INSIGHTS INTO ENDOSYMBIONT-MEDIATED DEFENSE OF *DROSOPHILA* FLIES AGAINST PARASITOID WASPS

A Dissertation

by

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ABSTRACT

Maternally-transmitted associations between endosymbiotic bacteria and insects are diverse and widespread in nature. To counter loss by imperfect vertical transmission, many heritable microbes have evolved compensational mechanisms, such as manipulating host reproduction and conferring fitness benefits to their hosts. Symbiont-mediated defense against natural enemies of hosts is increasingly recognized as an important mechanism by which endosymbionts enhance host fitness. Members of the genus *Spiroplasma* associated with distantly related *Drosophila*, are known to engage in either reproductive parasitism (i.e., male killing, MSRO strain) or defense against natural enemies (a parasitic wasp and a nematode). My previous studies indicate the *Spiroplasma* hy1 enhances survival of *Drosophila hydei* against the parasitoid wasp *Leptopilina heterotoma*, but whether this phenomenon can contribute to the long-term persistence of *Spiroplasma* is not clear. Here, I tracked *Spiroplasma* frequencies in fly lab populations repeatedly exposed to high or no wasp parasitism throughout ten generations. A dramatic increase of *Spiroplasma* prevalence was observed under high wasp pressure. In contrast, *Spiroplasma* prevalence in the absence of wasps did not change significantly over time; a pattern consistent with random drift. Thus, the defensive mechanism may contribute to the high prevalence of *Spiroplasma* in *D. hydei* populations despite imperfect vertical transmission.

A male-killing strain of *Spiroplasma* (MSRO), closely related to strain hy1, associates with the model organism *D. melanogaster*, and co-occurs

with *Wolbachia* (strain *w*Mel) in certain wild populations. We examined the effects of *Spiroplasma* MSRO and *Wolbachia w*Mel, on *Drosophila* survival against parasitism by two common wasps, *L. heterotoma* and *L. boulardi*, that differ in their host ranges and host evasion strategies. The results indicate that *Spiroplasma* MSRO prevents successful development of both wasps, and confers a small, albeit significant, increase in larva-to-adult survival of flies subjected to wasp attacks. We modeled the conditions under which defense can contribute to *Spiroplasma* persistence. *Wolbachia* also confers a weak, but significant, survival advantage to flies attacked by *L. heterotoma*. This additive protective effect of *Spiroplasma* and *Wolbachia* may provide conditions for such co-transmitted symbionts to become mutualists. Occurrence of *Spiroplasma*-mediated protection against distinct parasitoids in divergent *Drosophila* hosts implies a general protection mechanism.

DEDICATION

To my Family

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1. INTRODUCTION

Heritable (e.g., maternally transmitted) associations between endosymbiotic bacteria and their insect hosts are ubiquitous in nature. These associations are quite intimate and considered an important force of evolutionary and ecological diversification (Moran *et al.*, 2008). For example, association of the Gammaproteobacterium *Buchnera* with aphids (an endosymbiotic event that occurred ~150–120 million years ago) enabled aphids to exploit plant phloem, a nutritionally poor food source lacking essential amino acids (Douglas, 1998; Baumann *et al.*, 1999; Baumann, 2005). Both the host and endosymbiont are completely dependent on each other for survival and/or reproduction. Such reciprocally-dependent associations are known as obligate or primary. Primary endosymbionts are maternally transmitted with high fidelity, and host populations are usually fixed for endosymbiont presence.

Not all heritable endosymbionts of insects are absolutely required for the host survival or reproduction. These facultative endosymbionts, commonly referred to as secondary endosymbionts, usually exhibit imperfect maternal transmission and their infection prevalence varies largely among host populations. Nevertheless, these facultative endosymbionts are widespread. For instance, the Alphaproteobacterium *Wolbachia* has been found in ~40~66% arthropods species and infection frequency can be as high as > 90% in some host species, and as low as < 10% in others (Hilgenboecker *et al.*, 2008; Zug & Hammerstein, 2012). The success of *Wolbachia* can be attributed, at least partially, to its ability to modify the reproduction of its host to enhance its own

transmission. This reproductive parasitism exists in several forms: cytoplasmic incompatibility (CI); male-killing; parthenogenesis; and male feminization (Hurst *et al.*, 1999; Werren *et al.*, 2008). All of these are expected to increase the relative frequency of endosymbiont-infected to uninfected females in the host population, thereby enhancing the endosymbiont's own persistence and spread in the host population. Indeed numerous and taxonomically diverse endosymbionts are reproductive parasites of insects and other arthropods (reviewed in Moran *et al.*, 2008). Nevertheless, not all heritable facultative endosymbionts are reproductive parasites, so alternative mechanisms to counter their loss due to imperfect vertical transmission must exist.

In theory, in the absence of horizontal and paternal transmission, persistence of an endosymbiont in its host population requires the ability of infected females to produce infected daughters to be greater than the ability of uninfected females to produce uninfected daughters (Bull *et al.*, 1992) . This is a relative fitness measure, also known as ‘‘parasite host fitness’’ (Ebbert, 1991), which should be greater than 1 for the endosymbiont to persist. Besides reproductive manipulation, one way for heritable facultative endosymbionts to achieve this is by conferring a fitness advantage to the host (i.e., a mutualistic association). Unlike primary endosymbionts, most secondary endosymbionts are not essential for their hosts in terms of nutrition, development, or reproduction. However, some secondary endosymbionts can defend their hosts against natural enemies, such as predators, parasitoids, parasites, and RNA viruses (Oliver & Moran, 2009 ; Hurst & Hutchence, 2010). For example, *Hamiltonella defensa* (Gammaproteobacteria), a secondary endosymbiont of aphids, has been found to

enhance its host's survival against the parasitoid wasp *Aphidius ervi*, by killing the developing wasp larvae (Oliver *et al.*, 2005). This defensive phenotype requires a toxin-encoding bacteriophage APSE-3 found in some strains of *H. defensa* (Oliver *et al.*, 2009). Symbiont-mediated protection is also found in the association between *Wolbachia* and dipterans (e.g., *Drosophila* flies and *Aedes* mosquitoes), whereby *Wolbachia* protects its host against infection by several RNA viruses, including Dengue virus (Hedges *et al.*, 2008; Moreira *et al.*, 2009; Osborne *et al.*, 2009). Symbiont-mediated protection of insects is not restricted to the class Alphaproteobacteria (phylum Proteobacteria), and has been reported in highly divergent bacterial groups including *Streptomyces* (class Actinobacteria, phylum Actinobacteria) and *Spiroplasma* (class Mollicutes, phylum Tenericutes) (Currie *et al.*, 2003; Jaenike *et al.*, 2010b). For example, *Spiroplasma* has been reported to enhance the fitness of *Drosophila neotestacea* and *D. hydei* from parasitism by nematodes and parasitoid wasps, respectively (Jaenike *et al.*, 2010b; Xie *et al.*, 2010).

Spiroplasma are wall-less bacteria that are very common in arthropods. They are most abundant in the host hemolymph (Ota *et al.*, 1979; Hurst & Hutchence, 2010). Seventeen species of *Drosophila* from two divergent subgenera (*Sophophora* and *Drosophila*) have been reported to harbor this secondary endosymbiont (Montenegro *et al.*, 2005; Mateos *et al.*, 2006; Haselkorn *et al.*, 2009; Watts *et al.*, 2009). Phylogenetic studies indicate that *Drosophila*-associated *Spiroplasma* strains fall into four separate clades whose closest relatives include strains not associated with *Drosophila* (poulsonii, citri, tenebrosa and ixodetis). Therefore, each clade represents an independent invasion

event into *Drosophila* (Haselkorn *et al.*, 2009). Several strains in the poulsonii clade (i.e., MSRO, NSRO and WSRO) are male-killers, whereas other strains in the same clade do not exert the male-killing phenotype (hy1 in *D. hydei*, neo in *D. neotestacea*, and a strain associated with *D. simulans*).

In a previous study, we showed that the poulsonii-clade strain hy1 confers protection to its host *D. hydei* against the cosmopolitan parasitoid wasp *Leptopilina heterotoma* (Eucoilinae, Figitidae). *Spiroplasma* hy1 reduced fly larva-to-adult mortality induced by *L. heterotoma* (Xie *et al.*, 2010). Although a greater proportion of *Spiroplasma* infected flies survived a wasp attack, they still suffered reduced survival as adults and reduced fecundity, compared to flies that had not experienced a wasp attack (Xie *et al.*, 2011). Taking all of these into account, under our experimental conditions, which included high wasp parasitism (e.g., > 95% of fly larvae undergoing wasp oviposition), the estimated "parasite-host fitness" was greater than one (Xie *et al.*, 2010), and thus, potentially relevant to the persistence of *Spiroplasma* in nature. If so, *Spiroplasma* is expected to increase in frequency over time, at least under high wasp parasitism conditions, unless other fitness costs not detectable in our short-term experiment exist. Indeed, fitness costs of harboring the endosymbiont have been detected in the *Hamiltonella*-aphid system in the absence of wasp parasitism (Oliver *et al.*, 2003b). Therefore, the first study of this dissertation (Section 2) examines whether fitness costs and imperfect vertical transmission associated with *Spiroplasma* infection might reduce "parasite host fitness" in a multi-generation assay.

