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The Effect of Nonrandom Mating on *Wolbachia* Dynamics: Implications for Population Replacement and Sterile Releases in *Aedes* Mosquitoes

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Abstract. *Wolbachia* bacteria are known to cause deviations from random mating and affect sperm competition (SC) in some of their arthropod hosts. Because these effects could influence the effectiveness of *Wolbachia* in mosquito population replacement and suppression programs, we developed a theoretical framework to investigate them and we collected relevant data for the *w*Mel infection in *Aedes aegypti*. Using incompatibility patterns as a measure of mating success of infected versus uninfected mosquitoes, we found some evidence that uninfected males sire more offspring than infected males. However, our theoretical framework suggests that this effect is unlikely to hamper *Wolbachia* invasion and has only minor effects on population suppression programs. Nevertheless, we suggest that mating effects and SC need to be monitored in an ongoing manner in release programs, given the possibility of ongoing selection for altered mating patterns.

INTRODUCTION

In many arthropods infected with *Wolbachia*, the maternally inherited endosymbiotic bacterium can cause cytoplasmic incompatibility (CI), which is observed as infertility in uninfected females when mated only to infected males (reviewed in Hoffmann and Turelli[1] and Engelstädter and Telschow[2]). *Wolbachia*-infected females can produce viable, infected offspring when mated with uninfected or infected males, which equates to a fitness advantage over uninfected females. The fitness advantage gained by *Wolbachia*-infected females gives *Wolbachia* infection an edge in invading a population, and this can contribute to the rapid spread of *Wolbachia* in natural populations.[3,4] However, uninfected females could be selected to reduce the effect of incompatibility. This may occur through direct selection for alleles in host genomes that increase compatibility between crosses of uninfected females to infected males and indirect selection on *Wolbachia* alleles to decrease deleterious fitness effects,[5] or else by selection for mating isolation such that there is lower probability of uninfected eggs being fertilized by sperm from infected males than by sperm from uninfected males. Such mating isolation may be due to uninfected females preferentially mating with uninfected males[6] and/or sperm competition (SC) such that sperm from infected males is less likely to fertilize the egg when compared with sperm from uninfected males.[7,8] By reducing the effective level of incompatibility, these factors have potential to reduce the likelihood of *Wolbachia* infections spreading in host populations.[1]

Wolbachia introduced into *Aedes aegypti* block transmission of dengue and other viruses.[9–12] As the *Wolbachia* also cause CI in *Ae. aegypti*,[13–15] *Wolbachia* infections are good candidates for dengue control by means of mosquito population replacement. *Wolbachia* infections have also been proposed for population suppression using release of infected male mosquitoes to induce CI[16] akin to the sterile insect technique (SIT). *Wolbachia*-infected mosquitoes have now been used in field releases involving different strains but particularly *w*Mel,[17] as well as in trial SIT releases in Singapore (http://www.nea.gov.sg/public-health/environmental-public-health-research/wolbachia-technology) and elsewhere. Quality control is essential to understand the challenges that may impact *Wolbachia* utility in achieving population replacement or suppression. Quality control includes activities from laboratory fitness studies[15,18–20] to field evaluation[21] and evaluating patterns of genetic variation in *Wolbachia* and mtDNA.[22] Only a small number of mating isolation studies have been performed within the context of *Wolbachia* infection for population control,[23,24] and a greater understanding of mosquito mating behavior is needed.

Aedes aegypti females are thought to be monandrous (inseminated once),[25–28] whereas the males seek multiple mates.[29,30] During insemination, which usually occurs in a copulation event of > 6 seconds,[27] a substance within the male accessory gland appears to render females refractory to further insemination,[25,26] but females can still be inseminated within 24 hours after exposure to the male accessory gland.[25] There is also molecular evidence for some multiple insemination in the field.[31] This raises the question whether *Wolbachia* dynamics could be altered through SC and interrupted mating. Evidence for *Wolbachia* effects on SC of infected male relative to uninfected male range from a positive effect,[32] or no significant effect,[7,33] to a negative effect.[34]

Deviations from random mating may occur in *Ae. aegypti* for a number of reasons. Female size can affect male mate choice,[35] with mature males preferring larger females. Wing beat frequency, which allows some level of species recognition[36] during mating, appears to exhibit harmonic convergence when males and females mate.[37] With regard to mating success, Cator et al.[38] found that the ability to converge harmonically in wing beat frequencies could be crucial and represents a heritable trait that also increases mating success in male offspring. Each of these factors could be influenced by *Wolbachia*, given that there are known size and behavioral effects on *Ae. aegypti* associated with this infection.[20,39]

Here, we define a deterministic framework for investigating the effects of nonrandom mating and SC. We performed mating assays, exposing uninfected females to a mixture of infected and uninfected mosquitoes. Egg hatch rates were measured as a proxy for assessing whether females were mated to an uninfected (compatible) male or infected (incompatible)

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male. We use the framework and data to derive estimates of the magnitude of nonrandom mating and SC. Results are used to predict potential effects on population replacement and population suppression outcomes when releasing *Wolbachia*-infected mosquitoes.

MATERIALS AND METHODS

We first describe our empirical work testing for nonrandom mating at different *Wolbachia* infection frequencies. We then outline a model for nonrandom mating and SC and consider the implications for the mosquito control strategies before linking the empirical data to these predicted effects.

Mosquito lines. A laboratory-uninfected line and a *w*Mel-infected line of *Ae. aegypti* on a genetic background from Cairns, Far North Queensland, Australia, were used in this experiment. The uninfected line came from several hundred eggs collected around the Cairns region that were subsequently maintained as a mass bred population of several hundred adults. The *w*Mel-infected line was obtained from Gordonvale near Cairns where a field release of *w*Mel-infected mosquitoes had successfully led to *Wolbachia* invasion in the region.[17] The line had been held for three to seven generations in the laboratory at the time of the experiments. All cultures and experiments were maintained at 26°C, with a relative humidity of 70–80%, and 12:12 (hours) light to dark cycle, with about an hour of dim light before and after the light phase, to simulate dawn and dusk.

