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# Fitness of *w*AlbB *Wolbachia* Infection in *Aedes aegypti*: Parameter Estimates in an Outcrossed Background and Potential for Population Invasion

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*Abstract.* *Wolbachia* endosymbionts are potentially useful tools for suppressing disease transmission by *Aedes aegypti* mosquitoes because *Wolbachia* can interfere with the transmission of dengue and other viruses as well as causing deleterious effects on their mosquito hosts. Most recent research has focused on the *w*Mel infection, but other infections also influence viral transmission and may spread in natural populations. Here, we focus on the *w*AlbB infection in an Australian outbred background and show that this infection has many features that facilitate its invasion into natural populations including strong cytoplasmic incompatibility, a lack of effect on larval development, an equivalent mating success to uninfected males and perfect maternal transmission fidelity. On the other hand, the infection has deleterious effects when eggs are held in a dried state, falling between *w*Mel and the more virulent *w*MelPop *Wolbachia* strains. The impact of this infection on lifespan also appears to be intermediate, consistent with the observation that this infection has a titer in adults between *w*Mel and *w*MelPop. Population cage experiments indicate that the *w*AlbB infection establishes in cages when introduced at a frequency of 22%, suggesting that this strain could be successfully introduced into populations and subsequently persist and spread.

## INTRODUCTION

*Wolbachia* bacteria are endosymbionts of insects that form the basis of novel approaches for suppressing disease transmission by mosquitoes. In particular, *Wolbachia* from *Drosophila* transferred to *Aedes aegypti* mosquitoes are being utilized in current releases aimed at suppressing dengue transmission.[1,2] The main reason for this suppression comes from the fact that the presence of the bacteria reduces virus titer, particularly in tissues that the virus needs to invade for transmission between people.[3,4] In addition, *Wolbachia* can influence disease transmission by causing deleterious effects on its host and triggering embryo mortality or cytoplasmic incompatibility (CI) when *Wolbachia*-infected males mate with uninfected females, potentially reducing vector population size.[5,6] Effects on viral transmission interference and host fitness may last if the *Wolbachia* infection reaches a high and stable frequency in host populations after invasion, which has now been achieved in some field populations of *Ae. aegypti*.[7]

The ability of *Wolbachia* strains to generate viral blockage and influence host fitness depends on the nature of the *Wolbachia* strain and background host genome.[4,8,9] This has become evident from research in *Drosophila* where multiple combinations of hosts and *Wolbachia* have now been generated and challenged with viruses.[10,11] So far, in *Ae. aegypti* mosquitoes, three *Wolbachia* strains have been stably introduced and characterized: the *w*Mel and *w*MelPop infections from *Drosophila melanogaster*[12,13] and the *w*AlbB infection from *Aedes albopictus*.[14] As in *Drosophila*, there are likely to be differences in fitness effects and blockage exhibited by these strains, which requires the strains to be compared in the same genetic background. The *w*MelPop strain generates very high viral blockage, whereas blockage by *w*Mel is somewhat weaker.[4,13] Blockage by *w*AlbB appears to be strong,[15] but a direct comparison to the other strains has not yet been undertaken. The *w*MelPop infection generates large deleterious effects on adult longevity, egg viability particularly after quiescence, larval viability under high density, and other traits.[12,16,17] These deleterious effects are either much weaker or absent in *w*Mel.[13] Less comprehensive data for *w*AlbB also suggest limited deleterious effects on a genetic background different from that used in experiments with *w*Mel and *w*MelPop.[14] The magnitude of deleterious effects as well as viral blockage may be partly related to bacterial titer and tissue distribution, with the *w*MelPop infection occurring at a higher titer and having a wider tissue distribution than *w*Mel.[13]

Although strong viral blockage is clearly desirable from the perspective of curtailing disease transmission, large deleterious effects may be sufficient to prevent *Wolbachia* from invading into wild *Aedes* populations[16] and subsequently spreading into surrounding areas.[18] Invasion of uninfected populations depends on the process of CI, where infected males cause the death of embryos or immature stages when they mate with uninfected females (or females carrying an incompatible *Wolbachia* strain). Although CI is very strong for all three *Wolbachia* infections introduced into *Ae. aegypti* albeit with different genetic backgrounds,[12–14] the presence of large deleterious effects can mean that invasion by *w*MelPop is difficult because of a high invasion threshold, particularly in the dry season when eggs have to persist in a quiescent phase.[6] This is borne out by the difficulty of achieving invasion by this infection in semi-field cages[13,19] and in field releases into relatively isolated areas.[20] On the other hand, invasion by *w*Mel has now been repeatedly achieved, and results in high and stable frequencies.[7] The *w*AlbB infection has not yet been used for invasion in field releases although it has been shown to invade small laboratory population cages.[14]

In this article, we consider the three infections for fitness effects on a common genetic background with the aim of comparing their invasibility and also potential use in population suppression.[8] We focus particularly on the *w*AlbB infection that has not been characterized in detail previously, and contrast its host effects and bacterial density with that of the *w*Mel and *w*MelPop infections. We also provide estimates of

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maternal transmission and CI to help assess the likely frequency of *w*AlbB in natural populations after invasion.

## MATERIALS AND METHODS

**Colony maintenance.** *Aedes aegypti* infected with *w*AlbB, *w*Mel, or *w*MelPop were reared in a laboratory at 26 ± 1°C with a 12:12-hour (day:night) photoperiod, which included 1-hour dusk/dawn periods. Colonies of 450–500 adults (not differing significantly from a 1:1 sex ratio) were housed in 19.7-L BugDorm-1® cages (MegaView Science Co., Ltd., Taichung City, Xitun District, Taiwan), covered with plastic bags to maintain high humidity (~85%). Adults were provided with access to a 10% sucrose solution supplied by capillary action through a cotton wool–braided cord (7 × 0.5 cm) inserted through the lid of a 30-mL cup. Females were blood-fed by human volunteers for 15 minutes, 8 days after eclosion, to allow maturation and fertilization. Females oviposited on Norton® Master Painters P80 sandpaper (3.8 × 18 cm; Saint -Gobain Abrasives Pty. Ltd., Thomastown, Victoria, Australia) for routine maintenance or conical filter paper (Whatman® qualitative circles—15 cm Ø; GE Healthcare Australia Pty. Ltd., Parramatta, New South Wales, Australia) lining the inside of a plastic cup containing 150 mL reverse osmosis (RO) water. Eggs were conditioned by removing excess moisture with paper towel for 30 seconds on the second day post-oviposition, and then almost completely dried on the third day. Egg strips were then sealed in plastic zip-lock bags with a moist paper towel square (2 × 2 cm) to prevent desiccation. Egg hatching occurred in RO water (3 L for colony maintenance, but see specific methods for experimental volumes), deoxygenated with active dried yeast to stimulate hatching (~0.02 mg/L), and containing crushed TetraMin® fish food tablets (Tetra, Melle, Germany; hereafter referred to as hatching water). Immature stages were fed *ad libitum* with the fish food. Colonies were maintained by controlling the density of second instar larvae at 450–500 individuals per 4 L of RO water using a glass pipette and clicker counter. Colony pupae were collected 5 days later into 500 mL fresh RO water and placed in 19.7-L BugDorm-1 cages for eclosion. Colonies were routinely screened for *Wolbachia* to confirm their infection status (see section *Wolbachia* detection and primers) and maintained at a size of several hundred individuals.

