Monitoring long-term evolutionary changes following *Wolbachia* introduction into a novel host: the *Wolbachia* popcorn infection in *Drosophila simulans*

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Wolbachia may act as a biological control agent for pest management; in particular, the *Wolbachia* variant *wMelPop* (*popcorn*) shortens host longevity and may be useful for dengue suppression. However, long-term changes in the host and *Wolbachia* genomes can alter *Wolbachia* spread and/or host effects that suppress disease. Here, we investigate the phenotypic effects of *wMelPop* in a non-native host, *Drosophila simulans*, following artificial transinfection approximately 200 generations ago. Long-term rearing and maintenance of the bacteria were at 19°C in the original I-102 genetic background that was transinfected with the *popcorn* strain. The bacteria were then introgressed into three massbred backgrounds, and tetracycline was used to create uninfected sublines. The effect of *wMelPop* on longevity in this species appears to have changed; longevity was no longer reduced at 25°C in some nuclear backgrounds, reflecting different geographical origin, selection or drift, although the reduction was still evident for flies held at 30°C. *Wolbachia* influenced productivity and viability, and development time in some host backgrounds. These findings suggest that long-term attenuation of *Wolbachia* effects may compromise the effectiveness of this bacterium in pest control. They also emphasize the importance of host nuclear background on *Wolbachia* phenotypic effects.

Keywords: *Wolbachia*; *popcorn*; *Drosophila simulans*; longevity; evolution; host genetic background

1. INTRODUCTION

Wolbachia is an intracellular bacterium that has been shown to cause numerous phenotypic effects in its hosts and may potentially be useful as a biological control agent (Sinkins & O'Neill 2000; Brownstein *et al.* 2003; Rasgon *et al.* 2003; Rasgon & Scott 2004). One *Wolbachia* strain that holds particular promise in this respect is *popcorn* (*wMelPop*), which has been found to over-replicate within the cells of its host, *Drosophila melanogaster*, causing a reduction in host longevity (Min & Benzer 1997). Using this infection, the age structure of target host populations might be modified to prevent disease transmission (Brownstein *et al.* 2003; McMeniman *et al.* 2009). Apart from influencing longevity, *wMelPop* also causes other effects. In *D. melanogaster*, this infection causes cytoplasmic incompatibility (CI) involving a reduction in egg hatch rate following mating between infected males and uninfected females, and also delays egg-to-adult development (Reynolds *et al.* 2003).

Following identification in *D. melanogaster*, the *popcorn* infection was successfully transferred to *D. simulans* (McGraw *et al.* 2001) and more recently to *Aedes aegypti* (McMeniman *et al.* 2009). In both *D. simulans* and *A. aegypti*, the infection reduces the longevity of its host substantially while inducing CI. In *D. simulans*, *wMelPop* effects on longevity and other traits appear to have changed over time. McGraw *et al.* (2002) initially found that while the *wMelPop* infection caused strong CI, there

was also a significant reduction in reproductive fitness owing to a decrease in fecundity and egg hatch rates. However, these effects appeared to attenuate with time; by generation 22 after transinfection, hatch rate in compatible crosses returned to control levels, although there was still a significant effect of the infection on fecundity. McGraw *et al.* (2002) also showed that egg production in the *wMelPop*-infected line declined after only 5 days.

For *Wolbachia* infections, theory predicts a shift towards a decrease in the virulence of the bacteria in its host, while perfect maternal transmission and high levels of CI are expected to be maintained unless there is a cost (Turelli 1994). Attenuation of virulence of the *wMelPop* infection was recently identified in the native *D. melanogaster* host, through indirect selection on host longevity (Carrington *et al.* 2009). This shift in longevity was mainly due to host nuclear background, although interactions with host and bacterial genomes were also identified. There are several other cases where the evolution of phenotypic effects generated by *Wolbachia* symbionts have been documented. These include the evolution of a fecundity benefit in Californian *D. simulans* infected with the *Wolbachia* Riverside (*wRi*) strain (Weeks *et al.* 2007), as well as the suppression of the male-killing phenotype induced by the *wBoll* strain in the butterfly *Hypolimnas bolina* (Hornett *et al.* 2006). Both of these examples involve evolutionary changes that have occurred in natural populations.

In this study, we examine the *Drosophila simulans* and *Wolbachia popcorn* (*wMelPop*) symbiosis several years after transinfection (McGraw *et al.* 2001), to test for potential attenuation of *Wolbachia* effects compared with

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tetracycline-treated controls. We estimate this time to be greater than 200 generations, during which time the infection was maintained in the original background and under laboratory conditions. Longevity effects, levels of CI and several life-history traits are considered. Potential changes in phenotypic effects are assessed in three outcrossed nuclear backgrounds compared with treated controls and, where possible, we further contrast these results to those previously described several generations after transinfection (McGraw *et al.* 2002). Results are discussed within the context of effective management strategies for biological control of pest species using this *Wolbachia* strain.