Mosquito rearing. Eggs were hatched in 3 L of reverse osmosis (RO) water with yeast (~0.09 mg) and one crushed tablet (~300 mg) of TetraMin® Tropical Fish Food tablet Rich Mix (Tetra Holdings Inc., Blacksburg, VA) in plastic trays (20 cm × 28.5 cm × 9 cm). Two days later, density was controlled to 225 larvae at approximately second instar in 4 L of RO water in plastic trays (42.7 cm × 31.2 cm × 7.2 cm) (Modulab Systems, Gratnell Ltd., Harlow, United Kingdom). Each density-controlled tray of larvae was supplied with one tablet of TetraMin® (300 mg). Further food was added in the next 5 days whenever food became scarce. Care was taken to make sure there was always some food left in the trays to prevent starvation. Under this regimen, the size of males and females does not differ significantly between *w*Mel-infected and uninfected mosquitoes.[40]

Seven days after the day of hatching, approximately 80–90% of the larvae were expected to have pupated, with a small percentage of males being expected to have eclosed. Each tray of 225 mosquitoes was transferred into 500 mL round containers with approximately 400 mL RO water. Pupae were sexed by size (females are larger than males) (cf.[41]). Virgin females and males were separated into several temporary cages (12.5 L space) for at least 3 days before the experimental setup. Each cage was supplied with a wick connected to 25 mL 10% sucrose solution.

Mating test. Because the number of females required to be assessed would be too large if individual females were used, we pooled 25 females at a time. To further increase the power of detection, we competitively mated uninfected females with a male population with a range of *Wolbachia* infection proportions, namely, 0, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1, with 0.1 omitted because of logistical constraints. We replicated the controls (0 and 1) three times, treatments 0.5 and 0.6 seven times, and the other treatments eight times (see next section for relevant power analysis). By using a range of infection proportions, we could use regression to assess patterns (described in the next section) rather than pairwise comparison of treatments. Competitive mating was performed in 3 L rectangle containers housing 25 uninfected females and 30 males with the aforementioned infection proportions. The density of mosquitoes used in such containers was higher than that usually observed in the field (e.g., < 10 females per house[42]), but the density in the field varies locally and can be high during the wet season.[43] We chose to include more males as it has been suggested that the operational sex ratios (OSRs) (sexually mature males to female ratios) are male biased.[44]

Adult females were exposed to males for 10–14 days before egg collection. Adult females were provided blood from human volunteers 7–10 days after exposure to males. Eggs were collected four times over four consecutive days on filter papers from a pool of 25 females for each replicate. Filter papers with eggs were kept wet for 3 days and then partially dried (moist to touch). Photos of eggs were taken and eggs were then counted using the ImageJ program (https://imagej.net) by placing marks on each egg (although where egg counts were low (< 50), they were counted directly by scanning the paper). Within a week of drying, filter papers with eggs were submerged in 1 L of water in a plastic container with excess (300 g per 225 larvae) Tetramin® fish food tablets. Eggs collected on different days were hatched separately to avoid overcrowding. Although we did not control for density, care was taken to avoid food depletion (or an oversupply of food which promotes anaerobic conditions) by monitoring containers and adding additional food as required. We did not detect an effect of larval density on hatch rate (see Results). Hatch rates were estimated from the total number of third instar larvae (5–6 days after hatching) divided by the total egg count. Larvae were counted with the help of a glass dropper pipette with a rubber bulb, by taking up a small number of larvae and releasing these into a container of water before counting the larvae with a manual counter as they exited the pipette. Before the regression analysis, we checked for fecundity bias by testing whether fecundity was associated with proportion of infected males in the experiment based on a pilot experiment (see Supplemental Material 1). We validated results by also using a linear model to test whether egg numbers in this experiment was associated with frequency of infected males. We assessed whether density of larvae hatched in the container affected the hatch rate by running a linear model with density, replicate, and treatment (frequency of infected males) as the independent variable and arcsine-transformed hatch rate as the dependent variable.

Estimating relative fitness of infected males. We let 1) β be the relative fitness of infected males to uninfected males, 2) H_{obs} be the observed hatch rate of eggs from uninfected females when exposed to *Wolbachia*-infected and uninfected males, 3) p_I be the *Wolbachia* infection frequency in males, 4) H be the hatch rate of eggs resulting from incompatible crossing (*Wolbachia*-infected male crossed with uninfected female) to account for incomplete CI, and 5) h be the average hatch rate of eggs from completely compatible crosses, to account for absolute fecundity/hatch rate that was not 100%. From the mating experiment, H_{obs} was estimated via the larvae counts divided by egg counts, H was the hatch rate of the control with proportion infected males = 1, and h was the hatch rate of the control with proportion infected males = 0. β was the parameter

that we were interested in estimating. All parameters are probability values except β (β ≥ 0). β < 1 signifies lower fitness in infected males, whereas β > 1 signifies greater fitness.

$$H_{\text{obs}} = \frac{(1-p_{\text{I}})^{*}h + \beta p_{\text{I}} H}{\beta p_{\text{I}} + (1-p_{\text{I}})} \quad (1)$$

The numerator of equation (1) signifies all the possible viable offspring, whereas the denominator is the total probability of all possible matings, given the relative fitness β. We rearranged equation (1) such that those parameters could be treated as the slope of a linear regression model as described in equation (2).

$$(H_{\text{obs}} - h)^{*}(1-p_{\text{I}}) = \beta^{*}p_{\text{I}}{}^{*}(H - H_{\text{obs}}) + 0, \quad (2)$$

where

$$\beta = \frac{(h - H_{\text{obs}})}{(H_{\text{obs}} - H)} {}^{*} \frac{1-p_{\text{I}}}{p_{\text{I}}}. \quad (3)$$

The linear regression model treats $(H_{\text{obs}} - h)^{*}(1 - p_{\text{I}})$ as the *y* axis and $p_{\text{I}}{}^{*}(H - H_{\text{obs}})$ as the *x* axis, forcing the *y* intercept to zero. We ran Shapiro–Wilk's tests on those axis values to test the normality assumption.

