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# A *Wolbachia*-associated fitness benefit depends on genetic background in *Drosophila simulans*

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The α-proteobacteria *Wolbachia* infect a number of insect species and influence host reproduction to favour the spread of infected females through a population. The fitness effect of this infection is important in understanding the spread and maintenance of *Wolbachia* within and among host populations. However, a full elucidation of fitness effect requires careful control of host genetic background. Here, I transferred a single clone of *Wolbachia* (the *w*Ha strain) into three genetically distinct isofemale lines of the fly *Drosophila simulans* using microinjection methodology. These lines carried one of the three described mitochondrial haplogroups (*si*I, *si*II or *si*III) and differ in nuclear genome as well. Population cage assays showed that *w*Ha-infected *si*III flies enjoyed a dramatic fitness benefit compared to uninfected *si*III. In contrast, *w*Ha did not affect the fitness of *si*I or *si*II flies. This study points to the importance of host-by-symbiont interaction terms that may play an important role in organismal-fitness.

**Keywords:** *Drosophila simulans*; *Wolbachia*; fitness; genetic background; microinjection

## 1. INTRODUCTION

It is well appreciated that symbionts can influence the fitness of their hosts. For vertically transmitted symbionts, the fitness of their host is an important determinant of their spread and maintenance within host populations (Lipsitch *et al.* 1995). Teasing apart fitness contributions of host and symbiont has proven to be a challenge in some groups. Furthermore, fitness effects may depend not on the particular host or symbiont type, but on an interaction between the two. *Wolbachia*, a group of vertically transmitted α-proteobacteria, are a highly successful group of symbionts that infect 17–76% of all insect species (Werren *et al.* 1995; Jeyaprakash & Hoy 2000). These bacteria manipulate host reproduction in ways that favour their spread through populations (Hoffmann & Turelli 1997). Fitness costs or benefits conferred by the symbiont also hinder or promote spread of the infection (Caspari & Watson 1959; Turelli & Hoffmann 1991; Turelli 1994). Fitness-effect experiments have shown that *Wolbachia* run the gamut from parasite to commensal to mutualist. However, it is not well understood how fitness effects are manifested differently on alternative host genetic backgrounds within the same species.

In some cases, *Wolbachia* cannot be associated with statistically significant fitness effects (Nigro & Prout 1990; Turelli & Hoffmann 1995; Hoffmann *et al.* 1996; Charlat *et al.* 2004). However, both fitness costs and benefits have been found in a number of different insect species. Infected *Tribolium* beetles show a 37% reduction in overall fitness relative to uninfected beetles (Stevens & Wade 1990). Infected *Drosophila* females experienced a 29% reduction in the number of progeny (Hoffmann & Turelli 1988), and a follow-up experiment showed a 10–20% reduction in fecundity (Hoffmann *et al.* 1990). Infected male *D. melanogaster* produced significantly fewer sperm and suffered a reduction in fertility (Snook *et al.* 2000). Artificially infected *D. serrata*, a species not normally infected with *Wolbachia*, experienced significant reduction in egg-to-pupa viability and egg-to-adult viability (Clancy & Hoffmann 1997).

*Wolbachia* may also be associated with a fitness benefit to their hosts. In fact, a fitness benefit was indirectly inferred to explain the maintenance of *Wolbachia* in wild *D. melanogaster* populations (Hoffmann *et al.* 1998). This prediction was later supported in studies where infected flies had higher fecundity (Olsen *et al.* 2001) and showed significantly longer survival (Fry & Rand 2002). In *D. simulans*, infected females showed an increase in overall productivity when compared to their antibiotic-cured counterparts, although this fitness advantage appeared to be transient (Poinsot & Merçot 1997). In mosquitoes, infected females lived longer, produced more eggs and had higher hatching rates than uninfected females (Dobson *et al.* 2002). If the wasp *Asobara tabida* is cured of its *Wolbachia* infection, oogenesis fails (Dedeine *et al.* 2001). *Wolbachia* may also affect male reproductive success; sperm from infected *Tribolium* beetles out-competed sperm from uninfected males (Wade & Chang 1995).

All of these studies compared infected to uninfected individuals. There are two common ways that uninfected individuals are derived. First, one can collect naturally uninfected individuals; however, this strategy prevents the standardization of host genetic background compared to the infected individuals. Furthermore, some species are fixed for infection. A second strategy is to cure infected lines with antibiotics to create an uninfected subline with the same genetic background. However, it is possible that antibiotic treatment itself affects fitness. An alternative method to these two methods is to artificially infect a subset of individuals from a naturally uninfected line. This third strategy was employed here, in an attempt to control host genetic background without the use of antibiotics.