2. MATERIAL AND METHODS

The strains used in this experiment are described first, followed by a description of the life-history trait assays.

(a) *Strains and maintenance*

The original *D. simulans* I-102 strain, transinfected with *Wolbachia popcorn* by McGraw *et al.* (2001), was maintained under laboratory conditions for several years, at 19°C. We then generated three outcrossed infected populations in mid-2007. The *wMelPop* infection from the I-102-infected females was backcrossed for two generations to males from *D. simulans* massbred populations originating from Sydney (latitude 33°50' S) and Sorrell (42°46' S) in Australia, and Irvine (33°39' N) in CA, USA. These massbred populations were originally set up from 10 females and 10 males from each of 10 isofemale lines, established from field females collected in April 2007. Backcrossing was conducted within five generations of the massbreds being created. These populations represent genetically distinct backgrounds that will be influenced by both geographical origin and subsequent changes occurring in the laboratory environment (owing to drift and selection). Once generated, all populations were reared at 25°C on cornmeal–yeast–dextrose media under a rapid turnover regime and continuous light, unless otherwise stated (see experimental descriptions for productivity and the second longevity assay).

At the time all populations were placed at 25°C, the planned temperature for the experiments, it was noted that there were difficulties with rearing the infected I-102 line. Multiple attempts were made to rear I-102 at the higher temperature; however, with each generation we observed a significant reduction in adult progeny, and it was then impossible to reach a second generation (data not shown). The inability to rear this line was investigated in the productivity assay (see below), with results clearly confirming that the infected I-102 line has a severe productivity deficit at the higher temperature. We therefore excluded this line from the remainder of the study.

The experiments were undertaken in two stages. The initial stage was conducted seven generations after backcrossing and included all assays except for the second longevity assay, which was conducted in a second stage (21 generations after the first). Immediately prior to each stage, the infected populations were treated with tetracycline to cure the *Wolbachia* infection. Uninfected subpopulations from each of the massbred populations were created by treating larvae for two generations with tetracycline (0.033% v/v) added to the media. At least 200 flies per background were used to create each subpopulation, which was then cultured for at

least two generations without tetracycline, to control for any negative effects of antibiotic treatment and allow natural gut flora to regenerate (Ballard & Melvin 2007). Additionally, infected and treated populations from the same background location were then reciprocally crossed *en masse* for one generation (using a minimum of 75 males and virgin females), to re-homogenize the host background and control for any differences that might have developed between treated and untreated massbred populations. Because *Wolbachia* is maternally transmitted, reciprocally crossing infected and treated lines results in progeny that have maintained each infection status. However, reciprocal crossing did not control for nuclear background effects owing to sex linkage. To overcome the incompatibility created in the cross between infected males and uninfected females, we used older males (approx. 6–8 days old), whose ability to induce CI had declined. The resulting uninfected populations are henceforth referred to as I-102-T, Sydney-T, Sorrell-T and Irvine-T. Males of approximately the same age were also used in the reciprocal crosses, and the resulting infected populations are referred to without the -T suffix. At the end of the treatment, flies were confirmed uninfected by PCR (see below).

To generate flies for the experiments, larvae were reared at a low density during development. To control for density, inseminated adult females were allowed to lay eggs onto plastic spoons containing approximately 2 ml of a treacle-based medium, with a light covering of a yeast suspension to enhance oviposition. From these spoons, 40 eggs were picked and placed into a fresh vial with 10 ml of cornmeal medium, where they were allowed to develop. Once eclosed, flies were collected for use in experiments.

(b) *Infection status*

To test for the presence of *Wolbachia*, PCR was used to amplify *Wolbachia*-specific DNA extracted from single flies. To extract DNA, individual females were ground in 5 µl of Proteinase K (4 mg ml⁻¹), and 150 µl of 5 per cent Chelex was subsequently added (McColl *et al.* 1996). Samples were heated at 37°C for 30 min, and then at 95°C for 4 min to inactivate the Proteinase K. Prior to PCR, the extract was spun at 13 000 r.p.m. for 2 min and 1 µl of the supernatant was added to a 15 µl PCR reaction mix. PCR was conducted with *Wolbachia*-specific primers (*wsp*81F and *wsp*691R; Zhou *et al.* 1998), as well as primers that amplify a *Drosophila*-specific gene, *Suppressor of Sable* (*Su(s)*), as positive controls for each reaction (Voelker *et al.* 1991). Infection status was confirmed in lines prior to all experiments.

