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Larval Competition Extends Developmental Time and Decreases Adult Size of *w*MelPop *Wolbachia*-Infected *Aedes aegypti*

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Abstract. The intracellular endosymbiont *Wolbachia* has been artificially transinfected into the dengue vector *Aedes aegypti*, where it is being investigated as a potential dengue biological control agent. Invasion of *Wolbachia* in natural populations depends upon the fitness of *Wolbachia*-infected *Ae. aegypti* relative to uninfected competitors. Although *Wolbachia* infections impose fitness costs on the adult host, effects at the immature stages are less clear, particularly in competitive situations. We look for effects of two *Wolbachia* infections, *w*Mel and *w*MelPop, on intra-strain and inter-strain larval competition in *Ae. aegypti*. Development of *Wolbachia*-infected larvae is delayed in mixed cohorts with uninfected larvae under crowded-rearing conditions. Slow developing *w*MelPop-infected larvae have reduced adult size compared with uninfected larvae, and larvae with the *w*Mel infection are somewhat larger and have greater viability relative to uninfected larvae when in mixed cohorts. Implications for successful invasion by these *Wolbachia* infections under field conditions are considered.

INTRODUCTION

Wolbachia are intracellular endosymbiotic bacteria that infect as many as two-thirds of insect species.[1,2] *Wolbachia* are transmitted maternally[3] and commonly cause cytoplasmic incompatibility in their hosts; a mechanism resulting in embryonic lethality when an infected male mates with a female that is not infected. This incompatibility provides *Wolbachia*-infected females with a fitness advantage relative to uninfected females.[4,5] The reproductive manipulation induced by *Wolbachia* enables the infection to proliferate rapidly, potentially leading to replacement of naturally uninfected populations.[6–8]

Although many mosquito species are infected with *Wolbachia*, the container-breeding *Aedes aegypti*, the principal dengue vector, does not harbor a natural *Wolbachia* infection.[9] The *w*MelPop and *w*Mel strains of *Wolbachia*, originally from *Drosophila melanogaster*, have been artificially transinfected into *Ae. aegypti*.[10,11] These *Wolbachia* strains inhibit the replication of dengue virus in *Ae. aegypti*, preventing its transmission.[11,12] Thus, *Wolbachia* may be used as an effective biological control for dengue that may eliminate the need for mosquito eradication.

The potential for *Wolbachia*-infected *Ae. aegypti* to replace natural populations and block dengue transmission depends on their ability to survive and reproduce in the field.[8,13] The *w*Mel infection is relatively benign; it primarily localizes to the mosquito salivary glands and gonads, and does not cause any substantial deleterious effects on fitness.[11] The ability of *w*Mel to invade natural mosquito populations has been demonstrated; in trial releases of *w*Mel-infected *Ae. aegypti* at two locations near Cairns, Queensland, Australia, infection frequency approached 100% after two months of releases.[14] Conversely, *w*MelPop is relatively virulent towards *Ae. aegypti* because it proliferates throughout the entire mosquito.[11,12,15] The *w*MelPop infection reduces adult longevity by as much as 50% and decreases egg viability, fecundity,[10,16,17] and blood feeding success of females.[18]

Although the costs of *w*MelPop infection on adult *Ae. aegypti* are well documented, studies that examine the effects of *Wolbachia* infection on immature development are limited, particularly those that evaluate direct competition with uninfected larvae under stress. Gavotte and others[19] provided an example in *Ae. albopictus*. During field release *Wolbachia*-infected *Ae. aegypti* larvae will encounter competition with uninfected larvae for limited resources and space, as suitable habitats for oviposition are scarce.[20] Larval crowding in *Ae. aegypti* causes delayed development rates,[21] elevated mortality[22] and reduced adult size.[23] These deleterious effects on fitness may be explained by physical interference,[24] increased levels of pollution caused by natural waste released into the environment,[23,25] or through increased aggression leading to higher biting frequencies and increasing the incidence of disease transmission.[26] Larval competition between species has been proposed as the primary mechanism for recently observed shifts in species distributions of *Ae. aegypti* and *Ae. albopictus*.[27,28] Larval competition between infected and uninfected strains of *Ae. aegypti* may also play a critical role in the success of *Wolbachia* invasion.