The fly *D. simulans* offers an ideal model system to ask how *Wolbachia*-associated fitness effects are manifested,

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while controlling host genetic background. *D. simulans* serves as a host to four genetically distinct *Wolbachia* strains, called *w*Ri, *w*Au, *w*Ha and *w*Ma (Hoffmann & Turelli 1988; O'Neill & Karr 1990; Nigro 1991; Turelli & Hoffmann 1995; Ballard 2004). These four strains differ by over 12% at the nucleotide level at the *wsp* locus (Zhou *et al.* 1998) but some are invariant at the 16S locus (O'Neill *et al.* 1992). Interestingly, these four genetically distinct strains of *Wolbachia* are non-randomly associated with three genetically distinct mitochondrial haplogroups in the host *D. simulans* (Ballard 2004). The three mitochondrial haplogroups, called *si*I, *si*II and *si*III, differ by ~2% at the nucleotide level, while there is ~0.1% nucleotide polymorphism within each of these haplogroups (Solignac *et al.* 1986; Ballard & Kreitman 1994; Ballard 2000*a*,*b*). In contrast, nuclear genetic variation is random with respect to the structure seen in mitochondria and/or *Wolbachia* (Ballard 2000*a*; Ballard *et al.* 2002; Dean *et al.* 2003; Dean & Ballard 2004).

To directly test how host genetic background affects *Wolbachia*-associated fitness within a species, I transferred the *w*Ha strain into three uninfected lines that differ in their nuclear genomes and carry one of the three distinct mitochondrial haplogroups (Ballard 2000*a*; Ballard *et al.* 2002; Dean & Ballard 2004). Of the hundreds of *D. simulans* flies collected from over 30 countries, the *w*Ha strain has only been found infecting *si*I-carrying flies (Ballard 2004), so this experiment also offers a glimpse into the effects of disrupting naturally occurring *Wolbachia*/mitochondria complexes. Effects on fitness were identified using a population cage assay. Compared to its uninfected counterpart, the infected *si*III-carrying line showed a dramatic fitness increase. No effect of infection was seen in *si*I- or *si*II-carrying lines. These results underscore the importance of a host×symbiont interaction term in the description of fitness costs and benefits of infection within a single species.

## 2. MATERIAL AND METHODS

### (a) *Lines used*

I transferred the *w*Ha strain, which in *D. simulans* has only been found in hosts with *si*I mitochondria (Ballard 2004), into three genetically distinct host backgrounds. The donor *si*I/*w*Ha isofemale line was originally collected from Hawaii (line DSH from O'Neill & Karr 1990). DSH was chosen as the donor line because the *si*I mitochondrial type is apparently unable to replace or exist in a heteroplasmic state with incumbent *si*II or *si*III (de Stordeur *et al.* 1989; de Stordeur 1997). It should be noted that other cytoplasmic elements may be transferred during this process.

The uninfected *si*I recipient line was derived from DSH through stochastic loss of infection in the laboratory, which occurs rarely (Poinsot *et al.* 2000). Two additional, naturally uninfected recipient isofemale lines carried the *si*II (line DSW from Hoffmann & Turelli 1988) or *si*III mitochondrial type (line MD111 from Ballard 2000*a*). Nuclear heterozygosity within each host line is unlikely to contribute detectable variance. Before commencing this study, all lines were full-sib-mated for five generations to reduce nuclear variation. Furthermore, the DSH and DSW lines were maintained in the laboratory for over 10 years, while the MD111 line was maintained for over 5 years. There were many differences in both mitochondrial and nuclear sequences among these three lines (Ballard 2000*a*; Ballard *et al.* 2002; Dean *et al.* 2003; Dean & Ballard 2004). Although nuclear genomes among these three fly lines differ, I refer to them as *si*I, *si*II and *si*III throughout the remaining part of this manuscript for simplicity.

Two *D. melanogaster* lines, CyO/Sp and w118, were used as common competitors in population cage experiments, as described below. CyO/Sp displays a curly winged mutation. On an average, 50% of CyO/Sp eggs do not hatch. The w118 line is marked with a recessive white-eyed mutation.

