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THE *POPCORN* WOLBACHIA INFECTION OF *DROSOPHILA MELANOGASTER*: CAN SELECTION ALTER WOLBACHIA LONGEVITY EFFECTS?

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Wolbachia *popcorn* (*w*MelPop), a life-shortening strain of Wolbachia, has been proposed as an agent for suppressing transmission of dengue fever following infection of the vectoring mosquito *Aedes aegypti*. However, evolutionary changes in the host and Wolbachia genomes might attenuate any life span effects mediated by *w*MelPop. Here we test for attenuation by selecting strains of *Drosophila melanogaster* infected with *w*MelPop for early and late reproduction in three independent outcrossed populations. Selection caused divergence among the lines in longevity. This divergence was mostly associated with the host genetic background rather than the Wolbachia infection, although there were also interactions between the host and Wolbachia genomes. Development time, viability, and productivity were not altered by selection. The implications of these results are discussed in light of the intended use of *w*MelPop for suppressing disease transmission.

KEY WORDS: ***Drosophila melanogaster*, host background effects, longevity, *popcorn*, Wolbachia.**

Wolbachia pipientis is a maternally inherited intracellular bacterium with a broad host range across many insects, arthropods, and nematodes. Wolbachia has potential as a biological control agent in medical applications particularly through altering its hosts or acting as a vector for transgenes (Turelli and Hoffmann 1999; McGraw et al. 2002; Brownstein et al. 2003; Sinkins 2004; Sinkins and Gould 2006). One particular strain of Wolbachia, named *popcorn* (*w*MelPop), reduces longevity of its host (Min and Benzer 1997). This virulent strain has generated interest in vector control strategies because of its potential in altering disease transmission, in particular by manipulating the age structure of *Aedes aegypti* populations, the primary vector of dengue fever (Rasgon and Scott 2004; Sinkins and Gould 2006; McMeniman et al. 2009).

In original work on the *w*MelPop strain in *Drosophila melanogaster* by Min and Benzer (1997), all infected flies held at 29°C died within 14 days. However, when longevity was assessed at 25°C, infected flies lived twice as long, suggesting that the virulence of *w*MelPop depended on temperature. Longevity effects of *popcorn* also depend on host background; Reynolds et al. (2003) found that when the *popcorn* infection was introduced into different outbred backgrounds of *D. melanogaster* and held at 25°C, infected flies took longer to die when compared to an inbred background. Nevertheless, in all backgrounds the *popcorn* infection still reduced longevity substantially when compared to uninfected controls, highlighting its potential in altering the age structure of host populations.

Effects of *popcorn* on other traits including development time, fecundity, and levels of cytoplasmic incompatibility (CI) induction has also been assessed in both the native *popcorn* host, *D. melanogaster,* and a novel host, *Drosophila simulans*, after transinfection with the bacterium (McGraw et al. 2002; Reynolds

et al. 2003). The *w*MelPop infection extended development time in *D. melanogaster*, and also influenced fecundity (Reynolds et al. 2003). In both *D. melanogaster* and *D. simulans*, the *w*MelPop infection induced strong levels of CI, which helps promote its spread through populations (McGraw et al. 2002; Reynolds et al. 2003).

These studies define fitness effects of *popcorn* and conditions that influence its spread, but do not indicate the long-term fate of *popcorn* introductions into outbred populations. When Wolbachia are introduced into new hosts, there is the potential for evolution in both the Wolbachia and host genomes that modifies the way Wolbachia influences traits. Theory predicts that a host and symbiont will evolve to change levels of transmission and fitness effects associated with the infection (Turelli 1994). Given that *w*MelPop is virulent by nature, there may be an "attenuation" of *popcorn* effects progressing toward a relationship less detrimental to the host. An example of virulence attenuation of the intracellular bacterium Wolbachia was documented by McGraw et al. (2002). In *D. simulans* newly transinfected with *w*MelPop, the virulent effect of *w*MelPop on longevity at generation 20 had attenuated in the following nine generations to levels similar to that seen in the native host, *D. melanogaster*. There is also field evidence of attenuation of Wolbachia effects. In *D. simulans*, a Wolbachia strain (Riverside; *w*Ri) changed over a short 20-year period from producing a 20% deficit to host fecundity in wild populations to inducing a 10% increase in fecundity (Weeks et al. 2007).

Any attenuation of longevity effects might involve both nuclear and cytoplasmic factors that alter the longevity of populations (Maklakov et al. 2006; Rand et al. 2006; Toivenen et al. 2007). In *D. simulans*, particular mitochondrial haplotypes are monophyletic in their distribution with Wolbachia strains (James and Ballard 2000), and mtDNA haplotypes may influence the life span of male *D. simulans* (James and Ballard 2003). Rand et al. (2006) found that mitochondrial DNA variation can influence longevity in *D. melanogaster* as well as *D. simulans* through interactions between nuclear and cytoplasmic factors. Evolutionary changes in longevity following infection by *popcorn* might therefore involve the host nuclear genome, mitochondrial genome, Wolbachia genome, or interactions between them. Changes in any one of these components might be sufficient to attenuate any shifts in age structure of host populations mediated by the *popcorn* infection.

Here we test the potential for evolution of the longevity phenotype in outbred *D. melanogaster* lines infected by *w*MelPop. Given that the effects of Wolbachia *popcorn* have already been established (Reynolds et al. 2003), we do not consider lines cured of Wolbachia in our experiments. We instead focus on the maximal response possible using a selection regime for early and late reproduction in lines infected by *popcorn*. This selection regime is expected to indirectly influence longevity as well as other life-history traits (Sgrò and Partridge 1999). We test for changes in a number of life-history traits, and we examine the relative importance of nuclear background and Wolbachia (or other cytoplasmic) effects in any observed changes. Results are discussed in the context of using Wolbachia infections to suppress longevity, the implications this poses for future research into modifying the age structure of target populations, and thus the potential to modulate disease transmission in natural populations through bacterial infections.

