Stable phenotypic effects of an unstable deleterious *Wolbachia* infection

Running title: *w*MelPop phenotypic stability

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Author contributions

AAH and PAR conceived the study. PAR performed the phenotypic stability experiments. AGC and JKA performed the maternal transmission experiment. JKA and PAR performed the nuclear background evolution experiments. JKA and AGC performed the *Wolbachia* mating transmission experiments. JKA and AGC performed the routine colony monitoring. AGC and KMR performed the *Wolbachia* screening for all experiments. PAR and AAH analyzed and visualized the data. AAH performed simulations for the loss of *Wolbachia*. AAH, PAR, JKA and AGC wrote the paper. All authors read and approved the final manuscript.

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Conflict of interest statement

The authors declare that no competing interests exist.

Abstract

Wolbachia are being used to reduce dengue transmission by *Aedes aegypti* mosquitoes around the world. To date releases have mostly involved *Wolbachia* strains with limited fitness effects but strains with larger fitness costs could be used to suppress mosquito populations. However, such infections are expected to evolve towards decreased deleterious effects. Here we investigate potential evolutionary changes in the *w*MelPop infection transferred from *Drosophila melanogaster* to *Aedes aegypti* more than ten years (~120 generations) ago. We show that most deleterious effects of this infection are stable despite strong selection to ameliorate them. The *w*MelPop infection is difficult to maintain in laboratory colonies, likely due to the persistent deleterious effects coupled with occasional maternal transmission leakage. Furthermore, female mosquitoes can be scored incorrectly as infected due to transmission of *Wolbachia* through mating. Infection loss in colonies was not associated with evolutionary changes in the nuclear background. These findings suggest that transinfections with deleterious effects may have stable phenotypes which could ensure their long-term effectiveness if released in natural populations to reduce population size.

Keywords

Wolbachia, deleterious effects, attenuation, cytoplasmic incompatibility, maternal transmission, mating transmission

Introduction

There is increasing interest in using *Wolbachia* bacterial infections for suppressing dengue transmission by mosquitoes, with field releases aimed at both replacing existing natural mosquito populations with those infected by *Wolbachia* (Hoffmann et al. 2011; O'Neill et al. 2018) and suppressing these populations through sterility induced by *Wolbachia*-infected males (Zheng et al. 2019). Replacement releases can be effective because the presence of *Wolbachia* in mosquitoes reduces transmission of arboviruses (Moreira et al. 2009a; Walker et al. 2011; Ant et al. 2018). In addition, *Wolbachia* decreases the fitness of its mosquito hosts (Ross et al. 2019c). While this might have a suppressive effect on dengue transmission, for instance, by shortening mosquito lifespan (Cook et al. 2008), it can make the

infections more difficult to introduce into populations because the initial *Wolbachia* frequency has to be higher for the population to be invaded by *Wolbachia* (Hoffmann and Turelli 1997).

The *w*MelPop infection, which originated from a laboratory strain of *Drosophila melanogaster*, was one of the first *Wolbachia* strains successfully introduced into *Aedes aegypti* (McMeniman et al. 2009) where it is very effective at blocking transmission of dengue and other arboviruses (Moreira et al. 2009a). This strain reduces longevity in *D*. *melanogaster* (Min and Benzer 1997) and also in mosquitoes where it has additional deleterious effects, including reduced viability of eggs maintained in a quiescent state (McMeniman and O'Neill 2010; Yeap et al. 2011). The *w*MelPop infection was released in field trials in Vietnam and Australia but failed to establish (Nguyen et al. 2015), although it successfully invaded semi-field cages (Walker et al. 2011). Because of these deleterious effects, *w*MelPop may represent an effective tool to reduce or even eliminate mosquito populations (Ritchie et al. 2015), particularly in isolated populations experiencing seasonal rainfall (Rašić et al. 2014).

One of the challenges in using *w*MelPop is that the strain can be difficult to maintain under laboratory conditions, with the infection occasionally being lost from colonies. For instance, on one occasion we found that 95.5% (43/45) of our colony was infected based on RT-PCR screening but this declined to 6.7% (2/30) four months later. Although the infection causes strong cytoplasmic incompatibility and shows near-complete maternal transmission, which allow *Wolbachia* infections to invade populations once an unstable equilibrium frequency dictated by deleterious fitness effects is exceeded (Walker et al. 2011), the infection may still be lost for unknown reasons even when it is detected at a high frequency with molecular assays. Environmental effects might reduce infection frequencies since high temperatures and low levels of antibiotics can clear *Wolbachia* infections (Ross et al. 2017b; Endersby-Harshman et al. 2019). However, there is normally careful control of temperature and antibiotics in laboratory cultures. Other factors that may contribute to infection loss are inappropriate storage of eggs coupled with sporadic incomplete maternal transmission.

While *Wolbachia* infections like *w*MelPop and *w*Au (Ant et al. 2018) reduce host fitness, their effects are expected to attenuate over time because any *Wolbachia* or host alleles that decrease deleterious fitness effects should be favoured by selection (Turelli 1994; Hoffmann and Turelli 1997). Evidence for such a process has been obtained for the *w*Ri infection of *Drosophila simulans* where an initially deleterious effect on offspring production has attenuated to the extent that *w*Ri infected *D*. *simulans* now have a higher production rate than uninfected females (Weeks et al. 2007). This could undermine any strategy that relies on maintaining deleterious fitness effects after *Wolbachia* are established in novel hosts, a

process that has been documented for *w*MelPop after transfer to *D*. *simulans* (Carrington et al. 2009; Carrington et al. 2010). Evolutionary changes in the nuclear background may also suppress the phenotypic effects of *Wolbachia*, as demonstrated by the evolution of male-killing suppression in butterflies (Hornett et al. 2006). However, it is unknown if the deleterious effects of *w*MelPop on *Ae*. *aegypti* and its ability to cause cytoplasmic incompatibility are stable.

To investigate these issues, we here consider whether there have been evolutionary changes in *w*MelPop or its *Ae*. *aegypti* host in the 10-year period since the infection was established by comparing recent and past data on phenotypic effects of the infection. We also investigate factors that may confound monitoring of *w*MelPop and contribute to instability of the infection in laboratory cultures.

Methods

Mosquito strains and colony maintenance

We performed experiments with our laboratory populations of *w*MelPop-infected (McMeniman et al. 2009), *w*Mel-infected (Walker et al. 2011), *w*AlbB-infected (Xi et al. 2005) and uninfected *Ae. aegypti* mosquitoes. The *w*MelPop transinfection in *Ae. aegypti* was derived from *D. melanogaster* (Min and Benzer 1997) and was passaged in a mosquito cell line before being introduced into *Ae. aegypti* through embryonic microinjection (McMeniman et al. 2009). *w*MelPop-infected mosquitoes were collected from Babinda, Queensland, Australia in 2012, three months after releases commenced (Nguyen et al. 2015) and maintained in the laboratory since collection. All *Wolbachia*-infected populations were backcrossed to a common Australian nuclear background for at least five generations to ensure that backgrounds were >98% similar (Yeap et al. 2011). Stock populations were maintained through continued backcrossing to North Queensland material every six generations. Mosquitoes were reared in a temperature controlled laboratory environment at 26 °C ± 1 °C with a 12 hr photoperiod according to methods described previously (Axford et al. 2016; Ross et al. 2017a). An uninfected population (denoted *w*MelPop negative) was derived from *w*MelPop females that had lost their *Wolbachia* infection in June 2019. The *w*MelPop negative population was used in phenotypic stability experiments and to test for nuclear background evolution.