(c) *Strain confirmation*

The *wsp* gene of the *Wolbachia* in each of the infected outcrossed populations (Irvine, Sorrell and Sydney) was sequenced to verify that the strain of *Wolbachia* was in fact *popcorn*. DNA was extracted for sequencing using a phenol–chloroform method from single females (from generation 22), and the *wsp* gene was amplified. We used exonuclease-Shrimp Albumin Protein (ExoSAP-IT, USB Corporation) for PCR cleanup. DNA sequencing was conducted at the UCDNA Sequencing Facility (University of California at Davis, CA, USA). All strains tested showed 100 per cent sequence identity to the published *Wolbachia pipiensis wMelPop* outer surface protein gene precursor (GenBank accession number AF338346.1).

(d) Productivity and fecundity

Owing to the difficulties in obtaining enough progeny from the I-102-infected line to undertake planned experiments at 25°C, we investigated total productivity for all populations. As we were unable to prepare for the experiment at 25°C, we reared all of the flies at 19°C, so as to control for rearing temperature between populations. When the flies had eclosed, they were immediately placed at 25°C for testing. Pairs of virgin males and females (three per population) were set up in vials, which were changed every 24 h. Productivity was assessed every day for 9 days. Productivity was calculated by counting the number of adult flies that eclosed from each vial at each age. Sexes were counted separately but, as there was no effect of infection/background on sex ratio, only data pooled across sexes are considered further. The results of the assay confirmed the observations and we therefore excluded both I-102 and its treated counterpart, I-102-T, from all subsequent assays.

The fecundity of the remaining populations was assessed over a 10-day period when females were 12–36 h post-eclosion. All flies were reared and tested at 25°C. The number of eggs laid by individual females each day was counted as a measure of the fecundity for each population, with 20 replicate females individually assayed for each. Every female was paired with a male, and males that died were replaced during the assay. Only females that survived the entire 10-day period were included in the fecundity assessment. Eggs were laid on a treacle medium held on a plastic spoon coated with a live yeast suspension to trigger egg laying. Spoons were replaced every 24 h and the eggs counted.

(e) Development time and viability

This was assessed using eggs obtained from adults 3–5 days post-eclosion. After a 2 h laying period, eggs were picked and placed in vials with 12 ml of medium (20 eggs per vial). Egg-to-adult development time at 25°C was scored separately for the sexes every 6 h, starting at 168 h (7 days) after the middle of the laying period. There were 20 replicates for each of six populations (infected and uninfected of each of Sydney, Sorrell and Irvine). Viability was also estimated from this assay.

(f) Longevity

Two longevity assays were conducted. The first assay tested whether the *popcorn* longevity phenotype was present some 200+ generations after transinfection. All three outcrossed backgrounds were tested at 25°C. The second assay was conducted approximately 20 generations after the first and compared the effect of temperature (30°C versus 25°C) on longevity effects of the *popcorn* infection, using flies from two of the outcrossed backgrounds (Sydney and Irvine).

The first assay was set up with flies from the development time assay (described above). Longevity was assessed for all combinations of host background, infection status, sex and mating effects. Flies tested were either virgin or once mated, because previous studies have shown a trade-off between mating/reproduction and longevity (Chapman *et al.* 1993; Sgrò & Partridge 1999). To obtain once-mated individuals, single pair matings were set up when flies were 2 days post-eclosion. Mating pairs were identified over several hours, and sexes were separated after mating was complete. Longevity was scored every 2 days (within an hour of the same time every day), when flies remaining in a vial were placed into a fresh vial. Between three and five

replicates for each combination were tested, with a replicate consisting of 10 individuals per vial containing 6 ml of medium.

In the second longevity assay, the effect of the *Wolbachia* infection at 25°C or 30°C in two outcrossed populations (Sydney and Irvine) was assessed. Owing to the extended period between longevity assays, infected flies were re-treated with tetracycline for two generations. The newly cured and infected populations were reciprocally crossed prior to commencement of the assay as described above. Because male sterility in *Drosophila* is induced at high temperatures (David *et al.* 1971), we were unable to rear flies at 30°C; hence, all flies, including those for the 30°C treatment, were reared at 25°C. Newly eclosed virgin flies were collected every 6 h, grouped into replicate vials and placed at the appropriate temperature for subsequent ageing. Again, mortality was scored every 2 days, when the remaining flies were transferred to new vials with fresh medium. For each temperature, there were three to five replicates of each combination of factors (background, infection status and sex), containing 10 individuals. Mating effects were not considered in this second assay because they were not detected in the first assay (see below).

