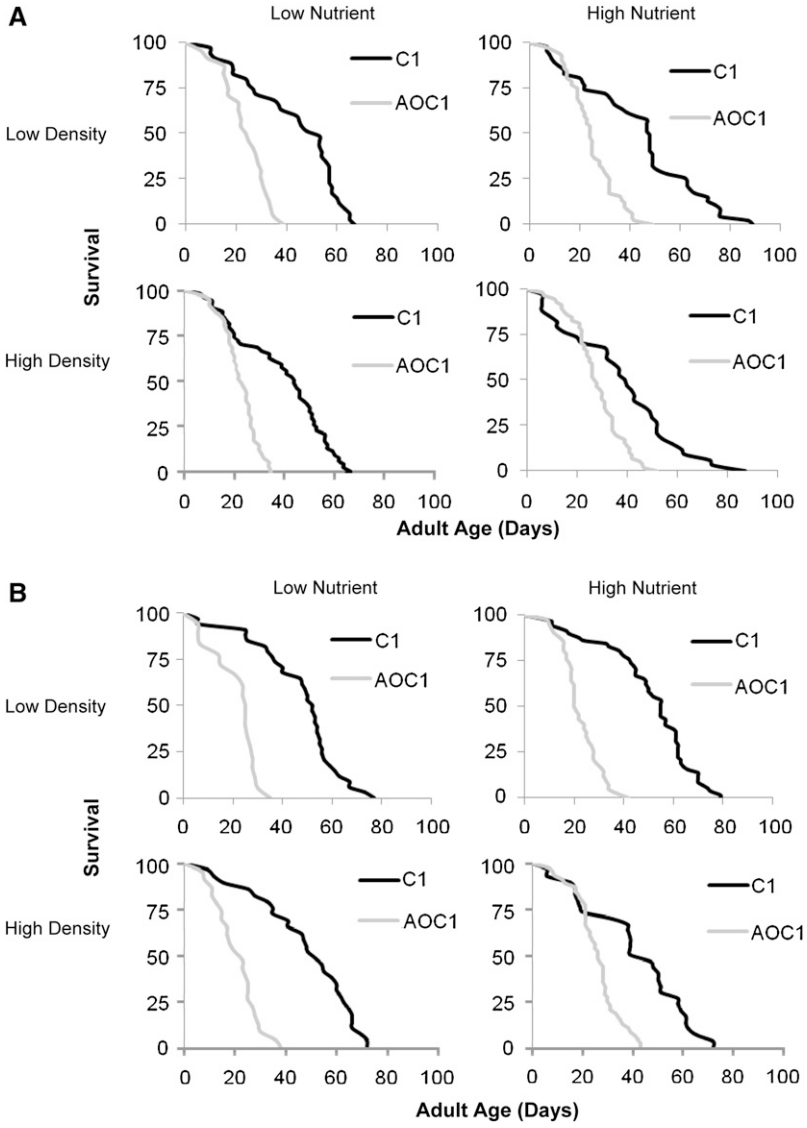

FIGURE 5.—Adult life span of male (A) and female (B) *Aedes aegypti* reared under varying larval nutrition and densities. Shaded lines, infected AOC1 line; solid lines, uninfected C1 line.

were only weakly affected except at a high temperature. There was a particularly sharp decline of hatch rates at 18° as noted previously (McMeniman and O'Neill 2010). This could lead to infected individuals being at a large fitness disadvantage in situations where eggs dry out and need to persist until hatching is induced when containers are refilled. *Ae. aegypti* breed in a variety of containers in North Queensland including roof gutters, garden accoutrements, discarded household items, rubbish, subsoil drain sumps, and rainwater tanks (Montgomery and Ritchie 2002; Montgomery *et al.* 2004; Kearney *et al.* 2009). This mix of containers shifts between the wet and dry seasons (Montgomery and Ritchie 2002). Models suggest that egg desiccation acts as a key trait in limiting distribution and abundance of *Ae. aegypti* in Australia (Kearney *et al.* 2009). Because of the potentially large fitness costs due to a low hatch rate, it is likely that an increase in Wolbachia infection frequency in the wet season would be countered by a reduction in frequency in the dry season, particularly near the distribution margins of the mosquito.