*Aedes aegypti* strains had been transinfected with the *w*AlbB, *w*Mel, and *w*MelPop strains of *Wolbachia* by embryonic microinjection as described elsewhere.[12–14] However, both the *w*Mel and *w*MelPop cultures used in the experiments had been sourced from field material subsequent to releases.[16] Colonies infected with each strain were maintained in our laboratory alongside an uninfected colony (CNS), sourced from eggs oviposited around Cairns. Host nuclear background effects[9] were controlled by backcrossing[16] females from all infected lines to CNS males for six generations before experimentation.

**Fecundity.** Reductions in female fecundity because of the *w*AlbB infection were tested relative to uninfected CNS females. Colonies were blood-fed by a single human volunteer. Twenty engorged females from infected and uninfected colonies were then isolated into 70-mL specimen cups with mesh lids using an aspirator. Access to a 10% sucrose solution was provided through a soaked cotton wool bud placed on the mesh. Cups were lined with a sand paper strip (2 × 12 cm, see section Colony maintenance) and filled with 20 mL water from larval rearing trays to promote oviposition. Eggs were collected daily and counted by eye under a dissecting microscope using a clicker counter.

**Quiescent egg viability.** The long-term viability of *w*AlbB-infected eggs in quiescence was assessed in comparison to eggs from the uninfected CNS colony. Eggs were collected daily en masse for 4 days from each line on a filter paper substrate (see section Colony maintenance). On the third day post-oviposition (day 0), filter papers were stored in a plastic environmental chamber, sealed with Blu-Tack (Bostik, Thomastown, Victoria, Australia). Relative humidity (RH) inside the chamber was maintained at 75% using a saturated solution of sodium chloride in a cup, which was monitored for 1 week with a hygrochron (1-wire; iButton.com) before the introduction of eggs. Ten replicate batches of at least 25 eggs from each line were hatched at days 0, 3, 10, 17, 24, 31, 61, 90, and 124. All batches were captured with a digital camera just before hatching and eggs were counted using the Cell Counter plugin[21] in ImageJ.[22] To avoid underestimating viability, eggs that hatched early (egg cap clearly detached) before immersion were removed from the analysis. Batches were immersed in plastic cups containing 140 mL hatching water (see section Colony maintenance). After 6 days, all individuals (dead or alive) were counted using a glass pipette and clicker counter.

**Larval development time, survival, and adult body size.** To test for any effects of *Wolbachia* on immature development, we reared cohorts of *w*AlbB-infected and uninfected CNS larvae under both high- and low-stress conditions. Eggs from both lines were submerged synchronously in RO water, and first instar larvae were added to treatments within 6 hours of hatching. Cohorts of 200 *w*AlbB-infected or 200 uninfected larvae were reared independently in either 4,000 mL (low density, one larva per 20 mL) or 200 mL (high density, one larva per 1 mL) of RO water. Containers were provided with either 0.25 mg per larva (high nutrition) or 0.05 mg per larva (low nutrition) of crushed TetraMin tablets daily. Each combination of density, nutrition, and *Wolbachia* infection status was replicated six times.

Cohorts were monitored to determine mean development time, survival, and adult body size. Pupae were transferred to separate containers of RO water as they appeared, and emerging adults were collected twice daily, in the morning and evening, and stored in absolute ethanol. Adults that emerged around the median development time for a particular level of nutrition and density were measured for their wing length to provide an estimate of body size.[23,24] At least 25 males and 25 females sampled across all containers were measured for each treatment. The right wing was dissected from each adult and fixed under a 10-mm coverslip with Hoyer's solution (distilled water:gum arabic:chloral hydrate: glycerin in the ratio 5:3:20:2).[25] The distance between the alular notch and the intersection of the radius 3 vein and outer margin (excluding the fringe scales) provided a measure of wing length.[20] Two independent measurements of each wing were averaged to give the final length. Damaged or folded wings were not measured.

**Adult survival in groups.** Adult survival of *w*AlbB, *w*Mel, and CNS was assayed using groups of 50 individuals (1:1 sex ratio), replicated eight times. Pupae were sexed and added to 25 mL of RO water in plastic cups and allowed to eclose in 3-L plastic containers with stocking lids and mesh sides. A filter paper oviposition site and 10% sucrose solution were provided and refreshed twice a week (see section Colony maintenance).

To prevent desiccation, containers were maintained at high humidity (~85% RH) in white plastic garbage bags. Females were blood-fed weekly for the duration of the experiment, and mortality was scored for males and females three times a week until at least 50% of females from the longest surviving line had died.

**Mating.** The mating success of *w*AlbB males competing with CNS males for mates was estimated. Infected and uninfected males were established in 19.7-L cages before the introduction of 80 virgin CNS females in the following groups: 1) 80 CNS males (negative control, 0% infected), 2) 40 *w*AlbB and CNS males (treatment, 50% infected), and 3) 80 *w*AlbB males (positive control, 100% infected). Each group was replicated five times. Males and females were allowed to mature for 1 week before release. During the release, cages were tapped to ensure males were distributed throughout the cage. Mosquitoes were left to mate for 1 week before providing females with a blood meal. Eggs were collected en masse. Because *Ae. aegypti* females are known to exhibit skip oviposition,[26,27] three sandpaper oviposition sites per cage were provided (see section Colony maintenance). Eggs were collected 2 and 4 days later, and counted digitally on the third day post-oviposition using a Canon LiDE 110 flatbed scanner (Canon Australia, Macquarie Park, New South Wales, Australia) and the Cell Counter plugin[21] in ImageJ.[22] Egg strips were hatched separately within 1 week of oviposition in 3 L hatching water (see section Colony maintenance) and all individuals were counted 6 days later using a glass pipette and clicker counter.