Mating isolation in the context of *Wolbachia*-infected and uninfected males could be either attributed to 1) nonrandom mating due to a fitness difference between infected and uninfected males (NR); 2) nonrandom mating due to assortative mating (AM), or the nonrandom mating of similar individuals, that is, infected × infected or uninfected × uninfected; or 3) SC where sperm from a male with one infection status is more successful at fertilizing the egg. If only one of the three types of mating isolation occurs, the estimate of β would be reflective of the strength of that particular mating isolation (see Table 1), but these effects are otherwise difficult to separate in *Ae. aegypti*. Nonrandom mating could be evaluated by observing the frequency of different combinations of mating pairs, but this was difficult in *Ae. aegypti* because of their short copula duration. It might be possible to approximate nonrandom mating effects if a separate experiment using sequential mating could be performed, but to mate female *Ae. aegypti* to two males in sequence can be challenging because, following formation of the first mating pairing, the second copulation might not lead to successful sperm transfer. As we were unable to disentangle nonrandom mating and SC effects, the estimate of β is the composite effect of the relative fitness of infected males to uninfected males.

Under random mating and no SC, the average reduction in the hatch rate observed in uninfected females would reflect the frequency of infection among males. We performed a power analysis to determine the sample size required to estimate β using equations (1)–(3). The power analysis was based on the null hypothesis that the hypothetical reduction in the hatch rate is equivalent to the infection frequency (as in the complement of equation [1]), whereas with the alternate hypothesis, we were interested in detecting β = 0.5, that is, the effective frequency of infected males is halved. For example, if $h = 1$ and $H = 0$, and if the null hypothesis, H_{o}, states that if $p_{\text{I}} = 0.75$, the average reduction in hatch rate = 0.75, then, the alternate hypothesis, H_1, should be that the average reduction in hatch rate = 0.6 (the complement of equation [1], with β = 0.5).

For uninfected female sample sizes, *n* of 100, 120, 130,150, 180, and 200, we simulated the reduction in the hatch rate due to incompatible mating (at infection frequencies, p_{I} of 0.05–0.95). We simulated *n* individual females assuming normally distributed egg numbers with mean and standard deviation determined in a fecundity assay of uninfected females (see Supplemental Material 1). We then simulated for each individual whether they would encounter an infected male with frequency, p_{I} (0.05–0.95). If they encounter an infected male, the female will have no offspring. Then, the overall hatch rate of these *n* individuals was computed. These *n* individuals were simulated 10,000 times, resulting in 10,000 computations of simulated overall hatch rate. The overall hatch rates are partitioned into quantiles 0–100% at an interval of 5%. Say H_0: average reduction in hatch rate = $p_{\text{I}}(1)$, the 5% quantile would mean that there is only a 5% chance that the observed reduction in hatch rate is due to an effective infection frequency, $p_{\text{I}}(1)$. This 5% quantile is the critical region for rejection (α = 0.05) of the null hypothesis. If (say) under an alternative hypothesis, the true effective reduction in hatch rate is $p_{\text{I}}(2) < p_{\text{I}}(1)$ and the 5% quantile for $p_{\text{I}}(1)$ fell within the 90–95% quantile of $p_{\text{I}}(2)$, then the power of detection (accepting the alternative hypothesis) would be in the range of 90–95%. Using this, we found that we needed to assess egg hatch from 150 to 200 uninfected females if we wanted more than 90% power of detecting that infected males were only half as likely as uninfected males to mate successfully with uninfected females (β < 0.5) (see Supplemental Material 2).

We benchmarked the analytical method to further evaluate the type 1 and 2 errors of the experimental design (25 uninfected females in each treatment, three replicates of each

TABLE 1

Mating isolation scenarios, experimental method to estimate their effect via equations (1)–(3), associated assumptions, and what β means in each context

Mating isolation scenario	Experimental method	Assumptions	What does β signify?
Nonrandom mating due to fitness difference between infected and uninfected males	Via measuring the hatch rate of eggs from uninfected females that were placed in an arena of infected and uninfected males	Females mate only once, probability of encountering males reflects infected/uninfected frequencies	Relative fitness of infected males to uninfected males
Nonrandom mating due to assortative mating	Via measuring the hatch rate of eggs from uninfected females that were placed in an arena of infected and uninfected males	Females mate only once, probability of encountering males reflects infected/uninfected frequencies	Relative preference of females for males of different infection status to males of the same infection status
Sperm competition	Via measuring the hatch rate of eggs from uninfected females that were sequentially force-mated to two males of different infection status, $p_{\text{I}} = 0.5$	Each male contributes the same number of sperm. No effect of order of mating	Relative fitness of sperm from infected males to sperm from uninfected males

control, 0% and 100% infected males, seven replicates of 50% and 60% infected males and eight replicates of 20%, 30%, 40%, 70%, 80%, and 90%). The experimental design used in this study was simulated 10,000 times and each of the simulated datasets was subjected to the regression analysis described in equations (1)–(3), testing the null hypothesis that the relative fitness of infected to uninfected males is equal or $\beta = 1$. From this, we found that we needed to adjust the P value to 5×10^{-5} to have less than 5% type 1 error and this adjusted P value had > 95% power (type 2 error < 5%) of detecting a relative fitness of infected to uninfected, $\beta \leq 0.8$ (see Supplemental Material 3)