The observed protection conferred by *Spiroplasma* hy1 to its host against parasitoids may explain *Spiroplasma*'s persistence in nature, as well as its relatively high prevalence in certain *D. hydei* populations (~66%, Kageyama *et al.*, 2006), despite an inability to manipulate host reproduction. There is no reason to believe, however, that reproductive parasites are precluded from engaging in host protection. Indeed, recent studies have shown that despite its ability to induce CI, several *Wolbachia* strains protect their hosts against viruses (see above). Given that hy1 is closely related to the strain that protects *D. neotestacea* from nematode parasitism (neo), and to several male-killing strains in the poulsonii clade, it is possible that these male-killing strains also protect their hosts against natural enemies. Male-killing itself in *Drosophila* is hypothesized to enhance *Spiroplasma* transmission as a result of the resources released by the dead brothers to their *Spiroplasma*-infected sisters, who are competing for resources during larval development (Hurst, 1991). Evidence for faster larval development, earlier reproduction, and earlier mating propensity has been reported for several male-killing *Spiroplasma* strains of *Drosophila* (Sakaguchi & Poulson, 1963; Malogolowkin-Cohen & Rodriguespereira, 1975; Ebbert, 1991; Martins *et al.*, 2010), but it is unclear whether these alone explain persistence of *Spiroplasma* in natural populations. The second study of the present dissertation (Section 3) examines whether the male-killing strain native to *D. melanogaster* (Melanogaster Sex Ratio Organism; MSRO) also confers protection to its host against wasp parasitism. If so, models of persistence of male-killers will have to incorporate the effects of defensive mutualism. Furthermore, discovery of *Spiroplasma*

protection in *D. melanogaster* will enable the use of this model system to better understand the mechanism and evolution of this protection.

Leptopilina heterotoma is considered a generalist because it can use several distantly related members of *Drosophila* as hosts (Schlenke *et al.*, 2007), including *D. melanogaster*. Another cosmopolitan member of the genus *Leptopilina*, *L. boulardi*, specializes on *D. melanogaster* and its close relatives. In Section 3, I also examine whether MSRO confers protection against both the generalist and specialist parasitoids, which use different strategies to counter host defenses.

In addition to harboring *Spiroplasma*, *D. melanogaster* is a common host to *Wolbachia*. To date, *Spiroplasma* and *Wolbachia* have been found to coexist in natural populations of only two species of *Drosophila*, *D. melanogaster* and *D. neotestacea* (Montenegro *et al.*, 2005; Jaenike *et al.*, 2010a). Co-occurrence of the two heritable endosymbionts may lead to competition or cooperation between them. Based on the non-random association of *Wolbachia* and *Spiroplasma* observed in *D. neotestacea* populations, Jaenike *et al.* (2010a) suggest that mutualism between *Wolbachia* and *Spiroplasma* has evolved, although no evidence of the cooperation mechanism has been found. Similarly, Montenegro *et al.* (2006) found no evidence of cooperation between the two endosymbionts in *D. melanogaster*. However, it is possible that cooperation occurs in the defense against parasitoid wasps. Therefore, in the Section 3, I examine the effects of *Spiroplasma* and *Wolbachia*, together and separately, on the fitness of *D. melanogaster* upon attack by parasitoids.

As stated above, I have shown that *Spiroplasma* hy1 enhances fitness of *D. hydei*

upon attack by the parasitoid *L. heterotoma*. The mechanism by which *Spiroplasma* confers protection against parasitoid wasps is not known, but my work indicates that the growth of wasp larvae is stalled when *Spiroplasma* is present in the host, precluding the wasp from completing development (Xie *et al.*, 2011). In the second study (Section 3), I examine the development of generalist and specialist wasps in the *D. melanogaster* with four different endosymbiont infection states (*Spiroplasma* infected only, *Wolbachia* infected only, double infected and uninfected) at different time points to uncover the potential mechanism of the protection.

2. RAPID SPREAD OF THE DEFENSIVE ENDOSYMBIONT *SPIROPLASMA* IN *DROSOPHILA HYDEI* UNDER HIGH PARASITOID WASP PRESSURE

2.1 Introduction

Numerous and diverse insects and other arthropods associate with maternally transmitted endosymbiotic bacteria (Moran *et al.*, 2008). The ecological and evolutionary consequences of harboring such symbionts are diverse and far-reaching (Moran *et al.*, 2008). Many heritable insect-bacteria associations involve perfect maternal transmission of the symbiont. These are typically ancient obligate associations of a nutritional nature, in which both partners are completely dependent on each other for survival, and thus, symbiont infections are fixed in host populations. Nevertheless, many other heritable insect-bacteria associations exhibit more variable distribution in time and space, as well as imperfect vertical transmission, which presents challenges to symbiont persistence. To counter loss by imperfect transmission, many of these facultative heritable endosymbionts manipulate host reproduction in ways that enhance the relative frequency of symbiont-infected to symbiont-uninfected females: cytoplasmic incompatibility; male-killing; parthenogenesis induction; and male feminization (O'Neill *et al.*, 1997).

Not all facultative heritable endosymbionts manipulate host reproduction, however. Therefore, their persistence despite imperfect vertical transmission must be the result of horizontal transmission and/or enhancing host fitness. A growing body of literature indicates that fitness benefits to the host are common, but typically context-

dependent, including: enhanced ability to utilize a particular resource (Brownlie *et al.*, 2009; Hosokawa *et al.*, 2010); enhanced fitness in the face of abiotic stressors (Burke *et al.*, 2010a; Burke *et al.*, 2010b; Brumin *et al.*, 2011), and enhanced tolerance or resistance against natural enemies (reviewed in Haine, 2008; Jaenike, 2012; Oliver *et al.*, 2013). Reported cases of symbiont-mediated defense against natural enemies are numerous and involve a broad taxonomic diversity of hosts, symbionts, and natural enemies. Such natural enemies include parasitoid wasps (Oliver *et al.*, 2003a; Xie *et al.*, 2010), parasitic nematodes (Jaenike *et al.*, 2010b), RNA viruses (Teixeira *et al.*, 2008), and fungi (Scarborough *et al.*, 2005; Lukasik *et al.*, 2012). Experimental evidence that defensive endosymbionts can rapidly spread in a host population under selection pressure from a natural enemy has been reported in two systems. Prevalence of the endosymbiont *Hamiltonella defensa* rapidly increases in lab populations of the aphid *Acyrthosiphon pisum* exposed to the parasitoid wasp *Aphidius ervi* (Oliver *et al.*, 2008). Similarly, frequency of the endosymbiont *Spiroplasma* (strain neo) rapidly increases in lab populations of *Drosophila neotestacea* exposed to parasitism by the nematode *Howardula aoronymphium* (Jaenike & Brekke, 2011).

Spiroplasma strain hy1 (belonging to the poulsonii clade; Haselkorn *et al.*, 2009), a facultative endosymbiont of *Drosophila hydei*, achieves relatively high frequency in nature, but it is not fixed (23–66% in Japan, Kageyama *et al.*, 2006; and 24.7–60% in North America, Watts *et al.*, 2009). The vertical transmission rate of *Spiroplasma* hy1 varies widely among individuals and environmental conditions: low temperatures can drastically reduce transmission efficiency (Osaka *et al.*, 2008; Osaka *et al.*, 2013a). In a

previous study (Xie *et al.*, 2010), we demonstrated that *Spiroplasma* hy1 confers protection to lab populations of its host *D. hydei* against the cosmopolitan parasitoid wasp *Leptopilina heterotoma* (Eucoilinae, Figitidae; hereafter also referred to as Lh). *L. heterotoma* is a solitary endoparasitoid that oviposits into the hemocoel of first- and second- instar *Drosophila* larvae. If it successfully evades or suppresses host defenses, the wasp larva hatches and feeds within the host during the host larva–prepupa stage. Upon host pupation, the wasp larva exits and kills the fly pupa, and continues development within the host puparium (Carton *et al.*, 1986). Overall, wasp success rate in *Spiroplasma*-free hosts (measured as the number of emerged wasps over the total number of emerged adults) is close to 90%, at least for the highly virulent wasp strain Lh14. In contrast, in *Spiroplasma*-infected hosts, wasp success rate decreases to 6%, and larva-to-adult survival of flies exposed to Lh is greatly enhanced, but not completely restored (Xie *et al.*, 2010). Furthermore, our subsequent study (Xie *et al.*, 2011) showed that *Spiroplasma*-infected flies surviving a wasp attack suffered reduced adult longevity and fecundity, compared to flies not exposed to wasps. Despite these costs, *Spiroplasma* was estimated to confer a ~3.5-fold advantage in the face of high wasp pressure (Xie *et al.*, 2011), and no fitness costs associated with *Spiroplasma* infection in the absence of wasps have been detected (Xie *et al.*, 2010; Xie *et al.*, 2011; Osaka *et al.*, 2013a). Nonetheless, the aforementioned studies relied on experimental setups involving *Spiroplasma*-infected and uninfected host lines reared separately over at most two generations, which might have limited their power to detect subtle differences in fitness (e.g. Oliver *et al.*, 2008). Consequently, a multi-generation study, in which infected and

uninfected host lines are reared together, is needed to better assess the potential for the defensive mechanism to contribute to *Spiroplasma* persistence in natural populations.

In the present study, we tracked the infection prevalence of the defensive symbiont *Spiroplasma* hy1 in its native host *D. hydei*. A population cage setting was used to compare lab fly populations repeatedly exposed to wasps over ten generations, to control populations lacking wasps. Based on the approach of Ballard and James (2004), the trend in *Spiroplasma* prevalence over time was used to distinguish between selection for (or against) *Spiroplasma* infection and drift under different wasp pressure, and hence provide a more reliable estimate of the overall fitness advantage or cost associated with *Spiroplasma* infection in *D. hydei*.

2.2 Materials and Methods

2.2.1 Fly strains

Drosophila hydei females were collected with banana baits in College Station, TX, USA (March 2012). Five females were used to establish five isofemale lines (hereafter isolines; i.e., mating only allowed among descendants of each female). At least three females derived from each isoline were examined for infection by heritable endosymbionts. This was achieved by sterile dissection of ovaries, followed by DNA extraction, and PCR amplification with three bacterial universal 16S rRNA primer pairs, as well as *Wolbachia*- and *Spiroplasma*-specific primers (Table 2. 1). All PCR reactions in this study were carried out with appropriate positive and negative controls. To date,

infection by *Wolbachia* has not been reported in *D. hydei* or any other member of the *repleta* species group, to which *D. hydei* belongs (Mateos *et al.*, 2006).