Methods

The selection experiment to test for attenuation of *w*MelPop infection is first described, followed by experiments designed to test whether any attenuation effects are due to the host or Wolbachia.

FLY STOCKS AND SELECTION

To generate lines for selection, we started with the laboratory *w1118* line infected with *w*MelPop, also used in Min and Benzer (1997) and Reynolds et al. (2003). We created *w*MelPop infected flies on different outbred nuclear backgrounds by introgressing the *popcorn*-infected *w1118* cytoplasmic background (crossing through the maternal lineage) for four generations into three mass bred populations. These mass bred populations were initiated with flies collected from three locations along the east coast *D. melanogaster* cline of Australia, which has been scored for multiple genes and traits (reviewed in Hoffmann and Weeks [2007]); populations were from northern (Innisfail, 17°33′S, 146°05′E), middle (Brisbane, 27°25′S, 153°02′E), and southern (Sorrell, 42°47′S, 147°34′E) locations along the cline, hereafter referred to as the Northern, Middle and Southern mass bred (MB) backgrounds. The mass bred lines had each been initiated with offspring from 100 inseminated field females, which had then been reared at a census size of at least 2000 individuals for six generations prior to backcrossing. Each of these newly infected populations was split into five replicate cage populations, which was then subdivided into two populations, selected for either early or late reproduction at 25°C (i.e., 30 cages were maintained in total). Flies were reared on a standard cornmeal-based diet without extra yeast. All flies used in experiments were controlled for rearing density prior to testing to remove potential competition effects during the larval development stages. Groups of 20 eggs were collected from a treacle-based media with a yeast suspension to promote egg laying, and transferred to a fresh vial containing 10 mL of cornmeal medium.

Selection for early reproduction involved collecting eggs laid by flies unto and including three days of age ("Early" selected lines), up to sufficient fly numbers (approx. 500 eggs) were obtained for each generation. Selection for late reproduction involved collecting eggs from flies when they were 15 days of

age ("Late" selected lines). Where sufficient egg numbers were not obtained from flies from this age group, we supplemented egg numbers with those from older flies. Eggs were then reared to adulthood, and used to start the following generation. Based on productivity results (see below), the intensity of selection was increased for Late selection after 15 generations. This involved collecting eggs from females between 20 and 25 days of age. We targeted 25 days, but when there were insufficient eggs laid, we used eggs laid from an earlier period (no less than 20 days) to increase egg numbers to 500. After five generations of this selection regime, we recharacterized the response in a second longevity assay.

Lines were tested for Wolbachia infection on a number of occasions, at generations 14 and 17 for the Late lines, and generations 20 and 25 for the Early lines. To test for the presence of Wolbachia, the Wolbachia-specific protein (*wsp*) gene (Zhou et al. 1998) was amplified in a minimum of 14 individuals from each line, to calculate the specific infection frequencies that may impact on phenotypic differences. DNA was extracted using a modified Chelex extraction protocol (McColl et al. 1996). Briefly, single flies were homogenized in Proteinase K, and 150 μl of Chelex was added. Samples were incubated at 65°C for 30 min., and then boiled for a further 8 min. The subsequent supernatant was used in all PCR reactions thereafter. A control set of primers were also used in all reactions, amplifying a *Drosophila* nuclear genome specific target, the single-copy gene *Supressor of Sable* (*Su(s)*) (Voelker et al. 1991), to confirm that uninfected results were in fact uninfected, and not a PCR dropout. An established PCR protocol was followed for Wolbachia *wsp* screening (Zhou et al. 1998).

Differences among lines emerged after 10 (Late) and 18 (Early) generations in the Middle populations (see below). To test whether the divergence was due to nuclear genes or cytoplasmic effects, target populations were backcrossed before being tested in a longevity assay. The aim of this experiment was to elucidate whether line divergence was due to Wolbachia (or other cytoplasmically transmitted elements), the host nuclear genome, or an interaction between these genomes. Two independent backcrosses were performed for three generations (to achieve greater than 90% nuclear background similarity), to create a Late selected Wolbachia on both an Early and a Late selected host nuclear background, and an Early selected Wolbachia on both an Early and a Late selected host background. In the backcrosses, at least 200 flies were used per cross.

TRAITS

Longevity

Flies were reared at a controlled density prior to assaying longevity at 25°C. After a 5 h laying period on treacle-based medium, 10 replicate vials each containing 20 newly collected eggs were reared to adulthood. Four longevity assays were performed, and for all assays, each replicate consisted of 10 females and five males (as per Reynolds et al. 2003). Each of our 30 cages had between three and five replicate vials. Females were examined only in the assays, due to their ability to transmit the bacteria between generations, however males were also maintained in the vials because in nature both sexes are present and male presence decreases life span (Chapman et al. 1993). A 2:1 female:male ratio was maintained throughout the duration of the assay: where required, excess males were removed as required to maintain this sex ratio in each vial. Mortality was recorded every 48 ± 3 h, when flies were transferred into new vials. The first longevity assay tested the Early lines at generation 18, and the Late lines at generation 10. The second assay retested these lines at generation 32 and generation 20, respectively.

Our initial productivity assay suggested that the intensity of selection in the Late lines could be increased. We obtained a substantial number of eggs from females older than those used to maintain the initial selection intensity (around 15 days), and we therefore increased the selection pressure on our Late lines for a further five generations, before conducting another longevity assay. Our aim was to select eggs laid as close to 25 days of parental age, although this was reduced to 20 days of parental age (see above).