### (b) *Microinjections and fly husbandry*

Microinjection methodology followed Santamaria (1987) with modifications. Embryos less than 1 h old were retrieved from donor and recipient lines and dechorionated in 50% bleach for 1 min. They were then washed in distilled water, followed by washing in 0.005% Triton X solution. Embryos were placed on 3 M double stick tape (Whiteley & Kassis 1993) and covered in Halocarbon 700 oil.

Under a dissecting microscope, cytoplasm was drawn from infected DSH embryos into a needle, pulled from 1 mm diameter glass capillaries. A small amount of this cytoplasm was injected into uninfected recipient embryos with a pneumatic pump. Injected embryos were placed in a sealed chamber with high-humidity and oxygen. After 24–36 h, surviving larvae were removed from the oil and placed in a vial containing standard medium. Any surviving adult females were placed individually in fresh vials with 3–6 males from her same genetic background.

Several generations after establishing an isofemale line from injected females, infection status was checked with polymerase chain reaction (PCR; following Dean *et al.* 2003). In most cases, lines deemed uninfected were rechecked a few generations later. In no case was an uninfected line later found to be infected. A region of mtDNA was amplified and sequenced (following James *et al.* 2002) from transfected lines 5–10 generations later to confirm the absence of contaminating *si*I mitochondria. The *si*I, *si*II and *si*III mitochondria differ by 26 single nucleotide polymorphisms and three length variants in this region, allowing unambiguous identification of each mitochondrial type (Ballard 2000*a*). Transfected lines were also sequenced for the *Wolbachia wsp* locus to further confirm the presence of *w*Ha in a homoplasmic state (following Zhou *et al.* 1998).

### (c) *Population cage experiments*

Following transfections, six unique genetic combinations existed—three host types (carrying *si*I, *si*II or *si*III mitochondria, as well as nuclear differences) and two infection states (infected with *w*Ha or not). It is possible that other unknown symbionts or genetic factors were transferred during microinjections.

Each genetic combination of *D. simulans* was competed against a common *D. melanogaster* mutant in three replicate perspex cages approximately 35×20×20 cm. The *D. melanogaster* mutant CyO/Sp was chosen since there is no meaningful gene flow between *D. simulans* and *D. melanogaster* (reviewed in Davis *et al.* 1996). More specifically, hybridization results in unisexual sterile hybrids. Differences in cuticular hydrocarbons form intense premating barriers between these two species (Coyne *et al.* 1994). The CyO/Sp mutant is easily distinguished from *D. simulans* by the presence of curly wings.

Under two different temperature regimes, 20 and 25 °C, three replicate cages were run for each *D. simulans*+CyO/Sp combination, for a total of 36 cages. A number of developmental, behavioural, and phenotypic characters differ among flies raised at different temperatures (Neat *et al.* 1995; Partridge & French 1996; Gilchrist *et al.* 1997; James *et al.* 1997). To seed each cage, 250 first instar larvae from each *D. simulans* and CyO/Sp line were placed in each of two bottles containing standard medium, for a total of 500 larvae per bottle and 1000 larvae per cage. Because egg-to-adult viability declines daily with the age of the mother (Hercus & Hoffmann 2000), mothers of these first instar larvae were the same age across all lines.

Cages were given 23 days of development at 20 °C and 13.5 days of development at 25 °C. After this period, adults were emptied into a perspex cage containing four fresh bottles and given 48 h to lay eggs. Adults were then frozen for later processing. Each cage was maintained in this manner until it became fixed for either *D. simulans* or CyO/Sp, or until the tenth generation.

All the adults were sorted and counted from each cage every second generation. The proportion of *D. simulans* was calculated and arcsin-transformed (Sokal & Rohlf 1995). This number was taken as a proxy for the competitive ability of each *D. simulans* line. The data were then analysed using a mixed model ANOVA, using host genetic background, infection status, temperature and their four possible interaction terms as the main factors. Since the adults of one generation seeded the next, data may not be independent among generations. Therefore, generation number was considered as a first-order autoregressive source of covariation. Since the very first generation (generation 0 in figure 1) measured larval competitive ability only, it was excluded from statistical analyses. All the statistical analyses were performed using SAS v. 9.0.