(g) Cytoplasmic incompatibility

CI is expressed when infected males mate with uninfected females. Egg hatch is reduced due to an incompatibility between the sperm and egg. The hatch rate for compatible and incompatible crosses was measured for all three outcrossed backgrounds. The four combinations resulting from crosses between infected (I) and uninfected (U) flies ($U\text{♀} \times U\text{♂}$, $U\text{♀} \times I\text{♂}$, $I\text{♀} \times U\text{♂}$ and $I\text{♀} \times I\text{♂}$) were considered, as well as effects of male age on CI.

Males were tested at 1, 3, 5, 7 and 9 days post-eclosion, or until male stocks were depleted (as occurred in the Sorrell background). Background and incompatibility effects were assessed independently for each age class. For ages 1–7 days, mean hatch rates were calculated from 12 replicates per population-cross combination. For age 9 days, 6–12 replicates were used. For each replicate, a virgin female (3–5 days old) was paired with a virgin male of the required age. Males had been aged in a vial with no more than 15 other males. Pairs were placed onto treacle-based medium with a yeast suspension and allowed to mate for 24 h. Males were removed after 24 h, while the female continued to lay eggs for a further 24 h on new medium. At the end of the 48 h laying period, eggs were allowed 24 h to hatch. The total number of eggs laid and the proportion that hatched were recorded. Replicates with fewer than 10 eggs were not included in analyses of hatch rate.

(h) Maternal inheritance

The efficiency of *Wolbachia* transmission by PCR-positive infected females was assessed for the three outcrossed backgrounds. Males and females were mated in pairs at generation 20 (three pairs per population) and allowed to lay eggs for 7 days. Parents were then frozen at –80°C to check infection status. Between 19 and 25 female progeny from each cross were collected and tested for infection status with the DNA extraction and PCR method described above.

(i) Analysis

All analyses were conducted using SPSS (v. 15). Longevity data were analysed with Kaplan–Meier (KM) log rank tests, to compare rates of mortality of populations and

Figure 1. Mean productivity for all populations for each day. (a) I-102; (b) Irvine; (c) Sorrell; (d) Sydney. White bars represent uninfected populations, while grey bars represent infected populations. Error bars indicate s.e. ± 1 of the mean.

assess effects of host nuclear background, sex and mating status. Mean longevities for each population were also analysed using analysis of variance (ANOVA). Mean longevities were normally distributed based on Kolmogorov–Smirnov tests. For life-history measurements and CI induction at each age class, ANOVAs were carried out to assess the effect of host background and infection status on traits (again, traits were untransformed because they met conditions of normality). The effect of age on CI was independently tested across all backgrounds using a Kruskal–Wallis test. Age and sex were also included as factors in some assays (see below), and viability was included as a covariate when analysing development time, testing for differences in elevation.

3. RESULTS

(a) Productivity and fecundity

For productivity, we initially included I-102 and the three outcrossed backgrounds, but then also re-analysed the results without I-102 because of the low productivity of this background, particularly when flies were infected (figure 1). In both analyses, infection, age and background all affected productivity, and there were also significant interaction terms (table 1). The presence of the infection led to a reduction in productivity in all backgrounds, while productivity in the Sydney background was lower than in the other outcrossed backgrounds (figure 1*b–d*). Productivity initially increased with age, but then decreased again (figure 1). The significant

interaction between background and infection reflected a greater reduction in productivity caused by the infection in the Sorrell and Sydney backgrounds compared to the Irvine background. The interaction between infection and age was due to a relatively larger difference between the infected and uninfected populations at intermediate ages, while the background and age interaction reflected a smaller difference between populations at intermediate ages (figure 1).

In the fecundity assay, the ANOVA indicated significant effects of infection and age, but not background (table 1). The presence of the infection resulted in fewer eggs. Mean eggs per female per day ($\pm$ s.e.) was 33.26 ± 0.67 for uninfected populations and 23.86 ± 0.79 for their infected counterparts.

(b) Development time and viability

The ANOVA indicated a significant effect of infection status on viability (table 2). Uninfected populations had a higher viability ($85.3\% \pm 2.2$) than infected populations ($65.5\% \pm 2.0$). A significant background effect (due to geographical origin, selection and/or drift) was also found, with Sydney having the lowest mean viability ($64.2\% \pm 3.5$), in contrast to Sorrell ($84.3\% \pm 1.9$) and Irvine ($78.1\% \pm 2.6$).