For invasion to occur, the frequency of *Wolbachia* infection in a population must reach or exceed a certain threshold.[29,30] This required frequency depends on variables, such as rates of maternal transmission and levels of cytoplasmic incompatibility induced by the *Wolbachia* infection. The fitness of infected mosquitoes relative to uninfected mosquitoes in the field is also an important factor; deleterious effects associated with the *w*MelPop infection mean a higher infection threshold must be reached for invasion to occur.[13] An important component of this is relative larval viability.[31] However, the competitive ability of *Wolbachia*-infected larvae relative to uninfected larvae under stress is poorly understood. Recent studies suggest *Wolbachia* infection may have deleterious effects on immature host survival and development,[16,17,32] and studies on the naturally infected *Ae. albopictus* suggest that *Wolbachia* infection reduces fitness under high stress conditions.[33] A minor cost to survival of *Wolbachia*-infected larvae relative to uninfected larvae could render invasion difficult.[31] Therefore, it is important to assess the effect of

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*w*MelPop infection on competition for resources with uninfected larvae under stress.

In this study, we tested the effect of larval crowding on the fitness of *Wolbachia*-infected *Ae. aegypti* larvae relative to an uninfected strain of *Ae. aegypti*. We monitor *w*MelPop-infected and uninfected larvae competing directly for the same resources when present in different frequencies. Effects are examined in terms of survivorship, developmental time, sex ratio and wing length (a proxy for body size[34]). We repeated this assay with the *w*Mel strain. The results are discussed in terms of implications for facilitating invasions of *Wolbachia* strains in competitive environments.

MATERIALS AND METHODS

Mosquito strains and colony maintenance. Three lines of *Ae. aegypti* were used. Wild-type uninfected C14 *Ae. aegypti* were collected from Cairns, Queensland, Australia, in June 2012 and maintained under laboratory conditions for at least one generation. The *w*MelPop-CLA-infected and *w*Mel-infected lines backcrossed to the Cairns background[17] were generated before each experiment to maintain the same background as the uninfected strain. These infected lines were established from field-sourced mosquitoes from populations established near Cairns after releases. Lines were maintained in a controlled laboratory environment at 26°C ± 1°C and 80–90% relative humidity, with a 12:12 light: dark photoperiod. Mosquito colonies were maintained according to methods described by Yeap and others.[17] Female mosquitoes were blood-fed on a single human volunteer, as approved by the University of Melbourne Human Ethics Committee (approval 0723847).

Larval competition. Preliminary experiments were conducted to determine a level of larval crowding that increased developmental time and reduced adult size. Larvae were provided with a fixed amount of crushed TetraMin® fish food tablets (Tetra, Melle, Germany) daily (0.25 mg/larvae) and reared at the following densities: 50, 100, 200, 400, and 800 larvae in 200 mL of reverse osmosis (RO) water (0.25, 0.5, 1, 2 and 4 larvae/mL, respectively). *Wolbachia*-infected and uninfected larvae were tested in pure cohorts, in which each strain was reared in separate containers, and also in mixed cohorts where infected and uninfected larvae were present in the same container. Development rate, size, and survivorship decreased with greater larval densities, concordant with previous studies.[22,23,35] A larval density of 2 larvae/mL affected these traits relative to the lowest larval density, increasing development time by approximately 1.5 days, decreasing wing length by nearly 10%, and decreasing survival by approximately 10%, and was chosen for the following experiments.

Experiments were conducted at 26°C ± 1°C and 80–90% relative humidity with a 12:12 light: dark photoperiod. Treatments, where *Wolbachia*-infected and uninfected larvae were present (mixed cohorts), comprised the following numbers of *w*MelPop or *w*Mel to uninfected larvae: 300 to 100, 200 to 200, and 100 to 300. Pure cohorts of 400 infected or uninfected larvae were also included as controls. Eggs were hatched synchronously and first instar larvae were transferred to plastic containers with mesh lids (9.5–11.5 cm radius, 7 cm height) holding 200 mL of RO water. Containers were topped up with RO water daily to counter evaporation. A total of 100 mL of water from each experimental container was removed every second day, and replaced with 100 mL of RO water to reduce the build-up of toxic waste products. Adult eclosion was scored twice a day; adults were released into cages, chilled, and then stored in absolute ethanol. Experiments were terminated when all mosquitoes had eclosed or died.

Each treatment of the *w*MelPop versus uninfected experiment was replicated four times. Larvae were provided with TetraMin®: 0.15 mg, 0.18 mg, 0.3 mg, 0.35 mg, and 0.35 mg per larva on days 1, 3, 4, 5, and 6 of the experiment, respectively. In the *w*Mel versus uninfected experiment, treatments were replicated five times. Larvae were provided with TetraMin®: 0.08 mg, 0.08 mg, 0.32 mg, 0.37 mg, and 0.37 mg per larva on days 1, 2, 3, 4, and 5 of the experiment, respectively. A few containers were contaminated with bacteria (cloudy appearance) and were excluded from the experiments.