### (d) *Identification accuracy*

To assess the accuracy in scoring flies, an allele-specific PCR was designed that selectively amplified either a 1139 bp fragment from *D. melanogaster* or a 437 bp fragment from *D. simulans* mtDNA (Ballard 2000*a*). Flies were sampled from three stages of the experiment. First, three randomly chosen adult flies from each of the 36 cages, originally identified as *D. simulans* were assayed from generation 0. Second, three flies originally identified as *D. melanogaster* were assayed from generation 0. Third, three flies originally identified as *D. simulans* were assayed from either generation 10 or from the generation in which *D. simulans* reached fixation.

### (e) *Potential loss of infection*

After approximately four generations of the population cage experiment, flies from each cage were assayed to confirm that they were still infected or uninfected as expected. Loss of infection may occur at low frequency in the laboratory (Poinsot *et al.* 2000), but should be selected against in a cage experiment.

## 3. RESULTS

### (a) *Microinjections*

5984 embryos were injected, from which eight transfected lines were successfully established. Among the 5984 embryos injected, 770 survived injections. Of these, 202 reached adulthood. Among these, 102 were males and not useful in establishing infected lines since *Wolbachia* is a maternally transmitted symbiont. Of the 100 females, 13 successfully established new isofemale lines. Of these, eight lines tested positive for the presence of *Wolbachia*, three of which were used in this study. These

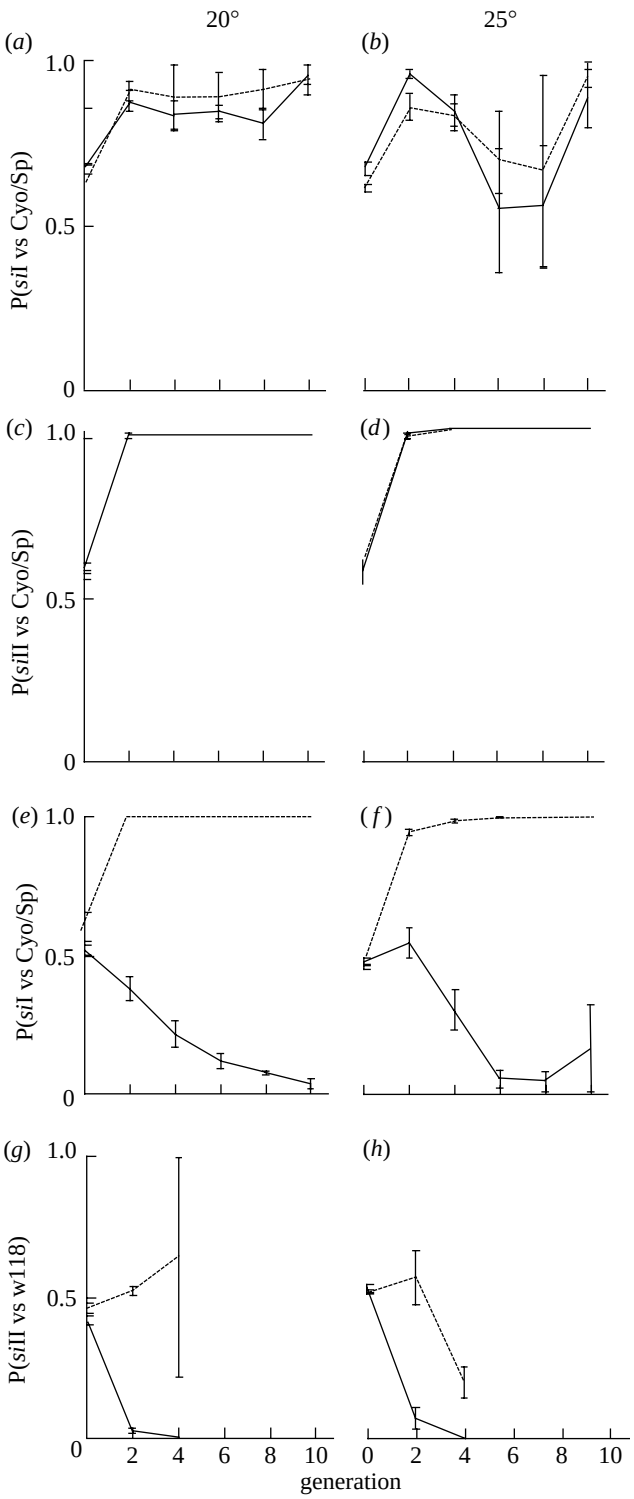


Figure 1. Proportion of *D. simulans* versus CyO/Sp through 10 generations at two temperatures. The first set of population cages involved competition between CyO/Sp and (*a*) *si*I at 20 °C or (*b*) 25 °C; (*c*) *si*II at 20 °C or (*d*) 25 °C; *si*III at (*e*) 20 °C or (*f*) 25 °. The second set of population cages involved competition between w118 and (*g*) *si*III at 20 °C or (*h*) 25 °C. Straight lines indicate an uninfected host line; dashed lines indicate transfected counterparts.