For development time, we included viability as a covariate in the analysis. Females and males were analysed separately because they had different mean development times; however, for both sexes the effects of infection and background were similar. We found that both host

Table 1. ANOVA testing the effects of infection, age and background on productivity and fecundity.

terms	productivity including I-102				productivity excluding I-102				fecundity excluding I-102			
	d.f.	MS	<i>F</i>	<i>p</i>	d.f.	MS	<i>F</i>	<i>p</i>	d.f.	MS	<i>F</i>	<i>p</i>
infection	1	137 959	1313.23	<0.001	1	110 298	892.24	<0.001	1	5176.3	31.79	<0.001
background	3	18 568	176.75	<0.001	2	5779	46.75	<0.001	2	48.8	0.30	0.741
age	8	3575	34.04	<0.001	8	3559	28.79	<0.001	9	684.8	4.21	<0.001
infection	1	1650	15.71	<0.001	2	2257	18.26	<0.001	2	162.2	1	0.370
× background												
age × infection	8	1977	18.82	<0.001	8	1448	11.72	<0.001	9	306.8	1.88	0.052
age × background	24	628	5.98	<0.001	16	362	2.93	<0.001	18	76.7	0.47	0.970
age × infection	24	323	3.08	<0.001	16	298	2.41	0.002	16	105.6	0.65	0.844
× background												
error	360	105	—	—	270	124	—	—	585	162.81	—	—

Table 2. ANOVA testing the effects of background and infection for development time and viability assays.

terms	viability				development time, female				development time, male			
	d.f.	MS	<i>F</i>	<i>p</i>	d.f.	MS	<i>F</i>	<i>p</i>	d.f.	MS	<i>F</i>	<i>p</i>
infection	1	1.15	55.19	<0.001	1	320.99	7.57	0.006	1	617.37	13.57	<0.001
background	2	0.41	19.94	<0.001	2	482.48	11.38	<0.001	2	487.42	10.72	<0.001
infection × background	2	0.03	1.31	0.273	2	663.20	15.64	<0.001	2	937.78	20.62	<0.001
viability	—	—	—	—	1	1506.83	35.53	<0.001	1	1795.75	39.48	<0.001
error	113	0.0207	—	—	906	42.41	—	—	914	45.49	—	—

nuclear background and infection influenced development time (table 2). Irvine flies eclosed earlier than those from the other backgrounds, while *Wolbachia*-infected populations tended to develop faster than their uninfected counterparts (figure 2). An interaction between infection status and background was also observed; this reflected the slower development time of infected populations from Sydney when compared to the uninfected population, but an opposing pattern in the Sorrell background (figure 2). Viability influenced development time; vials with a longer development time tended to come from vials with a higher viability.

(c) Longevity

In the first longevity assay, there was no overall effect of the infection on survival curves according to KM analyses ($\chi^2 = 2.62$, d.f. = 1, $p = 0.105$), and also no effect of background ($\chi^2 = 3$, d.f. = 2, $p = 0.233$). Within backgrounds, however, there were differences between the infected and treated populations (Irvine: $\chi^2 = 4.67$, d.f. = 1, $p = 0.031$; Sydney: $\chi^2 = 3.87$, d.f. = 1, $p = 0.049$; Sorrell: $\chi^2 = 6.62$, d.f. = 1, $p = 0.010$). Infected flies from both Sorrell and Irvine had a reduced longevity compared with uninfected populations, as expected based on published data. However, infected flies from Sydney had an increased longevity relative to Sydney-T (figure 3a–c). No overall effect of mating was observed on the longevity curves for all populations ($\chi^2 = 1.81$, d.f. = 1, $p = 0.178$). However, an overall effect of the infection was seen only in virgin flies ($\chi^2 = 7.07$, d.f. = 1, $p = 0.007$), reflecting a reduction in longevity in infected populations. This effect was not seen in mated flies ($\chi^2 = 0.189$, d.f. = 1, $p = 0.660$). Sex influenced longevity, with males living a shorter time than females in both infected and uninfected treatment

groups ($\chi^2 = 7.52$, d.f. = 1, $p = 0.006$ and $\chi^2 = 17.46$, d.f. = 1, $p < 0.001$, respectively).

Mean longevity for each population was also assessed using ANOVAs. Neither background nor infection status influenced longevity; however, there was an interaction between these two variables (table 3). This reflected the decrease in mean longevity in infected populations from Irvine and Sorrell, compared with an increase in the infected population originating from Sydney (table 4).