A subset of adults was collected on three occasions for wing measurement. Males were collected on days 8, 10, and 12, and females were collected on days 9, 11, and 13 to cover a range of development times. For each treatment, at least six adults per replicate were collected on each day; mosquitoes were screened for *Wolbachia* infection status (see below). All pure cohorts were also screened for *Wolbachia* to confirm infection status.

Wing length measurements. Both wings were removed from each adult, placed under a 10-mm coverslip, and fixed with Hoyer's solution (distilled water: gum arabic: chloral hydrate: glycerin in the ratio 5:3:20:2).[36] Wing length was calculated as the distance from the alular notch to the wing tip, excluding the fringe scales.[37] The lengths of both wings of a mosquito were averaged, and each measurement was repeated. Thus, length represented the average of four measurements. Damaged wings were excluded from the analysis.

DNA extraction and *Wolbachia* screening. Genomic DNA was extracted from adult mosquitoes by using a Chelex® 100 resin (Bio-Rad Laboratories, Hercules, CA) extraction method.[38] Mosquitoes were ground in 3 μL of proteinase K (20 mg/mL) (Roche Diagnostics Australia Pty. Ltd., Castle Hill New South Wales, Australia) and 250 μL of 5% Chelex® solution, incubated at 65°C for 1 hour, followed by incubation for 10 minutes at 90°C and storage at –20°C.

Wolbachia infection status was determined by using methods developed by Lee and others[39] for a Roche LightCycler 480. Quantitative real-time polymerase chain reaction (PCR) was used to amplify three markers with three primer sets: a mosquito primer set to detect mosquitoes from the *Aedes* genus, *Ae. aegypti* specific primers to differentiate *Ae. aegypti* from other *Aedes* species, and *Wolbachia* specific primers to determine *Wolbachia* infection status, as well as density. Diagnosis was based on crossing-point values for the PCR and melting temperature from high-resolution melt analysis. More details of the screening method are provided by Yeap and others[40] and Lee and others.[39]

Statistical analyses. Chi-square tests were used to assess deviations from the expected numbers of infected and uninfected offspring surviving until adulthood, and to test departures from an expected 1:1 male to female ratio. Contingency analyses were performed to assess differences in developmental time between infections in mixed cohorts, accounting for differential survivorship. Analyses of variance (ANOVA) and linear regressions were conducted using PopTools version 3.2 for Microsoft (Redmond, WA) Excel to test for the effect of varying infection frequencies on size and development time.

Survival data were arcsine square-root transformed, and development time data were square-root transformed for normality as determined through Kolmogorov-Smirnov tests. Tukey's honest significant difference post-hoc tests were conducted for pairwise comparisons between strains and treatments using SPSS version 19 (SPSS, Inc., Chicago, IL) after finding significant effects with ANOVAs.

RESULTS

Survivorship. Survival to adulthood was scored to determine any differences in overall larval viability between treatments. No significant effect of initial *Wolbachia* proportion on overall survivorship was found between treatments in the *w*MelPop versus uninfected experiment (overall mean viability ± SE = 0.892 ± 0.013, by one-way ANOVA, $F_{4,13} = 1.136$, $P = 0.382$) or the *w*Mel versus uninfected experiment (0.916 ± 0.010, by one-way ANOVA, $F_{4,17} = 0.691$, $P = 0.608$). Note that these comparisons address overall survivorship rather than the infection status of individual larvae. There were also no significant deviations from a 1:1 sex ratio in any of the treatments.

A subset of eclosing adults from each treatment was screened with PCR to differentiate between *Wolbachia*-infected and uninfected adults (Table 1). In the *w*MelPop versus uninfected experiment (Table 1A), no deviations from the expected number of *Wolbachia*-infected to uninfected adults were significant for any individual treatment (all $P > 0.09$), or when all mosquitoes were pooled across treatments and sex ($\chi^2 = 3.016$, degrees of freedom = 1, $P = 0.0824$). In the *w*Mel versus uninfected experiment (Table 1B), a significantly greater number of adults were positive for *Wolbachia* than expected when sexes and treatments were pooled ($\chi^2 = 14.341$, degrees of freedom = 1, $P = 0.0002$), suggesting higher relative survival of the infected strain when in a competitive environment.