Two further longevity assays assessed cytoplasmic versus nuclear effects that were each identified in the first and second assays. In both cases, we used two Early Middle MB lines and two Late Middle MB lines (the same lines in both assays). The first of these assays involved independent reciprocal backcrosses for three generations. The second assay involved one backcross (after crossing parental strains). This essentially resulted in four classes of flies that we were testing: 100%, 75%, 25%, and 0% Early background, with Early selected Wolbachia on the 100% and 25% backgrounds, and Late selected Wolbachia on the 75% and 0% backgrounds. Both of these assays were conducted in the same way as the above.

Productivity

This was assessed at 25°C for every line on nine occasions when flies were 3–33 days of age. Nine replicates of each line were assessed on each occasion, at generation 18 for the Early lines, and generation 10 for the Late lines. For the assay, individual females were placed into fresh vials for 48 ± 2 h. Eggs from each female were then reared to adults, and the number of emerging progeny was recorded.

Development time and viability

Larvae were reared at 25°C under controlled density in these experiments. For each replicate, groups of 10 eggs were picked after a 3 h laying period, and placed into fresh vials, with five

replicates per line. Newly eclosed flies were collected every 6 h from day 7 to day 13. Viability was estimated from the total number of flies that eclosed per replicate. The assay tested flies from the Early lines at generation 21 and from the Late lines at generation 12.

ANALYSES

All analyses were conducted using SPSS (version 15; SPSS Inc, Chicago, IL). A Kaplan–Meier (KM) test was applied to the longevity data. KM analyses assess the rate of mortality (as opposed to just the mean longevity of individual lines) using log-rank tests. For the longevity data, we examined the effects of various genetic backgrounds and selection regimes on the lines. We also ran a nested analyses of variance (ANOVA) on mean longevity values to examine the effects of the host nuclear and Wolbachia genomes directly on mean life span.

For the correlated life-history traits, ANOVA was used to assess the effect of selection regime (turnover), host background (origin), and infection frequency (as a covariate) on average productivity per day, mean development time, and viability, based on analyses of line means. Line effects (nested within turnover by origin) were assessed in separate ANOVAs on the vial/adult data rather than line mean data. For the productivity data obtained across nine age intervals, the number of offspring in cages tended to increase initially before decreasing again, producing a parabolic relationship for each line (see Fig. 5). This relationship was initially estimated for each line with a nonlinear regression by fitting the relationship $p_i = a + b_1 x_i + b_2 x_i^2$ where p_i is the productivity at age i, a is the intercept, b_1 is the linear regression coefficient, and b_2 is the quadratic regression coefficient. We then ran ANOVAs on the resulting 30 values for a (the intercept for $pi = 0$, reflecting the predicted age when reproduction falls to 0) and p at age $i = 3$ (reflecting early reproduction at age three days) to test whether there were effects of origin, turnover, and their interactions on predicted early productivity at age 3 and late productivity as determined by the age at which it was expected to decline to 0. We predicted that Late lines would have a lower early productivity but higher intercept reflecting higher egg production later in life, and we also were interested in testing if early or late reproduction altered total productivity of the lines.

Results

Selection on the three backgrounds took place from May 2006 and is still being maintained. Population size was maintained at around 100 mating pairs per generation. The results from the longevity assays are first described here, which is then followed by the Middle MB background backcrossing experiments aimed at determining the effect of host and Wolbachia genomes (or other cytoplasmic effects) on line divergence for longevity. Finally, measurements of other life-history traits are presented.

LONGEVITY

A total of four longevity assays were conducted. The first and second assays measured the longevity of all 30 lines on two separate occasions. The third longevity assay was designed to elucidate the cause of variation identified between Early and Late selected lines in the Middle MB background. The fourth (last) assay also considered causes of variation in longevity identified in the second longevity assay, where the Middle and Northern MB populations had diverged in longevity as a consequence of selection.

First assay

Longevity was assessed with females from all lines, when Early selected lines were at generation 18, and Late selected lines at generation 10. Kaplan–Meier tests demonstrated that there was a difference between the Northern, Middle, and Southern populations in longevity ($\chi^2 = 10.426$, df $= 2$, $P = 0.005$), with the Middle lines having the greatest life span. Mean life span for these populations was 42, 44.8, and 41.2 days, respectively (Table 1). There was no difference between the Early and Late selection regimes in the Northern and Southern backgrounds. However, in lines from the Middle background, the Early selected lines had a shorter mean life span of 43.7 days, and a survival curve was significantly different from that of the Late selected lines, which had a mean life span of 46.1 days (KM: $\chi^2 = 4.79$, df $= 1$, $P = 0.029$) (Fig. 1).

Second assay

This was conducted when the Early lines were at generation 32 and the Late lines were at generation 20, after five generations of intensified selection on the Late lines. The original *w*MelPop infected *w1118* line was also included in this assay for comparison.

There was a highly significant effect of genetic background on survival curves of the four origins (KM: $\chi^2 = 55.49$, df $= 3$, $P < 0.001$). Kaplan–Meier pairwise log-rank tests showed that the Southern population had a significantly shorter life span (mean of 35.5 days) than both Northern (43.8 days) ($\chi^2 = 30.22$, $P < 0.001$) and Middle (42.8 days) ($\chi^2 = 39.23$, df $= 3$, $P < 0.001$) populations, but not the *w1118* line (38.9 days) ($\chi^2 = 0.0034$, df $= 3$, $P = 0.954$) (Table 1). The Northern and Middle populations were also different from the *w1118* line (Northern; $\chi^2 = 14.87$, $P < 0.001$ and Middle; $\chi^2 = 12.14$, $P < 0.001$) (Fig. 2).