In the second longevity assay, there were significant differences between the 25°C and 30°C temperature treatments in both the Sydney and Irvine populations ($\chi^2 = 253.68$, d.f. = 1, $p < 0.001$ and $\chi^2 = 246.74$, d.f. = 1, $p < 0.001$, respectively). The *Wolbachia* infection influenced longevity at 30°C ($\chi^2 = 7.666$, d.f. = 1, $p = 0.006$), but not at 25°C ($\chi^2 = 0.144$, d.f. = 1, $p = 0.704$). The variation at 30°C was due to a reduction in longevity in the infected Sydney background ($\chi^2 = 13.444$, d.f. = 1, $p < 0.001$), as no difference was seen between infected and uninfected populations from Irvine ($\chi^2 = 0.640$, d.f. = 1, $p = 0.431$). No effect of *Wolbachia* was identified in the 25°C treatment in either the Sydney or Irvine populations ($\chi^2 = 0.021$, d.f. = 1, $p = 0.884$ and $\chi^2 = 0.059$, d.f. = 1, $p = 0.807$, respectively).

Mean longevity, assessed by ANOVA, showed a highly significant effect of temperature and sex (table 3). There was also an interaction between infection and temperature. Females lived longer than males (table 4), and the effect of the infection on longevity changed for the Sydney population between 25°C and 30°C (figure 4).

(d) Cytoplasmic incompatibility

The level of CI induced by virgin males aged 1, 3, 5, 7 and 9 days was measured against young virgin females. An overall effect of age was found to influence

Figure 2. Mean number of hours to eclosion from egg laying for each background. Infected populations are represented by grey bars, uninfected populations are shown in white bars. Error bars indicate s.e. ± 1 of the mean.

hatch rate, identified using a Kruskal–Wallis test ($\chi^2 = 118.89$, d.f. = 4, $p < 0.001$). CI induction decreased as male age increased. As expected, the CI-inducing cross ($U\phi \times I\sigma$) had the lowest mean hatch rate, which ranged between 0.27 and 0.35 across all backgrounds. Hatch rate was significantly reduced in incompatible crosses at ages 1–5 days, while at ages 7 days and 9 days there was no difference between the compatible and incompatible crosses (figure 5). No effect of background was apparent at any age (table 5), and no interactions were observed between background and compatibility, except at age 7 days.

(e) Maternal transmission

Transmission of *Wolbachia* from single females (in triplicate) into approximately 20 female progeny of the successive generation was tested for each infected population. Perfect transmission was observed for all females tested. The upper 95 per cent binomial confidence limit for this result was 0.016; the maternal leakage rate was therefore likely to be 0 but certainly less than 0.016.

4. DISCUSSION

The original characterization of the *D. simulans/wMelPop* symbiosis (McGraw *et al.* 2002) was the point of reference for this study. However, comparisons have turned out to be difficult because of the weakness of the original infected I-102 strain. This line was unable to be reared at 25°C, and was therefore not included in the longevity assessment. Consequently, we have been unable to test whether the longevity phenotype is still present in this background, a limitation in testing for long-term evolutionary change. Nevertheless, longevity-shortening effects appear to have attenuated in the outbred backgrounds, which are likely to be used in any releases with *popcorn* for the purposes of disease suppression. The infected individuals from the McGraw *et al.* (2002) study previously had mean longevity values of 20.8 days for females and 18.3 days for males. These values were significantly lower than values for the uninfected controls. In addition, Min & Benzer (1997) found that, in the original *D. melanogaster* host, control lines exhibited

Figure 3. Rates of mortality for each of the three outcrossed backgrounds assessed at 25°C for the first longevity assay. (a) Irvine; (b) Sorrell; (c) Sydney. For each graph, infected flies are represented by black diamonds, and uninfected flies by white squares. All differences between populations were significant to $p < 0.05$.

100 per cent mortality after 28 days. In contrast, the mean longevity values of the infected outcrossed populations in the present study (at the same temperature) were 53.8 and 51.4 days for females and males, respectively (combined across the two assays), as opposed to uninfected population values of 54.2 and 51.5 days.