2.2.2 Establishment of *Spiroplasma*-infected fly strains

To generate the five *Spiroplasma*-infected (S^+) isolines corresponding to the five naturally uninfected isolines (S^-), artificial infections (transfections) were performed by adult-to-adult hemolymph transfer (as described in Xie *et al.*, 2010) from the *Spiroplasma*-infected *D. hydei* isoline TEN104-102 (Mateos *et al.*, 2006). Experiments were carried out three generations after transfection.

2.2.3 Experimental setting

To track *Spiroplasma* prevalence over ten fly generations in the presence and absence of wasps, we set up 14 replicate fly populations. Each replicate was carried out in a half-pint glass bottle filled with ~80ml Opuntia-Banana Media. The replicate populations were established by combining equal numbers of flies (five females and five males) from each of the five *Spiroplasma*-infected and five *Spiroplasma*-free isolines, to a total of 100 adults (Figure 2. 1 and Table 2. 2), to achieve an initial *Spiroplasma* prevalence of ca. 50% in each replicate. The adult flies used to establish each generation were ~8–12 days-old; *D. hydei* age to maturity is 3 and 9 days for females and males, respectively (Markow, 2005). Seven of these replicate populations (hereafter S^+Lh^+) were subjected to parasitism by *L. heterotoma* (Lh) (strain 14 used in previous studies; Schlenke *et al.*,

2007; Kacsoh & Schlenke, 2012), whereas seven were not subjected to wasps (hereafter $S^{+}Lh^{-}$).

Table 2. 1 PCR primers used in this project.

Primer pair (5' to 3')	Target gene (Fragment size)	Target group	Annealing temp (ºC)
10F: AGTTTGATCATGGCTCAGATTG[a] 1507R: TACCTTGTTACGACTTCACCCCAG[a]	16S rRNA (~1500 bp)	Most bacteria	60
27F: GAGAGTTTGATCCTGGCTCAG[b] 1492R: GGTTACCTTGTTACGACTT[b]	16S rRNA (~1470 bp)	Most bacteria	55
559F: CGTGCCAGCAGCCGCGGTAATAC[c] 35R: CCTTCATCGCCTCTGACTGC[d]	16S-ITS-35R (>1000 bp)	Most bacteria (not Wolbachia)	58
p58IV_F: AAAGGTTTACATTCACCAAGTCG[e] p58IV_R: ATTGTTCATTAACTTTATCTTGTGG[e]	P58 (362 bp)	*Spiroplasma*	53
wspF: TGGTCCAATAAGTGATGAAGAAACTAGCTA[f] wspR: AAAATTAAACGCTACTCCAGCTTCTGCAC[f]	wsp (~600 bp)	*Wolbachia*	Touchdown 65–55
HCO2198: TAAACTTCAGGGTGACCAAAAAAT[g] LCO1490: GGTCAACAAATCATAAAGATATTG[g]	COI (~709 bp)	Most invertebrates	45

a Munson *et al.* (1991)
b Lane (1991)
c Russell *et al.* (2003)
d Mateos *et al.* (2006)
e Xie *et al.* (2010)
f Jeyaprakash and Hoy (2000)
g Folmer *et al.* (1994)

Table 2. 2 Number of flies per isoline, *Spiroplasma*-infection state, and sex used to stock the initial generation of each bottle (replicate).

Isoline ID	1				…				5				Total
Spiroplasma infection state	S^+		S^-		S^+		S^-		S^+		S^-		
Sex	F	M	F	M	F	M	F	M	F	M	F	M	
No. individuals in S^+ population	5	5	5	5	5	5	5	5	5	5	5	5	100
No. individuals in S^- population			10	10			10	10			10	10	100

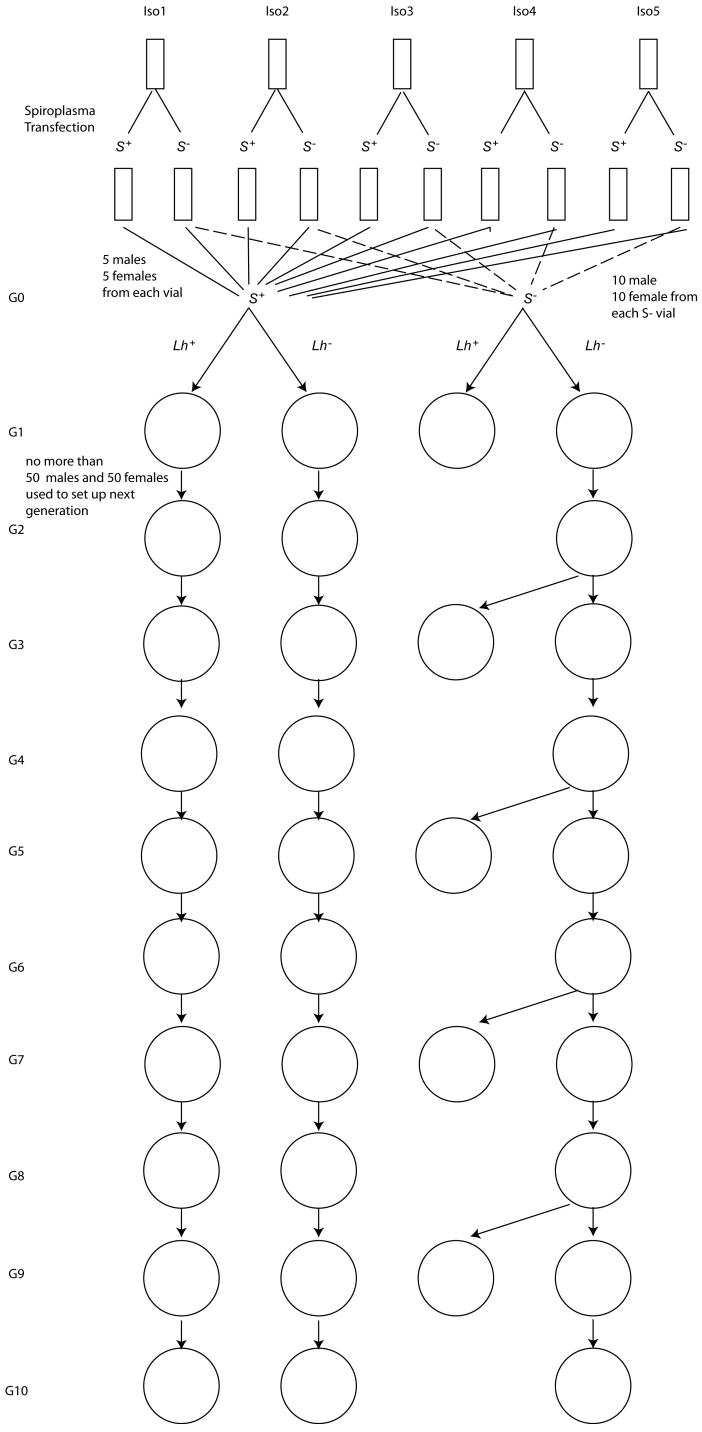

Figure 2. 1 Experimental Setting. Column indicates fly vials and circle represents population bottle. *S* = *Spiroplasma* infection; *Lh* = *Leptopilina heterotoma* treatment.

In addition, two *Spiroplasma*-free (S^-) control treatments were included. To establish these treatments, simply ten females and ten males from each of the five *Spiroplasma*-free (S^-) isolines were combined to a total of 100 per replicate. One of these treatments lacked wasps (hereafter S^-Lh^-) throughout the experiment, and was used as a control for environmental conditions that could affect fly fitness and vary among generations (seven replicates). The other treatment (hereafter S^-Lh^+) was subjected to the same wasp pressure as treatment S^+Lh^+, and was used to control for environmental factors affecting wasp oviposition. The flies used for this treatment, however, were derived from the S^-Lh^- treatment in the immediately preceding generation for two reasons: (1) to prevent selection on flies resulting from exposure to wasps in previous generations; and (2) because survival in the S^-Lh^+ was too low to sustain subsequent generations (see Results). This S^-Lh^+ treatment was run every other generation starting in G1.

The initial 100 flies (i.e., Generation 0) per bottle were allowed to mate and oviposit for two days, after which they were removed. For treatments S^+Lh^+ and S^+Lh^-, 20 of these G0 females per replicate (except for one replicate per treatment; which was used for the vertical transmission assay conducted every generation; see below), were immediately frozen, for the surveys of *Spiroplasma* prevalence (*I*) in the corresponding generation (e.g. I_0), via the PCR assay described below. Flies in the S^-Lh^- treatment were also screened for *Spiroplasma* to confirm that the control populations had not been inadvertently contaminated with *Spiroplasma*-infected flies. Flies from the S^-Lh^+

treatment were not screened for *Spiroplasma*, because they were directly derived from the $S^{-}Lh^{-}$ treatment starting at G3 (Figure 2. 1).

2.2.4 Assessment of *Spiroplasma* prevalence

At least 10 flies per replicate for the $S^{+}Lh^{+}$ and $S^{+}Lh^{-}$ treatments were screened for *Spiroplasma* infection. DNA was isolated from flies individually with the "squish" procedure (Gloor *et al.*, 1993), and used in a multiplex PCR with both, *Spiroplasma*-specific primers (P58IV; expected amplicon length 362bp; Table 2. 1) and host-specific primers (COI; expected amplicon length 709bp; Table 2. 1). We used the EmeraldAmp® MAX PCR Master Mix (Takara) and an annealing temperature of 54ºC. The host-specific gene was used as a control for DNA quality. PCR products were run on agarose gels and visualized with Ethidium Bromide.