Model of mating. Nonrandom mating (in females) due to a fitness difference between infected versus uninfected males (NR), and SC could be considered as a bias for or against males (and their sperm) of different *Wolbachia* infection status, whereas nonrandom mating due to AM could be considered a bias for or against mating events involving male and female pairs with a different infection status. We let β_{nr} be the ratio of preference for *Wolbachia*-infected males to uninfected males, β_{am} be the ratio of mating pair with dissimilar mating pair to mating pair where both males/females have the similar infection status, and β_{sc} be the ratio of preference for sperm of *Wolbachia*-infected males to uninfected males. These parameters can take values from 0 toward positive infinity. Where 1) β_{nr}, β_{am}, and β_{sc} values = 1, there are no NR, AM, and SC, respectively; thus, the effective infection frequency is the same as the actual infection frequency. 2) Where β_{nr} and $\beta_{sc} < 1$, there is a bias against infected males, and while $\beta_{am} < 1$ implies females having greater preference for males of the same infection status, 3) β_{nr} and $\beta_{sc} > 1$ implies a bias for infected males, or $\beta_{am} > 1$ implies a bias for males of dissimilar infection status. Under conditions where male preference is the same regardless of female infection status, we can estimate the effective infection frequency in males under NR, p_{nr}:

$$p_{nr} = \frac{\beta_{nr} p_I}{\beta_{nr} p_I + (1 - p_I)}, \tag{4}$$

where p_I is the actual infection frequency in males. When considering nonrandom mating due to AM, it is not possible to estimate effective male infection frequency as the effect is also dependent on female infection status (see Table 2), unless we were looking at only one female infection state. The effective male infection frequency under SC, p_{sc}, is defined as

$$p_{sc} = \begin{cases} p_I, & n = 1 \\ p_I(1 - p_m) + p_m p_I^2 + 2 p_m p_I (1 - p_I)\left(\dfrac{\beta_{sc}}{\beta_{sc} + 1}\right), & n = 2 \\ \displaystyle\sum_{k=1}^{n} \binom{n}{k} p_I^k (1 - p_I)^{n-k} p_m^{n-1} \left(\dfrac{\beta_{sc} k}{\beta_{sc} k + [n - k]}\right) + & \\ \qquad \displaystyle\sum_{j=1}^{n-1} \sum_{k=1}^{j} \binom{j}{k} p_I^k (1 - p_I)^{j-k} p_m^{j-1} (1 - p_m) \left(\dfrac{\beta_{sc} k}{\beta_{sc} k + [j - k]}\right), & n \in N, \end{cases} \tag{5}$$

where p_m is the remating frequency. For simplicity, we consider a maximum of two matings in polyandrous females. The effective infection frequency under SC, p_{sc} in (2), assumes equal sperm contribution from all males mated to the polyandrous females. It also assumes that each remating event is independent of the previous mating, and all males have an equal opportunity to mate, but SC is modified by the β_{sc} parameter.

Effect of nonrandom mating and SC on population replacement. To evaluate the impact of nonrandom mating and SC on population replacement, it was necessary to evaluate the infection unstable equilibrium frequency, as this is the frequency that defines whether the infection will increase (when infection frequency is above the equilibrium) or decrease (below the equilibrium). The formulation of the infection frequency difference equation can easily be derived from the Punnett square (Table 2). The next-generation probability of each cell is given by first multiplying the corresponding female

TABLE 2

Punnett squares with proportion of each cross resulting in viable offspring under different models

	Female		
	$1 - p_I$ (uninfected)	p_I (infected)	
Male	Uninfected offspring	Uninfected offspring	Infected offspring
Nonrandom mating due to difference in male fitness			
$1 - p_I$ (uninfected)	1	$\mu^*(1 - s_f)$	$(1 - s_f)^*(1 - \mu)$
p_I (infected)	$\beta^*(1 - s_h)$	$\beta^*\mu^*(1 - s_f)$	$\beta^*(1 - s_f)^*(1 - \mu)$
Nonrandom mating due to assortative mating			
$1 - p_I$ (uninfected)	1	$\beta^*\mu^*(1 - s_f)$	$\beta^*(1 - s_f)^*(1 - \mu)$
p_I (infected)	$\beta^*(1 - s_h)$	$\mu^*(1 - s_f)$	$(1 - s_f)^*(1 - \mu)$
Sperm competition			
$1 - p_{sc}$ (uninfected)	1	$\mu^*(1 - s_f)$	$(1 - s_f)^*(1 - \mu)$
p_{sc} (infected)	$(1 - s_h)$	$\mu^*(1 - s_f)$	$(1 - s_f)^*(1 - \mu)$

p_{sc} is defined in equation (5).

and the male frequencies and the relative viability within the given cell. This is then divided by the summed value across all cells. The s_h term refers to the reduction in viability due to infected sperm fertilizing an uninfected egg, s_f is the relative reduction in fecundity in infected females to uninfected females, and μ is the probability of offspring of infected females not acquiring *Wolbachia* infection, that is, transmission leakage. For nonrandom mating due to the difference in male fitness (Table 2), it should be apparent if all cells were divided by $\beta p + (1 - p)$, the β parameter could be absorbed into the male frequencies; hence, the model can be derived with the effective male infection frequency described in equation (4). It is not possible to factor out the β parameter into one of male/female frequencies in the AM model (Table 2). We derived the difference equation assuming discrete generations so that the unstable equilibria are mathematically tractable. We assumed there was no maternal transmission leakage, $\mu = 0$. Although there has been a reported case of maternal transmission leakage in the field[45] and transmission leakage can be induced under certain stressful temperature conditions,[19] under constant rearing temperature of 26°C, maternal transmission leakage has not been detected for this infection[14,19] (see Discussion).