Developmental time. Males (mean ± SE = 9.756 ± 0.051 days) developed around a day earlier on average than females (10.886 ± 0.051 days, $P < 0.0001$, by two-tailed t test). The proportion of *Wolbachia*-infected larvae had no effect on mean developmental time for experiments with *w*MelPop (males: one-way ANOVA: $F_{4,13} = 0.808$, $P = 0.542$, females: one-way ANOVA: $F_{4,13} = 0.331$, $P = 0.852$) or *w*Mel (males: one-way ANOVA: $F_{4,17} = 0.581$, $P = 0.681$, females: one-way ANOVA: $F_{4,17} = 0.361$, $P = 0.833$) when infection status of individual larvae was not considered.

Mosquitoes from the mixed cohorts, in which infected and uninfected larvae were present, were collected at three times for each sex and screened for *Wolbachia* to compare development rates of infected and uninfected larvae. If no differences in developmental time exist between strains, the infection frequency of adults emerging on each day would be expected to match expected numbers computed from the total number of infected and uninfected mosquitoes. Contingency analysis on numbers across days indicated significant differences in developmental time between *Wolbachia*-infected and uninfected larvae (Table 2). Relative to expectations, fewer mosquitoes that eclosed on day 8 (males) and day 9 (females) were infected with *w*MelPop (Table 2A). In contrast, on days 12 and 13, *w*MelPop-infected adults were overrepresented. This pattern is evident in males and females in all mixed cohorts (Table 2), and suggests that the development of infected larvae is delayed relative to uninfected larvae.

A similar but smaller delay in development was evident in the *w*Mel experiment (Table 2B). The *w*Mel-infected adults occurred less commonly than expected in the sample of early developers, and were more common than expected in late developers.

Wing length. Length differed significantly ($P < 0.0001$, by two-tailed t test) between males (mean ± SE = 2.106 ± 0.003 mm, n = 828) and females (2.743 ± 0.005 mm, n = 825), and was also positively associated with developmental time for males ($R^2 = 0.277$, $P < 0.0001$) and females ($R^2 = 0.425$, $P < 0.0001$) (Figures 1 and 2).

For the *w*MelPop experiment, wing lengths of infected adults that emerged on days 12 and 13 were generally smaller relative to uninfected mosquitoes that eclosed on the same day. In contrast, they were similar for the earlier emerging mosquitoes (Figure 1). This wing length disparity in late developers was most pronounced in females when infected and uninfected strains were reared separately; infected females that eclosed on day 13 had a mean ± SE wing length of 2.762 ± 0.016 mm compared with the uninfected mean of 2.942 ± 0.017 mm ($P < 0.0001$, by two-tailed t test). Differences tended to be reduced in mixed cohorts (Figure 1).

For the *w*Mel experiment, wing lengths of infected adults from mixed cohorts (males: mean ± SE = 2.147 ± 0.006 mm, females = 2.791 ± 0.012 mm) were greater than that of uninfected adults from mixed cohorts (males = 2.089 ± 0.007 mm, $P < 0.0001$, by two-tailed t test; females = 2.688 ± 0.009 mm, $P < 0.0001$, by two-tailed t test). This observation can partially be explained by an overrepresentation of the *w*Mel infection in slow developers, which generally have larger wings (Figures 1 and 2). However, *w*Mel-infected mosquitoes also had significantly larger wings in some comparisons with uninfected adults on the same day of eclosion (Figure 2), suggesting an effect of *w*Mel infection on wing length independent of developmental time.

***Wolbachia* density.** *Wolbachia* density, an estimate of the number of copies of *Wolbachia* DNA relative to *Ae. aegypti* DNA, was higher on average in the *w*MelPop strain relative to the *w*Mel strain (Figure 3), concordant with previous studies.[11] *Wolbachia* density increased with developmental time in both infected strains, and for both sexes (Figure 3). This trend

TABLE 1

Relative number of *Wolbachia*-infected to uninfected *Aedes aegypti* adults emerging in total from each treatment

*w*MelPop-infected: Uninfected					
		Females		Males	
Treatment*	Expected†	Observed†	P‡	Observed†	P‡
300 to 100	54:18	50:22	0.2763	54:18	1
200 to 200	36:36	32:40	0.3458	31:41	0.2386
100 to 300	24:72	17:79	0.0990	25:71	0.8137
*w*Mel-infected: Uninfected					
		Females		Males	
Treatment*	Expected†	Observed†	P‡	Observed†	P‡
300 to 100	67.5:22.5	70:20	0.5428	73:17	0.1806
200 to 200	45:45	50:40	0.2918	54:36	0.0578
100 to 300	13.5:40.5	17:37	0.2714	23:31	**0.0028**

*Initial proportion of *Wolbachia*-infected to uninfected larvae.

†Expected and observed number of *Wolbachia*-infected to uninfected eclosing adults pooled across all days of eclosion. Males were sampled on days 8, 10, and 12, and females were sampled on days 9, 11, and 13.