Second, we found a reduction in hatch rate of eggs produced by older females. This also concurs with observations in McMeniman and O'Neill (2010). Previous experiments demonstrated that *w*MelPop-CLA mosquitoes aged for several weeks suffer from a reduction in biting ability and movement (Evans *et al.* 2009; Turley *et al.* 2009). The reduction in hatch rate in eggs from older females may be related to the fact that older infected females are unable to acquire nourishment for egg synthesis due to lower biting ability (Turley *et al.* 2009) or perhaps through an increased density of Wolbachia in eggs from older mothers. In contrast, the hatch rate of eggs from older uninfected females remained high.

Third, we detected effects of the infection on larval development time. Under low nutrition levels, there was a delay in development time in both infected and uninfected mosquitoes. However, infected larvae de-

veloped relatively faster than uninfected larvae, especially at high larval density. The faster development time is likely to increase fitness (and overall rate of population growth) because it allows larvae to complete development before a container dries out. This positive effect may reflect a nutritional mutualism between Wolbachia and the host, which has been demonstrated for iron metabolism in *D. melanogaster* (Brownlie *et al.* 2009). Positive effects have also been reported in closely related naturally Wolbachia-infected *Ae. albopictus*, which confers increased fecundity (Dobson *et al.* 2004). In contrast, at high nutrition levels, the uninfected mosquitoes developed approximately half a day faster than infected mosquitoes.

Infected mosquitoes tended to be larger than uninfected ones except when females were reared at a high density. Larger males are known to inseminate females with more sperm, which may improve their chances of fertilization, while larger females may have a higher chance of being inseminated (Ponlawat and Harrington 2009). However, the spread of Wolbachia may be compromised under high density conditions due to a relatively lower rate of insemination of infected females.

These findings suggest that infection effects on larval fitness will depend on nutrition and density levels in the field. Nutrition levels in breeding containers are likely to be variable; exposed containers usually have lower nutrition levels compared to shaded containers and/or containers that collect fallen foliage (Tun-Lin *et al.* 2000). However, field mosquitoes tend to be smaller than laboratory-maintained populations, which may reflect low nutrition levels in the field, providing conditions where infected mosquitoes develop faster, but have a smaller body size.

Predicted population dynamics: When we assumed that there were 17 days of no rainfall, $\lambda_I < 1$, implying that the *w*MelPop-CLA–infected *Ae. aegypti* population will decline in numbers each generation and inevitably crash. Also, because $p \gg 0.5$, the infection is unlikely to spread in the population during the dry season (Turelli 2010). Therefore, once eggs need to persist through a period of drying, spread of the *w*MelPop-CLA infection in populations is unlikely. In contrast, in the wet season, $\lambda_I > 1$ and $p < 0.5$ regardless of whether this value is calculated with or without an age-dependent function, which suggests that infected *Ae. aegypti* can persist independently without population supplementation and may spread during humid conditions. The models used here do not allow for Wolbachia-induced positive fitness effects such as limiting infection of the host by other pathogens (Hedges *et al.* 2008; Kambris *et al.* 2009; Moreira *et al.* 2009a).

Information about fitness effects of *w*MelPop-CLA on *Ae. aegypti* helps to identify conditions when the infection is most likely to invade populations. Uncertainties remain around the likely impact of a shorter life span in the field (Rasgon and Scott 2004; Scott *et al.* 2008) as well as potential attenuation of *w*MelPop-CLA. These effects should be pursued through careful monitoring once releases have taken place. Augmentative releases of infected mosquitoes and prerelease suppression of field populations via insecticide are likely to boost the chances of a successful Wolbachia invasion. We also suggest using backcrossing with the genetic background of target field populations to minimize risk of failure due to unfavorable laboratory adaptation.

The deleterious effects may be utilized as a radical direct suppression of mosquito populations (McMeniman and O'Neill 2010). For example, if Wolbachia infection in a genetically isolated location is approaching fixation or fixed before the onset of the dry season, then population extinction might occur during the dry season. In this case, there might not be time for evolution of attenuation of *w*MelPop-CLA effects based on Drosophila models (Carrington *et al.* 2009). Any ecological impact of a reduction in numbers of mosquitoes should be examined prior to using this approach.