Effect on Incompatible Insect Technique. Akin to SIT, IIT is the inundation of the population with infected (incompatible) males rendering uninfected females sterile by CI. As the basis of IIT is male-only release, the impact of nonrandom mating effects and SC could be evaluated by looking at the effective male infection frequency. The effective male infection frequency in this case can be considered as the infection frequency when there is random mating and no SC. Thus, the infection frequency required to achieve the same effect as a given infection frequency under the random mating, and no SC, is given by making p_I the subject in equations (4) and (5).

The infection frequency required to achieve effective male infection frequency because of nonrandom mating effects is given by

$$p_I = \frac{p_{nr}}{p_{nr} + \beta_{nr}(1 - p_{nr})}. \tag{6}$$

Because IIT is a male-only release, the effect of nonrandom mating due to a difference in male fitness or AM should be the same because there are no infected females. The infection frequency required to achieve effective male infection frequency due to SC can be obtained by solving

$$p_I^2\left(p_m - 2p_m\left[\frac{\beta_{sc}}{\beta_{sc}+1}\right]\right) + p_I\left(1 - \left[p_m - 2p_m\left(\frac{\beta_{sc}}{\beta_{sc}+1}\right)\right]\right) - p_{sc} = 0. \tag{7}$$

To better reflect the magnitude of the release, we transformed the estimates of infection frequency, p_I, into release ratios as it provides a more direct view of how many incompatible males with respect to uninfected males will be required. For example, $p_I = 0.5$ equates to a ratio of one released *Wolbachia*-infected male to one uninfected wild male. The relationship of infection frequency, p, to the release ratio of infected factory produced males to uninfected wild males, R, is given by

$$R = \frac{p_I}{1 - p_I}. \tag{8}$$

We can use equations (6) and (7) to determine the infection frequency required to achieve the same effect as when there is no NR, AM, or SC (reflected in p_{nr} and p_{sc}). Then, the release ratio, R, can be determined by equation (8). To estimate the burden of NR, AM, and SC on the release program, we calculate the percentage increase, Δ, in number required to achieve the same effect as when there is no NR, AM, or SC, defined by

$$\Delta = \frac{R(p_I|\beta \neq 1) - R(p_I|\beta = 1)}{R(p_I|\beta = 1)} \times 100\%. \tag{9}$$

In fact, under the NR or AM model, equation (9) reduces into a constant independent of p_I.

$$\Delta_{nr} = \frac{1 - \beta_{nr}}{\beta_{nr}} \times 100\% \tag{10}$$

However, under the SC model, equation (9) is still dependent on p_I. In fact, increasing p_I under the SC model decreases the estimate of Δ when p_m and β_{sc} are held constant. With this model framework, we made an empirical estimation of the values of β under various nonrandom mating and SC effects to evaluate *w*Mel-infected *Ae. aegypti* as a candidate for population replacement and IIT.

RESULTS

Mating test. Based on incompatible control crosses (100% *w*Mel-infected males crossed with uninfected females), we estimated $H = 0$ or complete CI. Using the compatible cross-controls (0% *w*Mel-infected males), the average hatch rate of eggs, h, is around 0.8676. From a previous pilot experiment, there was no evidence that female fecundity was affected by the male infection status (see Supplemental Material 1). We further found no association between fecundity of uninfected females with proportion of infected males ($F_{1,65} = 0.014$, $P = 0.9069$). We found no association between hatch rates with either replicate ($F_{7,226} = 1.467$, $P = 0.1800$) or larval density ($F_{1,226} = 0.1138$, $P = 0.7362$). More specifically, when looked at treatments with the least infected males (0 and 0.2), there was no evidence that larval density affected the hatch rate ($F_{1,38} = 0.686$, $P = 0.4129$). We then ran a linear regression based on equation (2) and found that the estimate (and 95% confidence interval) for β was 0.6410 ± 0.0907 (Figure 1). The test for the null hypothesis of $\beta = 1$ yielded an unadjusted P value of 1.97×10^{-11} (which is far lower than threshold 5×10^{-5}) based on a t test with 66 degrees of freedom (there were 67 pools of females tested).

Effect of nonrandom mating and SC on population replacement. The unstable equilibrium under nonrandom mating due to difference in male fitness and nonrandom mating due to AM was

$$\hat{p} = \frac{s_f}{s_f + \beta_{nr}(s_h - s_f)}, \tag{11}$$

$$\hat{p} = \frac{\beta_{am}s_f + 1 - \beta_{am}}{2(1 - \beta_{am}) + s_f(\beta_{am} - 1) + \beta_{am}s_h}. \tag{12}$$

The unstable equilibrium under the NR model is nonzero and less than one if and only if $s_h > s_f$, and for the AM model, if and only if $\beta_{am} < (1 - s_f)^{-1}$ and $s_h > s_f^*(2 - s_f)$ or $\beta_{am} < (1 - s_f)/(1 - s_h)$ and $s_h < s_f^*(2 - s_f)$.