**Cytoplasmic incompatibility.** The *w*AlbB line was reciprocally crossed with CNS to test for CI. Reciprocal crosses between *w*AlbB and *w*Mel were included to test for bidirectional incompatibility. Incompatible crosses involved *w*AlbB males mated to *w*Mel or CNS females (or *w*Mel males mated to *w*AlbB females for the bidirectional test). Compatible crosses were performed between *w*AlbB females and CNS males and within each infected or uninfected parental line. All crosses were replicated eight times.

For each cross, 14 pupae were sexed (1:1 sex ratio) and added to 20 mL of RO water in 30-mL cups. Pupae eclosed within 1.5-L plastic containers with mesh sides and covered with a stocking. Adults were provided with access to a 10% sucrose solution (see section Colony maintenance). Adults were left to mate for 1 week; they were then fed, and 3 days later they were provided with a filter paper oviposition site. Eggs were collected daily over 4 days, photographed with a digital camera and counted in ImageJ[22] using the Cell Counter plugin[21] to determine the number of eggs. Within 1 week of embryonation, eggs were hatched in plastic containers with 500 mL hatching water (see section Colony maintenance). To test for age-related effects on CI, two further blood meals were provided weekly and hatch rates determined for three gonotrophic cycles in total.

**Maternal transmission.** The maternal transmission efficiency of the *w*AlbB infection was estimated by testing the proportion of infected offspring produced by an infected female. Females from the *w*AlbB line were crossed to CNS males en masse and blood fed by a single human volunteer within 1 week of emergence. Engorged females were isolated (see section Fecundity) and later stored in absolute ethanol at 4°C after completing oviposition. Within 1 week of collection, eggs were hatched in plastic trays containing 500 mL hatching water and larvae were reared to adulthood (see section Colony maintenance), then stored in absolute ethanol at 4°C. A minimum of 11 progeny each from 29 females was then tested to detect the presence of the *w*AlbB infection (see section *Wolbachia* detection and primers).

**Population cages.** The unstable equilibrium threshold (UET) is the proportion of infected to uninfected individuals in a population above which a particular strain of *Wolbachia* is likely to spread.[28] A UET of $\hat{p} = 0.15$ for the successful invasion of the *w*AlbB infection was estimated by Xi and others[14] based on an absence of maternal transmission leakage ($\mu = 0$) with perfect CI ($s_h = 1$) and a fitness cost ($s_f$) of 0.15. However, although our experiments have confirmed $\mu = 0$ and $s_h = 1$ (see Cytoplasmic Incompatibility and Maternal Transmission results), $s_f$ seemed to be somewhat lower; the mean relative egg viability for the first month for *w*AlbB was 0.97 with no reduction in fecundity ($s_f = 0.03$, see Fecundity and Quiescent egg viability results), giving rise to a UET lower than 0.15. The null hypothesis chosen for this experiment was therefore $H_0$: $\hat{p} = 0.15$ while the alternative was $H_1$: $\hat{p} = 0.03$. A power function was written in R 3.1.0.[29] to determine the likelihood of invasion given a UET of 0.03 with an initial invasion frequency ($p_0$) of 0.1. An existing model of *Wolbachia* invasion[30] was used within the power test, incorporating sample size stochasticity and discrete generations, which is applicable to laboratory colony cages (see Supplemental Appendix 1). The number of generations was set to 10 and population sizes ($N$) of 200, 400, 600, and 800 were included in the test. The criterion set for successful invasion was 1) the final infection frequency was $> p_0$ and 2) at least two-thirds of the 10 observed generations possessed an infection frequency $> p_0$. On the basis of $H_1$: $\hat{p} = 0.03$, establishing five replicates of 400 individuals (1:1 sex ratio) with $p_0 = 0.1$ and observing invasion within 10 generations was expected to provide sufficient power to reject the null hypothesis while keeping an appropriate type I error rate, $\alpha < 0.05$. For this set up, we set an observation of at least three invasions of five as the critical point of rejection as it has a probability of less than 0.05 if $H_0$: $\hat{p} = 0.15$ is true, but a probability near or above 0.8 if $H_1$: $\hat{p} = 0.03$ is true (see Supplemental Figure 1). We also established five replicate positive control cages with a $p_0$ of 0.22 (22% group) alongside the 10% treatment group.

Separate 12-L cages were populated with male or female pupae from each line. In the 10% group, *w*AlbB males and females totaled 20 each, whereas they totaled 44 each in the 22% group. Sex was confirmed post-eclosion and dead individuals were replaced to ensure that $p_0$ was exactly maintained, leading up to the initial invasion event. Adults matured for 1 week before an exposure of 4°C for 1 minute. Comatose males were combined from *w*AlbB and CNS into 19.7-L BugDorm-1 cages whereas females were combined into 500-mL containers. Once all adults were capable of flight again, females were released into the cages containing the males.

Colonies were maintained following the methods described earlier (see section Colony maintenance). After egg collection was complete, 50 females were sampled from each cage and stored in absolute ethanol at 4°C for *Wolbachia* detection (see section *Wolbachia* detection and primers). Discrete generations were maintained in new cages, which were established by randomly selecting 400 offspring from the previous generation.

***Wolbachia* detection and primers.** Tests for *Wolbachia* infection were conducted using a previously described quantitative real-time polymerase chain reaction assay[7] with the addition of *w*AlbB-specific primers (Table 1). Specific strains were

TABLE 1
Primers used in qPCR

| | Name | Sequence (5′-3′) | Reference |
|---|---|---|---|
| *Aedes* | mRpS6_F | AGTTGAACGTATCGTTTCCCGCTAC | 32 |
| | mRpS6_R | GAAGTGACGCAGCTTGTGGTCGTCC | |
| *Aedes aegypti* | aRpS6_F | ATCAAGAAGCGCCGTGTCG | 32 |
| | aRpS6_R | CAGGTGCAGGATCTTCATGTATTCG | |
| *w*Mel | w1_F | AAAATCTTTGTGAAGAGGTGATCTGC | 32 |
| | w1_R | GCACTGGGATGACAGGAAAAGG | |
| *w*MelPop | *w*Melpop_F | CTCATCTTTACCCCGTACTAAAATTTC | 19 |
| | *w*Melpop_R | TCTTCCTCATTAAGAACCTCTATCTTG | |
| *w*AlbB | *w*AlbB_F | CCTTACCTCCTGCACAACAA | * |
| | *w*AlbB_R | GGATTGTCCAGTGGCCTTA | |

qPCR = quantitative real-time polymerase chain reaction.
*Inaki Iturbe-Ormaetxe, personal communication.

detected based on the combinations of crossing point (*C*p) and melting point ($T_m$) values of the PCR products as determined in a Light Cycler 480 (Roche Applied Science, Castle Hill, New South Wales, Australia). *Wolbachia* load was also determined in 25 females sampled from the *w*AlbB, *w*Mel, and *w*MelPop colonies.