To test for potential contamination of the S^{-} controls with *Spiroplasma*-infected flies, we extracted DNA from ten pooled female flies per replicate per generation, and performed multiplex PCR as described above. A preliminary experiment indicated that this procedure allows for detection of *Spiroplasma* if the pooled sample contains at least one *Spiroplasma*-infected fly (results not shown). No *Spiroplasma*-infected flies in the S^{-} treatments were detected throughout the ten generations.

2.2.5 Wasp treatments

In the two wasp parasitism treatments ($S^{+}Lh^{+}$ and $S^{-}Lh^{+}$), 20 *Leptopilina heterotoma* wasp females were introduced to each population bottle immediately after adult flies

were removed. At this time, the bottle contained < 2d-old fly larvae (first-second instar G1 flies). Wasps used throughout the experiment were < 5d-old and derived from a *D. melanogaster* Canton-S culture. Wasps were allowed to oviposit for two days, after which they were removed and discarded. To assess wasp oviposition rate, immediately after wasps were removed, ten fly larvae per replicate were collected and dissected to determine presence/absence of wasp eggs or larvae. Wasp oviposition rate (i.e., proportion of fly larvae with one or more wasp egg or larva) was recorded in all generations and treatments subjected to wasps (i.e., G1–G10 for the $S^{+}Lh^{+}$ treatment; G1, G3, G5, G7 and G9 for the $S^{-}Lh^{+}$ treatment; see Figure 2. 1).

2.2.6 Establishment of subsequent generation

Eclosing G1 flies and/or wasps from each bottle were recorded during the first ~12 days of fly emergence. Fly sex ratio was recorded for the first seven generations. To establish the next generation, emerging flies were placed in fresh food vials (~50 flies per vial; separate sexes) to age for ~15 days, which allowed most adult flies to reach reproductive maturity (peak emergence occurred ~5 days after the first day of eclosion). To account for mortality during the aging period and ensure that enough adults were available to establish every subsequent generation of each replicate, we typically collected the first ~100 flies per sex that eclosed, with one exception: every other generation of the $S^{-}Lh^{-}$ treatment beginning G2, twice the number of flies were collected, because additional ones were needed to set up the $S^{-}Lh^{+}$ treatment. To establish every subsequent generation of each replicate, 100 aged flies (1:1 sex ratio)

were placed into a bottle following the same procedures described above for G0 flies. The only exception was the S^+Lh^+ treatment, in which the number of emerging G1 flies that survived to day 15 was < 50 per sex for several replicates (range: 8–50 males and 20–50 females; see Results). Thus, G2 flies for these replicates were derived from a smaller number of G1 flies.

2.2.7 Assessment of *Spiroplasma* maternal transmission rate

To compare the vertical transmission rate of *Spiroplasma* between wasp treatments and among generations, every generation, 20 females from one replicate each of the S^+Lh^+ and S^+Lh^- treatments (a different replicate was used every generation) were placed into separate vials with two males from their own bottle, and allowed to mate and oviposit. The females were later removed and subjected individually to the PCR procedure described above, to estimate the *Spiroplasma* prevalence (*I*) of their replicate. To assess vertical transmission, we collected 10 eclosing female progeny from each of five vials per replicate (out of the original 20 vials per replicate), and subjected them individually to the PCR assay (10 flies X 5 vials X 2 wasp treatments = 100 flies). The five vials were selected randomly among those vials in which the mother was *Spiroplasma*-infected, according to the PCR assay. Because G0 flies used to establish the S^+Lh^+ and S^+Lh^- treatments were equivalent (i.e., in the S^+Lh^+ treatment, it was the G1 flies as larvae that were exposed to wasps), the vertical transmission rate of G0 females was measured on the S^+Lh^+ treatment only (10 flies X 5 vials X 1 treatment = 50 flies).

2.2.8 Statistical analysis

To assess selection for or against *Spiroplasma* in *D. hydei* as a function of parasitoid attack, we used a General Linear Model (GLM, in JMP 9.0.0) to regress *Spiroplasma* prevalence (*I*) (logit-transformed) against generation (continuous variable, fixed), wasp treatment (fixed), and their interaction. Because values of 0 and 1 are undefined under the logit transformation, we substituted $I = 0.025$ and 0.975, respectively, in cases where *Spiroplasma* became fixed ($I = 1$) or was completely lost ($I = 0$). Furthermore, we only included data points up to the first generation in which $I = 0$ or 1 (i.e., for S^+Lh^+ up to G6; for S^+Lh^- all generations; see Results). A similar approach was used by Jaenike and Brekke (2011) and Oliver *et al.* (2008). In addition, because all variables including the interaction term were significant (see Results), we then regressed *Spiroplasma* prevalence vs. generation (continuous variable, fixed) separately for each wasp treatment, to examine the respective slopes.

For the S^+Lh^- treatment, in which few replicates achieved complete loss or fixation of *Spiroplasma* (see Results), we conducted an additional analysis including all of the data points and no transformation. A Generalized Linear Model (GzLM; SAS Enterprise Guide 4.2) was fitted to *Spiroplasma* prevalence (dependent variable; binomial distribution) and generation (discrete and fixed factor). This model was also used to conduct the following analyses. Firstly, we tested whether wasp oviposition frequency varied between *Spiroplasma* treatments (S^+Lh^+ vs. S^-Lh^+; fixed), generations (fixed), or their interaction. This analysis examined data from Generations 1, 3, 5, 7, and 9 (i.e., the only generations in which the S^-Lh^+ treatment was carried out). For the

treatment S^+Lh^+ alone, we also examined the effect generation, including all generations, on wasp oviposition frequency. Secondly, we tested the effect of generation (fixed) and wasp treatment (fixed) on the vertical transmission efficiency of *Spiroplasma* (treatments S^+Lh^+ and S^+Lh^-).

We then used a GLM model for the S^-Lh^+ treatment, in which we tested whether wasp success rate changed over time (i.e., generation; fixed), which could be indicative of inadvertent selection for enhanced resistance or tolerance of flies against wasps and whether it was correlated with wasp oviposition rate (fixed). Finally, for S^+Lh^+ treatment, we tested whether wasp success was correlated with *Spiroplasma* prevalence. This analysis however, was restricted to wasp success in G1 vs. *Spiroplasma* frequency in G0 (i.e., the mothers of G1), because both variables exhibited little variation in subsequent generations of this treatment.

As a proxy for female realized fecundity (i.e., the actual number of progeny surviving to adulthood), we examined the number of flies emerging over the first ~12 days of emergence, normalized by the number of potential mothers (i.e., 50 per replicate in all treatments and generations except for S^+Lh^+ in G1). Several GLM analyses were carried out to examine the effect of several variables (i.e., *Spiroplasma* and wasp treatment, generation, and wasp oviposition) on realized female fecundity.

2.3 Results

2.3.1 Effect of high wasp parasitism on *Spiroplasma* frequency

The *Spiroplasma* infection frequencies in the seven *D. hydei* lab populations subjected to parasitism by *Leptopilina heterotoma* (treatment S^+Lh^+) increased from a mean ± SE of 59.06 ± 6.46% (G0) to 93.38 ± 3.41% (G1) in a single generation (Table 2. 3, Figure 2. 2A). *Spiroplasma* prevalence reached 100% in all seven replicates by G6, and remained stable thereafter. In contrast, mean *Spiroplasma* prevalence in the populations not exposed to wasps (S^+Lh^-) exhibited a slight decrease from 66.61 ± 4.37 (G0) to 55.65 ± 15.17 (G10) over the course of the experiment, but the variation among replicates was high. One replicate lost the infection completely by G1, whereas in another replicate, *Spiroplasma* became fixed at G6 and remained fixed thereafter (Figure 2. 3).

For the logit-transformed data, the effect of wasp parasitism on *Spiroplasma* prevalence was highly significant ($F_{(1,1)}$=26.13, p <0.0001, Table 2. 4). The slopes of *Spiroplasma* change over time in the two treatments also differed significantly (as indicated by the significant wasp treatment X generation interaction: $F_{(1,1)}$=5.50, p =0.0215, Table 2. 4). The estimated slope of *Spiroplasma* prevalence over generations in the replicates exposed to wasps was 0.48 ± 0.15, and significantly different from zero ($F_{(1,1)}$=9.18, p =0.0066, Table 2. 4). In contrast, for the replicates not exposed to wasps, the estimated slope was 0.03 ± 0.06, and not significantly different from zero ($F_{(1,1)}$=0.2799, p =0.5987; Table 2. 4). The effect of generation remained non-significant in the treatment lacking wasps (S^+Lh^-), even when all data points were included (i.e., the GzLM analysis treating generation as a discrete variable).

Table 2. 3 Mean ± Standard Error (in percentage) per generation for *Spiroplasma* frequency, *Spiroplasma* vertical transmission rate, wasp oviposition rate, and wasp success rate (number of emerged adult wasps/total number of emerged adults).