The additional generations of selection led to further divergence between the lines. Overall, an effect of turnover was identified (KM: $\chi^2 = 14.50$, df $= 1$, $P < 0.001$), with Early lines having a mean life span of 41 days, and the Late lines 43.5 days (a 5.5% difference). Within MB backgrounds, an effect of selection was now observed in both the Northern (KM: $\chi^2 = 6.98$, df $= 1$, $P = 0.008$) and Middle (KM: $\chi^2 = 9.50$, df $= 1$, $P = 0.002$) populations. In both cases, the Early lines had a mean life span of around 2.5 days shorter than their Late selected counterparts. No

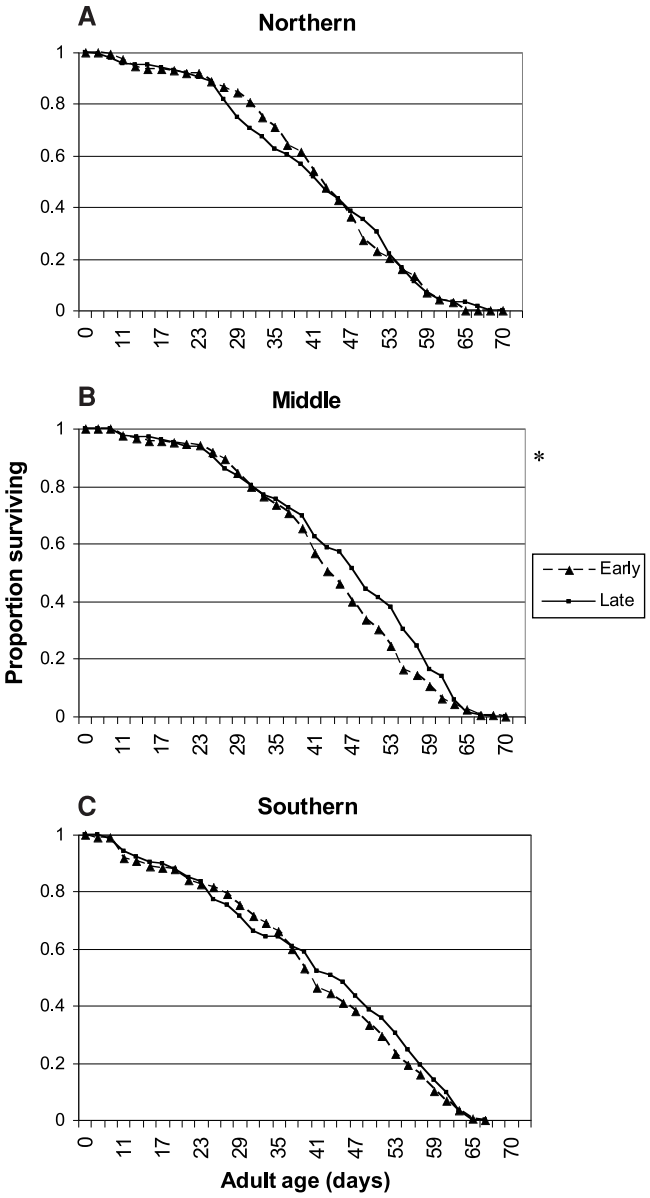

Figure 1. Survival curves for (A) Northern, (B) Middle, and (C) Southern MB populations for Longevity assay 1. Each graph depicts the differences between the Early and Late fecundity selection regimes at generations 18 and 10, respectively. * denotes $P = 0.029$.

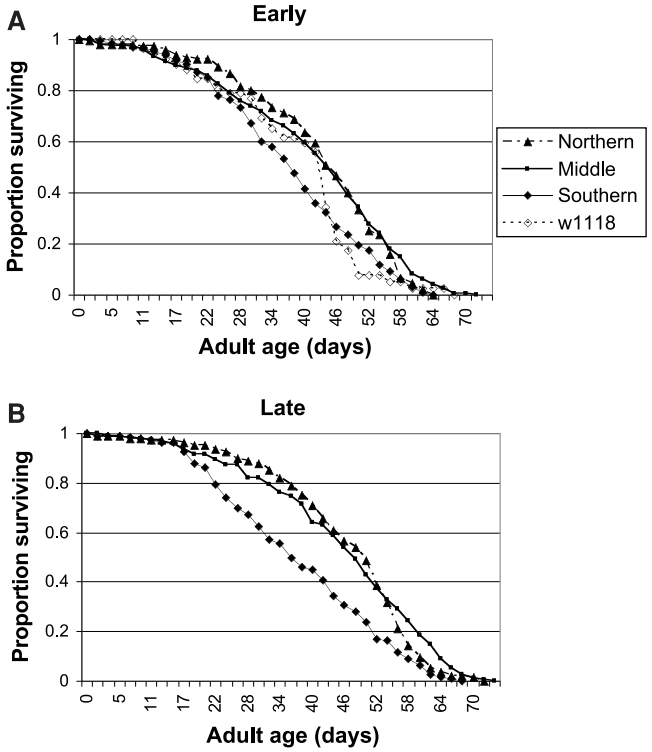

Figure 2. Survival curves for all origins for Longevity assay 2, conducted after several generation of intensified selection on the Late lines. (A) Early (generation 32) and (B) Late (generation 20) selected lines are shown separately, each portraying the differences in life span between origins.

effect of selection was observed in the Southern population (KM: $\chi^2 = 0.54$, df = 1, $P = 0.460$), with both Early and Late lines having a mean longevity within 1.5 days of each other (Fig. 3).