**Analysis.** All data were analyzed and graphed in R 3.1.0.[29] Fecundity was scored as the number of eggs laid per female, and egg viability (or hatch rate) was defined as the proportion of eggs that hatched by counting the number of third or fourth instar larvae. Proportional data were arcsine square root transformed if they failed Shapiro–Wilk tests of normality and tested again. We checked for heteroscedasticity between groups being compared using *F* tests. Depending on whether we could assume equal variance and normality, we used the Student's *t* test and Welch *t* test for comparing means. For data that could not be transformed to meet assumptions of parametric tests, we used nonparametric Mann–Whitney *U* tests.

Development time was calculated as the time in days from hatching to adult emergence while larval survival was defined as the proportion of larvae that reached adulthood. Larval development time, survival to adulthood, and wing length data were analyzed by analysis of variance (ANOVA) and Tukey's honest significant difference post hoc tests or by nonparametric Kruskal–Wallis tests depending on normality. To identify deviations from a 1:1 sex ratio, $\chi^2$ tests were run.

The equality of adult survival curves (separated by sex) was compared using the Cox regression procedure. Replicates were initially compared, and replicates detected as significant outliers were removed from the analysis. Strains were then compared for pooled data. CNS was set as the baseline, thus the hazard ratio ($e^{\beta}$) for each infected line is the average relative mortality rate of infected to uninfected CNS for the entire monitoring period. We tested the proportional hazards assumption for each line. If nonproportionality was suspected, time was partitioned into blocks. Blocks were decided based on survival curves by visually identifying cutoff time points where hazard ratios were expected to change. The final blocks met the proportional hazard assumption test.

The degree of CI induced by *w*AlbB males in crosses to CNS or *w*Mel females (and the reciprocal cross) was determined by computing the proportion of eggs that hatched (cages that laid ≤ 10 eggs were excluded). We also tested for the effects of cross and gonotrophic cycle (a proxy of female age), treated as factors, on hatch rates in an ANOVA if data were distributed normally or a Kruskal–Wallis test for nonnormal data.

Assortative mating data were analyzed by applying a linear model on mean hatch rates resulting from cages possessing 0%, 50%, or 100% infection rates. Hatch rate was used as a proxy for the proportionate contribution of infected versus uninfected males.[31] The mating competitiveness term, $\beta_{am}$, represents the deviation between observed and expected hatch rates given a particular rate of CI (see Supplemental Appendix 2). A Student's *t* test was then performed against the null model ($\beta_{am} = 1$) to test for significant deviations, where $\beta_{am} > 1$ or $\beta_{am} < 1$ indicates an advantage or disadvantage, respectively, in *w*AlbB male–mating success relative to CNS.

The rate of *w*AlbB maternal transmission from parent to offspring was calculated as the mean proportion of infected progeny produced by infected mothers. Binomial 95% confidence intervals (95% CIs) were also computed.

Bacterial density, an indicator of *Wolbachia* load, was quantified using $\Delta C\text{p}$ (= $C\text{p}^{Ae.\ aegypti} - C\text{p}^{Wolbachia}$), which corresponds to the difference in log concentration of template DNA between host and parasite.[32] Differences in *Wolbachia* load ($\Delta C\text{p}$) between the strains *w*AlbB, *w*Mel and *w*MelPop were compared with a Kruskal–Wallis test because of a lack of normality. A post hoc (Dunn) test for multiple comparisons was also performed.

## RESULTS

**Fecundity.** No difference was found in the number of eggs laid ($P > 0.9$, *t* test) between *w*AlbB females (mean = 60.14, standard deviation [SD] = 7.63, $N = 14$) and CNS females (mean = 59.72, SD = 12.16, $N = 18$, relative fecundity = 1.007).

**Quiescent egg viability.** Egg batches ranged from 25 to 78 eggs. Student's *t* tests were performed on hatch proportions (arcsine transformed). The average percentage of *w*AlbB eggs surviving at days 0, 3, 10, 17, 24, and 31 was not significantly different from equivalent time points for the CNS strain (Figure 1, $P > 0.05$). However, after day 31, the *w*AlbB infection caused a 27% reduction in mean egg hatch by day 61, 79% by day 90, and 99% by day 124, compared with CNS. The *w*AlbB egg hatch rates were significantly different from those for CNS (day 61: $t = 6.85$, df = 18, $P < 0.001$; day 90: $t = 25.39$, df = 18, $P < 0.001$; day 124: $t = 28.86$, df = 18, $P < 0.001$). In contrast, the *w*MelPop strain had a strong negative effect on quiescent egg viability in *Ae. aegypti* outbred to the CNS background (plotted in Figure 1 for comparison),[16] while *w*Mel-infected *Ae. aegypti* outbred to the same genetic background did not affect quiescent egg viability within a period of 1 month.[13]