Even so, the infection still seems to influence longevity, although not always in the predicted direction and in a way that is strongly dependent on nuclear background. The first longevity assay showed that the *popcorn*-infected Sydney population had a greater mean longevity than the uninfected population at 25°C, contrary to expectation. This suggests that the presence of the infection provided a benefit to the host, compared with the situation in the Irvine and Sorrell populations, where the infected populations showed reduced longevity relative to their uninfected counterparts. However, in the second assay at 30°C, *popcorn* negatively influenced the longevity of infected flies from Sydney, suggesting that longevity effects had a strong temperature dependency. Overall, our longevity assays suggest interactions between host, infection and temperature in *D. simulans*. Interactions between host background and *Wolbachia* on longevity have been noted in previous studies; both Reynolds

Table 3. ANOVA testing infection, background, temperature, sex and mating effects for the two longevity assays.

terms	longevity 1				longevity 2			
	d.f.	MS	F	p	d.f.	MS	F	p
infection	1	180.44	1.20	0.274	1	49.90	0.488	0.485
background	2	40.37	0.27	0.765	1	389.91	3.8133	0.051
temperature	—	—	—	—	1	63 670.03	622.684	<0.001
sex	1	2488.57	16.54	<0.001	1	3621.58	35.419	<0.001
infection × background	2	1407.58	9.36	<0.001	1	125.79	1.2302	0.2678
background × temperature	—	—	—	—	1	55.81	0.5458	0.4603
infection × temperature	—	—	—	—	1	529.62	5.1797	0.0232
infection × background × temperature	—	—	—	—	1	7.93	0.0775	0.7808
infection × sex	1	36.66	0.24	0.622	1	4.54	0.0444	0.8332
mating	1	0.59	0	0.950	—	—	—	—
error	1097	150.42	—	—	653	102.25	—	—

Table 4. Summary of the means (and ranges based on replicate means) for each population tested in the two longevity assays. The two treatment temperatures for the second assay are presented separately.

background	infection	longevity 1	longevity 2	
		25°C	25°C	30°C
Sydney	infected	55.9 (52.2, 61.5)	44.4 (32.7, 53.0)	22.2 (19.6, 25.3)
	uninfected	52.7 (24.8, 62.0)	44.5 (33.6, 52.5)	25.4 (20.4, 29.0)
Irvine	infected	52.5 (25.6, 60.2)	43.1 (34.6, 47.1)	22.2 (16.2, 26.8)
	uninfected	56.4 (46.4, 66.1)	40.7 (33.6, 49.2)	23.6 (21.0, 31.2)
Sorrell	infected	53.9 (47.8, 60.1)	not done	not done
	uninfected	56.2 (47.4, 64.2)	not done	not done

et al. (2003) and Carrington *et al.* (2009) found that host nuclear background influenced the longevity of *popcorn*-infected *D. melanogaster* populations. Host-dependent effects of *Wolbachia* have previously been noted in other systems, including the woodlouse *Porcellionides pruinosus* (Michel-Salzat *et al.* 2001) and butterfly *Hypolimnas bolina* (Hornett *et al.* 2006).

The present results also indicate that the *popcorn* infection continues to induce a cost to the reproductive fitness of its *D. simulans* hosts, consistent with McGraw *et al.* (2002). In the productivity assay, infected flies produced a significantly lower number of progeny in all backgrounds and at every age tested. The most severe reduction in productivity within populations was in the original infected I-102 strain. Multiple efforts to rear this strain at 25°C for more than one generation failed, owing to inadequate progeny numbers being produced for the successive generation. However, it was possible to rear this strain at lower temperatures (19 and 16°C). In contrast, I-102-T could be reared at all temperatures, suggesting a negative interaction between the host nuclear background, temperature and infection. For the outbred backgrounds, rearing was possible at a range of temperatures.

One of the most important traits induced by *Wolbachia* in this system is CI, as it is necessary for the intra-population spread of the bacterium. We found that CI induced in *D. simulans* remained strong, but was age-dependent, with CI effects diminishing within 7 days. This decrease in CI with increasing male age is consistent with results obtained with the *popcorn* infection in *D. melanogaster* (Reynolds *et al.* 2003), where the hatch

rate of incompatible crosses is indistinguishable from that of compatible crosses after 7 days. Age effects on CI have also been observed for *wRi* in *D. simulans* (Hoffmann *et al.* 1986, 1990; Weeks *et al.* 2007) and *wMel* in *D. melanogaster* (Reynolds & Hoffmann 2002). It remains to be seen what levels of CI might be present in the field as opposed to laboratory populations for *wMelPop*. However, under field conditions, CI is typically weaker than in the laboratory, at least in *wRi* (Hoffmann *et al.* 1990; Turelli & Hoffmann 1995) and *wMel* (Hoffmann *et al.* 1998). Therefore, the CI induction of *wMelPop* in the field will dictate the likelihood of *popcorn* spreading in natural populations when coupled with fitness effects and maternal transmission efficiency (Turelli 1994). Maternal transmission does appear to be high and perhaps perfect in the laboratory, although again lower rates of transmission can be found in the field, at least in *wRi* (Turelli & Hoffmann 1995).