	Spiroplasma Frequency		Vertical Transmission	Wasp Oviposition	Wasp Success	
Generation	S^+Lh^+	S^+Lh^-	S^+Lh^+ & S^+Lh^-	S^+Lh^+ & S^+Lh^-	S^+Lh^+	S^-Lh^+
0	59.06±6.46	66.61±4.38	100±0			
1	93.38±3.41	57.06±13.81	100±0	98.57±0.97	45.71±4.82	97.34±0.70
2	97.31±1.30	48.11±12.69	97.75±1.51	100±0	6.79±3.10	
3	96.15±1.91	41.18±10.98	98±1.33	99.29±0.71	1.01±0.33	91.91±4.24
4	98.53±0.95	52.72±12.05	100±0	100±0	1.46±0.79	
5	98.32±1.08	54.85±14.51	99±1	98.57±0.97	0±0	90.90±3.84
6	100±0	67.64±13.29	97±1.53	94.28±2.97	0.89±0.51	
7	100±0	52.98±14.52	100±0	94.28±5.00	0.27±0.11	92.48±1.82
8	100±0	66.99±13.07	100±0	92.86±4.21	0.35±0.12	
9	100±0	63.12±15.45	100±0	97.14±1.25	0±0	78.31±4.36
10	100±0	55.65±15.17		88.57±4.04	0±0	

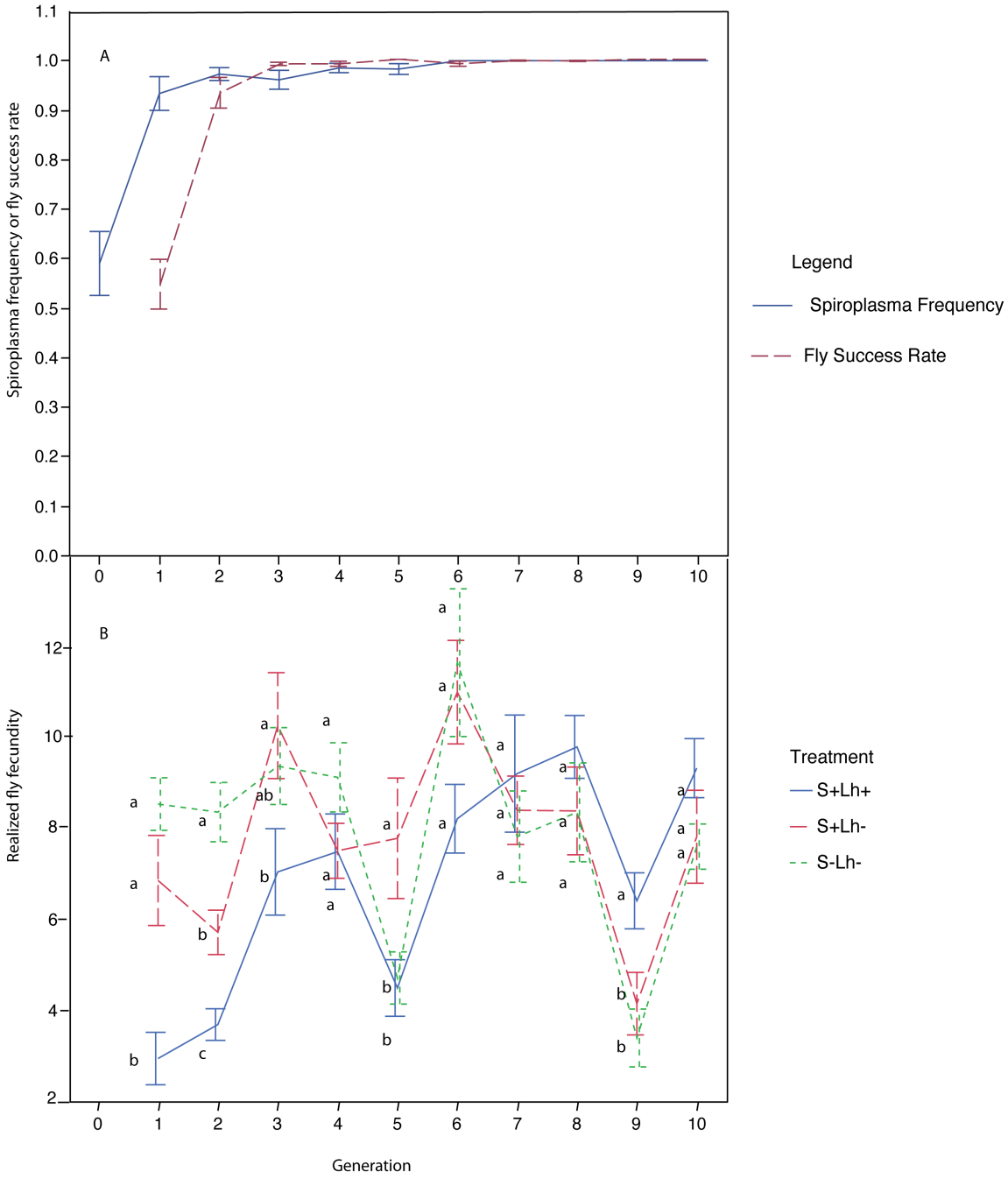

Figure 2. 2 The *Spiroplasma* frequency and fly fitness measures throughout the experimental period (*S* = *Spiroplasma*; *Lh* = *Leptopilina heterotoma* treatment). **A**. Blue solid line, *Spiroplasma* frequency in treatment $S^{+}Lh^{+}$ across ten generations; Red dashed line, Fly success rate (number of eclosed fly adult / (fly adults + wasp adults)) across ten generations. **B**. Fly realized female fecundity (number of eclosed fly adults / number of founder females used to found the respective generation) in the treatment of $S^{+}Lh^{+}$ (Blue solid line), $S^{+}Lh^{-}$ (Red dashed line), and $S^{-}Lh^{-}$ (Green dashed line). Different lower case letters indicate the significant ($p < 0.05$) post-hoc test comparing the three treatments within each generation. Error bars: standard error.

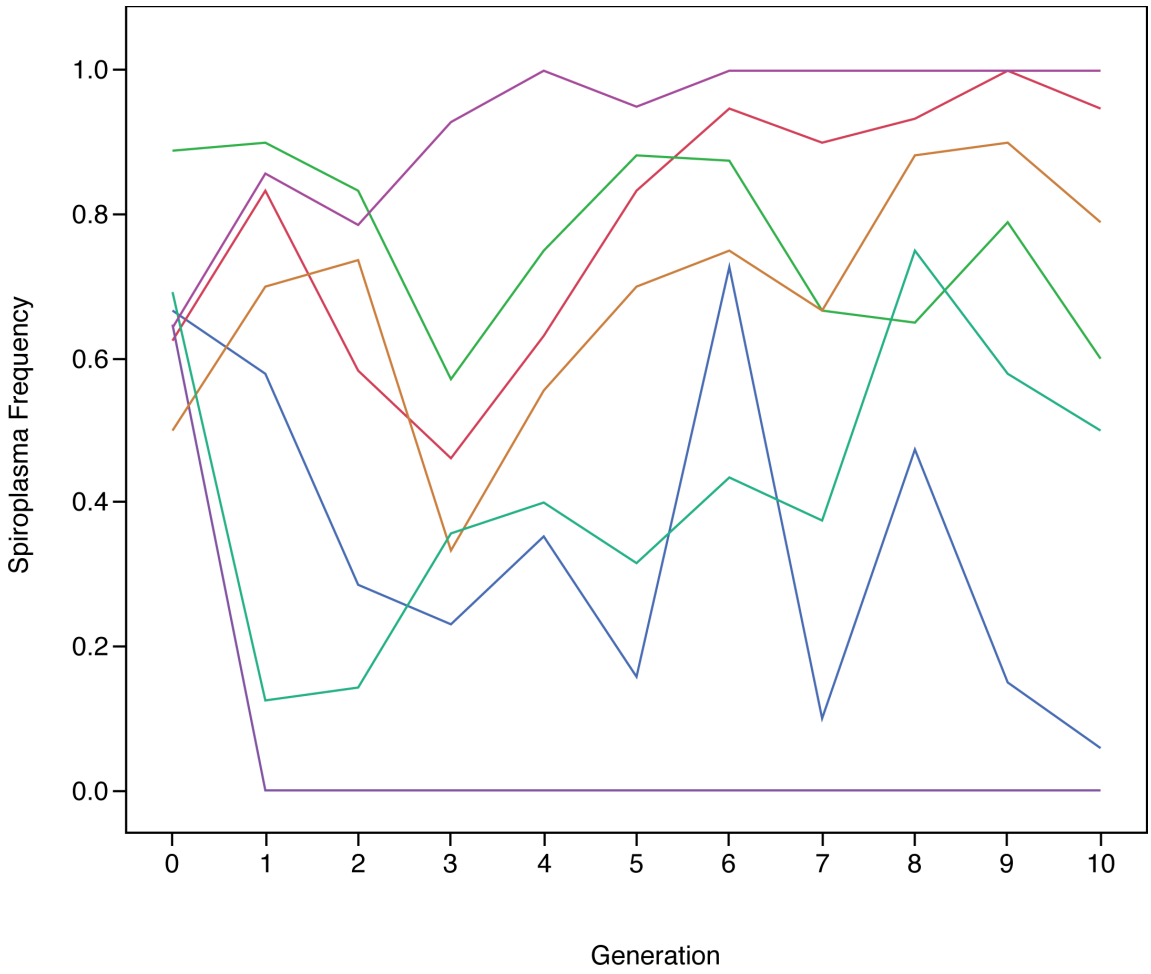

Figure 2. 3 The random distribution of the *Spiroplasma* frequency in the treatment $S^{+}Lh^{-}$ over ten generations. Each color indicates a single replicate bottle.

Thus, no evidence of selection for or against harboring *Spiroplasma* was observed in populations lacking wasps (Table 2. 4), where the pattern of *Spiroplasma* prevalence change over time appears to be random (Figure 2. 3).

Table 2. 4 Effect of wasp treatment and generation on *Spiroplasma* frequency.