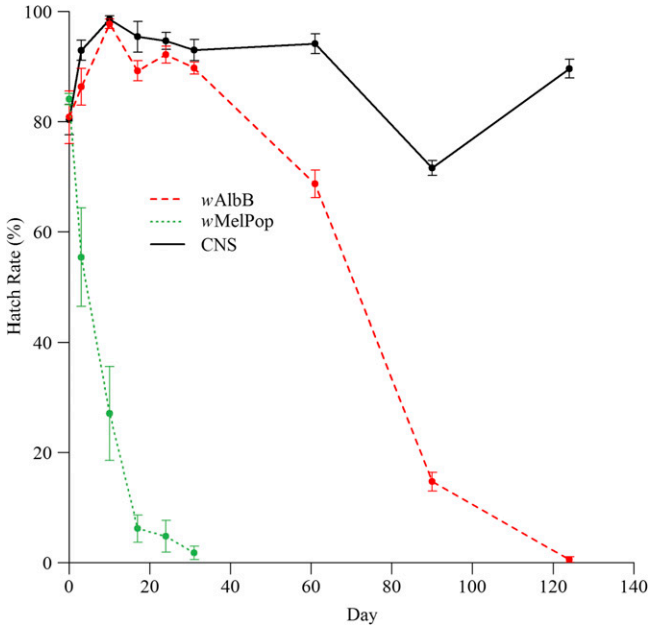


FIGURE 1. Percentage hatch rate of *w*AlbB-infected (red, dashed line), *w*MelPop-infected (green, dotted line), and uninfected CNS (black, solid line) *Aedes aegypti* eggs after 0, 3, 10, 17, 24, 31, 61, 90, and 124 days of quiescence. Error bars are standard error of means. Ten replicates per time point. Egg batches ranged from 25 to 78 eggs. The *w*MelPop curve, generated from eggs outbred to the Cairns genetic background, is taken from the work of Yeap and others.[16]

**Larval development time, survival, and adult body size.** Larval to adult survival was generally high (Table 2). In an analysis of the overall data, we found significant effects of nutrition ($F_{1,40}$ = 14.38, $P$ < 0.001) and larval density ($F_{1,40}$ = 10.50, $P$ = 0.002) but not *Wolbachia* infection status ($F_{1,40}$ = 0.37, $P$ = 0.548, relative *w*AlbB survival = 1.002) on survival to adulthood. There were no significant departures from a 1:1 sex ratio in both *w*AlbB-infected and uninfected adults for any treatment ($P$ > 0.05, $\chi^2$ test).

We also found that nutrition ($F_{1,80}$ = 3,082.52, $P$ < 0.001), larval density ($F_{1,80}$ = 295.36, $P$ < 0.001), and sex ($F_{1,80}$ = 144.95, $P$ < 0.001) but not *Wolbachia* infection status ($F_{1,80}$ = 0.02, $P$ = 0.891) affected larval development time. Relative to CNS, larval development time for *w*AlbB was 1.015 days for males and 0.99 days for females (average across nutrition and density). Low larval densities increased development time to a greater extent when nutrition was also low (Table 2). Males reached adulthood around 1.3 days earlier than females at high nutrient levels (mean ± standard error (SE) for males = 9.002 ± 0.151 days, females = 10.302 ± 0.142 days), while this difference was extended to 4.4 days in the low-nutrition treatments (males = 20.653 ± 0.759 days, females = 25.085 ± 0.800 days). No pairwise comparisons for development time between *w*AlbB-infected and uninfected larvae were significant (Table 2).

We estimated body size in the larval development experiment by measuring wing length. As expected, female wings (mean ± SE = 2.502 mm ± 0.011, $N$ = 213) were considerably larger than male wings (1.941 ± 0.006 mm, $N$ = 222, $F_{1,419}$ = 6,078.57, $P$ < 0.001). We also found a significant effect of nutrition on wing length ($F_{1,419}$ = 725.89, $P$ < 0.001), while *Wolbachia* infection ($F_{1,419}$ = 3.09, $P$ = 0.080) and larval density ($F_{1,419}$ = 0.91, $P$ = 0.340) had no significant effects. Relative to CNS, wing length for *w*AlbB males was 0.99 and 1.00 for females. Larval density affected size differentially at each level of nutrition. For the high nutrition level, the low-density treatment resulted in larger wings, while for the low-nutrition level, the high-density treatment resulted in larger wings (Table 2). No pairwise comparisons between *w*AlbB-infected and uninfected wings were significant, with the exception of the low-nutrition, high-density containers where uninfected females were significantly larger than *w*AlbB-infected females (relative *w*AlbB wing length = 0.97, Table 2).

**Adult survival in groups.** Shortly after CNS had passed 50% survival, this experiment was terminated at day 86. Log-rank tests on unpooled data identified CNS replicates 1 and 2 and *w*Mel replicate 5 as significant outliers, which were excluded before proceeding to analyze pooled data. Within infected lines, *w*Mel males survived the longest followed by *w*AlbB and then *w*MelPop (Figure 2A). Hazard ratios for males from each infected line remained proportional throughout the monitoring period. The mortality rate of *w*AlbB males differed significantly from CNS (Figure 2, $z$ = 3.24, $e^{\beta}$ = 1.43 [95% CI = 1.15–1.78], $P$ = 0.001), as did *w*MelPop ($z$ = 5.77, $e^{\beta}$ = 1.98 [95% CI = 1.57–2.50], $P$ < 0.001), whereas *w*Mel and CNS did not differ significantly ($z$ = −1.01, $e^{\beta}$ = 0.89 [95% CI = 0.71–1.12], $P$ = 0.312). Within the infected lines, *w*AlbB females performed the best up to around 40 days before a sudden increase in mortality rate, which led to a

TABLE 2

Larval survival, development time, and wing length (mean ± SE) for *w*AlbB-infected and CNS reared under two nutrition regimes and at two densities

| Nutrition* | Density† | Infection | Survival to adulthood (%)‡ | Development time (days)‡ | | Wing length (mm)‡ | |
|---|---|---|---|---|---|---|---|
| | | | | Male | Female | Male | Female |
| High | High | *w*AlbB | 98.92 ± 0.154 a | 8.32 ± 0.06 a | 9.53 ± 0.05 ab | 1.992 ± 0.012 c | 2.600 ± 0.015 f |
| | | CNS | 97.83 ± 0.792 ab | 8.30 ± 0.08 a | 9.77 ± 0.10 ab | 2.000 ± 0.008 c | 2.587 ± 0.015 f |
| | Low | *w*AlbB | 98.17 ± 0.803 a | 9.83 ± 0.09 ab | 10.99 ± 0.08 b | 2.027 ± 0.011 c | 2.678 ± 0.016 g |
| | | CNS | 97.33 ± 0.703 abc | 9.56 ± 0.09 ab | 10.93 ± 0.07 b | 2.017 ± 0.008 c | 2.655 ± 0.014 fg |
| Low | High | *w*AlbB | 96.50 ± 1.252 abc | 17.23 ± 0.29 c | 21.65 ± 0.44 d | 1.869 ± 0.010 ab | 2.379 ± 0.012 d |
| | | CNS | 97.75 ± 0.793 ab | 17.29 ± 0.31 c | 21.63 ± 0.45 d | 1.916 ± 0.012 b | 2.462 ± 0.013 e |
| | Low | *w*AlbB | 93.00 ± 1.494 bc | 24.46 ± 0.56 e | 28.21 ± 0.88 f | 1.837 ± 0.011 a | 2.344 ± 0.024 d |
| | | CNS | 93.00 ± 0.707 c | 23.64 ± 0.91 de | 28.85 ± 1.04 f | 1.864 ± 0.017 ab | 2.327 ± 0.025 d |

SE = standard error.
*High- and low-nutrition regimes consisted of 0.05 and 0.25 mg, respectively, of TetraMin per larva per day.
†High- and low-density treatments consisted of 200 larvae in 200 mL (one larva per 1 mL) and 200 larvae in 4,000 mL (1 larva per 20 mL), respectively.
‡For each trait, values with the same letter are not significantly different from each other ($P$ > 0.05, Tukey's honest significant difference test).

