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ORIGINAL ARTICLE

Loss of reproductive parasitism following transfer of male-killing *Wolbachia* to *Drosophila melanogaster* and *Drosophila simulans*

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Wolbachia manipulate insect host biology through a variety of means that result in increased production of infected females, enhancing its own transmission. A *Wolbachia* strain (*w*Inn) naturally infecting *Drosophila innubila* induces male killing, while native strains of *D. melanogaster* and *D. simulans* usually induce cytoplasmic incompatibility (CI). In this study, we transferred *w*Inn to *D. melanogaster* and *D. simulans* by embryonic microinjection, expecting conservation of the male-killing phenotype to the novel hosts, which are more suitable for genetic analysis. In contrast to our expectations, there was no effect on offspring sex ratio. Furthermore, no CI was observed in the transinfected flies. Overall, transinfected *D. melanogaster* lines displayed lower transmission rate and lower densities of *Wolbachia* than transinfected *D. simulans* lines, in which established infections were transmitted with near-perfect fidelity. In *D. simulans*, strain *w*Inn had no effect on fecundity and egg-to-adult development. Surprisingly, one of the two transinfected lines tested showed increased longevity. We discuss our results in the context of host-symbiont co-evolution and the potential of symbionts to invade novel host species.
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INTRODUCTION

The occurrence of *Wolbachia*, a group of maternally transmitted endosymbionts, in two-thirds of all species of insects renders them perhaps the most diverse group ever to inhabit the earth (Hilgenboecker *et al.*, 2008). Equally striking is the almost complete lack of phylogenetic congruence between insect host species and the *Wolbachia* lineages that infect them (for example, Werren *et al.*, 1995), indicating that colonization of the world's insect species by *Wolbachia* has occurred largely by lateral transmission from one species to another. The lack of phylogenetic congruence also indicates that these infections are short-lived on a macroevolutionary time scale (Werren *et al.*, 1995; Werren *et al.*, 2008).

Wolbachia spread within host species by increasing the relative fitness of infected cytoplasmic lineages, either by conferring direct fitness benefits (Vavre *et al.*, 1999) or by manipulating host reproduction via cytoplasmic incompatibility (CI), male-killing, feminization of genetic males or parthenogenesis (thelytoky) (Werren *et al.*, 2008; Saridaki and Bourtzis, 2010). The fitness advantage conferred to infected cytoplasmic lineages and the fidelity of maternal transmission jointly determine the dynamics of infection within a host species, including the tendency to increase following *Wolbachia*'s introduction to a new host and the eventual equilibrium prevalence of infection. Thus, the global association between insects and *Wolbachia* is continually reconfigured by the processes of lateral transmission between species and by the phenotypic effects and maternal transmission fidelity of *Wolbachia* within infected host species.

The development of a macroevolutionary theory of insect–*Wolbachia* associations requires understanding how the phenotypic effects and transmission fidelity of *Wolbachia* depend on host species or genotype, *Wolbachia* strain, environmental conditions and interactions among these factors. The fate of novel infections will be more predictable if the phenotypic effect of *Wolbachia* depends solely on host species or *Wolbachia* strain, rather than on idiosyncratic interactions between these factors. For instance, the 'popcorn' strain of *Wolbachia* causes CI and reduces adult lifespan both in its native host, *Drosophila melanogaster*, and in a very distantly related host, *Aedes aegypti*, to which it has been experimentally transferred (McMeniman *et al.*, 2009). In other cases, however, *Wolbachia* fail to express the original phenotype and sometimes express entirely novel phenotypes (for example, Grenier *et al.*, 1998; van Meer and Stouthamer, 1999; Sasaki *et al.*, 2002; Sasaki *et al.*, 2005; Jaenike, 2007). From an applied standpoint, predictability of *Wolbachia* phenotypic effects is highly desirable in programs using these

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endosymbionts for control or genetic manipulation of insect populations (Cook *et al.*, 2008). Given the growing interest in using *Wolbachia* for such purposes, it is clearly important to develop an understanding of the degree to which the phenotypic effects of a given *Wolbachia* strain are conserved across host species.

One way to distinguish the effects of *Wolbachia* strain from those of the host species involves transfer of a *Wolbachia* strain that has a particular phenotypic effect (for example, male-killing) in its native host to a novel host whose native *Wolbachia* has a different phenotypic effect (for example, CI). If the *Wolbachia* expresses the same phenotype in both hosts, this would indicate that the *Wolbachia* strain determines the phenotype. In contrast, if the recipient host species expresses the same phenotype with both its native and the introduced *Wolbachia* strains, this would indicate that *Wolbachia* phenotype is governed by the host species. Finally, if a novel (or no) phenotype is expressed, this reveals the importance of host species by *Wolbachia* strain interactions.