Wolbachia screening

Aedes aegypti females were tested for the presence of *Wolbachia* DNA using methods previously described with modifications (Lee et al. 2012; Axford et al. 2016). DNA extraction methods varied between experiments due to our research spanning several years. Mosquito DNA was extracted using 100-250 µL of 5% Chelex® solution (Bio-Rad Laboratories, Gladesville, NSW, Australia) and 2.5-5 µL of Proteinase K (20 mg/mL, Bioline Australia Pty Ltd, Alexandria, NSW, Australia) in either 96-well plates or 1.5 mL tubes. Polymerase chain reactions were carried out with a Roche LightCycler 480 system (384-well format, Roche Applied Science, Indianapolis, IN, USA) using a RT/HRM (real-time PCR/high-resolution melt) assay as described previously (Lee et al. 2012; Axford et al. 2016).

We used mosquito-specific (*mRpS6*), *Aedes aegypti*-specific (*aRpS6*) and *Wolbachia*-specific primers (*w1* primers for the *w*MelPop and *w*Mel infections and *wAlbB* primers for the *w*AlbB infection) to diagnose *Wolbachia* infections (Axford et al. 2016). All individuals were expected to have robust and similar amplification of the *mRpS6* and *aRpS6* primers. An individual was scored as positive for *Wolbachia* if its *w1* or *wAlbB* Cp (crossing point) value was lower than 35 and its Tm (melting temperature) value was within the expected range based on positive controls (approximately 84.3, but this varied between runs). An individual was negative for *Wolbachia* when Cp values were 35 or absent and/or Tm values were inconsistent with the controls. For experiments with the *w*MelPop infection, we assigned infected individuals to two categories: strongly positive (Cp ≤ 23) and weakly positive (Cp > 23). Based on the mating transmission experiments (see below), females that were strongly positive likely represented true infections, while weakly positive females were likely uninfected and had mated with a *Wolbachia*-infected male. Relative *Wolbachia* densities were determined by subtracting the *Wolbachia* Cp from the *aRpS6* Cp and then transforming this value by 2^n.

Phenotypic stability of deleterious effects

The *w*MelPop infection induces a range of deleterious effects, including life shortening, reduced fertility, impaired blood feeding success and reduced quiescent egg viability as outlined below. To evaluate the stability of these deleterious effects, we performed experiments with the *w*MelPop infection over 10 years after its introduction to *Ae. aegypti*. Before experiments commenced, the *w*MelPop-infected colony was purified by pooling the offspring of isolated females that were strongly positive for *Wolbachia* (see *Infection recovery*). Female offspring were crossed to uninfected males, and the progeny

were used in the following experiments. We compared fitness relative to two uninfected populations; a natively uninfected laboratory population (uninfected) and a population derived from uninfected individuals from the *w*MelPop colony that had lost their infection (*w*MelPop negative). Due to logistical constraints, the fertility experiment included the *w*MelPop and *w*MelPop negative populations only.

Longevity

Previous studies reported that *w*MelPop shortens adult lifespan by approximately 50% (McMeniman et al. 2009; Yeap et al. 2011). We performed longevity assays by establishing 8 replicate 3 L cages with 50 adults (25 males and 25 females) for each population. Cages were provided with 10% sucrose and water cups which were replaced weekly. Females were provided with blood meals for 10 minutes once per week and given constant access to an oviposition substrate. Mortality was scored three times per week by removing and counting dead adults from each cage until all adults had died. One replicate of *w*MelPop was discarded due to a sugar spill early in the experiment which caused high mortality. We used log-rank tests to compare adult longevity between populations. To evaluate *Wolbachia* density and infection frequencies with adult age, 16 females from separate cages that were 0, 7, 14, 21, 28 and 35 d old were screened for *Wolbachia*. We used a linear regression to test whether (log) *Wolbachia* density was affected by adult age. All data were analyzed using SPSS statistics version 24.0 for Windows (SPSS Inc, Chicago, IL).

Fertility

The *w*MelPop infection substantially reduces fertility as females age (McMeniman and O'Neill 2010); we therefore tested the fertility of *w*MelPop and *w*MelPop negative populations over successive gonotrophic cycles. We established two cages of approximately 500 individuals (equal sex ratio) for each population. Five-day old females (starved for 1 d) were blood fed and 35 engorged females were selected randomly from each population and isolated in 70 mL cups with sandpaper strips and larval rearing water to encourage oviposition. Eggs were collected 4 days after blood feeding, partially dried and hatched three days after collection. Fecundity and egg hatch proportions were determined by counting the number of unhatched and hatched eggs (hatched eggs having a clearly detached cap). Following egg collection, females were returned to their respective cages for blood feeding. Successive gonotrophic cycles were initiated every 4-5 days with females selected randomly from cages. Cages were provided with oviposition substrates, however no sugar was provided to isolated females or the population cage during the experiment because sugar feeding influences fecundity (Naksathit and Scott

1998). We tested fertility for a total of 9 gonotrophic cycles. Females from the *w*MelPop population that were still alive after 9 gonotrophic cycles were tested with qPCR to confirm *Wolbachia* infection. Effects of gonotrophic cycle on egg hatch proportions were compared for the *w*MelPop and *w*MelPop negative populations. Egg hatch proportions were not normally distributed and were therefore analysed with Kruskal-Wallis tests.

Quiescent egg viability

The *w*MelPop infection reduces the viability of quiescent eggs (McMeniman and O'Neill 2010; Yeap et al. 2011; Ritchie et al. 2015). For quiescent egg viability assays, eggs were collected from colonies on sandpaper strips and stored in a sealed container with a saturated solution of potassium chloride to maintain ~80% humidity. Nine replicate batches of eggs (40-98 eggs per batch) per population were hatched twice per week by submerging eggs in containers of water with a few grains of yeast. Egg hatch proportions were determined by dividing the number of hatched eggs by the total number of eggs. Larvae that had not completely eclosed and died in the egg were scored as unhatched. This experiment continued until eggs were 31 d old. To test for the potential loss of *w*MelPop infection with egg storage, we reared larvae hatching from 3, 13, 20, 24, 27 and 31 d old egg to adulthood and scored 16 females (< 24 hr old) for *Wolbachia* infection and density from each group. We used a linear regression to test whether (log) *Wolbachia* density was affected by egg storage duration.