Effects	*F*-ratio $_{(df)}$	*P*-value[†]
GLM on logit-transformed data for S^+Lh^+ and S^+Lh^- treatments		
Wasp treatment (fixed)	$26.13_{(1,1)}$	**<0.0001**
Generation (fixed)	$7.15_{(1,1)}$	**0.0091**
Wasp X Generation (fixed)	$5.50_{(1,1)}$	**0.0215**
GLM on logit-transformed data for the S^+Lh^+ and S^+Lh^- treatments separately		
S^+Lh^+		
Generation (fixed)	$9.18_{(1,1)}$	**0.0066**
S^+Lh^-		
Generation (fixed)	$0.2799_{(1,1)}$	0.5987
GzLM on the raw data of S^+Lh^- treatment (Binomial)		
Generation (fixed)	$0.40_{(10,65)}$	0.9423

2.3.2 Wasp oviposition rate and wasp success rate

Wasp oviposition rate was high throughout the experiment (overall mean 96.76 ± 0.86%), ranging from 88.57% in G10 to 100% in G2 and G4; Table 2. 3), and did not

differ significantly among treatments (S^+Lh^+ vs. S^-Lh^+; all generations) or among generations (Table 2. 5, Figure 2. 4). Wasp success rate (number of emerged wasps/total number of emerged flies and wasps) in the treatment lacking *Spiroplasma* (S^-Lh^+) ranged between 78.31 ± 4.36% (G9) and 97.37 ± 0.70% (G1, Table 2. 3, Figure 2. 5), with overall mean across generations of 90.19 ± 1.77%. No correlation was observed between wasp oviposition and wasp success rate (S^-Lh^+ treatment; Table 2. 5), which could be attributable to a lack of variance in oviposition rate. Interestingly however, in the treatment lacking *Spiroplasma* (S^-Lh^+), generation had a significant effect on wasp success rate. A post-hoc test indicated that the last generation tested (G9) was significantly lower than the other generations (G1, G3, G5, and G7; Figure 2. 5).

As expected, wasp success rate in the treatment containing *Spiroplasma*-infected flies (S^+Lh^+) was lower than in the treatment lacking *Spiroplasma*, and ranged from 45.71 ± 4.82% (G1) to 6.79 ± 3.10% (G2) to less than 1.5% in subsequent generations (Table 2. 3). Wasp success rate in G1 was significantly negatively correlated with the *Spiroplasma* frequency of the preceding generation G0 (Table 2. 5). This relationship could not be tested in subsequent generations due to lack of enough variation among replicates for both variables.

2.3.3 Vertical transmission rate

The overall vertical transmission rate of *Spiroplasma* hy1 was 99% throughout the experiment (range 97–100%; Table 2. 3), and did not differ significantly between the treatment exposed to wasps and the treatment lacking wasps (99.18% and 98.75%,

respectively). Vertical transmission also did not differ significantly among generations (Figure 2. 6; Table 2. 5).

Table 2. 5 Effects of different variables on wasp oviposition, wasp success, and vertical transmission of *Spiroplasma*.

Effects	*F*-ratio $_{(df)}$	*P*-value[†]
GzLM of wasp oviposition frequency (S^+Lh^+ and S^-Lh^+ in G1, 3, 5, 7, 9)		
Spiroplasma treatment (fixed)	$0.00_{(1,60)}$	0.9810
Generation (fixed)	$0.04_{(4,60)}$	0.9975
Treatments X Generation	$0.36_{(4,60)}$	0.8374
GzLM of wasp oviposition frequency (S^+Lh^+ in all generations; 4 outliers removed)		
Generation (fixed)	$0.90_{(9,56)}$	0.5302
GLM of wasp success rate (arcsine square root transformed; S^-Lh^+ in G1, 3, 5, 7, 9)		
Oviposition	$0.2638_{(1,1)}$	0.6114
Generation (fixed)	$4.6515_{(4,4)}$	**0.0050**
GLM of wasp success rate (arcsine square root transformed; S^+Lh^+ in G1)		
Spiroplasma frequency G0 (fixed)	$7.80_{(1,1)}$	**0.0364**
GzLM of *Spiroplasma* vertical transmission (S^+Lh^+ and S^+Lh^- in all generations)		
Generation (fixed)	$0.23_{(9,72)}$	0.9893
Wasp treatment (fixed)	$0.02_{(1,72)}$	0.8978

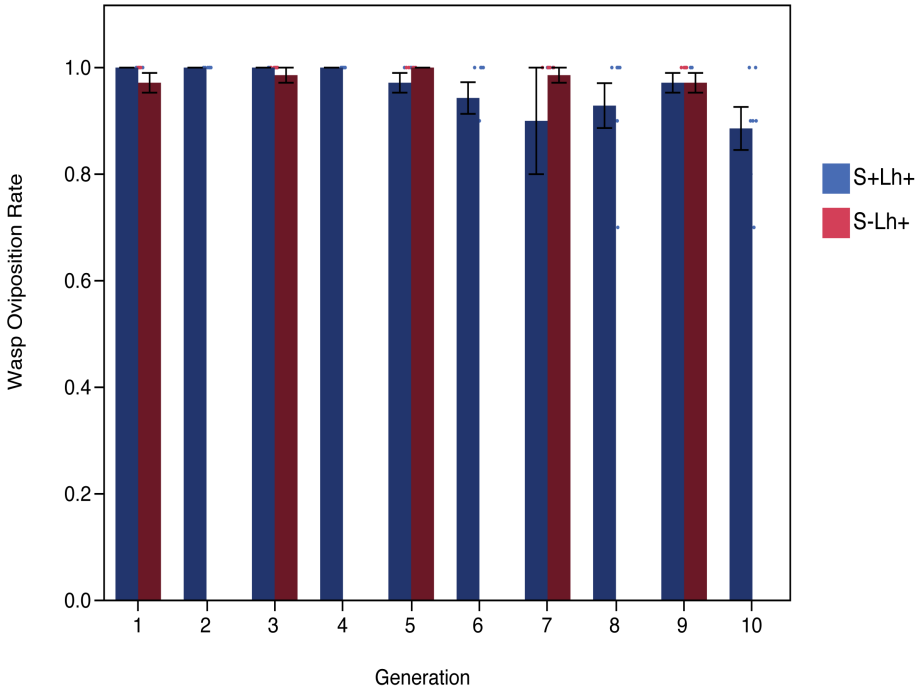

Figure 2. 4 Comparison of wasp oviposition frequency among fly larvae from the treatment of $S^{+}Lh^{+}$ (blue bar) and $S^{-}Lh^{+}$ (red bar) treatments. S = *Spiroplasma*; Lh = *Leptopilina heterotoma* treatment. Error bars: standard error.

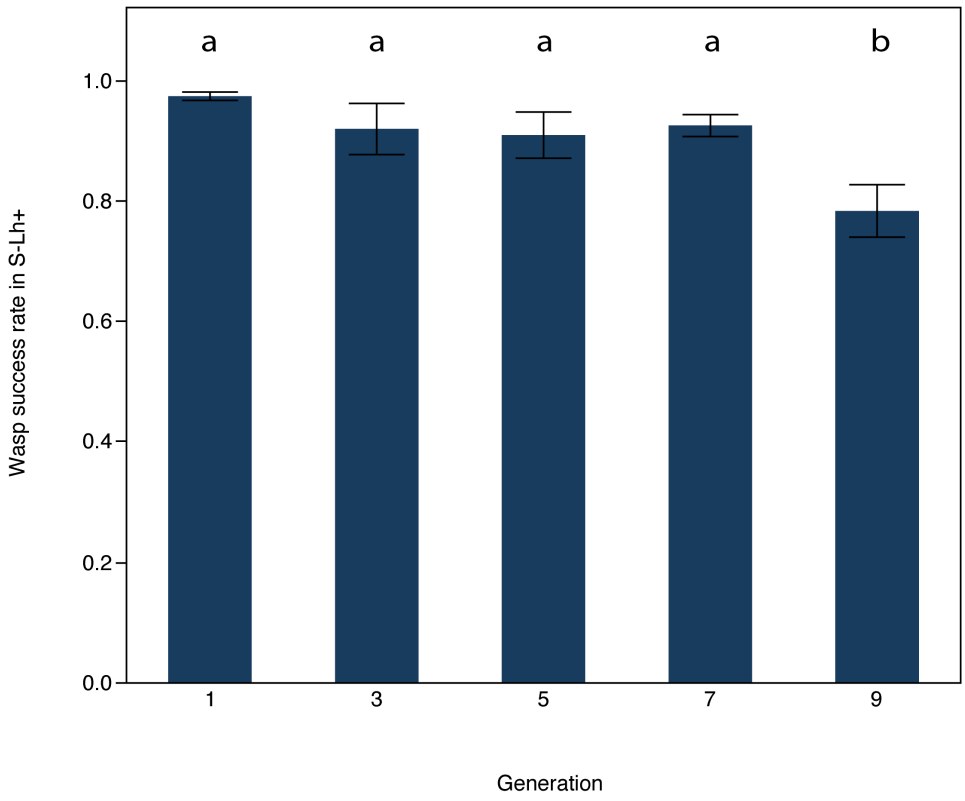

Figure 2. 5 Wasp success rate in the treatment of $S^{-}Lh^{+}$ treatment. Different lower case letters indicate significant ($p < 0.05$) post-hoc test among generations. *S* = *Spiroplasma*; *Lh* = *Leptopilina heterotoma* treatment. Error bars: standard error.