In the present study, we transferred *Wolbachia* from *D. innubila* to both *D. melanogaster* and *D. simulans*. *D. innubila* is a member of the quinaria group within the subgenus Drosophila, whereas *D. melanogaster* and *D. simulans* belong to the melanogaster group within the subgenus Sophophora. These two subgenera are thought to have split ~60 mya (Tamura *et al.*, 2004). *D. innubila* is naturally infected with a strain of *Wolbachia* (*w*Inn) that experiences nearly perfect maternal transmission and causes ~100% mortality of infected male embryos (Dyer and Jaenike, 2004). *D. melanogaster* and *D. simulans* both harbor natural *Wolbachia* infections that cause CI (Hoffmann *et al.*, 1986; Hoffmann and Turelli, 1988; O'Neill and Karr, 1990; Bourtzis *et al.*, 1996; Zabalou *et al.*, 2008). The expression of CI is particularly strong in *D. simulans*. Besides testing the relative roles of host species and *Wolbachia* strain on the expressed phenotype, the transfer of *Wolbachia* into *D. melanogaster* could allow in-depth genetic and developmental analyses of *Wolbachia*–host interactions, specifically, in this case, the mechanism by which *Wolbachia* brings about embryonic male killing.

A male-killing strain of *Wolbachia* very closely related to *w*Inn has been discovered in *D. borealis* (Sheeley and McAllister, 2009). *D. innubila* and *D. borealis* belong, respectively, to the quinaria and virilis groups of the subgenus Drosophila, which split over 30 million years ago (O'Grady and DeSalle, 2008; Tamura *et al.*, 2004). Thus, to cause male killing in these distantly related flies, the *Wolbachia* in *D. innubila* and *D. borealis* may target a highly conserved developmental mechanism in early embryonic development. If such a mechanism was conserved across the genus *Drosophila*, then male killing by these *Wolbachia* might be expressed in species of the melanogaster group.

The *w*Inn strain found in *D. innubila* is notable in another respect, as it belongs to the ST-13 strain complex of *Wolbachia* (Baldo *et al.*, 2006). Recent surveys have found that this strain complex has been extraordinarily successful in the recent colonization of innumerable species of Diptera, including *D. simulans* and *D. melanogaster* (Baldo *et al.*, 2006; Stahlhut *et al.*, 2010). This strain complex is also remarkable in the variety of reproductive phenotypes it can cause, including male killing (in *D. innubila*), parthenogenesis (in *Muscidifurax uniraptor*) and CI (for example, *D. simulans, D. melanogaster* and *Nasonia longicornis*). Thus, the experimental transfer of *Wolbachia* strain *w*Inn to novel host species may shed light on the mechanisms responsible for the success of this strain complex and the lability of its reproductive phenotypes.

Our results indicate that in the novel hosts *D. simulans* and *D. melanogaster* *w*Inn does not express the phenotype manifest in its native host (male killing) nor the phenotype expressed by the *Wolbachia* normally resident in *D. simulans* and *D. melanogaster* (CI). In fact, we found no reproductive manipulation at all. We did find that these novel infections are either benign or perhaps slightly beneficial in the new hosts. These findings have potentially important implications for the evolution of insect—*Wolbachia* associations.

MATERIALS AND METHODS

Establishment of the transinfected lines

Microinjections were carried out as previously reported (Zabalou *et al.*, 2004). Cytoplasmic donor was *D. innubila* infected with the *w*Inn male-killing *Wolbachia* strain (Dyer and Jaenike, 2004). Recipient lines were uninfected lines iso31 for *D. melanogaster* and STCP for *D. simulans* that have nearly homozygous genetic backgrounds and have been used repeatedly in our laboratory for *Wolbachia* transfers (Zabalou *et al.*, 2008).

Presence of *Wolbachia* in transinfected flies was checked every generation by STE/boiling method for DNA extraction and subsequent PCR using primers for 16S recombinant DNA or *wsp* genes (O'Neill *et al.*, 1992; Zabalou *et al.*, 2008). The *Wolbachia* strain(s) of the donor and of the transinfected Drosophila hosts were genotyped using a Multi Locus Sequence Typing system developed in Paraskevopoulos *et al.* (2006).