Blood feeding success

The *w*MelPop infection reduces female blood feeding success and affects probing behaviour, particularly in older females (Moreira et al. 2009b; Turley et al. 2009). We evaluated blood feeding traits in 5 and 35 d old females according to methods described previously (Ross et al. 2019b). We recorded pre-probing duration (time from landing to insertion of the proboscis), feeding duration, blood meal weight and proportion feeding. Females that did not feed within 10 minutes were scored as not feeding. The proportion of females exhibiting a bendy or shaky proboscis phenotype (Moreira et al. 2009b; Turley et al. 2009) was also recorded. Feeding trials were performed on individual females by three experimenters. At least 32 individuals per population and age group were tested across the three experimenters. To confirm the infection status of *w*MelPop females, we screened all 35 d old females for *Wolbachia* infection. Pre-probing duration, feeding duration and blood meal weight data were analysed with general linear models. Pre-probing and feeding durations were log transformed for

normality before analysis. Comparisons of proportional data (proportion feeding and the presence of a bendy or shaky proboscis) with previous studies were performed with two proportions Z-tests.

Loss of *Wolbachia* during colony maintenance

We carried out a series of experiments and monitoring exercises to understand the loss of the *w*MelPop infection in colonies during routine maintenance.

Infection recovery

In May 2019 we observed an apparent loss of *w*MelPop infection from our laboratory colony despite a high level of infection in previous generations. To return the population to a 100% infection frequency, one hundred blood-fed females were isolated for oviposition, screened for *Wolbachia*, then placed into categories of strongly positive, weakly positive or negative (see *Wolbachia* screening). We then pooled the offspring of females from each category and screened 30 offspring (15 males and 15 females) for *Wolbachia* per category. Female offspring from the strongly positive population were crossed to uninfected males before commencing the maternal transmission, nuclear background evolution and phenotypic stability experiments.

Maternal transmission

We estimated maternal transmission fidelity by crossing *w*MelPop-infected females to uninfected males, then screening ten offspring (4th instar larvae) from the first gonotrophic cycle of ten females that had been separated individually for oviposition. Maternal transmission fidelity was expressed as the proportion of infected offspring produced by infected mothers, for which 95% binomial confidence intervals were calculated.

Nuclear background evolution

Loss of *w*MelPop infection in laboratory colonies may be explained by the evolution of resistance to *Wolbachia* infection by uninfected mosquitoes. We performed crossing experiments to test whether the *w*MelPop infection was maintained across generations when *w*MelPop-infected females were crossed to natively uninfected males or uninfected males that had lost their *Wolbachia* infection (*w*MelPop negative). We established two replicate populations for each cross with 200 adults of each sex. Crosses were performed for four consecutive generations. Thirty individuals from each replicate population per

generation were then screened for *Wolbachia* infection. A *w*MelPop-infected colony (*w*MelPop-infected males crossed with *w*MelPop-infected females) was also monitored across the same time period.

To test for resistance to cytoplasmic incompatibility, we tested the ability of *w*MelPop-infected males to induce cytoplasmic incompatibility with uninfected and *w*MelPop negative females. For each cross, 30 males and 30 females were aspirated into a single 3 L cage. When adults were 5 d old, females were blood fed. Twenty females from each cross were isolated for oviposition and egg hatch proportions were determined according to the fertility experiment (see above).

Wolbachia *mating transmission*

Although *Wolbachia* in mosquitoes are maternally transmitted, it is possible that *Wolbachia* might also be transferred through seminal fluid, leading to the detection of *Wolbachia* in uninfected females that mate with infected males. To test for *Wolbachia* transmission through mating, we performed crosses between *Wolbachia*-infected males and uninfected females. Experiments were performed with the *w*MelPop, *w*Mel and *w*AlbB strains. Control crosses were also performed, where both sexes were either infected (positive controls) or uninfected (negative control). Crosses were established with 160 virgin adults of each sex (4 – 7 d old) in a single cage and left for two days to mate, after which males were removed. Females were blood-fed one week after crosses were established and provided with an oviposition substrate. Thirty females (whole adults) were stored 2, 9, 16 and 23 d after crosses were established and screened for *Wolbachia*. Females from the positive and negative controls were tested 2 and 23 d after crosses were established. Due to apparent differences in mating transfer between *Wolbachia* strains, this experiment was repeated with the *w*AlbB infection, but females were stored 5 d after crosses were established.

We conducted an additional cross between uninfected females and *w*MelPop-infected males to see if the detection of *Wolbachia* following transmission through mating was tissue-specific. Females and males were left to mate for five days, after which females were stored in ethanol. Heads and abdomens from 20 uninfected females were dissected and extracted separately for *Wolbachia* screening.

Relative fitness during laboratory maintenance

We compiled data on egg hatch proportions during our routine maintenance of *w*MelPop, *w*Mel, *w*AlbB and uninfected colonies from July 2012 to April 2018. Egg hatch proportions were determined by

hatching a subset of eggs collected from each colony during maintenance (>200 eggs per subset), then dividing the number of larvae counted by the number of eggs tested. We then divided the egg hatch proportions of *Wolbachia*-infected colonies by the egg hatch proportion of the uninfected colony to obtain relative egg hatch proportions. When multiple *Wolbachia*-infected colonies were maintained simultaneously, we included these as separate estimates. We used sign tests to compare relative hatch proportions of *Wolbachia*-infected and uninfected colony eggs. We used a general linear model to test for long-term changes in the relative egg hatch proportion of *w*MelPop-infected colonies.

Results

Phenotypic stability of deleterious effects

We re-evaluated the deleterious fitness effects induced by *w*MelPop to test for attenuation. In previous experiments conducted more than 10 years ago, *w*MelPop infection shortened adult lifespan by ~50% relative to uninfected populations (McMeniman et al. 2009; Yeap et al. 2011). Here, the *w*MelPop infection shortened median female lifespan by 22% compared to the uninfected populations (Log-rank: $\chi^2 = 116.310$, df = 2, $P < 0.001$), while male lifespan was unaffected by population ($\chi^2 = 4.722$, df = 2, $P = 0.094$, Figure 1). These results suggest that the effects of *w*MelPop on adult lifespan may have attenuated. Although adults from this experiment were not screened for *Wolbachia*, samples of colony females from the same generation aged 0-35 d (n = 101) all had strongly positive (Cp ≤ 23) infections, suggesting that this result was not influenced by incomplete maternal transmission. (log) *Wolbachia* density decreased with adult age (linear regression: $R^2 = 0.186$, $F_{1,86} = 20.837$, $P < 0.001$, Figure S1A), in contrast to *Drosophila* where *w*MelPop density increases with age (McGraw et al. 2002; Chrostek et al. 2013).