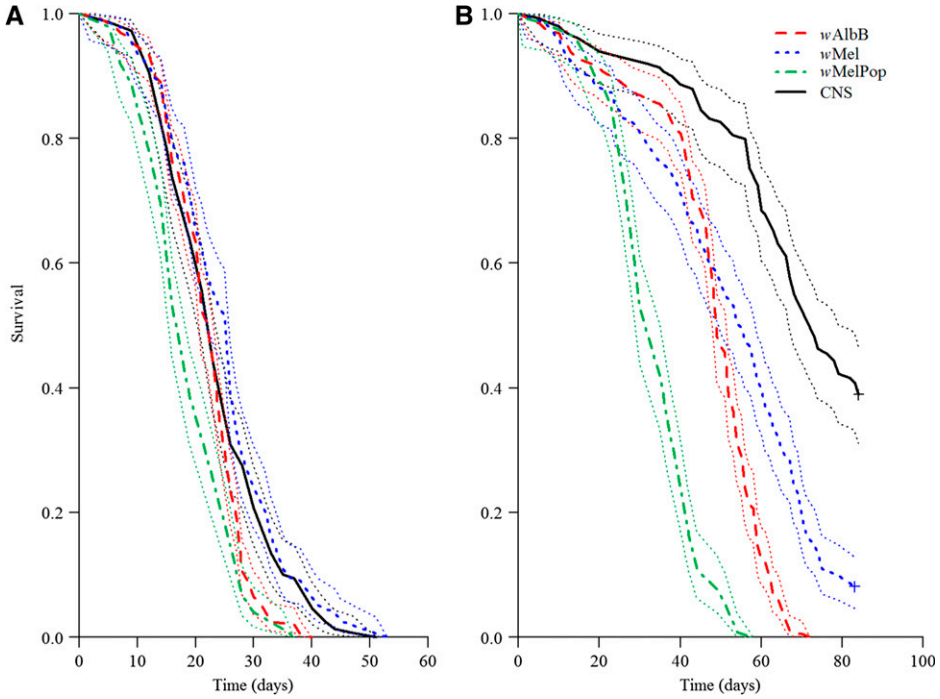


FIGURE 2. Survival of adult *Aedes aegypti* males (**A**) and females (**B**) infected with *w*AlbB (red, dashed line), *w*Mel (blue, dotted line), or *w*MelPop (green, dot–dash line) outcrossed to the Cairns genetic background, represented by CNS (black, solid line). "+" represents right-censored data. Thin dotted lines are 95% confidence intervals.

significantly lower survival overall compared with *w*Mel, but this was still higher than for *w*MelPop (Figure 2B, Supplemental Table 3). Hazard ratios were nonproportional for *w*AlbB and *w*MelPop females; however, *w*Mel was proportional throughout the monitoring period. Separate Cox regressions were performed on days 0–20 (block 1), 21–40 (block 2), and > 40 days (block 3) as the hazards appeared to be different across blocks (Figure 2, see Supplemental Table 3). The mortality rate of *w*AlbB females was significantly different from CNS in block 3 only ($z = 13.27$, $e^{\beta} = 8.54$ [95% CI = 6.22–11.73], $P < 0.001$), whereas *w*Mel females became significantly different from CNS in blocks 2 ($z = 3.34$, $e^{\beta} = 3.77$ [95% CI: 1.73–8.23], $P < 0.001$) and 3 ($z = 6.34$, $e^{\beta} = 2.64$ [95% CI = 1.95–3.56], $P < 0.001$). Females infected with *w*MelPop had the highest mortality rate, becoming significantly different from CNS in blocks 2 ($z = 8.29$, $e^{\beta} = 21.63$ [95% CI = 10.45–44.75], $P < 0.001$) and 3 ($z = 12.85$, $e^{\beta} = 21.87$ [95% CI = 13.66–35.01], $P < 0.001$) (see Supplemental Table 4).

**Mating.** The average number of eggs oviposited by CNS females in cages possessing 0%, 50%, and 100% infection frequencies was 2,364, 2,471, and 2,346, respectively, and their mean hatch rates were 0.01, 0.39, and 0.85, respectively (Figure 3). The number of eggs that hatched early and died on the paper (egg cap clearly detached) was negligible (< 40 per replicate). The relative mating success of *w*AlbB males to CNS males ($\beta_{am}$) was 1.114, but this did not differ significantly from the null hypothesis ($\beta_{am} = 1$) in a *t* test ($t = 0.89$, df = 14, $P = 0.195$). Therefore, there was no strong evidence for assortative mating in favor of males from either strain.

**Cytoplasmic incompatibility.** Complete CI was observed between *w*AlbB males and CNS females resulting in sterility, regardless of gonotrophic cycle (Table 3). Similarly, reciprocal crosses between *w*Mel and *w*AlbB exhibited complete

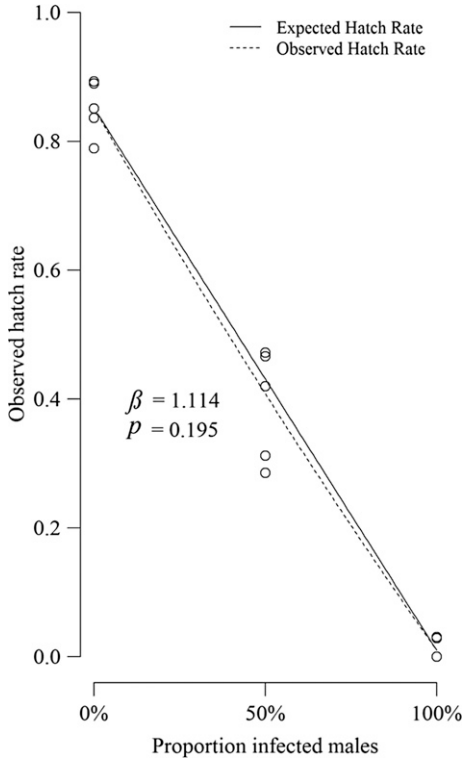


FIGURE 3. Mean hatch rates of *Aedes aegypti* eggs oviposited by 80 CNS females exposed to populations of males in the following groups: 0% infected (80 CNS males), 50% infected (40 *w*AlbB and CNS males), and 100% infected (80 *w*AlbB males). The solid line denotes the null model for the expected hatch rate (no difference in mating ability), whereas the dotted line represents the observed hatch rate and relative mating success ($\beta$) of *w*AlbB to CNS males and its probability ($p$).