Maternal transmission, sex ratio and CI assays

For each generation after injection, females were tested for *Wolbachia* infection and isofemale lines were set up accordingly. We measured the transmission efficiency by determining the proportion of their offspring that was infected. We simultaneously determined the offspring sex ratio and tested for CI several generations after injection, using previously described methods (Zabalou *et al.*, 2008). In brief, young virgin females and males were used in these experiments, mating was confirmed by visual inspection, eggs were collected for three consecutive days and egg hatch rates were then determined. The statistical analysis for the sex ratio was based on *t*-test comparisons of the proportion of females in the offspring of each infected female to the expected 50% proportion (one-sample *t*-test). These comparisons were performed separately for each female type. This kind of analysis was selected because it takes into account the sex ratio variability among infected flies. The statistical analysis for the CI was based on one-way analysis of variance (ANOVA) comparing the embryonic mortality among crosses. Groups of similar crosses were determined by Tukey's Multiple Comparisons Test.

Fitness measurements

Female fecundity, egg-to-adult viability and adult longevity of *Wolbachia*-infected *D. simulans* were measured 1 year after establishment of infection in the laboratory. Fecundity was scored for three consecutive days at three different periods during females' life span: early (days 1–3), middle (days 11–13) and late (days 21–23). For egg-to-adult viability, 15 eggs deposited through the 'early period' by these same females were added to food vials and the number of emerging adults was subsequently counted. Longevity of the flies was assessed by holding groups of 6 to 13 females and 6 to 13 males together in food vials, with 10 to 13 replicate vials per line, and monitoring the number of individuals that died every 5 days. The longevity experiment was repeated twice, at post-infection generation 45 in 2007 and generation 95 in 2009. The longevity data were analyzed with the Kaplan–Meier survival analysis, while the Mantel–Cox (log-rank) test was used for comparisons among vials (Kleinbaum and Klein, 2005). The latter analysis indicated that there were significant among-vial effects on longevity for both sexes of all three strains in both generations. Consequently, for each vial, we used median survival time (that is, when 50% of the flies were dead) of males and females as two data points. We then carried out two-way ANOVAs of these vial-level median survival times for each generation, searching for effects of line (including *Wolbachia* infection status) and sex.

Immunofluorescence

Eggs, testes and ovaries of *Wolbachia*-infected *D. melanogaster* and *D. simulans* were processed and stained with the anti-WSP antibody and propidium iodide with standard immunofluorescence techniques (Veneti *et al.*, 2004). For *D. innubila*, fixation and staining procedures were based on Ferree *et al.*

(2005). After dissection, ovaries were fixed and devitelinized in a 1 : 3 mixture of 4% paraformaldehyde in phosphate-buffered saline with 0.5% NP40 detergent and heptane on agitation for 20 min. The samples were then washed three times for 10 min in PBS-T (0.1% Triton) before overnight treatment with 10 mg ml^{-1} solution of RNase A. After washing 4–5 times over 2 h, samples were incubated in Alexa 488-conjugated phalloidin for 2 h and then washed a further 4–5 times over 2 h before mounting in propidium iodide-containing mounting media (10 μg ml^{-1} propidium iodide in solution of 70% glycerol in PBS). Samples were observed on a Leica SP5 inverted confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) using a ×63 oil objective.

RESULTS

Establishment of the transinfected lines

A total of 1840 eggs of the uninfected *D. melanogaster* iso31 line were injected with cytoplasm from *D. innubila* infected with *w*Inn. Out of 46 fertile females recovered, 16 gave positive PCR signals for infection. Seven of them were used to set up several isofemale lines and three of them transmitted the infection from G0 to the next generation (F1) with the following transmission rates: line 23 (28.5%; 4/14 females), line 35 (10%; 1/10 females) and line 46 (87.5%; 7/8 females). Line 23 lost the infection by generation 11, while the two other infected lines were maintained in the laboratory by selection until about generation 40.

For *D. simulans* 1600 microinjections were performed, and 10 out of 50 female flies were found to be fertile and positive for the infection. F1 females were used to establish 10 isofemale lines for each initially infected female. Transmission rates for the infection to the next generation (F1) were as follows: line 4 (100%), line 22 (0%), line 24 (40%), line 29 (30%), line 30 (60%), line 42 (0%), line 43 (0%), line 45 (50%), line 46 (0%) and line 48 (0%). From each infected isofemale line, several new lines were started and tested for infection. All isofemale sublines within lines 4, 24, 30 or 45 were positive for infection, while all of the isofemale sublines from line 29 were negative. No further loss of infection was found for lines 4, 24, 30 or 45 in subsequent generations. Multi Locus Sequence Typing analysis clearly indicated that all transinfected lines were infected with the *w*Inn *Wolbachia* strain that naturally infects *D. innubila*.