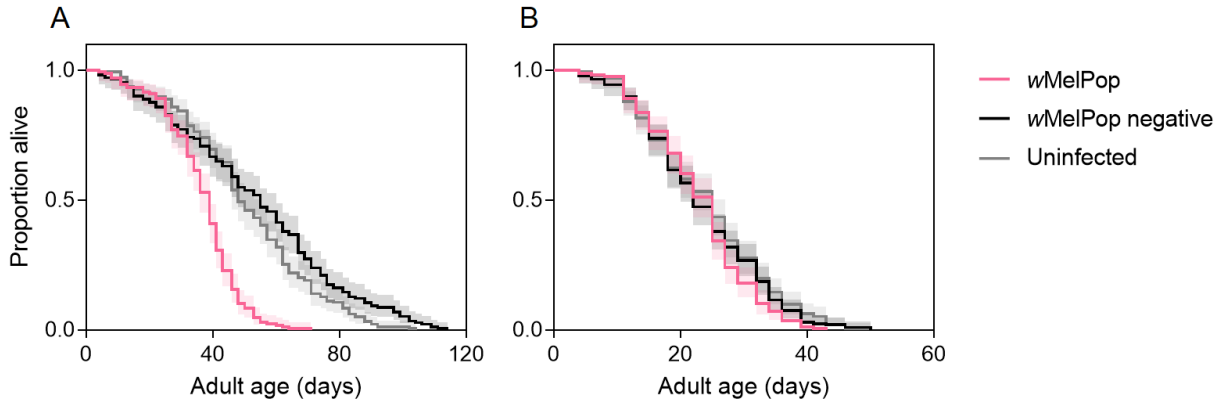

Figure 1. Longevity of female (A) and male (B) adult *Aedes aegypti* from *w*MelPop (pink lines), *w*MelPop negative (black lines) and uninfected (gray lines) populations. Lines represent the proportion of mosquitoes alive, while shaded regions show 95% confidence intervals.

In previous studies, *w*MelPop infection severely reduced fertility with increasing female age (McMeniman and O'Neill 2010) and egg storage duration (McMeniman and O'Neill 2010; Yeap et al. 2011). Patterns of fecundity (Figure 2A) and quiescent egg viability (Figure 2D) observed here closely matched a previous study (McMeniman and O'Neill 2010), indicating that these deleterious effects have remained stable for over 10 years after transinfection. Loss of female fertility with age was due to declining fecundity rather than egg hatch, which was stable across gonotrophic cycles for both *w*MelPop (Kruskal-Wallis: $\chi^2 = 4.654$, df = 7, P = 0.702) and *w*MelPop negative ($\chi^2 = 7.580$, df = 8, P = 0.476) females (Figure 2B).

As adult age increased, we observed an increasing proportion of *w*MelPop females that had a high fecundity but had zero eggs hatching (Figure 2C). We excluded these individuals from the results since they may represent uninfected mosquitoes that mated with *w*MelPop infected males. Uninfected individuals may result from incomplete maternal transmission and become increasingly represented throughout the experiment due to having a longer lifespan (Figure 1A). Only two of the seven *w*MelPop females surviving to the ninth gonotrophic cycle had a strongly positive (Cp ≤ 23) *Wolbachia* infection, indicating maternal transmission leakage. In contrast, all individuals hatching from quiescent eggs (storage durations of 3-31 d, n = 96) were strongly positive for *Wolbachia* (Fisher's exact test: P < 0.001), although adult *Wolbachia* density decreased with increasing egg storage duration (linear regression: $R^2 = 0.108$, $F_{1,83} = 10.087$, P = 0.002, Figure S1B).

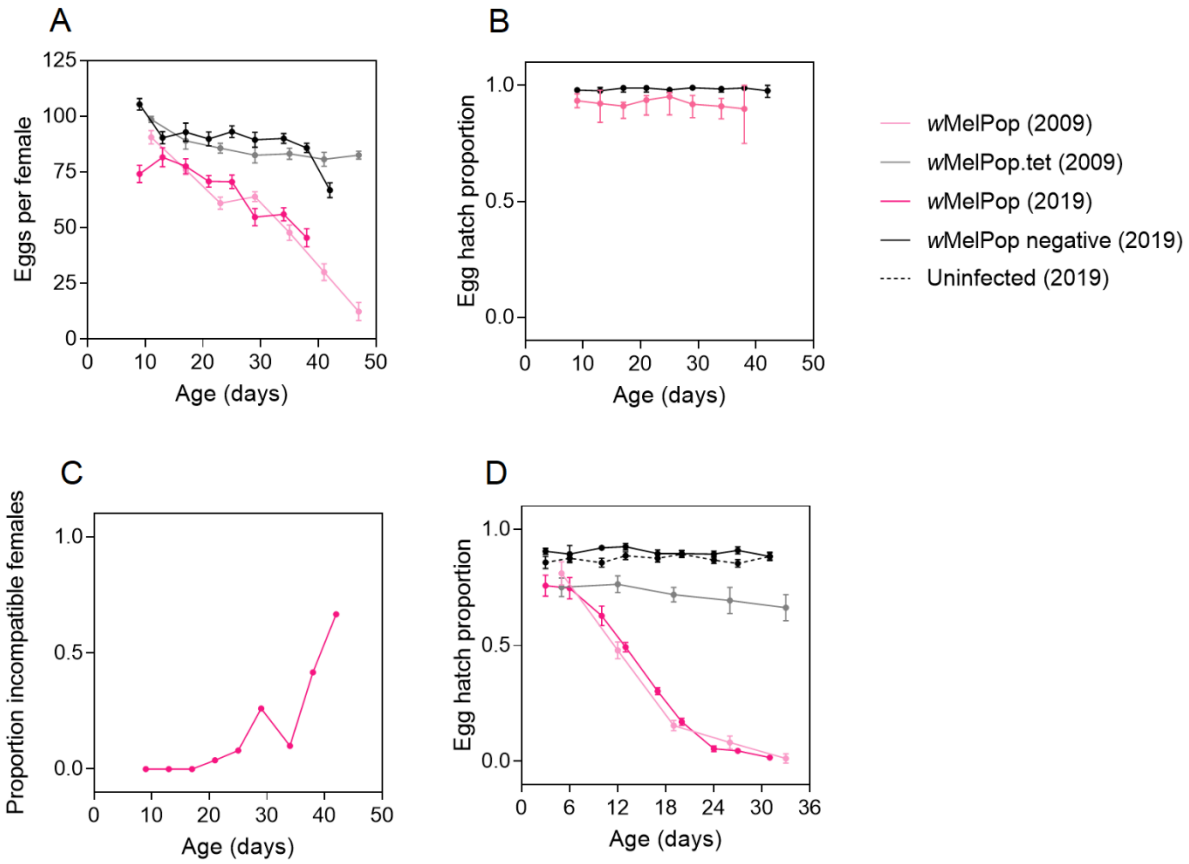

Figure 2. Fertility of *w*MelPop-infected and uninfected *Aedes aegypti* populations with increasing female age and egg storage duration. (A) Fecundity across gonotrophic cycles. (B) Egg hatch proportion across gonotrophic cycles. (C) Proportion of *w*MelPop-infected females with zero viable progeny across gonotrophic cycles. (D) Egg hatch proportion with different durations of egg storage. Data for 2009 (pale lines) are from McMeniman and O'Neill (2010). Dots and error bars are means and standard errors respectively, consistent with the original study.