TABLE 3
Crosses performed between infected and uninfected lines to test for CI

| | Gonotrophic cycle* | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Incompatible | | | |
| CNS (♀) × *w*AlbB (♂) | 0 (319.75) | 0 (334) | 0 (286.38) |
| *w*Mel (♀) × *w*AlbB (♂) | 0 (214.25) | 0 (221.75) | 0 (206.25) |
| *w*AlbB (♀) × *w*Mel (♂) | 0 (332.43) | 0 (294.75) | 0 (268.38) |
| Compatible | | | |
| CNS (♀) × CNS (♂) | 0.97 (484.86) | 0.99 (364) | 0.93 (434.25) |
| *w*AlbB (♀) × CNS (♂) | 0.94 (227.75) | 0.96 (241.13) | 0.94 (214) |
| *w*Mel (♀) × *w*Mel (♂) | 0.81 (286.38) | 0.81 (288.75) | 0.79 (200.13) |
| *w*AlbB (♀) × *w*AlbB (♂) | 0.88 (381.25) | 0.83 (384.63) | 0.84 (285.38) |

CI = cytoplasmic incompatibility.
*Mean hatch rate (mean number of eggs scored).

bidirectional incompatibility (Table 3). One-way ANOVAs on arcsine transformed hatch rates of the compatible crosses indicated a nonsignificant effect of gonotrophic cycle ($F_{2,88}$ = 0.35, $P$ = 0.708), but a significant effect of cross ($F_{3,88}$ = 28.16, $P$ < 0.001). Fecundity did not differ between incompatible and compatible crosses in a $t$ test ($t$ = 0.28, df = 141, $P$ = 0.782), but showed a consistent decrease between gonotrophic cycles 1 and 3, most likely due to age effects (Table 3). Average hatch rates of ≥ 79% were observed for all control crosses. Tests on Cairns outbred *w*Mel and *w*MelPop males crossed to Cairns wild-type females also indicated very strong CI.[7,13,16]

**Maternal transmission.** Out of the 319 offspring produced by 29 *w*AlbB-infected females, 319 were positively infected with the *w*AlbB strain (maternal transmission rate = 1, lower 95% CI = 0.99).

**Population cages.** As outlined in the Materials and methods section, replicates were terminated from four to seven generations after it was clear that the *w*AlbB infection had successfully invaded in either the 10% or 22% groups (Figure 4). All remaining colonies were terminated at generation 7 when the trajectory of the populations was clear. By the seventh generation, one of the five cages in the 10% group had successfully invaded. Because we observed less than three invasions, the critical threshold to achieve $\alpha < 0.05$, we could not reject the null hypothesis that $\hat{p}$ = 0.15 ($P$ = 0.482; Figure 4, Supplemental Figure 1). The remaining four cages in the 10% group failed to invade, with infection frequencies ranging from 0% to 23% over seven generations. All of the 22% control cages successfully invaded uninfected populations in four to six generations (Figure 4).

***Wolbachia* load.** The median difference in log concentration of template DNA between *Wolbachia* and its host genome ($\Delta Cp$) in order of highest to lowest was *w*MelPop (6.83), *w*AlbB (5.09), and *w*Mel (3.33) (Figure 5). Significant differences between groups were found in a Kruskal–Wallis

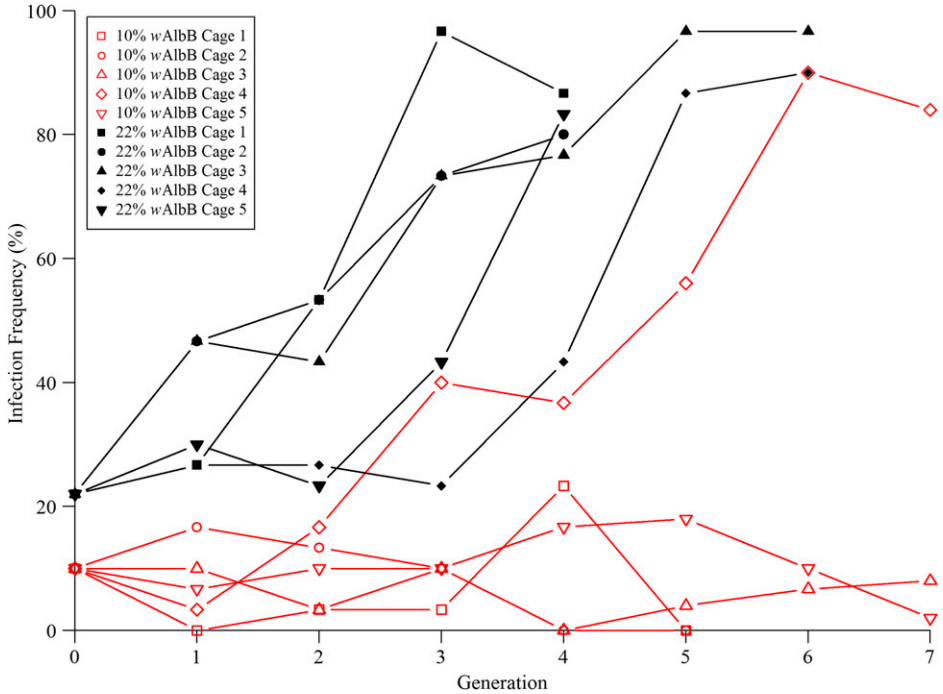


FIGURE 4. *Wolbachia* infection frequency per generation after an initial release of *w*AlbB females and males (red, open markers: 10%; black, solid markers: 22%) into 10 cages possessing CNS populations.