Table 1. Mixed model ANOVA results of population cage assays.

| competitor | effect | num d.f. | den d.f. | *F* value | *p* |
|---|---|---|---|---|---|
| CyO/Sp | temp | 1 | 168 | 4.12 | 0.04 |
| | host | 2 | 168 | 221.89 | <0.0001 |
| | infection | 1 | 168 | 298.23 | <0.0001 |
| | temp×host | 2 | 168 | 1.92 | 0.15 |
| | temp×infection | 1 | 168 | 2.45 | 0.12 |
| | host×infection | 2 | 168 | 246.07 | <0.0001 |
| | temp×host×infection | 2 | 168 | 0.86 | 0.42 |
| w118 | temp | 1 | 18 | 1.68 | 0.21 |
| | infection | 1 | 18 | 69.91 | <0.0001 |
| | temp×infection | 1 | 18 | 2.87 | 0.11 |

numbers are probably an overestimate of failure rate, as techniques were continually optimized throughout the experiment.

The three transfected lines used in the population cage experiments yielded 'clean' sequence from their incumbent mitochondria, confirming the absence of contamination by *si*I mitochondria. All transfected lines also yielded 'clean' *w*Ha sequence. Therefore, the transfected *si*II and *si*III lines represented a decoupling of natural host/symbiont associations.

### (b) *Population cage experiments*

For this part of the experiment, 191 367 flies were counted, for an average (standard deviation, s.d.) of 1031 (498) flies per counted cage per generation. When in competition with CyO/Sp, the proportion of *D. simulans* increased over time in all cages except those containing uninfected *si*III (figure 1*a–f*). Competitive ability differed significantly based on host genetic background ($F_{1,168}=221.89$, $p<0.0001$, table 1). The *si*II line increased most rapidly, reaching fixation by the fourth generation. At 25 °C, the *si*II line reached fixation by the second generation. At 25 °C, the *si*I and *si*III lines showed more fluctuation in the proportion of *D. simulans* compared to a more steady increase seen at 20 °C.

The presence of the *w*Ha strain of *Wolbachia* played a significant role in the competitive ability of these flies ($F_{1,168}=298.23$, $p<0.0001$, table 1), and interacted significantly with host genetic background ($F_{2,168}=246.07$, $p<0.0001$, table 1). Specifically, the presence of *Wolbachia* dramatically increased competitive ability in the *si*III host compared to uninfected *si*III flies, although it did not affect competitive ability in *si*I or *si*II hosts (figure 1*e* and *f*). Temperature significantly affected competitive ability ($F_{1,168}=4.12$, $p\approx0.0.04$), but this was probably due to the greater fluctuation seen at 25 °C (figure 1*b* and *f*). Temperature did not interact significantly with either host or symbiont (table 1).

The dramatic difference between infected and uninfected *si*III lines warranted further corroboration. The above experiment was repeated for the *si*III lines only using the alternative *D. melanogaster* mutant, w118. This mutant was marked by white eyes and, in contrast to CyO/Sp, was not 50% lethal. Therefore, w118 was expected to be a more intense competitor. For this experiment, 49 599 flies were counted for an average (s.d.) of 1459 (728) flies per counted cage. The *w*Ha strain significantly increased competitive ability of *si*III flies ($F_{1,18}=69.91$, $p<0.0001$; figure 1*g* and *h*, table 1). Temperature did not contribute to variation ($F_{1,18}=1.68$, $p\approx0.21$). Thus, in two independent experiments, the infected *si*III host showed an increased competitive ability against two independent *D. melanogaster* competitors.

### (c) *Identification accuracy*

Of the 232 PCR reactions, 229 confirmed the original identification. This constitutes an identification accuracy of nearly 99%. Among the three misidentifications, two were flies incorrectly identified as *D. melanogaster* and one was a fly incorrectly identified as *D. simulans*. Thus, misidentification is unlikely to contribute substantially to the analyses presented above.