The *w*MelPop infection reduces female blood feeding success and affects probing behaviour, particularly in older females (Moreira et al. 2009b; Turley et al. 2009). Here we found no effect of population on pre-probing and feeding duration or blood meal weight in 5 d old females (GLM: all $P > 0.05$, Figure 3). Conversely, in 35 d old females we observed costs of *w*MelPop infection for all traits, with significant effects of population for pre-probing duration ($F_{2,82} = 26.135$, $P < 0.001$), feeding duration ($F_{2,82} = 7.988$, $P = 0.001$) and blood meal weight ($F_{2,82} = 14.338$, $P < 0.001$, Figure 3). Substantial effects of experimenter

were also observed for all three traits tested (all $P < 0.01$), leading to differences of up to 0.37 mg (10.27%) in blood meal weight, 39.5 s (27.96%) in feeding duration and 100 s (113.64%) in pre-probing duration.

Effects of *w*MelPop infection on blood feeding traits were weaker in comparison to previous studies with similar methods. For instance, Turley et al. (2009) observed a 50.3% (95% confidence interval: 37.5-63.1%) reduction in blood meal weight in 35 d old females due to *w*MelPop infection, while we observed a 29.5% (95% confidence interval: 12.1-46.7%) reduction relative to the two uninfected populations. Aged *w*MelPop females had reduced feeding success (65% feeding compared to 91% for uninfected populations) and also displayed a bendy/shaky proboscis phenotype as characterized previously (Moreira et al. 2009b; Turley et al. 2009). However, these phenotypes occurred at a significantly lower frequency than previously reported (Moreira et al. 2009b) (proportion feeding: two proportions Z-test: $Z = 3.431$, $P < 0.001$, bendy/shaky proboscis: $Z = 4.288$, $P < 0.001$). Weaker effects relative to previous studies may result from methodological differences, human experimenter effects, effects of laboratory rearing, attenuation or incomplete maternal transmission. *Wolbachia* screening of 35 d old females showed that 6 females (20%) had a weakly positive ($Cp > 23$) infection which may indicate maternal transmission leakage.

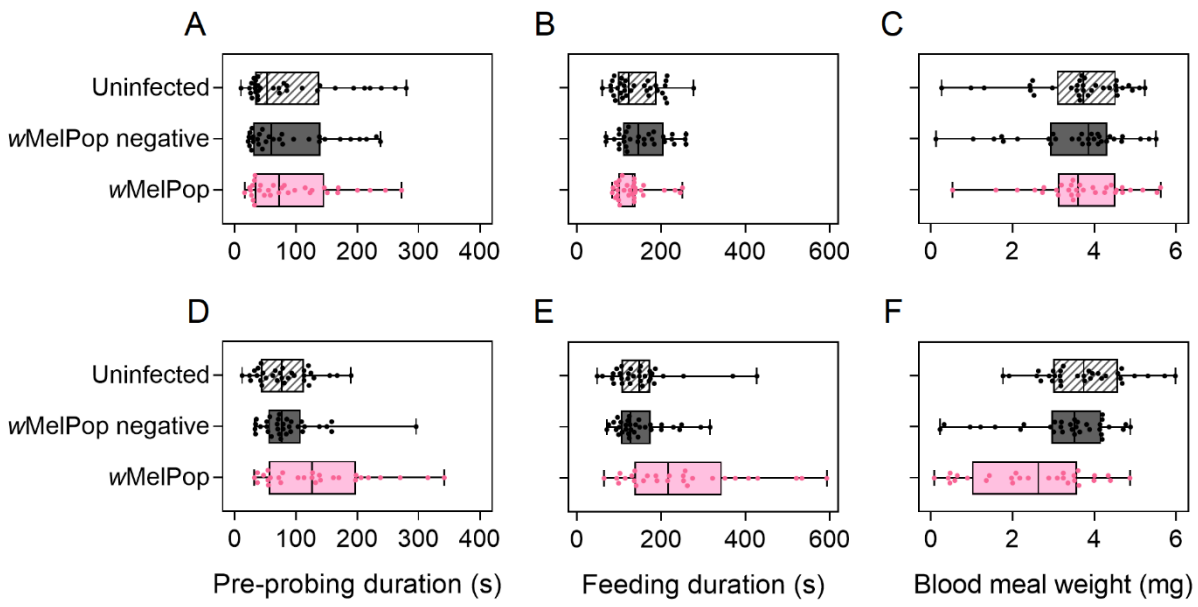

Figure 3. Pre-probing duration (A,D), feeding duration (B,E) and blood meal weight (C,F) of uninfected, *w*MelPop negative and *w*MelPop *Aedes aegypti* females aged 5 (A-C) or 35 d (D-F). Box plots show medians and interquartile ranges, with error bars representing minimum and maximum values. Data for individual females are shown by dots.

Loss of *Wolbachia* during colony maintenance

Infection recovery

Due to an apparent loss of *Wolbachia* from our *w*MelPop colony, we isolated females to restore the *w*MelPop infection in the population. Of the females that produced viable offspring, 20 were negative, 17 were strongly positive (median Cp 16.3, range 3.33) and 41 were weakly positive (median Cp 31.38, range 8.37). These results point to a polymorphic colony despite the colony having been scored as 100% infected prior to this time (all Cp values ≤ 23). All offspring tested from strongly positive females were strongly positive (females: median Cp 19.19, range 0.61), males: median Cp 19.12, range 5.83). No offspring from the weakly positive or negative females were infected (n = 30 each), thus females scored as weakly positive were unable to transmit *w*MelPop to the next generation.

Maternal transmission

We tested ten offspring from ten *w*MelPop-infected females and found that a single female produced two uninfected offspring, with an overall maternal transmission fidelity of 98% (binomial confidence interval: 92.96-99.76%). These results are consistent with previous studies that indicate a low level of maternal transmission failure (Yeap et al. 2011; Ross et al. 2017b).

Nuclear background evolution

We crossed *w*MelPop-infected females to *w*MelPop negative or uninfected males for four generations to see if the loss of *w*MelPop infection was associated with changes in the nuclear background. The *w*MelPop infection frequency declined in all four populations (Figure 4). In contrast, when *w*MelPop-infected females were crossed to *w*MelPop-infected males the infection frequency remained at 100%, likely due to cytoplasmic incompatibility. Loss of *w*MelPop infection does not appear to be strongly related to nuclear background since the infection declined in both sets of crosses. Rather, declines in infection frequency are likely due to a combination of incomplete maternal transmission and fitness costs.