Maternal transmission, sex ratio and CI assays

The average percentage of *Wolbachia* transmission to the next generation in the transinfected *D. melanogaster* line is shown in Table 1. PCR tests were carried out for line 23 from generations 2 through 11, at which point the infection was lost, while for lines 35 and 46 the assays were performed until the thirtieth generation. These assays revealed variable levels of maternal transmission with no evidence of fixation (prefect transmission). In *D. simulans*, the maternal transmission fidelity reached 100% two generations after injection and remained at 100% subsequently, as mentioned above.

The sex ratio was not significantly distorted from 1:1 in any of the generations or lines tested, indicating that *Wolbachia* strain *w*Inn does not cause male killing in either *D. melanogaster* or *D. simulans* (Tables 2 and 3).

Several generations after injection, transinfected lines were tested for CI expression. For *D. melanogaster*, ANOVA comparison of all crosses was significant ($F = 2.23$, degree of freedom (d.f.) = 9, 196, $P = 0.021$) owing to the difference observed in the comparison of the cross (Infected46 × Infected46) with that of [uninfected × uninfected] (Table 4). For *D. simulans*, ANOVA comparison of all crosses was also significant ($F = 2.98$, d.f. = 12, 200, $P < 0.01$) owing to the differences observed in the comparison of the crosses (uninfected × infected4), (infected4 × infected4) and (infected24 × infected24) with that of (uninfected × infected45) (Table 5). These results show that none of seven transinfected lines expressed elevated mortality in crosses between uninfected females and infected males, indicating that *Wolbachia* strain *w*Inn does not cause detectable levels of CI in either *D. melanogaster* or *D. simulans*.

Table 1 Transmission rate of *D. melanogaster* transinfected lines

	Offspring		
Female type	*Tested*	*Infected*	*Percentage infected (%)*
Infected23	317	254	80.1
Infected35	425	304	71.5
Infected46	476	333	70.0
Total	1218	891	73.2

Table 2 Sex ratio of *D. melanogaster* transinfected flies

		Progeny	
Female type	N	*Females (%), mean (s.d.)*	t-*test*, P-*values*
Infected23	46	50.27 (7.9)	0.280, NS
Infected35	160	50.42 (7.9)	0.506, NS
Infected46	156	50.31 (6.2)	0.529, NS

The sex ratio was determined in every generation from generation fourth to eleventh for line 23 and from generation fourth to thirtieth for lines 35 and 46.
N, number of infected females whose offspring sex ratios were determined.
Females (%), number of female offspring/total number of offspring.
t-test, one-sample *t*-test comparison with the assumed 50% proportion of females.
NS, not significant.

Table 3 Sex ratio of *D. simulans* transinfected flies

		Progeny	
Female type	N	*Females (%), mean (s.d.)*	t-*test*, P-*values*
Infected4	25	48.2 (9.6)	0.351, NS
Infected24	25	48.6 (6.7)	0.313, NS
Infected30	25	49.8 (6.9)	0.861, NS
infected45	25	49.3 (5.5)	0.518, NS

The sex ratio was determined in every generation from generation tenth to thirteenth for all four lines.
N, number of infected females whose offspring sex ratios were determined.
Females (%), number of female offspring/total number of offspring.
t-test = one-sample *t*-test comparison with the assumed 50% proportion of females.
NS, not significant.

Fitness effects of *Wolbachia* in transinfected *D. simulans*

No major consistent difference in female fecundity was observed at any age between transinfected and control (uninfected) STCP lines (ANOVA and Tukey test: early $F = 0.752$, $P = 0.476$; middle $F = 3.672$, $P = 0.034$; and late $F = 0.128$, $P = 0.880$). A difference was found between infected lines 24 and 30 for the middle period, but the rebound in fecundity of older line 30 flies suggests that their low fecundity in the middle period may have been anomalous (Table 6). No significant effect of infection upon egg-to-adult viability was found in the two transinfected lines tested (ANOVA, $F = 0.618$, $P = 0.54$; Table 7).

Kaplan–Meier survival analysis performed separately for sex, line and generation revealed significant differences among vials in all

Table 4 Egg mortality of *D. melanogaster* transinfected flies

Cross (female × male)	*Eggs*	*Number of crosses*	*% Mortality*	*Tukey groups*
Uninfected × infected23	1562	24	9.64 ± 1.2	ab
Uninfected × infected35	2160	27	10.59 ± 1.0	ab
Uninfected × infected46	2132	26	10.27 ± 2.0	ab
Infected23 × uninfected	761	9	11.94 ± 2.5	ab
Infected35 × uninfected	1350	16	11.38 ± 2.8	ab
Infected46 × uninfected	1139	13	13.45 ± 2.9	ab
Infected23 × infected23	1958	21	12.10 ± 1.3	ab
Infected35 × infected35	1629	21	15.46 ± 3.9	ab
Infected46 × infected46	1928	24	17.99 ± 2.5	b
Uninfected × uninfected	2011	25	7.26 ± 1.2	a

Tukey groups, different letters correspond to statistically significant differences at 5%.
The cytoplasmic incompatibility tests were performed in generation third, fifth, tenth and eleventh.