‡P for chi-square test (degrees of freedom = 1) testing the deviation of observed ratio from expected due to chance ($P < 0.05$ in bold).

TABLE 2
Number of *Wolbachia*-infected to uninfected adults emerging from each treatment, separated by day of eclosion*

			*w*MelPop-infected: Uninfected			
			Females		Males	
	Ratio	Eclosion†	*w*MelPop‡	Uninfected‡	*w*MelPop‡	Uninfected‡
No. *w*MelPop to uninfected (treatment)	300–100	8/9	10	14	14	10
		10/11	19	5	17	7
		12/13	21	3	23	1
		Expected§	16.67	7.33	18	6
		χ^2¶		**0.0012**		**0.0094**
	200–200	8/9	5	19	3	21
		10/11	8	16	11	13
		12/13	19	5	17	7
		Expected§	10.67	13.33	10.33	13.67
		χ^2¶		**0.0001**		**0.0002**
	100–300	8/9	2	30	4	28
		10/11	3	29	7	25
		12/13	12	20	14	18
		Expected§	5.67	26.33	8.33	23.67
		χ^2¶		**0.0015**		**0.0139**
			*w*Mel-infected: Uninfected			
			Females		Males	
	Ratio	Eclosion†	*w*Mel‡	Uninfected‡	*w*Mel‡	Uninfected‡
No. *w*Mel to uninfected (treatment)	300–100	8/9	20	10	19	11
		10/11	22	8	25	5
		12/13	28	2	29	1
		Expected§	23.33	6.67	24.33	5.67
		χ^2¶		**0.0353**		**0.0040**
	200–200	8/9	12	18	12	18
		10/11	14	16	21	9
		12/13	24	6	21	9
		Expected§	16.67	13.33	18	12
		χ^2¶		**0.0037**		**0.0235**
	100–300	8/9	5	13	2	16
		10/11	2	16	9	9
		12/13	10	8	12	6
		Expected§	5.67	12.33	7.67	10.33
		χ^2¶		**0.0148**		**0.0025**

*Expected proportions are computed from the number emerging on a day and the number of infected and uninfected individuals overall (i.e., corrected for survivorship differences between strains).
†Days after hatching to eclosion. Males were sampled on days 8, 10, and 12 and females were sampled on days 9, 11, and 13.
‡Observed numbers of *Wolbachia*-infected and uninfected adults on each day of eclosion.
§Expected numbers of adults emerging on each day, adjusted for survivorship differences between strains. Expected values are equal for each day of eclosion.
¶ *P* value for contingency table, χ^2 with degrees of freedom = 2, testing the deviation of observed ratio from expected due to chance ($P < 0.05$ in bold).

was not observed in the 100 to 300 *Wolbachia*-infected to uninfected treatments, most likely because of low sample sizes of *Wolbachia*-infected mosquitoes (Table 1).

DISCUSSION

We examined the ability of *Wolbachia*-infected larvae to compete with uninfected larvae at different frequencies under crowded conditions. Our results indicate that survivorship of *w*MelPop-infected larvae was not significantly reduced relative to uninfected larvae in either mixed cohorts or pure cohorts. However, even a 5% relative viability cost of *Wolbachia* infection can make invasion difficult.[31] The *w*MelPop-infected adults tended to be slightly underrepresented in mixed and pure cohorts, although whether this minor cost of infection to larval viability is sufficient to affect *w*MelPop invasion requires further investigation. In contrast, *w*Mel was significantly overrepresented in screened adults; *w*Mel infection appears to provide a viability benefit relative to uninfected larvae in mixed cohorts.

When strains were reared separately, neither *Wolbachia* infection had any significant effect on mean male or female developmental rate relative to the uninfected strain. However, development of *w*MelPop-infected and *w*Mel-infected larvae was delayed relative to uninfected larvae when competing with them directly. This was observed in all treatments; *Wolbachia* delays development to a similar extent regardless of the initial infection frequency, although the delay was more severe in the *w*MelPop strain. *Wolbachia* infection might therefore have several impacts on fitness under field conditions. Rapid development is particularly important for males because they can reach reproductive age faster and avoid competition for mates.[41] Nutrition is limited in the field[20]; slower developers risk food being depleted before they can complete development. Slow development also increases the duration of exposure to predation during immature stages[42] and reduces the likelihood of survival in temporary habitats where water evaporates.