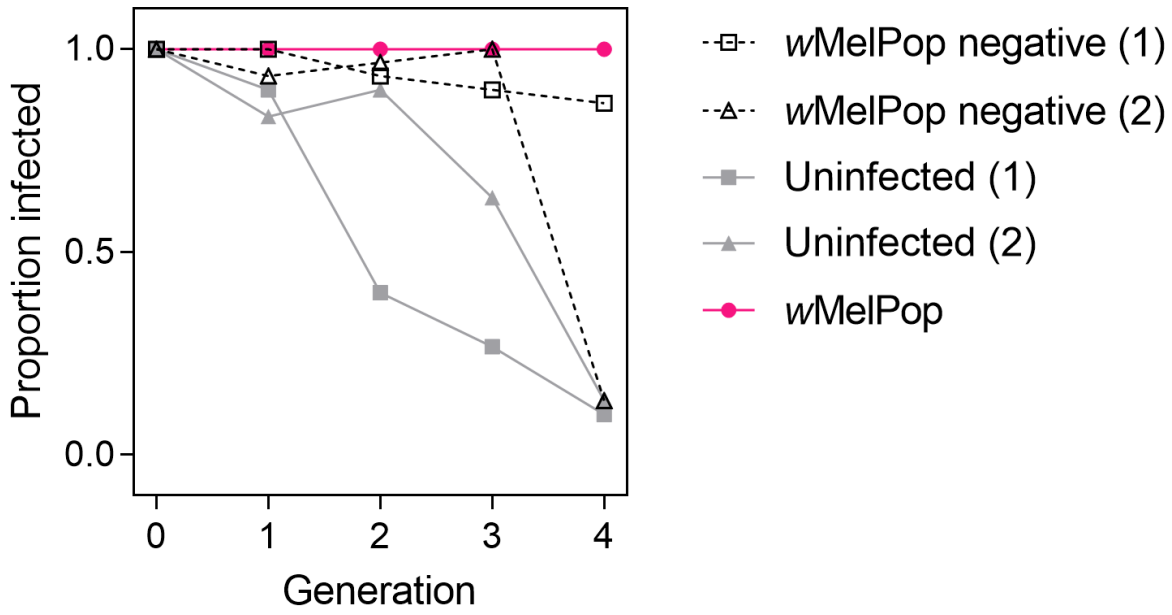

Figure 4. Loss of *w*MelPop infection in *Aedes aegypti* in the absence of cytoplasmic incompatibility. *w*MelPop-infected females were crossed to wMelPop negative (gray), uninfected (gray) or *w*MelPop (pink) males each generation for four generations. Infection frequencies were determined for 30 individuals per population, per generation.

*w*MelPop-infected males induced complete cytoplasmic incompatibility with uninfected females (no eggs hatching, Table 1), suggesting that this phenotype has remained stable since transinfection over 10 years ago (McMeniman et al. 2009). Compatible crosses exhibited high hatch proportions, showing that the *w*MelPop infection is self-compatible. *w*MelPop-infected males also induced complete cytoplasmic incompatibility with *w*MelPop negative females, indicating that this population has not evolved resistance to cytoplasmic incompatibility.

Table 1. Egg hatch proportions resulting from crosses between *w*MelPop, *w*MelPop negative and uninfected *Aedes aegypti* populations. Data are medians followed by 95% confidence intervals (lower, upper).

		Male		
		***w*MelPop**	**Uninfected**	***w*MelPop negative**
Female	***w*MelPop**	0.933 (0.903, 0.964)	0.988 (0.970, 1)	Not tested
	Uninfected	0 (0, 0)	0.936 (0.893, 0.969)	Not tested
	***w*MelPop negative**	0 (0, 0)	Not tested	0.980 (0.972, 0.984)

Wolbachia *mating transmission*

We crossed *Wolbachia*-infected males with uninfected females to test the potential for *Wolbachia* to be transferred through mating. In control crosses, *Wolbachia*-infected females had a 100% infection frequency and high densities (Figure 5), while *Wolbachia* were not detected when uninfected females were crossed to uninfected males. We detected *Wolbachia* in uninfected females that were crossed to *w*MelPop- (Figure 5A) and *w*Mel-infected (Figure 5B) males for up to 23 d post-mating, with the proportion scored as positive decreasing with time after mating. *Wolbachia* densities in uninfected females were distinctly lower than in females with a maternally-inherited *Wolbachia* infection. In an additional cross, we specifically tested for transfer of seminal fluid by crossing uninfected females to *w*MelPop-infected males and testing the heads and abdomens of females separately. All heads were negative for *Wolbachia*, while 19/20 abdomens were positive with a median Cp of 28.78 (range 4.44). Uninfected females can therefore be incorrectly scored as infected if they have mated with a *w*MelPop or *w*Mel-infected male.

In contrast to the other two infections, we did not detect *Wolbachia* in any uninfected females that were crossed to *w*AlbB-infected males (Figure 4C). We detected no *Wolbachia* in a second independent experiment, indicating that this *Wolbachia* strain is not transferred through mating.

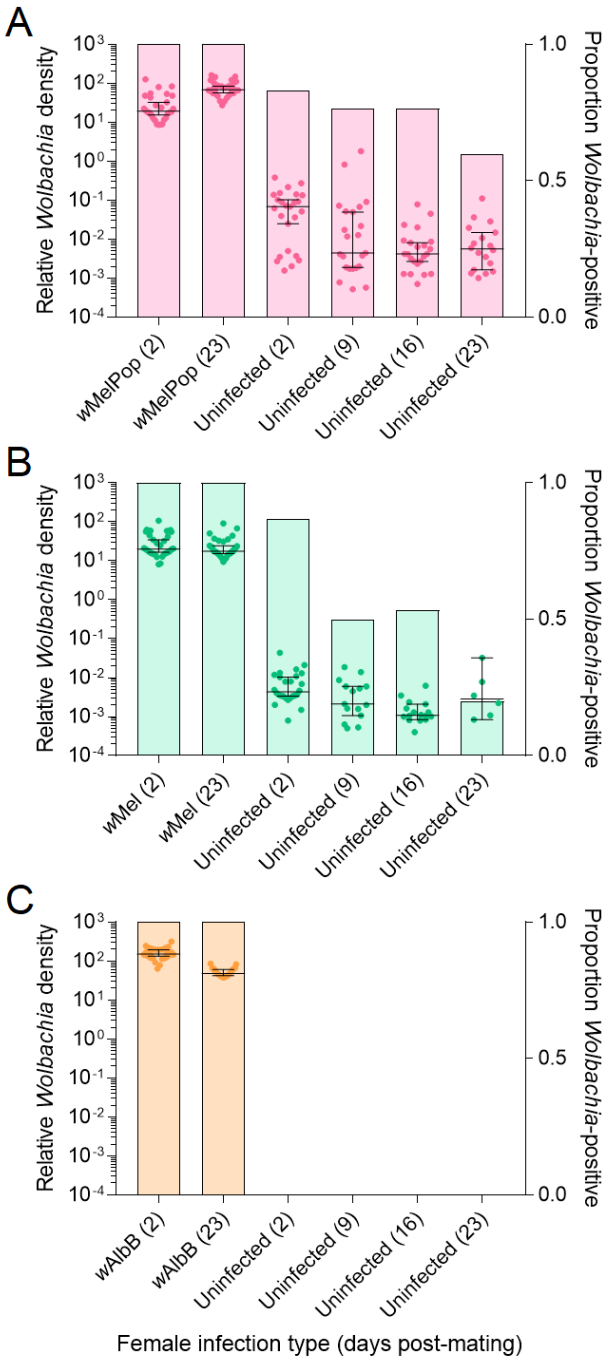

Figure 5. Detection of *Wolbachia* in uninfected *Aedes aegypti* females via seminal fluid from *Wolbachia*-infected males. Males were infected with the (A) *w*MelPop, (B) *w*Mel or (C) *w*AlbB *Wolbachia* strains. Dots show *Wolbachia* densities of individual females (left y-axis), while horizontal lines and error bars are medians and 95% confidence intervals respectively. Shaded bars show proportions of females (n = 30) from each group that tested positive for *Wolbachia* (right y-axis).