Table 5 Egg mortality of *D. simulans* transinfected flies

Cross (female × male)	*Eggs*	*Number of crosses*	*% Mortality*	*Tukey groups*
Uninfected × infected4	875	16	1.62 ± 0.6	a
Uninfected × infected24	881	16	3.77 ± 0.9	ab
Uninfected × infected30	1551	18	5.25 ± 1.2	ab
Uninfected × infected45	1319	17	6.22 ± 1.9	b
Infected4 × uninfected	1000	16	1.84 ± 0.4	ab
Infected24 × uninfected	1349	21	2.40 ± 0.6	ab
Infected30 × uninfected	827	13	4.00 ± 1.4	ab
Infected45 × uninfected	1173	18	3.65 ± 1.1	ab
Infected4 × infected4	1214	18	1.35 ± 0.3	a
Infected24 × infected24	1047	15	1.02 ± 0.2	a
Infected30 × infected30	1234	14	1.86 ± 0.5	ab
Infected45 × infected45	1162	17	4.49 ± 0.8	ab
Uninfected × uninfected	1313	14	2.77 ± 0.8	ab

Tukey groups, different letters correspond to statistically significant differences at 5%.
The cytoplasmic incompatibility tests were performed in generations eleventh to fourteenth.

Table 6 Fecundity of *D. simulans* transinfected flies

	Line		
Female age	*Infected24*	*Infected30*	*STCP (uninfected)*
Early	156 ± 34.69 (21)	160.87 ± 23 (23)	169.16 ± 40.42 (16)
Middle	91.57 ± 25.36 (14)	53.94 ± 47.51 (17)	71.58 ± 34.71 (12)
Late	95.63 ± 53.56 (8)	83.88 ± 52.3 (8)	70.13 ± 43.09 (8)

Entries are the mean number of eggs laid ± s.d. (sample size) over a 3-day period. The fecundity was determined in generation fourtieth for both lines.

possible combinations (Log-rank test—$P<0.05$, Supplementary Table 1). The median survival time for flies in each vial (days) was determined. These medians were subjected into two-way ANOVA using sex and line as fixed factors for both experiments in generation 45 (in 2007) and 95 (in 2009). In both cases, the interaction between sex and line was not significant ($P=0.09$ for 2007 and $P=0.31$ for generation 95). On the other hand, a highly significant difference was found among the lines for both generations ($F=6.23$, d.f. = 2, 71, $P=0.003$ for generation 45 and $F=6.30$, d.f. = 2, 59, $P=0.003$ for generation 95). *Post-hoc* Tukey test revealed that the *D. simulans* transinfected line 24 exhibited longer median adult survival than the control line in the two independent experiments performed in generations 45 and 90 (Table 8). In addition, males exhibited longer adult survival times than females for all lines. In generation 45, this difference is highly significant ($F=23.58$, d.f. = 1, 71, and $P<0.001$), while in generation 95, the difference is marginal ($F=4.05$, d.f. = 1, 59, and $P=0.049$).

Table 7 Egg-to-adult viability of *D. simulans* transinfected flies

Line	*Infected24*	*Infected30*	*STCP (uninfected)*
	11.9 ± 2.91 (21)	12.39 ± 2.44 (23)	12.88 ± 2.53 (16)

Entries are the mean number of adults emerged from 15 eggs deposited by each of the original flies used for fecundity measures ± s.d. (number of laying females). The egg-to-adult viability was determined in generation fourtieth for both lines.

Table 8 Survival analysis of *D. simulans* transinfected flies

Line	N	*Survival days (mean (s.d.))*	*Tukey groups*
Generation 45			
STCP	26	29.23 (12.06)	a
Insim30	22	31.59 (6.97)	a
Insim24	24	37.92 (11.32)	b
Generation 95			
STCP	26	44.50 (8.26)	ab
Insim30	22	39.75 (6.68)	a
Insim24	24	48.00 (7.68)	b

N, total number of vials per line (males and females).
Tukey, different letters correspond to statistically significant differences at 5%.