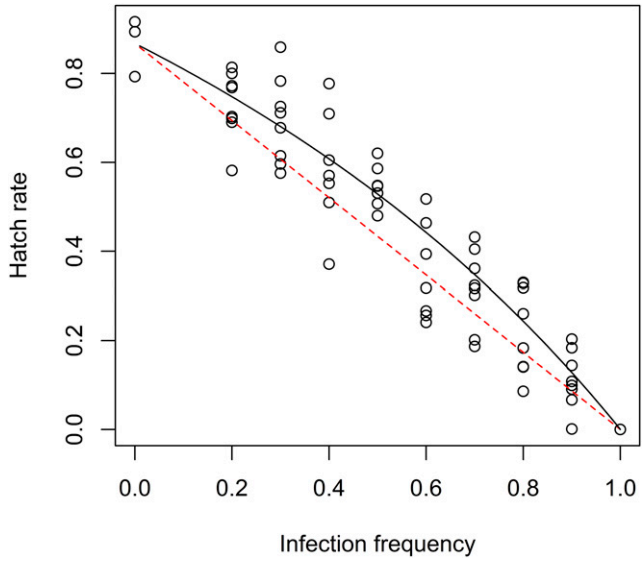

FIGURE 1. Hatch rate of eggs from replicate pools of 25 uninfected female *Aedes aegypti* exposed to a corresponding frequency of *w*Mel-infected males. Each circle represents a single replicate pool of 25 uninfected females. The dotted line is β = 1 (no difference in infected and uninfected male contribution) and β = 0.6410 is the estimated value from the regression. This figure appears in color at www.ajtmh.org.

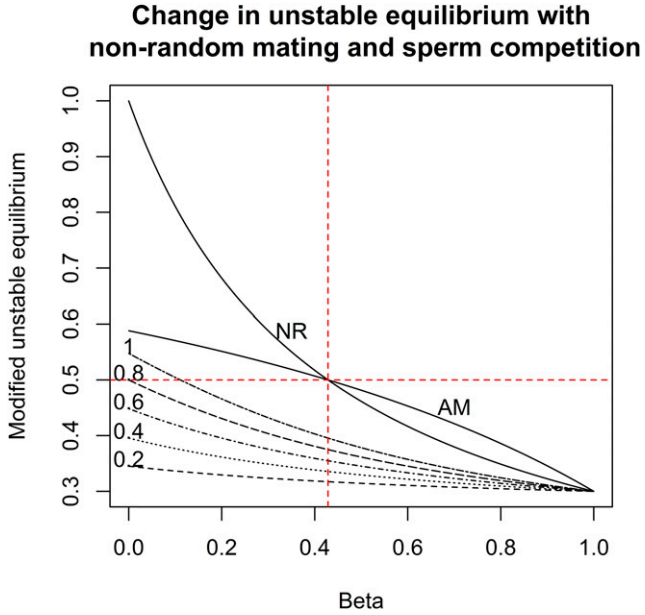

FIGURE 2. Modifications to the unstable equilibrium under non-random mating due to difference in male fitness (NR), assortative mating (AM), and sperm competition (SC) (with remating frequency, p_m, labeled on the graph). The estimate of unstable equilibrium when there is random mating and no SC was assumed to be 0.3, given by s_f and s_h of 0.3 and 1, respectively. β in general (except for AM) refers to relative fitness of infected to uninfected males (*Aedes aegypti*) under NR, β_{nr}, or SC, β_{sc}. NR occurs under the scenario when infected males are β_{nr} times likely than uninfected males to mate with females (infected or uninfected). The lines labeled with values (just above the line) are under the SC only model for which the values equate to the remating frequency. Under the AM model (note that the line above the number 1 is part of the AM model), β is the relative frequency of mate pairing between nonidentical infection states, to mate pairing between identical infection states. At β = 0.4286 (vertical dotted line), both NR and AM models modify the unstable equilibrium to 0.5. This figure appears in color at www.ajtmh.org.

Under SC with maximum two mates only, the unstable equilibrium is the positive solution to equation (13) bounded by 0 and 1, where $s_h > s_f$,

$$\hat{p}^2\left(s_h p_m\left[\frac{1-\beta_{sc}}{1+\beta_{sc}}\right]\right)+\hat{p}\left(s_h+s_h p_m\left[\frac{\beta_{sc}-1}{1+\beta_{sc}}\right]\right)-s_f=0. \quad (13)$$

As should be expected, when $\beta_{nr} = \beta_{am} = \beta_{sc} = 1$, that is, random mating or no SC, the unstable equilibrium reduces to s_f/s_h which is the unstable equilibrium under random mating and no SC. In all cases, the stable equilibrium frequencies are 0 and 1. Decreasing the value of β increases the estimate of the unstable equilibrium. Under the two nonrandom mating scenarios, it can be shown that $\beta_{nr} = \beta_{am} = s_f/(s_h - s_f)$ when the unstable equilibrium is 0.5 (Figure 2) for any scenario of s_f and s_h (see Supplemental Material 4). The unstable equilibrium under the NR and SC models (equations [11] and [13]) is in fact the same as applying equations (6) and (7), replacing p_{nr} and p_{sc} with the unstable equilibrium under random mating and no SC (see Supplemental Material 5). This means that the effect of β_{nr} and β_{sc} will be the same for any combination of s_f and s_h which gives rise to the same values of an unstable equilibrium under random mating and no SC, that is, s_f/s_h. This, however, only works when we assume no maternal transmission leakage, μ = 0 (see Supplemental Material 6).

Say if s_f and s_h were 0.3 and 1, respectively, the unstable equilibrium under random mating and no SC is 0.3. If β was less than 0.5 (or more specifically < 0.4286) for both nonrandom mating scenarios (NR and AM), the unstable equilibrium would increase beyond 0.5. However, the effect of SC appears to be less than that of NR, as it depends on the remating rate (Figure 2, see Supplemental Material 7) and also on the number of male mates, *n*. We showed numerically that when remating frequency is 1 and *n* is large, the effect of SC will be similar to that of AM for the same β values (see Supplemental Material 8). Given it is biologically unforeseeable that a female could have very large numbers of mates especially if reproductive lifespan is short, it is safe to say that SC will never surpass the effect of nonrandom mating due to difference in male fitness, given the same relative fitness of infected to uninfected types. It is important to reiterate that this assumes equal contribution of male mates without any sperm exclusion strategy.