Relative fitness during laboratory maintenance

We monitored egg hatch proportions of our *Wolbachia*-infected laboratory colonies across multiple generations to assess variance in fitness costs. *w*MelPop-infected (Sign test: $Z = 6.197$, $P < 0.001$) and *w*Mel-infected ($Z = 3.900$, $P < 0.001$) colonies tended to have lower egg hatch proportions relative to uninfected colonies (Figure 6). *w*AlbB-infected colonies had similar hatch proportions to uninfected colonies overall ($Z = 1.000$, $P = 0.317$), though the sample size for this infection was much lower. For the *w*MelPop infection, relative egg hatch proportions were as low as 40% which may contribute to the loss of infection from colonies. Because data were collected over nearly a 6-year period, we could test for changes in egg hatch across time. For *w*MelPop, where the most data were available, there was no temporal difference in relative egg hatch (General linear model: $F_{17,42} = 1.727$, $P = 0.076$), suggesting that there has been no change in relative fitness during this period.

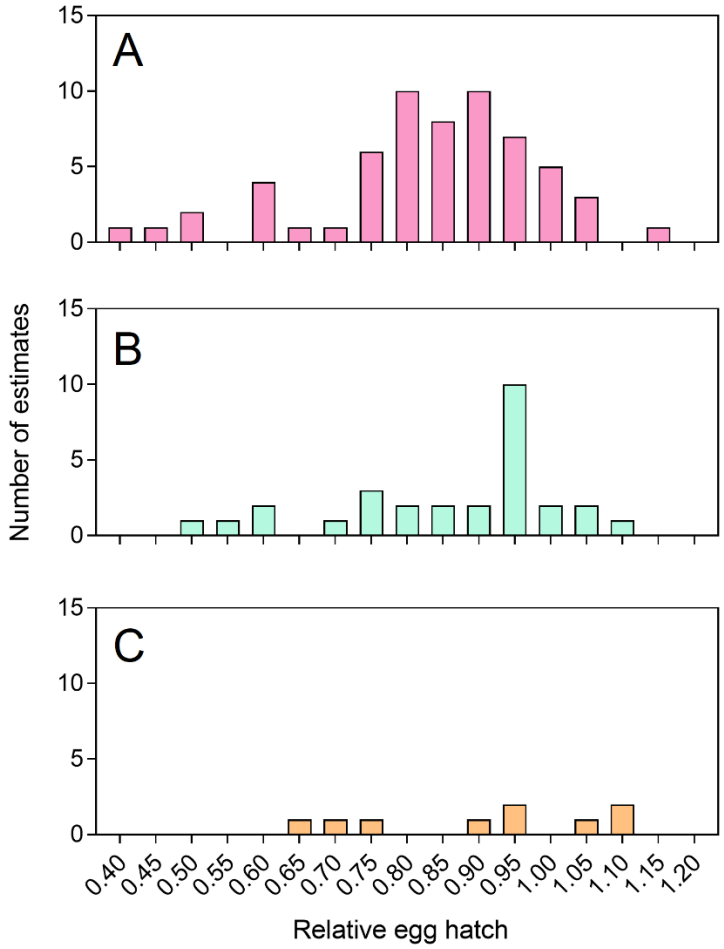

Figure 6. Histograms of egg hatch proportions of (A) *w*MelPop, (B) *w*Mel and (C) *w*AlbB colonies relative to uninfected colonies during routine laboratory maintenance. Each estimate was undertaken on a different laboratory generation or colony from at least 200 eggs.

Discussion

Here we show that there has been little evolutionary attenuation of deleterious effects in *w*MelPop, either through changes in the host nuclear genome or the *Wolbachia* genome. This is despite an elapsed period of more than ten years or ~120 generations of rearing in the laboratory (and with an additional short period in the field). This contrasts sharply with the attenuation of *w*MelPop seen in *D. simulans* following its transfer from *D. melanogaster*. As in its native host, *w*MelPop reduced longevity when transferred to *D. simulans* (McGraw et al. 2001), *Ae. aegypti* (McMeniman et al. 2009) and *Aedes albopictus* (Suh et al. 2009). Other deleterious effects in *D. simulans* were also detected; however, many

of these attenuated after around 20 generations, including effects on egg hatch (McGraw et al. 2002). Moreover, after around 200 generations, *w*MelPop-infected *D. simulans* lines no longer showed a decrease in longevity in some genetic backgrounds (Carrington et al. 2010).

It is unclear why most deleterious effects in *Ae. aegypti* have not attenuated. It may be due to the nature of the mutation causing the high virulence; a duplication of a gene region has been identified in *Drosophila* (Chrostek and Teixeira 2015) which may be difficult to reverse. Instead, attenuation may require evolutionary changes in the host nuclear background. Selection experiments for increased quiescent egg viability in *w*MelPop-infected *Ae. aegypti* found evidence for attenuation due to nuclear background evolution but not *Wolbachia* evolution (Ritchie et al. 2015). Compared to studies performed over ten years ago, some deleterious effects of *w*MelPop appear weaker, particularly blood feeding traits (Moreira et al. 2009b; Turley et al. 2009) and male longevity (McMeniman et al. 2009; Yeap et al. 2011). Although this may indicate nuclear background evolution, direct comparisons with previous studies are difficult due to methodological differences and potential confounding effects of inbreeding, drift and laboratory adaptation that can occur during colony maintenance (Ross et al. 2019a).

Because *Wolbachia* are maternally inherited, selection acts to increase maternal transmission fidelity and not the ability of males to induce cytoplasmic incompatibility (Turelli 1994). Novel *Wolbachia* infections tend to induce much stronger cytoplasmic incompatibility than natural infections, suggesting that these effects can attenuate (Hoffmann et al. 2015). Furthermore, theory predicts that resistance to cytoplasmic incompatibility may evolve if maternal transmission is incomplete (Bull and Turelli 2013). Although hosts may evolve resistance to the effects of *Wolbachia* on reproduction, such as male killing in *Hypolimnas bolina* (Hornett et al. 2006) and cytoplasmic incompatibility in *D. melanogaster* (Hoffmann and Turelli 1997), effects can also remain stable despite intense selection pressure (Jaenike and Dyer 2008; Carrington et al. 2011). Over ten years after *w*MelPop was introduced to *Ae. aegypti*, the infection still induces complete cytoplasmic incompatibility. We therefore find no evidence to suggest that cytoplasmic incompatibility has attenuated or that *Ae. aegypti* has evolved to suppress cytoplasmic incompatibility. In crossing experiments, the *w*MelPop infection was lost from colonies regardless of whether infected females were crossed to uninfected males or males that had lost the *w*MelPop infection, suggesting that loss of *w*MelPop was not due to paternal factors that affect *Wolbachia* maternal transmission.