NUCLEAR VERSUS WOLBACHIA EFFECTS

Third assay

To ascertain the source of the difference between the Early and Late selected lines in the Middle MB background, we backcrossed lines for three generations to generate an Early selected Wolbachia (or other cytoplasmic factor) onto a Late selected nuclear background, and a Late selected Wolbachia onto an Early selected nuclear background. Backcrosses began when Early lines were at generation 30 and Late lines were at generation 18. Kaplan–Meier analyses and plots for this longevity assay show that the host nuclear background dictated a large and significant part of the longevity reduction ($\chi^2 = 40.82$, df = 2, $P < 0.001$) (Fig. 4). When both Early and Late selected Wolbachia were placed into the opposing background, the Early selected host background consistently had a shorter mean life span (31.9 days) than the Late selected background (38.5 days), reflecting an effect of nuclear background rather than Wolbachia (or other cytoplasmic) effects. A nested ANOVA further supported the significant effect of the host nuclear background on mean longevity ($F = 39.15$, df = 1, 496, $P < 0.001$).

Although no overall effect of Wolbachia background was observed in the Kaplan–Meier analyses, we did identify a difference between Wolbachia sources in the Late selected host background (KM: $\chi^2 = 6.85$, df = 1, $P = 0.008$). In this host background, Wolbachia (or other cytoplasmic effects) sourced from Early selected hosts tended to survive longer (mean 41.3 days) than the Late selected Wolbachia (mean 36.6 days). An ANOVA also revealed this effect of Wolbachia origin on longevity ($F = 6.95$, df = 1, 496, $P = 0.008$). However, Wolbachia did not influence

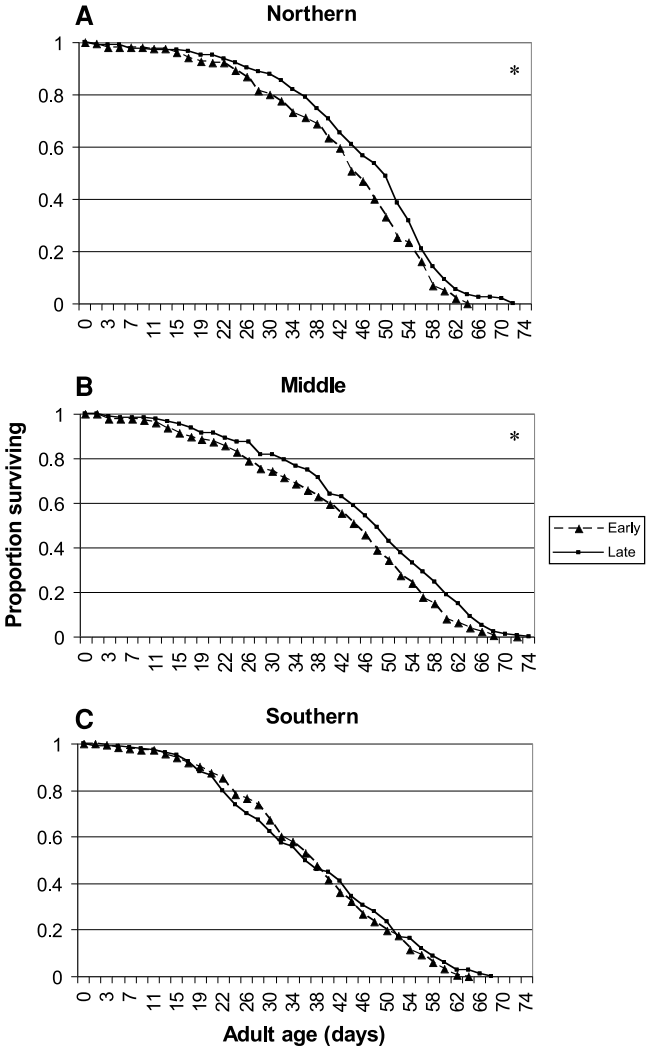

Figure 3. Survival curves for (A) Northern, (B) Middle, and (C) Southern MB populations for Longevity assay 2, conducted after several generation of intensified selection on the Late lines (compare with Fig. 1). Each graph depicts the differences between the Early and Late fecundity selection regimes at generations 32 and 20, respectively. * denotes $P < 0.01$.

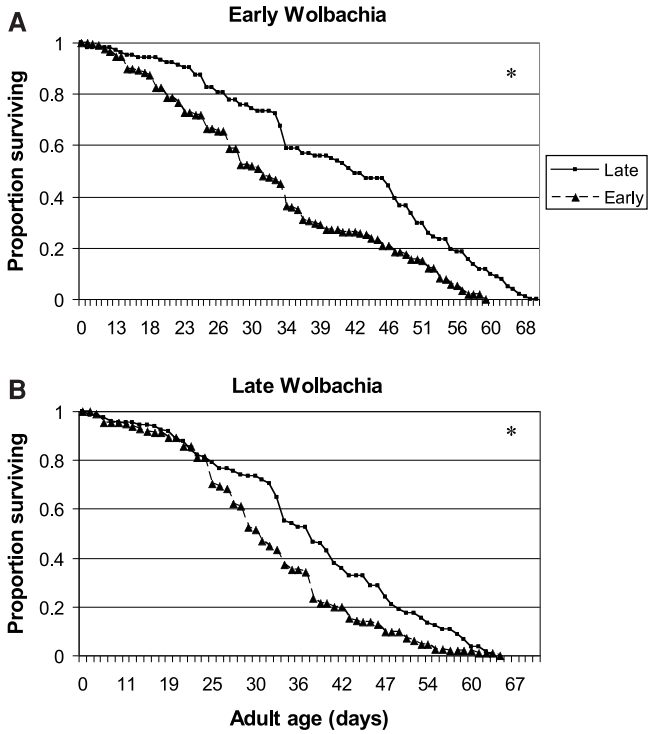

Figure 4. Survival curves for Middle MB backcrossed lines in Longevity assay 3. Two Early and Late selected Middle MB lines were independently backcrossed, to determine the source of variation identified in these lines in Longevity assay 1. The curves illustrate the effect of host background on the proportion of flies surviving. (A) Early selected Wolbachia; (B) Late selected Wolbachia. * denotes $P < 0.001$.