Effect on IIT. It has been suggested that release ratios of sterile males to wild female numbers be in the magnitude of 1.7 up to 150 times[46–49] to achieve suppression of the target insect. IIT in *Aedes* species is being based on a relative ratio of 5–20 (Zhiyong Xi, personal communication). As stated in the previous section, the effect of SC was likely lower than that of NR/AM; thus, we use the effect of NR on the release ratios as the upper bound which is a constant dependent only on β, as in equation (10).

It was apparent that if β = 0.5, then the release ratio would need to be increased by at most 100% (i.e., twice the effort) (Figure 3). This meant that if the ratio of release was forecast to be 5:1 when AM/NR is not considered, then for β = 0.5, the ratio of release needs to be increased to 10:1 to achieve the same suppression effect. If a release ratio of 5:1 was necessary and the production line was able to tolerate an increase

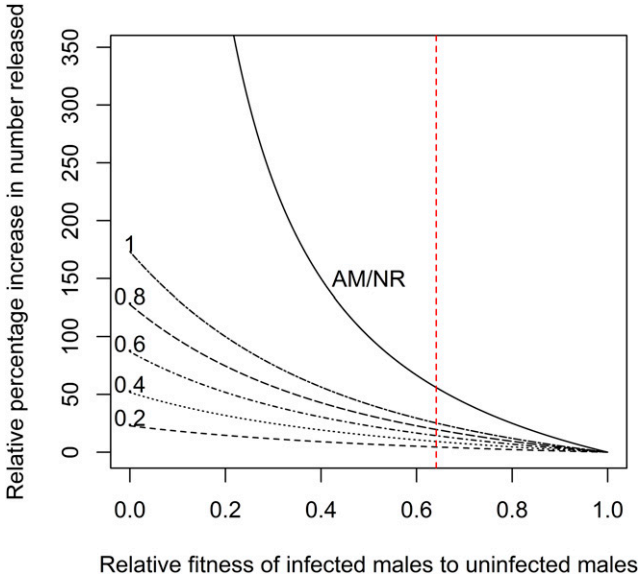

FIGURE 3. Percentage increase in number of infected male *Aedes aegypti* required to achieve the same suppression levels as when there is assortative mating or sperm competition (SC) effects, $\beta = 1$. Assortative mating/NR is the curve for the effects of nonrandom mating, whereas the other curves are for SC effects under varying remating frequencies (given on lines). This figure appears in color at www.ajtmh.org.

up to 20:1 (increase of 300%), then based on equation (10), the lowest possible β is 0.25.

Linking models to empirical data. Because it was not possible to make an empirical estimate of β_{sc}, the value of $\beta = 0.6410 \pm 0.0907$ represents the composite effect of nonrandom mating and SC. As we observed that the nonrandom mating due to difference in male fitness model reflected the greatest change to the effective infection frequency and also the unstable equilibrium (see also Supplemental Materials 7 and 8), we evaluated the results based on that model.

The unstable equilibrium frequency for the *w*Mel infection in *Ae. aegypti* based on previously estimated fitness parameters was around 0.3.[17] Assuming no maternal transmission leakage, the result in Figure 2 will be reflective of the *w*Mel infection for all values of β_{nr} between 0 and 1. The estimated value β should not lead to an unstable equilibrium that will exceed 0.5. In the IIT context, the recommended increase in production of *w*Mel-infected males was 56.0% to achieve the same effect as the initial estimates when AM/NR was not considered (Figure 3, and equation [10]). If the maximum capacity of the production facility was also the same as the recommended release ratio when AM/NR was not considered, say 20 infected males to one wild uninfected male, we found that the extra time required to achieve the same level of suppression was only at most 17.9% longer (see Supplemental Material 9).

DISCUSSION

Here, we defined three separate contexts for nonrandom mating (difference in male fitness, AM, and SC). We used these contexts to estimate relative fitness of *Wolbachia*-infected males to uninfected males based on hatch rates of eggs from uninfected females that were exposed to different proportions of infected and uninfected males. We defined a separate framework for assessing SC based on the assumption of no sperm displacement. As this represents postmating isolation, it is dependent on the females mating multiple times. Disentangling the effects of nonrandom mating (premating effects) and SC required remating frequencies to be estimated along with the incidence of forced remating to estimate SC effects separately. However, it was impossible to estimate remating frequency using only offspring hatch rate data, which required the assumption of no AM. Also, forced remating may not be testable when cage environments restrict the ability of females to counter males by moving away. This led to estimates based on the composite effect of nonrandom mating and SC.

The empirical method we used to estimate the composite relative fitness of infected to uninfected males is identical to Fried's competitive index[50] or similarly the competitive index based on the relative sterility index (CRSI), which was proposed as a measure to evaluate sterile male quality. The Fried competitive index, I, is given by

$$I = \frac{H_N - H_o}{H_o - H_S} * \frac{N}{S}, \tag{14}$$

where N and S are the number of non-sterile and sterile males, respectively, whereas H_N, H_o, and H_S are the average hatch rates of non-sterile crosses, observed hatch rate, and average hatch rate of eggs of sterile males crossed with wild females. When compared with the relative sterility index (RSI), which is also often used in sterile male evaluation, RSI can be computed by taking $I/(I+1)$. Values of RSI, and CRSI in the context of sterile tephritid fruit flies, are directly estimated from identifying capturing mating pairs. However, in the context of *Ae. aegypti*, studying mating couples is practically impossible because of short copula duration,[27] whereas mating isolation studying egg hatch rate is actually well established in *Ae. aegypti*, and has been used in monitoring releases of *Wolbachia*-infected mosquitoes.[17,51]

When compared with equation (3), it should be apparent that N/S is the same as $(1 - p_I)/p_I$, and h and H are analogous to H_N and H_S, respectively. The composite effect should provide the most pessimistic view of the effect of nonrandom mating and SC because it is based on the nonrandom mating model which provides the worst-case scenario for the relative fitness of infected to uninfected males. In general, we were most interested in knowing if nonrandom mating causes the unstable equilibrium to exceed 0.5, as this was the conservative estimate at which the infection will not spatially spread.[52] Both models were identical when determining the nonrandom mating parameter values (β_{nr} and β_{am}) at which the unstable equilibrium exceeds 0.5. However, in most other cases, both models affected the unstable equilibrium slightly differently. To fully disentangle the type of nonrandom mating, it was, therefore, necessary to study mating pairing probability for all combinations of infected/uninfected males and females.