Overall, our data provide parameter estimates for predicting invasion by a Wolbachia infection for dengue suppression when placed on an outbred background. Outbreeding ensures that release material has a genetic background that matches the target population. Our parameter estimates suggest that *w*MelPop invasion is possible under humid conditions. However, because of deleterious effects associated with *w*MelPop-CLA, invasion under dry conditions will be difficult, so it will be important to explore other Wolbachia strains that may suppress dengue in mosquitoes (Moreira *et al.* 2009a), particularly if they have lower unstable equilibrium points.

The research conducted in this article required human volunteers for mosquito blood feeding as approved by the University of Melbourne Human Ethics Committee (Approval 0723847) and the University of Queensland Human Ethics Committee (Approval 2007001379). This research was funded by a grant from the Foundation for the National Institutes of Health as part of the Grand Challenges in Global Health Initiative of the Bill and Melinda Gates Foundation. A.A.H. was supported by an Australian Laureate Fellowship from the Australian Research Council.

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Communicating editor: C. H. Langley

GENETICS

Supporting Information
http://www.genetics.org/cgi/content/full/genetics.110.122390/DC1

Dynamics of the "Popcorn" Wolbachia Infection in Outbred *Aedes aegypti* Informs Prospects for Mosquito Vector Control

H. L. Yeap, P. Mee, T. Walker, A. R. Weeks, S. L. O'Neill, P. Johnson, S. A. Ritchie, K. M. Richardson, C. Doig, N. M. Endersby and A. A. Hoffmann

DOI: 10.1534/genetics.110.122390

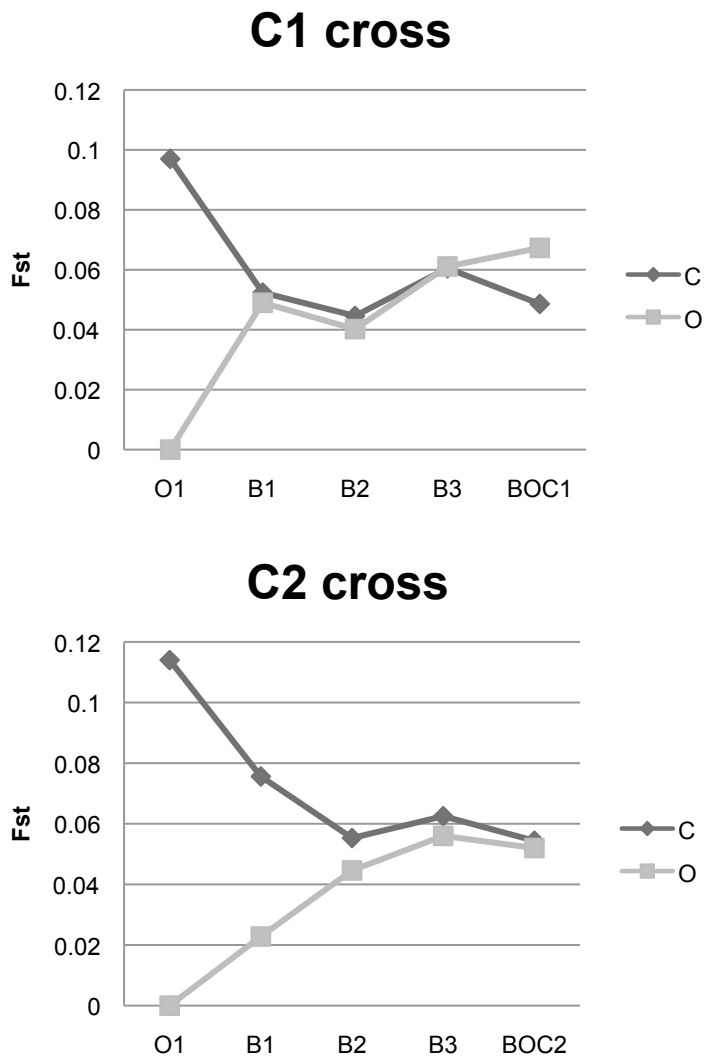

FIGURE S1.—Effect of successive generations of backcrossing of *Aedes aegypti* under Scheme B on genetic divergence between lines at microsatellite markers. Means for the C1/C2 derived lines and O line are shown.