Wolbachia density

Immunofluorescence data of transinfected eggs, testes and ovaries overall suggested the presence of lower levels of bacteria in *D. melanogaster* than *D. simulans* (Figure 1). Bacteria were evenly distributed throughout the embryos of both *D. melanogaster* and *D. simulans* transinfected flies with considerable intra-line variation in density and *D. simulans* having, on average, 10 times more bacteria. The even distribution of *Wolbachia* throughout the embryos of both species suggested that the bacteria may not exhibit a special affinity for the germ plasm at this stage.

Testes of transinfected *D. melanogaster* flies contained very few bacteria of somatic origin and no infected cysts, while *D. simulans* testes showed a few heavily infected cysts. Finally, *Wolbachia* were abundant in the ovaries, especially in the early stages of oogenesis of both *D. melanogaster* and *D. simulans* transinfected flies.

DISCUSSION

The first requirement for successful colonization of a new host species is that, following a lateral transfer event, *Wolbachia* must be transmitted from infected mothers to their offspring. More specifically, the relative selective advantage of infection to a cytoplasmic lineage (s) must, to a first approximation, exceed the proportion of uninfected offspring produced by an infected female (u). We observed only fair transmission of *w*Inn within *D. melanogaster*, averaging only 73.2% (Table 1). To spread within *D. melanogaster*, the *Wolbachia* would have to confer a fitness advantage sufficient to overcome this imperfect transmission, that is, on the order of 30%. This is much

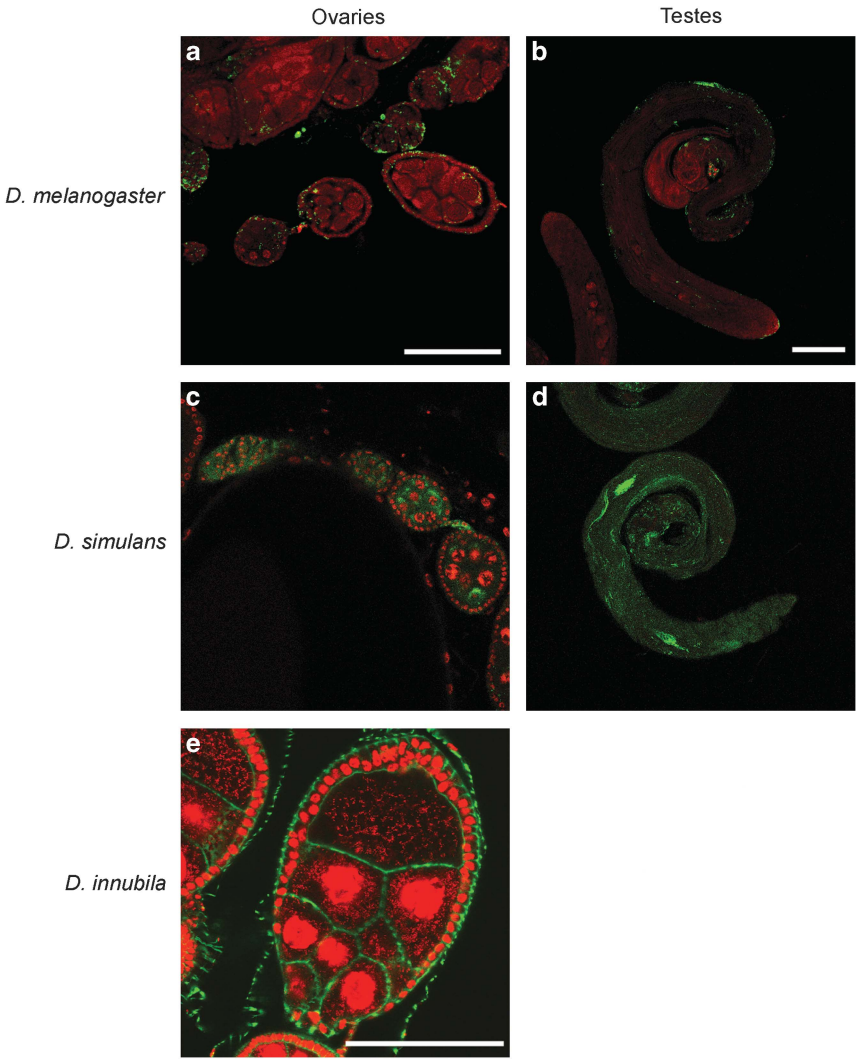

Figure 1 Representative ovaries and testes of adult transinfected *D. melanogaster* (**a** and **b**) and transinfected *D. simulans* (**c** and **d**), respectively. *Wolbachia* are stained green and nuclei red (propidium iodide). Representative *D. innubila* egg chamber (**e**), the red stain is propidium iodide (which stains host and *Wolbachia* DNA), and the green is phalloidin (which stains F actin). Scale bars are 100 μm.

greater than the 4–5% selective advantage that has been inferred in the native host, *D. innubila* (Dyer and Jaenike, 2004).