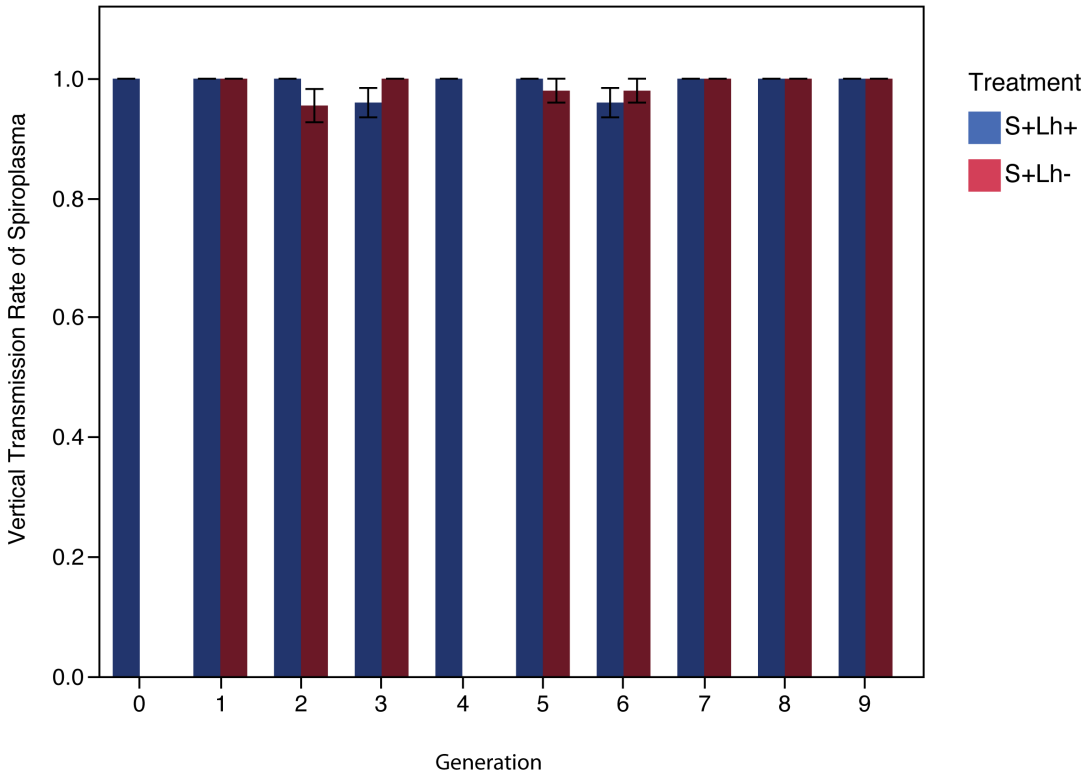

Figure 2. 6 Vertical transmission efficiency of *Spiroplasma* in females from the $S^{+}Lh^{+}$ and $S^{+}Lh^{-}$ treatments in all generations. *S* = *Spiroplasma*; *Lh* = *Leptopilina heterotoma* treatment. Error bar: standard error.

2.3.4 Realized female fly fecundity

As a measure of realized female fecundity, we recorded the number of flies emerging per bottle each generation, measured over the first ~12 days of fly emergence and standardized by the number of potential mothers (typically 50). Realized female fecundity varied significantly among treatments and generations. In the absence of the *Spiroplasma*, this measure was significantly larger in the treatment lacking wasps than in

the treatment exposed to wasps (Table 2. 6), consistent with the high wasp oviposition and wasp success described above. A significant effect of generation and generation X wasp treatment was observed (Table 2. 6). A closer look indicates that female fecundity in the treatment lacking wasps ($S^{-}Lh^{-}$) varied significantly among generations (Figure 2. 7), oscillating within ~3–11 progeny/female (Figure 2. 2B). A negative density dependence effect is apparent, because when female fecundity reached a threshold of ~10–11 progeny per female (e.g. G3 and G6; Figure 2. 2B), it decreased relatively rapidly in subsequent generations. This phenomenon may be explained by reduced reproductive fitness resulting from flies being exposed to high competition as larvae. Female fecundity in the treatment exposed to wasps ($S^{-}Lh^{+}$) appeared to increase slightly over time from 0.15 ± 0.04 in G1 to 0.64 ± 0.13 in G9, with the last two generations examined (G7 and G9) significantly higher than the first three (G1, G3, and G5; Figure 2. 7). This observation is somewhat consistent with the lower wasp success rate observed for G9 (see above), and cannot be explained by differences in wasp oviposition, which was high and not significantly different among generations.

Table 2. 6 Effects of different variables on realized female fecundity, measured as the number of emerged flies (during the first ~12 days of fly emergence) over the total number of potential mothers.

Effects	*F*-ratio $_{(df)}$	*P*-value[†]
GLM of realized female fecundity ($S^{-}Lh^{+}$ and $S^{-}Lh^{-}$ in G1, 3, 5, 7, 9)		
Wasp treatment (fixed)	$353.27_{(1,1)}$	**<0.0001**
Generation (fixed)	$10.32_{(4,4)}$	**<0.0001**
Treatment X Generation	$12.87_{(4,4)}$	**<0.0001**
GLM of realized female fecundity ($S^{+}Lh^{+}$, $S^{+}Lh^{-}$, and $S^{-}Lh^{-}$ in all ten generations)		
Spiroplasma and wasp treatment (fixed)	$4.24_{(2,2)}$	**0.0159**
Generation (fixed)	$12.82_{(9,9)}$	**<0.0001**
Treatment X Generation	$3.71_{(18,18)}$	**<0.0001**
GLM of realized female fecundity ($S^{-}Lh^{+}$ in G1, 3, 5, 7, 9; one outlier removed)		
Wasp oviposition frequency	$0.5436_{(1,1)}$	0.4671
Generation (fixed)	$5.8343_{(4,4)}$	**0.0015**

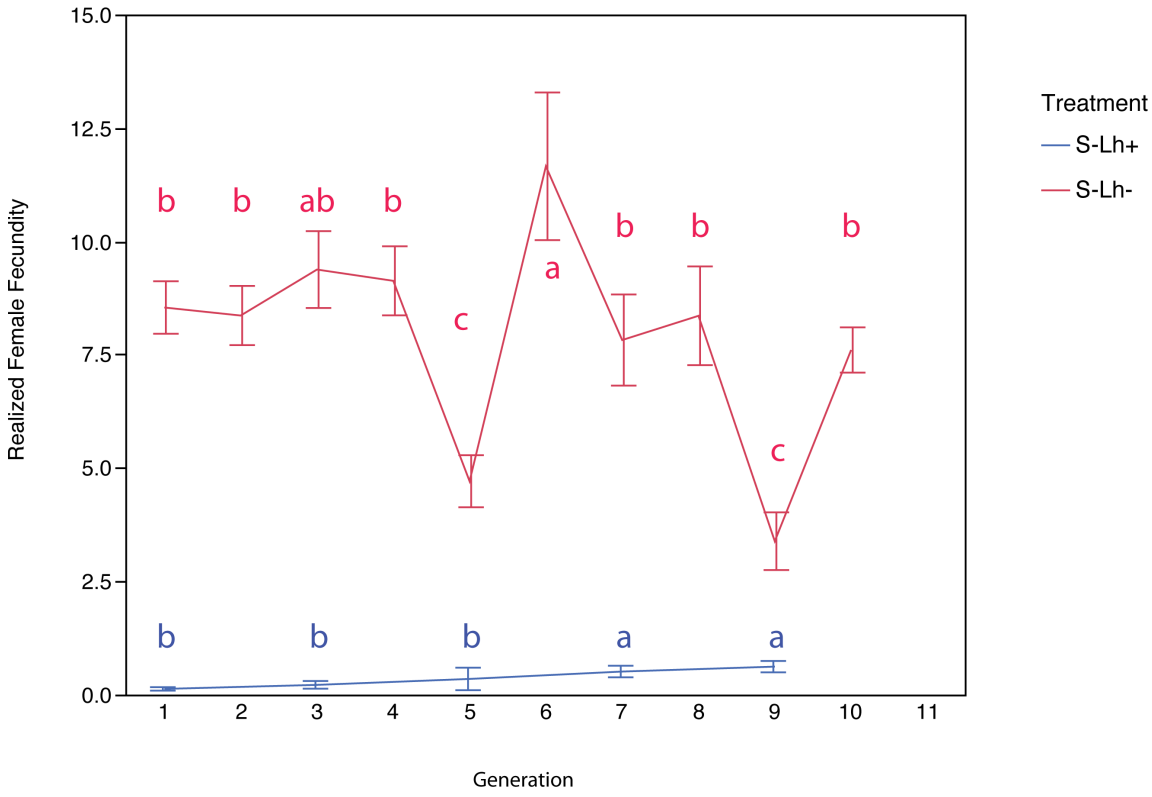

Figure 2. 7 Female fly realized fecundity in $S^{-}Lh^{+}$ (Blue) and $S^{-}Lh^{-}$ (Red) treatments. *S* = *Spiroplasma*; *Lh* = *Leptopilina heterotoma* treatment. Different lower case letters indicate significant ($p < 0.05$) post-hoc test between generations within each treatment. Error bar: standard error.

Realized female fecundity also varied across generations in the treatments containing *Spiroplasma*-infected flies ($S^{+}Lh^{+}$ and $S^{+}Lh^{-}$). To better understand the influence of *Spiroplasma* infection frequency on realized female fecundity and account for any effects of generation, we compared the two treatments lacking wasps ($S^{+}Lh^{-}$ and $S^{-}Lh^{-}$) and the $S^{+}Lh^{+}$ treatment in a single GLM analysis (Table 2. 6). Treatment, generation, and treatment X generation interaction were significant. Realized female

fecundity in the S^+Lh^+ treatment was significantly lower than in the treatments lacking wasps (S^+Lh^- and S^-Lh^-) in the first generation (G1; Figure 2. 2B), when *Spiroplasma* frequencies of the mothers (G0) were relatively low (59%). It gradually increased in the next three generations, and by G4, it was not significantly different from the S^+Lh^- and S^-Lh^- treatments. In none of the subsequent generations, was the S^+Lh^+ significantly lower than both the S^+Lh^- and S^-Lh^- treatments. In one case (G9), female fecundity in S^+Lh^+ was actually significantly higher than in both the S^+Lh^- and S^-Lh^- treatments. The initial increase in realized female fecundity of the S^+Lh^+ treatment is consistent with the rapid increase in *Spiroplasma* frequencies, but realized fecundity exhibited an apparent delay with regard to *Spiroplasma* frequencies. Mean *Spiroplasma* frequencies of G1 flies were ~93% (Table 2. 3, Figure 2. 2A). Given the high transmission rate (~99%), eggs laid by G1 females must have had a comparable *Spiroplasma* frequency (e.g. 93% x 99% = 92%). Nevertheless, the number of progeny produced by G1 flies was still relatively low compared to subsequent generations, in which changes in *Spiroplasma* prevalence were less drastic. This discrepancy cannot be explained by a change in wasp success, as wasp success in the S^+Lh^+ treatment was consistently low (< 7%) from G2 to G10. Therefore, the delay in realized female fecundity might be related to fitness costs associated with surviving a wasp attack (e.g. reduced number of eggs laid per female and reduced male fecundity; (Xie *et al.*, 2011)), and/or an allee effect.