In our scenario, we had a male-biased OSR in swarms which is thought to be the norm for mosquitoes,[44] although *Ae. aegypti* exhibits variation in swarming behavior.[53,54] Male-biased OSR and monandrous females may tend to select for more aggressive males that can encounter more virgin females and also select for female choice because females have only one chance to maximize their fitness. Laboratory experiments may not relate to field conditions well but offer a way of studying fitness across multiple treatments. In a confined

space such as a cage, females may be unable to escape as easily from males as in the field, reducing the opportunity for mate choice. Also, less aggressive males are less likely to be disadvantageous compared with aggressive males in confined spaces. This means that inferences about relative fitness in the context of AM based on cage studies need to be made with caution. Results from larger tent enclosures considering the relative fitness of *Wolbachia*-infected males at one male density are consistent with those obtained from cages[23] but it is not easy to scale-up such experiments with the type of replication required to establish frequency dependent patterns.

In this study, we have omitted *Wolbachia* maternal transmission leakage from the models, which is normally low for *w*Mel even under field conditions[45] except under hot conditions.[19] The estimation of relative fitness of infected males to uninfected males does not require any knowledge of maternal transmission leakage. However, it does affect the estimation of the unstable equilibrium under the population replacement scenario. If we assume that relative fitness of males is independent of maternal transmission leakage, then transmission leakage of *Wolbachia* will lead to a higher unstable equilibrium, as in worst outcome for population replacement. Say if the lower relative fitness of infected males to uninfected males was due to a mitochondrial defect that hitchhiked with the *Wolbachia* infection, the resultant unstable equilibrium may decrease as lower fitness uninfected males are generated, but this is unlikely to be any worse than in the previous example for the *Wolbachia* infection. In terms of population suppression with infected males, maternal transmission leakage will not have any effect because there are no infected females, unless the females that gave rise to the infected males were reared under suboptimal conditions.

In the experiments, we showed that we needed to measure at least 175 females to detect $\beta_{am} < 0.5$ with 90% power when rejection probability is < 0.05. Moreover, we formulated a linear model in the form of equation (2) to allow a large amount of replicated data with a spectrum of infection frequencies to be analyzed and avoid multiple comparison concerns when estimating β, increasing the power of detection. Because of logistics of observing hatch rates from a large number of individual females, pooling females into equal pool sizes was performed. This may be prone to bias contribution from females that were more fecund; for example, if more fecund females by chance mated to a particular infected male, then the pool hatch rate will likely become lower than expected. However, the total number of females tested (1,550 across all treatments and 75 for each control, i.e., 0% and 100% infected male) should overcome any stochastic effects of infection status of male mates (see Supplemental Material 3). It was necessary to ensure that uninfected female fecundity was not associated with infection status of the male mate (see Supplemental Material 1). Because hatch rates were determined at a later juvenile stage, we needed to ensure that the estimation of the hatch rate was not affected by larval competition (due to varying densities) and we did not detect confounding effects of larval density. However, in future experiments it may be possible to control larval density at an earlier instar stage by placing larvae in a larger volume of water.

In our case study using the *w*Mel infection in *Ae. aegypti*, we found that there was a statistically significant disadvantage in the composite effect of AM and SC. However, the effect on the unstable equilibrium for population replacement programs and relative release ratios for IIT programs was relatively minor and unlikely to affect efficacy of those programs. In the context of population replacement programs, the unstable equilibrium did not exceed 0.5 when including the composite effect, which meant that spatial spread of the infection is unlikely to be affected.[52] The increase in the production rate required to achieve the desired suppression effects is unlikely to tip the balance of the economic benefit from releases. Still, in concert with other fitness costs that were not considered in the evaluation of *w*Mel infection, such as temperature-dependent mortality[19] and larval performance under starvation conditions,[18] any disadvantage for infected males can complicate release programs.

Although we did not find large effects of the infection on mating or SC in this study, it is possible that such effects might evolve over time. Evolutionary changes are unlikely in cases where the infection rapidly increases to a high frequency following releases because there will be little opportunity for evolutionary changes to occur before the entire population becomes infected. However, if there is a high degree of maternal transmission leakage, uninfected females that mate with uninfected males will have a massive fitness advantage over randomly mating uninfected females, creating a strong selection pressure for mate recognition (or SC). Strong selection pressures may also exist following releases when adjacent areas with high and low infection frequencies occur side by side because of dispersal barriers, as seen in areas of North Queensland following *Wolbachia* releases.[55,56] For this reason, it may be prudent to monitor mating behavior across time.

CONCLUSION

We developed a way to evaluate the efficacy of *Wolbachia* infection for population replacement or IIT when hatch rate data are easy to obtain. In the context of population control, studying the composite effects of nonrandom mating and SC may be sufficient to assess the effects of mating isolation. In the context of population replacement or IIT using releases of both sexes, knowing male relative fitness might be sufficient to evaluate spatial spread capability of the infection. If possible, it is advisable to study mating isolation in both sexes (i.e., all possible crosses within one enclosure replicated many times). In the *w*Mel infection, there was no strong evidence to suggest that the estimated reduction in efficacy of infected versus uninfected males would significantly impair population replacement and IIT programs.

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