In contrast, the *w*Inn infection was either quickly lost or quickly established with essentially perfect transmission in *D. simulans*. Such quick establishment indicates that the selective advantage required for spread would not have to overcome an initially low rate of maternal transmission, and thus that a relatively minor selective benefit would suffice for invasion. *D. simulans* may well be particularly conducive to *Wolbachia* transmission, as it has been successfully colonized at least four times by different *Wolbachia* strains in nature (Ballard, 2004). In *D. innubila*, *w*Inn cells are dispersed within oocytes, rather than being localized at the posterior pole, as they are in *D. simulans*. This indicates that *Wolbachia* strain *w*Inn can experience very high rates of maternal transmission, even when their patterns of localization are very different, as they are in *D. innubila* and *D. simulans*.

Studies of *Wolbachia* localization in late stage (10–14) oocytes have revealed strain-specific differences in localization patterns (Serbus and Sullivan, 2007). Specifically, the *w*Mel strain was shown to localize to the posterior cortex in its native host and after transfer to *D. simulans*. In contrast, the *w*Ri strain native to *D. simulans* does not exhibit localization to the posterior cortex in its native host. It is plausible that cortical localization is a feature important for efficient transmission of *Wolbachia* in *D. melanogaster*, owing to some unknown feature of the biology of this species. As *w*Inn does not localize to the cortex of stage 10–14 oocytes in *D. innubila*, the low transmission efficiency of *wInn* in *D. melanogaster* relative to *D. simulans* could be linked to the absence of cortical localization.

Although *w*Inn experiences moderate to high transmission in both *D. melanogaster* and *D. simulans*, this *Wolbachia* strain did not cause male killing in either of these novel host species (Tables 2 and 3). Given that *w*Inn causes nearly 100% male killing in its native host, *D. innubila*, it is clear that the expression of male killing is highly dependent on host genetic background. The nearly equal offspring sex ratios in *D. melanogaster* and *D. simulans* also indicate that these *Wolbachia* do not cause parthenogenesis or feminization in either of these species.

We do not know why *w*Inn does not cause embryonic male killing in the melanogaster group, nor why it does cause male killing in *D. innubila*. *Wolbachia* may target the dosage compensation system of

D. innubila, as male-killing *Spiroplasma* have been shown to do in *D. melanogaster* (Veneti *et al.*, 2005). As *Drosophila* males are XY, failure to upregulate transcription of their single X chromosome results in male lethality. Thus, interfering with the dosage compensation complex (DDC), which is responsible for such X chromosome upregulation in males, could bring about embryonic male killing. It is therefore notable that most or all of the genes encoding the proteins of the DDC have undergone rapid, positive selection in the melanogaster group (Levine *et al.*, 2007; Rodriguez *et al.*, 2007). Therefore, if *w*Inn targets a certain component of the DDC of *D. innubila*, it is possible that this component is sufficiently different in *D. melanogaster* and *D. simulans* to be unrecognizable. In fact, Rodriguez *et al.* (2007) postulate that rapid evolution of DDC genes in *D. melanogaster* may result from an arms race with the male-killing *Spiroplasma* that targets that complex. Thus, even though *D. melanogaster* is currently susceptible to *Spiroplasma*-induced male killing, this arms race may have rendered melanogaster group species resistant to the male-killing effects of *Wolbachia* strain *w*Inn.

Alternatively, perhaps male-killing *Wolbachia* attack males even earlier in the process of sexual differentiation although it should be noted that male killing does not necessarily target early processes, as shown in *Hypolimnas bolina* (Charlat *et al.*, 2007). Although the basic molecular mechanisms involved in sex determination are conserved across the genus *Drosophila*, there are some variations on the basic theme (Marín and Baker, 1998). The master gene at the top of the regulatory hierarchy, *Sxl*, is expressed only in females in species of the subgenus Sophophora (including *D. melanogaster* and *D. simulans*), whereas it is expressed in both males and females in species of the virilis group, including *D. borealis*, which belongs to the subgenus Drosophila (Bopp *et al.*, 1996). Intriguingly, the male-specific Sxl protein in the virilis group is somewhat smaller than the female-specific protein. Thus, *D. borealis* (and perhaps *D. innubila*) produce a male-specific protein in the sex determination pathway that is not produced by species of the melanogaster group. Perhaps *w*Inn and the closely related *Wolbachia* strain in *D. borealis* target this protein or something downstream to cause male killing. In any case, our study demonstrates that male killing, although perhaps less complex mechanistically than CI, is not a default phenotype for *Wolbachia* strain *w*Inn. Instead, the phenotypic expression of male killing by *w*Inn exhibits a higher level of host specificity than the potential host range of this strain.