### (d) *Potential loss of infection*

In the fourth generation of the population cages, all transfected lines were confirmed to be infected and all uninfected lines were confirmed to be uninfected. The *w*Ha strain of *Wolbachia* induces strong incompatibility in its natural *si*I host (O'Neill & Karr 1990; James & Ballard 2000), suggesting loss of infection would be selected against in a cage environment.

## 4. DISCUSSION

Competitive ability is probably a complex interaction between life-history characteristics such as fecundity, development time and longevity. The genome of any individual will obviously influence these characteristics, but interaction terms between symbiont and host may also be important. While this experiment showed a clear difference in competitive ability among infected and uninfected *si*III, it cannot necessarily reject differences between infected and uninfected *si*I or *si*II lines. Differences in competitive ability might be subtle and difficult to detect given the rapid increase in frequency of *si*I and *si*II lines during the population cage assay.

In terms of species interactions, *Wolbachia* run the gamut from parasite (Hoffmann & Turelli 1988; Hoffmann *et al.* 1990; Stevens & Wade 1990; Clancy & Hoffmann 1997; Snook *et al.* 2000) to commensal (Nigro & Prout 1990; Turelli & Hoffmann 1995) to mutualist (Wade & Chang 1995; Poinsot & Merçot 1997; Hoffmann *et al.* 1998; Dedeine *et al.* 2001; Olsen *et al.* 2001; Dobson *et al.* 2002; Fry & Rand 2002). By controlling host genetic background, this study points to the importance of host genetic background when describing fitness effects and the rate of spread within a single

species. In addition, it is the first explicit disruption of natural *Wolbachia*/mitochondrial complexes. A fitness increase was observed in an unnatural host (*si*III versus *si*I), supporting the hypothesis that association between particular *Wolbachia* strains and mitochondrial groups is not maintained directly by selection (Dean & Ballard 2005).

Unpublished data further support the conclusion that *Wolbachia* increases fitness of *si*III-carrying flies (A. C. James 2003, personal communication). After artificially curing the naturally *w*Ma-infected, *si*III-carrying line MD199 with tetracycline, significantly fewer progeny developed after 3 days of egg-laying compared to the original infected line. One interpretation is that tetracycline had a detrimental effect on the cured line. However, curing several other infected hosts that carried the *si*I and *si*II mitochondrial types showed no effect. Therefore, a fitness advantage associated with *Wolbachia* was observed independently in a different host and symbiont than those studied here. The significant increase in fitness of *si*III hosts due to *Wolbachia* infection may be a general phenomenon, at least in the laboratory.

How do these results apply to the distribution of *Wolbachia* in nature? In nature, *si*III hosts may be infected with the *w*Ma strain, which does not induce strong incompatibility (James & Ballard 2000). Theory predicts that without fitness benefits or improved transmission dynamics, the *w*Ma symbiont should be stochastically lost from populations (Turelli 1994). The interaction term observed here may help to explain why *Wolbachia* has not been lost from *si*III populations in nature. On the other hand, with such a dramatic increase in competitive ability, infection frequency might be expected to reach fixation. Instead, the *w*Ma strain attains a relatively low-equilibrium infection frequency of 0.17 (95% C.I.= 0.13–0.22, $n=245$ isofemale lines; combined data of Merçot & Poinsot 1998; James & Ballard 2000; Dean *et al.* 2003). Future field study should determine if and how competitive ability is affected in nature. It is also possible that some nuclear backgrounds of *si*III-carrying flies do not enjoy such a fitness advantage.

Clearly, one weakness of this study is that each transfected line was created once in the laboratory. Without multiple transfected lines, the generality of the results observed here cannot be further assessed. The low success rate of microinjections (see §3*a*) posed a technical limitation. Further experiments are needed to ask whether the fitness increase observed here is a general phenomenon.

An alternative hypothesis to explain the patterns observed here is that the uninfected *si*III parental stock acquired a deleterious mutation prior to commencement of the population cage experiments. This alternative hypothesis seems unlikely as it would require a strongly deleterious mutation to fix in the short time between transinfecting the subline of *si*III and commencing the population cages, a period of less than six months.

It is possible that the presence of *Wolbachia* simply marks the presence of other unknown symbiont or genetic factors that were the true cause of the interaction term observed here. Until *Wolbachia* can be cultured in isolation, any linkage to unknown symbionts cannot be adequately addressed. Regardless of true cause, the interaction term observed here reveals an additional layer of complexity when considering organismal fitness. Even within a single species, alternative host genetic backgrounds may manifest symbiont-associated fitness effects differently.

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