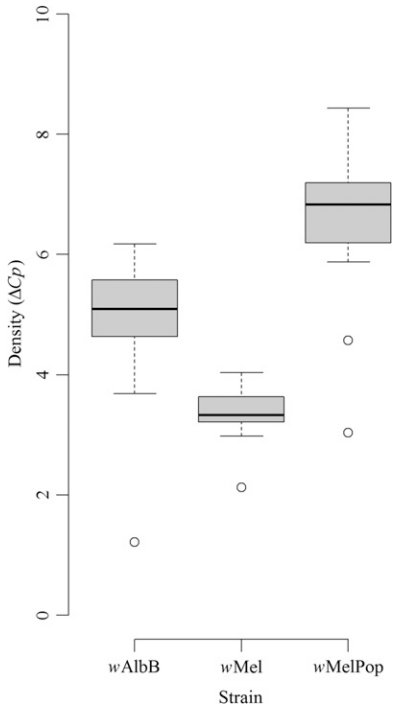


FIGURE 5. Box plot of *Wolbachia* load for *w*AlbB, *w*Mel, and *w*MelPop in laboratory-reared *Aedes aegypti* ($N = 25$). Outliers are represented as circles. Medians are indicated by horizontal lines.

test ($\chi^2 = 53.19$, df = 2, $P < 0.001$) and all pairwise comparisons between strains in a post hoc test ($P < 0.001$, Dunn test).

## DISCUSSION

We have characterized fitness and reproductive effects of the *w*AlbB infection in an outbred *Ae. aegypti* population. Previously, this infection was placed into an *Ae. aegypti* line originating from Texas[33] through microinjection after having been sourced from *Ae. albopictus*.[14] In this study, we have crossed *w*AlbB-infected *Ae. aegypti* into a common Australian background to allow comparisons to other *Wolbachia*-infected strains of *Ae. aegypti*. The effects of *w*AlbB on fitness appear to fall between those of *w*Mel and *w*MelPop, infections that both originated from *D. melanogaster*.[13,16,34] We find costs associated with the *w*AlbB infection on egg hatch when the eggs are maintained in a quiescent state for more than 31 days. These costs are greater than those imposed by the *w*Mel infection but the costs are not as severe as for *w*MelPop, where the majority of infected eggs do not last longer than 1 month.[19]

In terms of adult survival, we found that *w*AlbB shows a reduction in lifespan that is intermediate between the pattern for *w*Mel and *w*MelPop. Females infected with *w*AlbB reached 50% survival 29 days earlier than uninfected females, in contrast to *w*Mel and *w*MelPop, which reached the same point at 17 and 43 days earlier than uninfected females, respectively. We also found nonproportionality in the hazard ratio for *w*AlbB females due to an increase in mortality after around 40 days for five of eight replicates. This may represent an underlying feature of its growth within *Ae. aegypti* adults as they age; *w*AlbB densities tend to increase with adult age in both its native hosts[35] and in an experimental infection of *Anopheles stephensi*.[36] The nonproportional hazard ratio of females infected with *w*MelPop may reflect its overreplication in host cells, which causes early death as demonstrated in *Drosophila*.[37] The fitness effects of *Wolbachia* infections (but not their levels of CI) appear to be at least partly related to the density of the *Wolbachia* in tissues although only a modest number of infections have so far been tested.[8,10]

We confirmed the absence of any costs in terms of fecundity[14] and also male mating success for *w*AlbB; *w*MelPop does exhibit fecundity costs,[12,16] but neither this infection nor *w*Mel show mating costs.[31] We also confirmed the maternal transmission of *w*AlbB was 100%[14] and complete CI was shown in crosses with uninfected females from Cairns, which was also reported for *w*Mel and *w*MelPop.[13,16] Complete bidirectional incompatibility was found between *w*AlbB and *w*Mel; given the relative fitness effects found here, *w*Mel may outcompete *w*AlbB should these two infections be released in the same population at an equal frequency. However, a population that has been invaded by *w*AlbB is not expected to be invaded by *w*Mel because of bidirectional incompatibility and frequency dependence; CI will be induced in crosses between *w*AlbB males and *w*Mel females, and, when present at a low frequency, most *w*Mel females will inevitably mate with *w*AlbB males. If two infected strains are present in a population, the outcome will depend mostly on the nature of incompatibility patterns among the strains.[28] If strains are bidirectionally incompatible, both strains may persist when they have invaded different areas because common strains have an advantage in an area; females from the common strain will be more likely to engage in compatible matings with males from the same strain. If males from one strain show CI but males from the other strain do not, the CI-inducing strain is likely to invade as occurred in Australian populations of *Drosophila simulans* where the *w*Au strain that did not induce CI was replaced rapidly by the *w*Ri strain that did induce it.[38]

A decade ago, the *w*AlbB infection was reported as generating strong CI and perfect maternal transmission while having minimal effects on host fitness in terms of fecundity and egg hatch.[14] The *w*AlbB infection was shown to invade small population cages when introduced at a starting frequency of 20%.[14] We confirmed these results in a larger cage population with greater replication, suggesting relative fitness costs that are comparable to the frequency of *Wolbachia* required to obtain invasion[28] are most likely greater than 10% but lower than 20%. However, we found no significant fitness costs for male mating competitiveness, adult survival within 1 month, or larval development and survival to adulthood. Previous experiments on the natural host of the *w*AlbB infection, *Ae. albopictus*, suggest that the *w*AlbB infection in combination with the *w*AlbA infection can generate a fitness advantage for its host.[39] Our results suggest that releasing *w*AlbB-infected adults so that they comprise 20% of the target population may be sufficient to achieve rapid invasion, but this threshold may well be higher in natural populations, particularly given the costs we have found at the quiescent egg stage.

In summary, the *w*AlbB infection might be suitable for invasion into *Ae. aegypti* populations given that this infection appears to have moderate fitness costs that place it between *w*Mel, which can successfully invade populations where it is stably maintained,[1,7] and *w*MelPop, which can be invaded into semi-field populations[13,19] but which has substantial fitness costs that make invasion into natural populations

difficult. Given that *w*AlbB in *Ae. aegypti* can block arbovirus transmission, this strain may be suitable for release alongside *w*Mel, although its ability to block different serotypes of dengue and other viruses remains to be established (cf.[4]). Because it influences the hatch rate of quiescent eggs, the *w*AlbB strain may also have utility in releases aimed at suppressing or eliminating populations of *Ae. aegypti* during the dry season.[6,19]

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