FILE S1

Supporting Data

File S1 is available for download as compressed folder (.zip) at http://www.genetics.org/cgi/content/full/genetics.110.122390/DC1.

- Egg viability data.xlsx – Egg Viability Data
- Maternal Transmission.xls – Maternal Transmission Data
- Larval development time.xlsx – Larval Development Time Data
- General Longevity Organised_Figure2.xlsx – Longevity Data
- Molecular Marker_Dataset.xlsx – Molecular Marker Data
- Longevity_Density&Nutrition_Levels.xlsx – Longevity with Density Nutrition Data
- Cytoplasmic_Incompatibility.xlsx – Cytoplasmic Incompatibility Data
- Genetic differences developed.xlsx – Genetic Difference Data
- Wing_Size.xlsx – Wing Size Data

TABLE S1

Genetic variability parameters for the *Ae. aegypti* populations generated under Scheme A at backcross generation 3 (B3) and Scheme B at backcross generation 1 to 3 (B1 to B3) as well as parental lines.

Population	A_p	H_E	H_O	F_{IS}	F_{IS} (*P* value)	HW (*P* value)
C1 F3	3.429	0.479	0.474	0.009	0.4640	0.734
C2 F3	3.250	0.458	0.462	-0.009	0.6010	0.448
O1 G35	3.143	0.433	0.408	0.057	0.1770	0.178
AOC1 (B3)	3.375	0.487	0.543	-0.117	0.9950	0.997
AOC2 (B3)	3.875	0.447	0.440	0.015	0.3970	0.408
BOC1 (B1)	3.714	0.578	0.651	-0.129	0.9940	0.995
BOC2 (B1)	4.000	0.498	0.510	-0.024	0.7028	0.728
BOC1 (B2)	4.000	0.564	0.614	-0.092	0.9739	0.975
BOC2 (B2)	4.143	0.547	0.543	0.008	0.4722	0.615
BOC1 (B3)	3.714	0.541	0.511	0.056	0.1557	0.147
BOC2 (B3)	3.857	0.511	0.517	-0.013	0.5696	0.485
BOC1	3.429	0.549	0.586	-0.070	0.8986	0.942
BOC2	4.000	0.556	0.576	-0.037	0.7966	0.785

A_p is the mean number alleles per polymorphic locus; H_E is the expected heterozygosity; H_O is the observed heterozygosity; F_{IS} is the inbreeding coefficient or fixation index and its *P* value is randomized and adjusted; and HW (*P* value) is the probability for Hardy-Weinberg Equilibrium test.

TABLE S2

Analysis of Molecular Variance (AMOVA) comparing *Aedes aegypti* population subgroups

Source of variation	df	Sum of squares	Variance components	Fixation Indices	Percentage of Variation	*P*-value
A						
Among subgroups	3	48.102	0.067	0.03482	3.48 (F_{ct})	<0.010
Among populations within subgroups	8	36.185	0.046	0.02467	2.38 (F_{sc})	< 0.001
Within populations	700	1267.275	1.810	0.05863	94.14 (F_{ST})	< 0.001
B						
Among subgroups	1	6.743	0.011	0.00571	0.57 (F_{CT})	0.110
Among populations within subgroups	6	25.018	0.039	0.02062	2.05 (F_{SC})	< 0.001
Within populations	464	862.809	1.860	0.02622	97.38 (F_{ST})	< 0.001

A – AMOVA across eight markers for each *Ae. aegypti* population subgroup consisting of uninfected parental populations C1 and C2 (at the F3 stage), uninfected populations C1 and C2 (at the F6 stage), and backcross populations under Scheme A and Scheme B.

B – AMOVA across eight markers for each of the two population subgroups (*Ae. aegypti* backcross populations developed under Scheme A and Scheme B).