While there are several issues that this laboratory-based study has identified as potentially affecting the success of a control strategy based around longevity reduction by *Wolbachia*, attenuation in field populations may follow a different trajectory. Conditions in the field will differ from those in the laboratory owing to variable turnover, non-discrete generations and fluctuating environmental conditions. Further, the phenotypic changes seen in the *wMelPop* infection in *D. simulans* may also differ from those in infected mosquitoes like *A. aegypti*. Tissue tropism and density of the bacteria may differ between host species, and assessing the *wMelPop* infection in *D. simulans* may not necessarily reflect effects in an alternate host. For instance, the

Figure 4. Rates of mortality for (a) Sydney 30°C, (b) Irvine 30°C, (c) Sydney 25°C and (d) Irvine 25°C treatments in the second longevity assay. Infected flies are represented by black diamonds, and uninfected populations with white circles.

Figure 5. Mean hatch rate of eggs produced during matings of 3-day old females with males of different ages. Grey bars represent incompatible crosses, white bars compatible crosses. Error bars show s.e. ± 1 of the mean.

dynamics of *wMelPop* will differ between flies and *A. aegypti*, given the evidence of perfect maternal transmission and complete CI not influenced by male age in *A. aegypti* (McMeniman *et al.* 2009), in contrast to *D. simulans*. This may reflect bacterial density and tropism effects; it has recently been shown that higher bacterial density increases the penetrance of a male-killing *Wolbachia* strain in *D. innubila* (Unckless *et al.* 2009). Finally, even if attenuation of the longevity phenotype occurs, it may not influence the effectiveness of a control programme. Given that it has taken around 200 generations for this attenuation to occur, transmission of dengue will probably be eradicated before any attenuation

if all other traits associated with the infection are highly penetrant (CI to spread the *Wolbachia* into the population, maternal transmission to ensure a high infection frequency and sufficient longevity reduction).

Although the fate of the *popcorn* infection in natural *D. simulans* populations is likely to depend on the genetic background of the host, our experiments had limitations in predicting the precise trajectories on particular backgrounds. Background effects were noted not only for longevity but also for development time, with Sydney and Sorrell populations showing large differences between infected and treated populations in opposing directions, while there was no difference between the population from Irvine. It is possible that segregating genetic polymorphisms and drift between the generation of the infected/uninfected lines and the experiments (four generations) could have influenced the results, even though we performed a reciprocal cross immediately prior to the experiments to obviate these effects. Prediction about host phenotypic effects or the population dynamics of the infection in a particular background will therefore depend on thorough testing of *popcorn* in replicated populations with the target background of the host.

In conclusion, we have shown that the *wMelPop* infection is maintained successfully within *D. simulans* populations, and also continues to reduce longevity in different host backgrounds. However, host nuclear background influences the *Wolbachia*-induced phenotypes including longevity and fitness costs/benefits associated with the infection. Phenotypic effects may depend on temperature, as evident for the longevity phenotype in the Sydney background. This makes it pertinent to characterize *Wolbachia* effects in target backgrounds when developing models for its spread. Attenuation of

Table 5. ANOVAs testing background and compatibility (infection) effects for each of the five ages for which CI was assessed.

age (days)	background				compatibility				background × compatibility				error	
	d.f.	MS	<i>F</i>	<i>p</i>	d.f.	MS	<i>F</i>	<i>p</i>	d.f.	MS	<i>F</i>	<i>p</i>	d.f.	MS
1	2	0.01	0.26	0.773	1	5.86	111.2	<0.001	2	0.04	0.72	0.491	132	0.053
3	2	0.06	1.29	0.279	1	3.56	79.4	<0.001	2	0.07	1.51	0.225	132	0.045
5	2	0.17	2.02	0.136	1	2.94	35.35	<0.001	2	0.10	1.15	0.321	135	0.083
7	2	0.03	0.25	0.778	1	0.03	0.26	0.608	2	0.27	2.67	0.073	132	0.102
9	2	0.22	2.04	0.137	1	0.05	0.42	0.518	1	0	0	0.955	85	0.109

popcorn's longevity phenotype at 25°C appears to have occurred over the past few years, emphasizing the need for ongoing monitoring of any phenotypic effects. At present, it is not known if these issues will present problems for controlling dengue transmission in *A. aegypti* mosquitoes. The effect of the infection on longevity of the mosquitoes appears strong (McMeniman *et al.* 2009), and this also applies when the infection is tested in outbred backgrounds (K. Richardson 2009, personal communication). Nevertheless, attenuation might occur in this system through nuclear changes (Carrington *et al.* 2009) and interaction effects. This suggests the need for detailed investigations into target populations and their responses to the bacterial infection if long-term predictions are to be developed.

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