Although we were not able to determine the basis for this *Wolbachia*-induced developmental delay, recent studies suggest several potential explanations.[19,43] *Wolbachia* is known to modify adult behavior,[44] and *Wolbachia* might also reduce larval foraging capability, but this remains to be tested. In addition, immune up-regulation[12] or increased metabolism[45]

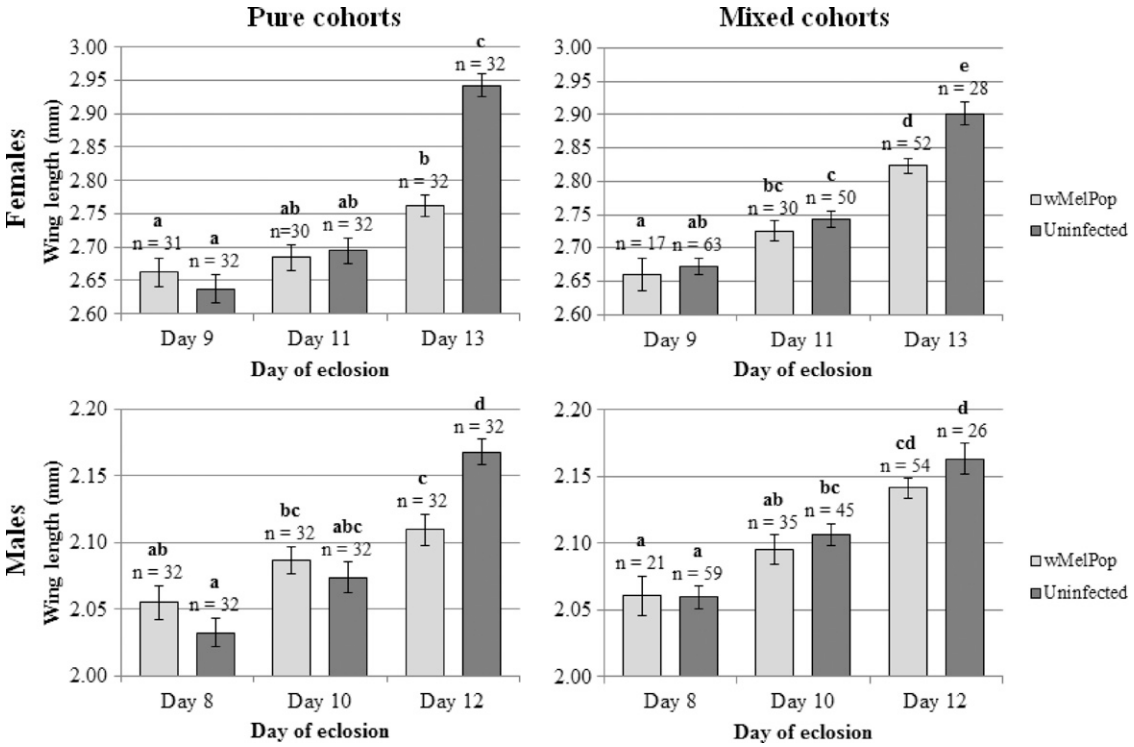

FIGURE 1. Mean wing length comparison between *w*MelPop-infected and uninfected *Aedes aegypti* for pure cohorts and mixed cohorts, and for females and males on three days of eclosion. Error bars indicate standard errors. Bars with the same letter (in bold) are not significantly different from each other ($P > 0.05$, by analysis of variance and Tukey's honest significance difference test). n indicates number of samples per bar.