longevity in the Early selected host background, as indicated by a KM test ($\chi^2 = 0.33$, df = 1, $P = 0.560$). A significant effect of line was also detected (KM: $\chi^2 = 53.73$, df = 11, $P < 0.001$), as well as a significant effect of vial on longevity ($F = 2.21$, df = 69, 496, $P < 0.001$), indicating divergence between some replicate lines. A breakdown of the independent backcrosses indicates that the mean longevity in one group of crosses is influenced by a Wolbachia by host background interaction ($F = 7.61$, df = 1, 181, $P = 0.006$), whereas the other group is not ($F = 1.46$, df = 1, 188, $P = 0.227$).

Fourth assay

A final longevity assay further clarified the effects of host nuclear and Wolbachia effects on longevity. Selection on the Late lines had been relaxed prior to this assay, and there was one generation of backcrossing, which led to host nuclear backgrounds being 75% of the backcrossed line. Backcrosses were conducted using flies from the Middle MB background, with Early flies from generation 45, and Late flies from generation 28. In the assay, there was an effect of host background on longevity (KM: $\chi^2 = 99.05$, df = 3, $P < 0.001$); flies from crosses with only the Early selected host background had the shortest longevity, while there was a sequential increase in longevity as flies carried more of the Late selected host background. In contrast, when the effect of Wolbachia was examined within the 75% Early and 75% Late backgrounds, no effect of Wolbachia (or other cytoplasmic factor) was identified (KM: 75% Early background $\chi^2 = 1.78$, df = 1, $P = 0.182$; 75% Late background $\chi^2 = 0.99$, df = 1, $P = 0.318$). There was also no overall effect of Wolbachia on longevity when tested across backgrounds (KM: $\chi^2 = 2.50$, df = 1, $P = 0.114$).

OTHER TRAITS

Productivity

Average productivity per female was 43.14 progeny per 48 h laying period. There was a significant effect of origin on this trait

Table 1. Average days until death for each origin and turnover, for longevity assays 1 and 2, where flies from three outcrossed backgrounds, each selected for either early or late turnover were assessed. Ranges for the replicate lines are presented in brackets. ****w1118***, the original infected *popcorn* strain, was not assessed in longevity assay 1.**

Origin	Turnover	Longevity 1	Longevity 2
Northern	Early	42.15 (39.7, 44,7)	42.72 (36.4, 46.3)
	Late	41.46 (36.2, 46.9)	45.22 (36.6, 49.7)
Middle	Early	43.70 (41.9, 46.0)	41.59 (36.6, 46.0)
	Late	46.23 (39.2, 48.5)	44.16 (38.6, 51.4)
Southern	Early	40.70 (33.0, 50.7)	34.89 (25.1, 41.9)
	Late	41.92 (35.9, 47.8)	36.33 (31.5, 46.7)
w1118	Early	—*	38.69
Average		42.69	40.51

but not turnover (Table 2). An effect of replicate line (nested) was also detected ($F = 4.07$, df = 24, 240, $P < 0.001$). Productivity peaked to a maximum at day 7, with more than 63 eggs being produced per female on average. At day 33, the average output was only 15 per female. Productivity was highest in the Middle background and lowest in the Northern background (Fig. 5). Analysis of total productivity showed that there was a significant effect of background origin ($F = 4.35$, df = 2, 23, $P = 0.024$; Fig. 6A), but not turnover ($F = 1.69$, df = 1, 23, $P = 0.205$). ANOVAs on regression parameters obtained for each line indicated that there was a significant effect of background origin ($F = 8.42$, df = 2, 24, $P = 0.001$; Fig. 6B) and turnover ($F = 7.81$, df = 1, 24, $P = 0.010$; Fig. 6C) on the intercept reflecting the predicted age at which productivity declined to 0. There was also a weak interaction between turnover and origin for the intercept data ($F = 3.55$, df = 2, 24, $P = 0.044$). For all backgrounds, the intercept of the Early lines had a lower value than that of the Late lines, but this was particularly evident in the Northern MB background (Fig. 6C). The difference between background origin was due to the lower values in the Northern background compared to the other backgrounds, reflecting the earlier point at which reproduction was predicted to cease in this background. For the estimated early productivity at age 3, we found a significant effect of background of origin ($F = 16.74$, df = 2, 23, $P < 0.001$) and also turnover ($F = 13.47$, df = 1, 23, $P = 0.001$). However, contrary to expectations early productivity was higher in the Late lines for the Middle and Southern MB backgrounds, and not the Northern background.

Development time and viability

For development time, we separated sexes for our analyses. ANOVAs indicated that our selection regimes had not influenced development time in either sex. There was also no effect of background origin (Table 2). Replicate line (nested) was also significant in an ANOVA on both females ($F = 17.16$, df = 24, 607, $P < 0.001$) and males ($F = 10.56$, df = 24, 450, $P < 0.001$). The mean development time for all lines was 226.9 h (9.45 days). The shortest development time was 174 h (7.25 days) in an Early line on the Middle MB background, whereas the longest was 294 h (12.25 days), in an Early Southern line.