The lack of attenuation of deleterious fitness effects may contribute to the occasional loss of the *w*MelPop infection from *Ae. aegypti* laboratory populations. Following Hoffmann *et al.* (1990) the change in frequency of the infection (p_f) in a population is given by

$$\boldsymbol{p_{(t+1)} = \frac{p_t(1-u)(1-s_f)}{1 - s_f p_t - s_h p_t(1-p_t) - u s_h p_t^2(1-s_f)}}$$

where u is the fraction of uninfected progeny produced by infected females, s_f is the fecundity deficit (representing a combination of the number of eggs laid and that hatch) and s_h is the incompatibility between infected and uninfected strains. In the presence of strong maternal transmission (u=0) the unstable point for invasion versus loss of the infection is given by the ratio of s_f/s_h (Hoffmann and Turelli 1997). This means that if incompatibility is very strong (s_h near 1) as is the case with *w*MelPop, it is normally very unlikely for a deleterious fitness effect to result in a loss of infection in a population.

However, we have observed a low level of maternal transmission failure in our *w*MelPop colony of 2%, with an upper estimate of 7%. When coupled with large deleterious effects, this level of leakage may be sufficient to trigger a loss of the *w*MelPop infection, particularly when there are issues around accurately monitoring the infection frequency in a population due to mating-based transmission. Based on the variance in egg hatch proportions and costs to fecundity, we estimate that the relative fitness of *w*MelPop-infected mosquitoes compared to uninfected mosquitoes may fall to as low as 28% during routine maintenance, or even lower if adults are aged or eggs are stored before hatching. This will produce a situation where $p_{(t+1)}$ is less than p, and the infection will continue to drop out unless relative fitness is increased.

Our detection of *Wolbachia* at low densities in uninfected females that had mated with *Wolbachia*-infected males was unexpected, given that *Wolbachia* are absent from mature sperm in other insects (Yen and Barr 1971; Binnington and Hoffmann 1989; Clark et al. 2008). However, a recent report in *Hylyphantes graminicola* spiders demonstrated sexual transmission of *Wolbachia*, both from males to females and from females to males (Su et al. 2019). Our results have implications for *Wolbachia* monitoring in laboratory and field populations because uninfected females might be incorrectly scored as infected. Assuming random mating, the incidence of false positive detections is equivalent to the frequency of infected individuals in the population. If a loss in infection occurs, it may not be detected immediately when an infection is monitored only by screening adult females. Moreover, because environmental conditions can affect *Wolbachia* density (Ross et al. 2017b; Endersby-Harshman et al.

2019), determining infection status based on a threshold *Wolbachia* density may be unreliable under field conditions. We therefore advise that during laboratory maintenance and field monitoring, infection frequencies are determined by screening immature stages or unmated adults. This issue appears to be specific to certain *Wolbachia* strains given that we found no evidence for the transmission through mating of *w*AlbB.

In conclusion, our findings have implications for the long-term effectiveness of *Wolbachia* releases and for the maintenance of *w*MelPop stocks in the laboratory. The stability of the deleterious effects detected here suggests that *w*MelPop can suppress populations for a long time once established. However, field trials with this infection suggest that long-term persistence in natural populations is unlikely (Nguyen et al. 2015). *w*MelPop is difficult to maintain even under benign laboratory conditions due to a combination of incomplete maternal transmission, deleterious effects and monitoring issues, but a strict rearing schedule and regular *Wolbachia* screening will help to ensure its persistence in a colony. Due to its fitness costs, *w*MelPop may be suitable for temporary suppression or elimination of populations rather than population replacement which is now taking place in field populations with the *w*Mel and *w*AlbB strains (Hoffmann et al. 2011; Nazni et al. 2019). Suppression through the release of *w*MelPop was proposed as a way of tackling mosquito incursions in isolated areas without the use of pesticides (Rašić et al. 2014). Establishing *w*MelPop in large semi-field cages and then imposing a dry period that required the persistence of quiescent eggs led to population elimination (Ritchie et al. 2015). This approach to suppression does not require sex separation unlike strategies that rely on cytoplasmic incompatibility (Mains et al. 2019) and could be effective even if the infection does not persist in the long-term. Although research has shifted away from this deleterious *Wolbachia* infection, *w*MelPop may still prove to be useful when seasonal population suppression is desirable.

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Supplementary information

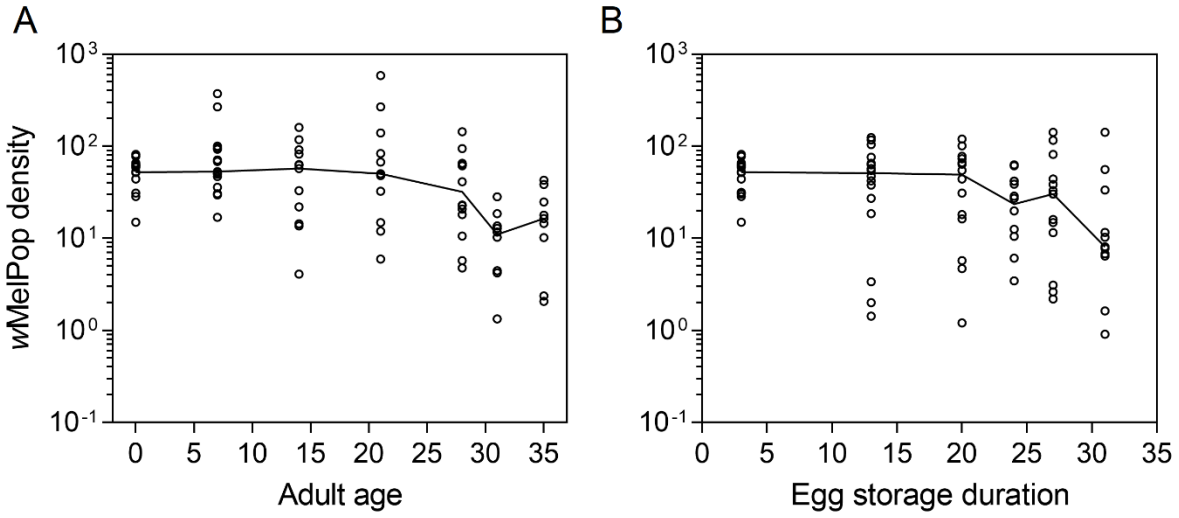

Figure S1. Relative *Wolbachia* density of *w*MelPop-infected females with increasing (A) adult age or (B) egg storage duration. Each dot represents the *Wolbachia* density of a single female, while solid lines join the median densities for each time point.