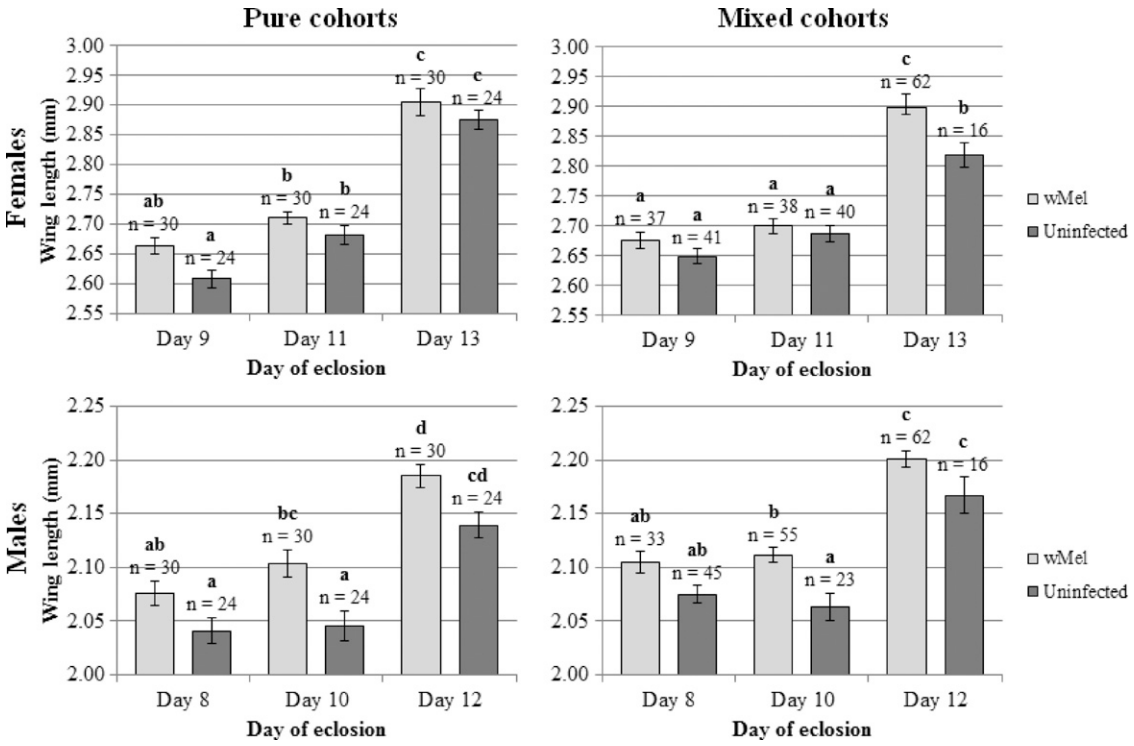

FIGURE 2. Mean wing length comparison between *w*Mel-infected and uninfected *Aedes aegypti* for pure cohorts and mixed cohorts, and for females and males on three days of eclosion. Error bars indicate standard errors. Bars with the same letter (in bold) are not significantly different from each other ($P > 0.05$, by analysis of variance and Tukey's honest significance test). n indicates number of samples per bar.

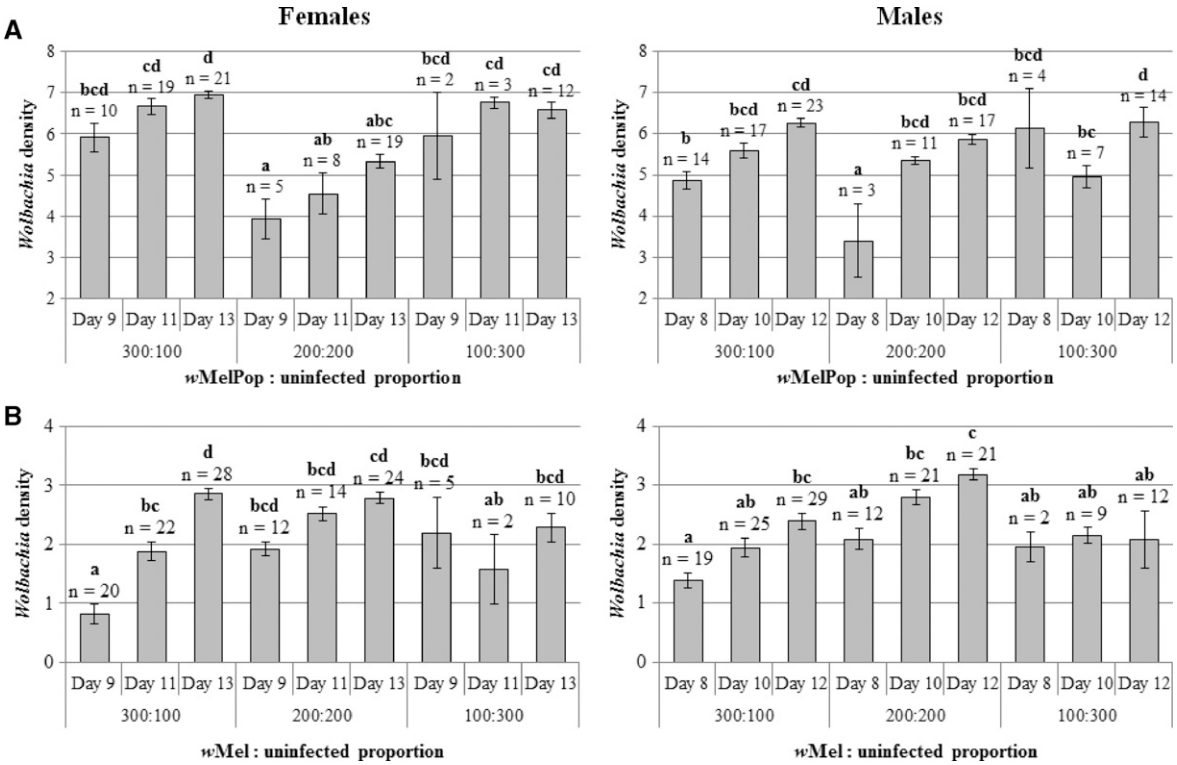

FIGURE 3. Relative *Wolbachia* density of *Wolbachia*-positive males and females of *Aedes aegypti* from **A**, *w*MelPop versus uninfected treatments and **B**, *w*Mel versus uninfected treatments. Mean values are given for each day of eclosion. Error bars indicate standard errors. Bars with the same letter (in bold) are not significantly different from each other ($P > 0.05$, by analysis of variance and Tukey's honest significance test). n indicates number of samples per bar.