Viability estimates were made from the number of flies that eclosed during the development time assay. Both background origin ($F = 9.25$, 2, 23, $P = 0.001$) and selection regime ($F = 5.55$, df = 1, 23, $P = 0.027$) had an effect on mean viability. No interaction between these terms was observed (Table 2) but there was an effect of the nested line term ($F = 2.45$, df = 24, 119, $P < 0.001$).

Infection frequency

The frequency of the infection within each of the 30 lines was checked on two occasions, around the same time that the first and second longevity assays were being conducted. Infection frequencies ranged from 69% to 100% between individual lines, with an average of 96.3%. The averages for each line were used as covariates in the analyses on line means.

Discussion

This study assessed whether the phenotypic effects of *w*MelPop on the longevity of *D. melanogaster* could be attenuated under selection for early and late reproduction. Recent experiments have suggested the feasibility of using *w*MelPop as a tool for biological

Table 2. Summary of ANOVA results for productivity, development time (DT), and viability assays. Significance values are as follows: ****P*<0.05, *****P*<0.01.

Term	df	Productivity		DT (female)		DT (male)		Viability	
		MS	*F*	MS	*F*	MS	*F*	MS	*F*
Infection	1	12	0.49	50	0.25	34	0.23	0.01	0.91
Origin (O)	2	108	4.35*	105	0.52	32	0.22	0.13	9.25**
Turnover (T)	1	42	1.69	263	1.30	110	0.74	0.08	5.56*
O × T	2	45	1.81	37	0.19	4	0.03	0.02	1.36
Error	23	24		202		149		0.01	

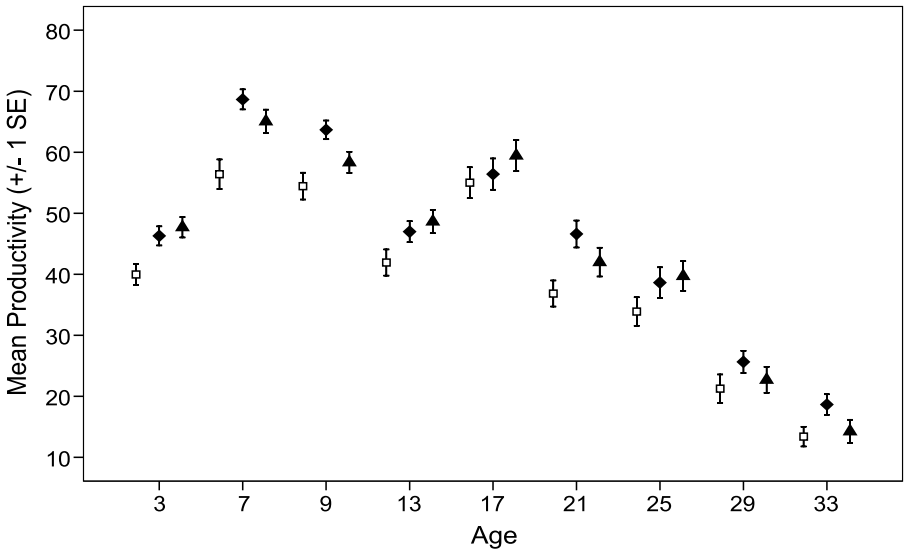

Figure 5. Average productivity of all lines over the nine age classes tested, separated by background origin. □, Northern MB; •, Middle MB; ▲, Southern MB populations. Error bars represent ± 1SE of the mean.

control of the spread of the dengue fever virus in mosquito vectors (McMeniman et al. 2009). In mosquitoes, longevity will depend on feeding and oviposition. Previous studies have shown that egg laying is strongly correlated with blood-feeding by *Ae. aegypti* (Judson 1967; Styer et al. 2007), and after blood-feeding, mortality of *Ae. aegypti* increases with the commencement of oviposition (Styer et al. 2007; Harrington et al. 2008). Nevertheless, the *Drosophila* data provide an indication of the potential for attenuation due to direct selection on patterns of offspring production, which may involve changes in host nuclear background, Wolbachia and/or other cytoplasmic factors such as mtDNA. The first of our four longevity assays indicated that the Middle MB population had responded to selection for early and/or late reproduction, whereas the Northern population also showed a response in later generations. This suggests that at least some populations carrying the *popcorn* infection have the potential to evolve and alter their longevity if there is selection for early or late reproduction. Although the intensity of such selection in nature is unknown, it is likely to be weaker than we have imposed here (see below). Any changes in longevity under selection might then impact on the ability of *popcorn* to alter the age structure of populations and thus affect rates of disease transmission.

Because we selected on lines that carried Wolbachia, we were able to separate the effects of Wolbachia from nuclear background effects. By backcrossing lines we showed that selection responses were the consequence of changes in the host nuclear background rather than the Wolbachia themselves (or other cytoplasmic factors). Although there was a small effect of the Early/Late selected Wolbachia observed in the Late selected host background, the majority of the longevity phenotype appeared to be dictated by the host background. These data support other findings suggesting that phenotypic effects of Wolbachia strains can depend on host background (Reynolds and Hoffmann 2002; Reynolds et al. 2003; Dean 2006; Rand et al. 2006).

Are the types of changes we have detected likely to represent an obstacle for the use of *popcorn* as an agent in altering the age structure of mosquitoes? In our experiments, host genetic background evolved under selection to produce a difference in longevity of around 5.5% due to selection. However this difference was only achieved through strong selection for early and late reproduction across a number of generations. In our late lines, no eggs laid before 20–25 days contributed offspring to the successive generations, whereas in nature eggs laid throughout life may contribute to the next generation. Therefore, the intensity of selection on infected hosts in nature will be much weaker than in our experiments. Moreover, although our experiments indicate divergence in longevity over tens of generations, we could not establish whether this change was due to a decrease in longevity in the early reproduction lines or an increase in the late reproduction lines (or a combination of both). This is primarily because we did not test these lines after a tetracycline treatment, or before selection began on these lines. Our focus was to compare phenotypic changes between selection regimes, and how these might differ between flies from various origins. Therefore, our results demonstrate the potential for longevity to be altered under selection in an infected population rather than the actual likely rate of change of longevity in natural populations.