We also found no evidence that *w*Inn causes CI in either *D. simulans* or *D. melanogaster* (Tables 4 and 5). The lack of CI in *D. melanogaster* may result from the failure of strain *w*Inn to colonize the testes of this species (Figure 1), presumably a necessary requirement for *Wolbachia*-mediated modification of sperm that underlies the expression of CI (Veneti *et al.*, 2003). The lack of CI in *D. simulans* is especially noteworthy, as several other strains of *Wolbachia*, including those that naturally infect this species, as well as some transinfected into *D. simulans* (Braig *et al.*, 1994; Poinsot *et al.*, 1998), do cause CI (all but wAu; (Hoffmann *et al.*, 1996)). Thus, although *D. simulans* appears to be particularly susceptible to CI, strain *w*Inn is unable to cause such effects in this species, even though these *Wolbachia* clearly colonize the testes (Figure 1). That strain *w*Inn does colonize and proliferate within the testes of *D. simulans* is particularly interesting. As *w*Inn is a male killer in *D. innubila*, this strain of *Wolbachia* very rarely occurs in males in its native host. Furthermore, because this strain has probably been a male killer for at least 15 000 years (Jaenike and Dyer, 2008), this suggests a very slow decay in the ability of *Wolbachia* to colonize the testes of host *Drosophila*.

Thus, the results of our experiments indicate that the strain of *Wolbachia* that causes male killing in *D. innubila* exhibits no reproductive manipulation in either *D. simulans* or *D. melanogaster*. In contrast, it is interesting to note that the male-killing strain of *Wolbachia* that infects the butterfly *H. bolina* expresses CI upon the evolution of resistance to male killing in the butterflies (Hornett *et al.*, 2006). Whether this occurs within *D. innubila* is not known, as this species does not appear to have evolved any resistance to male killing (Jaenike and Dyer, 2008).

Our assays of *D. simulans* fitness revealed that *w*Inn had no significant effect on either egg-to-adult viability or lifetime female fecundity. Relative to an uninfected line, adult survival was unaffected in one line of infected flies, and slightly, but significantly and consistently greater in another. Although the two recipient lines of *D. simulans* were derived from the same strain, it is possible that they were genetically slightly different, having been derived from different transinfected flies, or that they harbored different microbial gut communities (including, possibly, pathogenic microbial species), owing to the ecological independence of these lines during many generations of laboratory culture. Thus, the difference in survival between the lines might be owing to direct effects of differences between their gut microbiotas or genetic makeup or to an interaction between *Wolbachia* infection and either host genotype or gut microbiota. If the increased longevity is indeed owing to *Wolbachia*, the present findings have important implications for understanding *Wolbachia* dynamics. First, if this survival advantage occurs across multiple genotypes of *D. simulans*, then *w*Inn could spread deterministically as a mutualist, independently of any reproductive manipulation of its new host species. Furthermore, it has recently been found that *w*Inn has antiviral protective effects in *D. innubila* (Unckless and Jaenike, 2011). If such effects were manifest in another host species, such as *D. simulans*, this could provide the necessary selective advantage to spread in natural populations.

Second, suppose the dynamics of infection in a new host species were governed by a balance between weak beneficial fitness effects and imperfect maternal transmission. This could result in a low, but stable equilibrium, prevalence of infection. This might enable the *Wolbachia* population in this host species to persist in sufficient numbers and for sufficient time to accumulate mutations enabling it to become a reproductive parasite and achieve a higher prevalence of infection. Thus, the association could conceivably evolve from mutualism to parasitism.

Finally, consider the fate of a new *Wolbachia* strain that causes CI in *D. simulans*. If this strain has an adverse effect on female fitness, then the prevalence of *Wolbachia* infection must exceed a particular threshold frequency in order to spread (Caspari and Watson, 1959; Turelli and Barton, 1994). The lack of any adverse effects of *w*Inn infection on *D. simulans* means that, if it could cause CI, this infection could spread from an arbitrarily low initial frequency. Perhaps the success of the ST-13 strain complex results in part from the relatively benign effects of these *Wolbachia* on female hosts.

DATA ARCHIVING

Data have been deposited at Dryad: doi:10.5061/dryad.m78fk.

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Supplementary Information accompanies the paper on Heredity website (http://www.nature.com/hdy)