observed in adults may also influence larval development rate. Our results are consistent with earlier observations; delayed development of *w*MelPop-infected larvae was shown by McMeniman and O'Neill[16] and Yeap and others.[17] However, neither of these studies conducted experiments with mixed cohorts, when *Wolbachia*-infected and uninfected larvae compete for the same resources. During release periods, direct competition will occur between infected and uninfected larvae. We predict that *Wolbachia* infections will disadvantage immature stages in the field relative to the wild-type population under competitive situations.

Although *Wolbachia* infection causes a developmental delay during inter-strain competition, the implications for adult fitness under field conditions are less clear. We observed a size trade-off in fast developers; when larvae were crowded, slow developers were larger than mosquitoes that were quicker to emerge, presumably because of increased feeding time and reduced competition. This finding is in contrast to a study of *Ae. albopictus*,[19] in which delayed developers gained no size benefit when reared at a high density. Body size is an important indicator of female reproductive success; larger size is associated with greater fecundity,[41,46] blood feeding success[47] and mating success.[48] The *w*MelPop infection significantly reduced body size (wing length) in slow developers relative to uninfected *Ae. aegypti* adults. However, no size difference between strains was observed in quicker developers. Slow-developing *w*MelPop-infected larvae therefore appear less able to take advantage of an increased development period to grow larger. Although *w*MelPop caused a deleterious effect regardless of the initial infection frequency, the cost to size appears amplified during intra-strain competition.

A body size reduction was not observed in the *w*Mel infection; instead the infection was associated with a marginally increased size relative to uninfected mosquitoes, regardless of developmental time. We hypothesize that differential effects on size of *w*Mel and *w*MelPop infections might be caused by differences in the level of replication between the *Wolbachia* strains.[11] *Wolbachia* infection appears to be beneficial up until a certain density, but over-replication in host tissues becomes detrimental to size. We showed that *Wolbachia* density is highest in slow-developing *w*MelPop, in which we observed deleterious effects on size. However, *w*MelPop infection has no effect on the size of faster developers, in which *Wolbachia* density is lower. In the *w*Mel infection in which *Wolbachia* densities are half that of *w*MelPop, size is increased over the uninfected strain.

Prior studies have noted differential effects of *Wolbachia* infection between sexes in response to larval competition.[19,33] Sex-specific effects are expected in maternally transmitted endosymbionts because selection pressures acting on males are different than those acting on females. We found no clear sex-specific effects of either *Wolbachia* infection in our experiments. *Wolbachia* does not appear to cause differential survivorship between sexes because no deviations from a 1:1 male to female ratio in the surviving adults were significant. *Wolbachia* infection affected male and female developmental time in mixed cohorts equally, and wing length and *Wolbachia* density followed similar trends with respect to treatment and developmental time for both sexes.

Despite a developmental delay in the *w*Mel infection, this strain was able to invade two field populations,[14] and continues to persist.[49] For the *w*MelPop infection to become established in natural populations, strategies to counteract its deleterious effects must be used to maximize mosquito fitness. One potential strategy uses insecticides to crash target populations during release of insecticide-resistant *Wolbachia*-infected mosquitoes.[50] This strategy will reduce the release numbers required for the *Wolbachia* infection to exceed its threshold frequency and reach fixation in a population.

Although establishing the *w*MelPop infection in the field poses a challenge, there is continuing interest in the use of *w*MelPop for dengue control strategies; *w*MelPop provides a superior dengue blocking ability to *w*Mel,[12] and the life-shortening effect of *w*MelPop can further reduce potential dengue transmission (in the absence of its innate dengue protection) by killing the mosquito before the virus can be transmitted to a human host.[51,52] We showed that *w*MelPop infection has deleterious effects on developmental time and adult size in competition with uninfected larvae; ideally competition should be avoided during releases to minimize these fitness costs. These results are important for assessing the invasion prospects of the *w*MelPop infection in areas to which dengue is endemic.

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