However, the results suggest another important issue that needs to be addressed in any releases of *popcorn*-infected strains, which is the large potential difference between lines due to their origin. In the three MB backgrounds, we found a difference of 15% in mean life span. This large difference suggests the need to

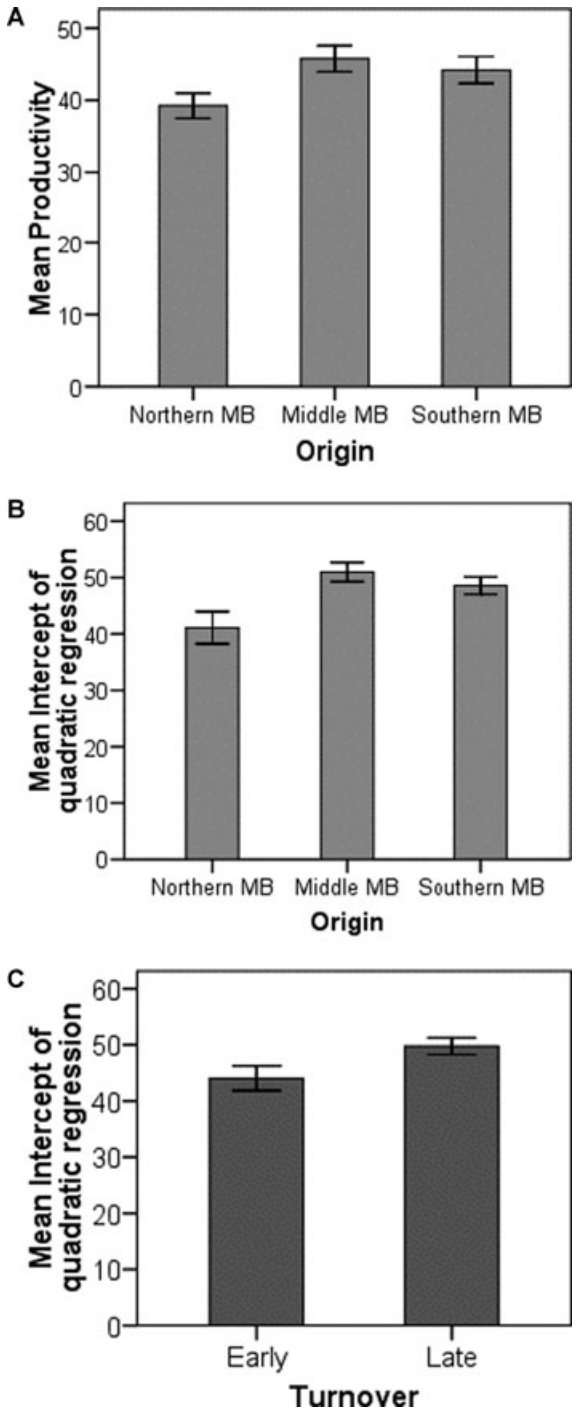

Figure 6. Effects of selection and background on productivity. (A) Mean number of progeny produced by individual flies per day for each origin. (B, C) Mean intercept of the regression for each origin, representing the variation observed between and within the three backgrounds (B) or turnover regime (C). Error bars represent ± 1 SE of the mean.

test the effects of *popcorn* infections on the background of target mosquito populations in which the infection is to be introduced. The interaction between Wolbachia (or other cytoplasmic effect) and the host genome we detected in one experiment also emphasizes that Wolbachia effects may depend on background nuclear genes.

Many studies have investigated the effects of increasing longevity on life-history traits, most commonly indirectly selecting for longevity via late fecundity selection regimes (Rose 1984; Sgrò and Partridge 1999) as we have also done here. Sgrò and Partridge (1999) found that there was a trade-off between early reproduction and increased survival at late ages associated with mating. Females have also been assessed for costs of reproduction, by mating with spermless males (Chapman et al. 1993), and it was found that the mating procedure itself contributed to a reduction in longevity. Results from our selection experiments support the notion that early and late reproduction leads to line divergence for longevity; this was the selection regime used and generated lines with an increased longevity and higher predicted productivity later in life. However, in our lines we did not find that there was a reduction in productivity early in life in the late selected lines. Thus, we did not observe patterns of decreased early fecundity seen in lines with extended longevity (Luckinbill et al. 1984; Rose 1984; Partridge et al. 1999). Perhaps our selection responses altered the effects of the *popcorn* infection rather than specifically changing underlying life-history patterns. In any case, it appears that the response to selection was due to late productivity that led to a higher reproductive output later in life, without any cost in terms of early productivity.

In conclusion, the results suggest that selection on host genetic backgrounds have the potential to alter longevity patterns in lines infected by *popcorn* Wolbachia. With the planned implementation of Wolbachia-based biocontrol of *Ae. aegypti*, we advocate that the effects of the introduced *popcorn* strain should be carefully assessed in target nuclear backgrounds. In *Ae. aegypti*, there is the potential for old females to produce large numbers of eggs, particularly directly after blood feeding (Styer et al. 2007), and this could produce selection for extending the life span of infected mosquitoes. It also seems worthwhile collecting longitudinal data on longevity effects in strains that have been developed for eventual release of *popcorn* into target natural populations.

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