2.3.5 Fly sex ratio

The proportion of male flies was not significantly different among any of the four treatments (S^+Lh^-, S^-Lh^-, S^+Lh^+ and S^-Lh^+) or among the first seven generations tested (Table 2. 7). The proportion of males averaged over treatments and generations was 47.31 ± 0.88%. These results indicate that infection by *Spiroplasma* does not alter the fly sex ratio. Furthermore, lack of an effect of wasp treatment on fly sex ratio implies that *D. hydei* males and females are equally susceptible to *L. heterotoma* parasitism.

Table 2. 7 Fly sex ratio, measured as males / (males + females).

Effects	*F*-ratio $_{(df)}$	*P*-value[†]
GzLMM of sex ratio		
Wasp x *Spiroplasma* treatment (fixed)	$1.76_{(3,164)}$	0.1572
Generation (Random)	$0.00_{(N/A)}$	0.9827

2.3.6 Estimate of the selection coefficients (*s*)

The selection coefficient (*s*) for infection by *Spiroplasma* hy1 in the *D. hydei* lab populations examined can be estimated from *Spiroplasma* prevalence changes over time by applying equation 1 (Section 3). In the present study, *Spiroplasma* frequency

changed most rapidly during the first generation, reaching a high frequency (93.38%), but not becoming fixed. Therefore, the parameters generated by this interval (which represents an unbounded space) likely enable the most accurate estimation of the selection coefficient (s) based on *Spiroplasma* frequency change. Based on the mean ± standard error *Spiroplasma* prevalence in G0 and G1, s = 0.7882–0.9632, assuming both vertical transmission (β) and wasp oviposition were 100% during this generation (Table 2. 8).

Table 2. 8 Calculation of the selection coefficient (s), based on *Spiroplasma* prevalence (I) change between G0 and G1. SE = Standard Error. β = vertical transmission efficiency. Equation 1 (see Section 3 for details): $s = (\beta\text{-}1) / (\beta I\text{-}1)$

	Mean	SE	mean – SE	mean + SE
I_0	0.5906	0.0646	0.526	0.6552
I_1	0.9338	0.0341	0.8997	0.9679
s (when β =1)	0.8977		0.7882	0.9632

Prevalence of *Spiroplasma* hy1 (a non-male killing strain) in natural populations of *D. hydei* reported to date ranges between 23–66% (Kageyama *et al.*, 2006; Watts *et al.*, 2009). To maintain an equilibrium frequency of 23–66%, when the vertical transmission rate (β) is 0.99 (as estimated in the present study throughout the 10

generations), *s* must range within ~0.0129–0.0289 (Table 2. 9). The estimated *s* in the present study based on the *Spiroplasma* frequency change (i.e., 0.7882–0.9632) is much larger than that needed to maintain frequencies reported in nature. Nevertheless, the vertical transmission of *Spiroplasma* hy1 is very sensitive to environmental temperature, and is completely blocked at 15ºC (Osaka *et al.*, 2008). Furthermore, broad variation in vertical transmission is observed among wild-caught females, even at optimal temperature for transmission (as low as 0.364 at 25°C; Osaka *et al.*, 2013a). If we consider this lower end of the vertical transmission range, then the required *s* to maintain equilibrium frequencies of 23–66% is much larger (0.6941–0.8371, Table 2. 9), and more similar to the selection coefficient estimated in the present study (i.e., 0.7882–0.9632).

Table 2. 9 Required selection coefficient (*s*) under *Spiroplasma* equilibrium prevalence for different vertical transmission (*β*) values.

	Spiroplasma equilibrium frequency	
Vertical Transmission (*β*)	0.23	0.66
1	0	0
0.99	0.0129	0.0289
0.364	0.6941	0.8371

2.4 Discussion

Exposure to high parasitism pressure from the parasitoid wasp *Leptopilina heterotoma* resulted in a rapid increase of *Spiroplasma* prevalence in lab populations of *Drosophila hydei*. This is consistent with our previous finding that larva-to-adult survival is higher in *Spiroplasma*-infected than -uninfected flies exposed to *L. heterotoma* (Xie *et al.*, 2010), due to the strongly negative effects that *Spiroplasma* exerts on wasp growth and eclosion rate (Xie *et al.*, 2011). Longevity and fecundity of *Spiroplasma*-infected females and males surviving a wasp attack is somewhat compromised (Xie *et al.*, 2011), however, raising the question as to whether populations exposed to high wasp parasitism are sustainable. Furthermore, although prior studies did not detect fitness costs associated with *Spiroplasma* infection (Xie *et al.*, 2010; Xie *et al.*, 2011; Osaka *et al.*, 2013a), their experimental setup comparing fitness measures of host lines reared separately over one or two generations, might have provided limited power to detect slight differences in fitness (Oliver *et al.*, 2008). Nonetheless, the present study corroborates previous findings that *Spiroplasma* infection in the absence of wasps is effectively neutral. It is possible however, that context dependent fitness costs to *Spiroplasma* infection exist. For example, Herren and Lemaitre (2011) reported that *D. melanogaster* infected with *Spiroplasma* strain MSRO are more susceptible to Gram-negative pathogens.

Our results represent another experimental demonstration that defensive endosymbionts can spread rapidly in a host population as a result of protection against natural enemies. Rapid spread of a defensive endosymbiont due to selection pressure

from natural enemies of its host has been reported in lab and natural populations (Oliver *et al.*, 2013). Oliver *et al.* (2008) reported that prevalence of *Hamiltonella defensa*, the symbiont that confers protection to the aphid *Acyrthosiphon pisum* against the parasitic wasp *Aphidius ervi*, increased rapidly in lab populations exposed to the parasitoid. Similarly, rapid spread of *Spiroplasma* strain neo, the symbiont that restores fertility of *D. neotestacea* females parasitized by the sterilizing nematode *Howardula aoronymphium*, is reported in both natural and lab fly populations exposed to nematodes (Jaenike *et al.*, 2010b; Jaenike & Brekke, 2011). Nevertheless, in the absence of the natural enemy, different patterns are observed. The prevalence of *H. defensa* exhibits a steady decline in the absence of the parasitoid, implying a fitness cost to infection (Oliver *et al.*, 2008). In contrast, the prevalence of *Spiroplasma* strain neo in lab populations of *D. neotestacea* not exposed to nematodes (Jaenike & Brekke, 2011) is similar to the prevalence of *Spiroplasma* strain hy1 in our lab populations not exposed to wasps, with no significant change in the mean prevalence over time, but large variation among replicates consistent with random drift of *Spiroplasma*-infected and -uninfected cytotypes. Lack of detectable fitness costs to *Spiroplasma* infection in *D. hydei* not exposed to wasps, suggests that any reductions in *Spiroplasma* frequency in natural populations would be mostly attributable to imperfect vertical transmission.

The selection coefficient (s) for *Spiroplasma* infection estimated from the frequency change in our lab populations (s = 0.7882–0.9632) is much higher than the s of ~0.0129–0.0289 required to maintain equilibrium frequencies of 23–66% (i.e., the range of *Spiroplasma* frequencies reported in natural populations of *D. hydei,* Kageyama

et al., 2006), assuming a highly efficient vertical transmission rate (β) of 0.99 (i.e., the rate estimated in the present study). Vertical transmission of *Spiroplasma* by wild caught *D. hydei*, however, is highly variable among individuals, even under an optimal temperature of 25°C, and may be as low as 0.36 (Osaka *et al.*, 2013a). Similarly, vertical transmission of *Spiroplasma* strain WSRO in *D. willistoni* is influenced by fly female age and genetic background (Ebbert, 1991). Under a lower vertical transmission value of 0.36, the selection coefficient (s) required to maintain an equilibrium frequency of 23–66% is much higher (0.6941–0.8371), and more similar to estimates based on our prevalence results.

Vertical transmission of *Spiroplasma* in lab populations of *D. hydei* and *D. melanogaster* is very sensitive to temperature (Montenegro & Klaczko, 2004; Anbutsu *et al.*, 2008; Osaka *et al.*, 2008). Whereas vertical transmission in *D. hydei* is nearly perfect at 25 and 28°C, it is partially suppressed at 18°C, and completely suppressed at 15°C (Osaka *et al.*, 2008). Due to the influence of ambient temperature, vertical transmission, and thus *Spiroplasma* prevalence, are expected to vary over time and space in natural populations. Indeed, *Spiroplasma* frequency is positively associated with increased temperatures in some natural populations of Japan, but this phenomenon does not hold for all years and populations examined (Osaka *et al.*, 2011). Therefore, other factors must interact with temperature in determining *Spiroplasma* frequencies.

Parasitoid abundances themselves exhibit spatial and temporal variation. Therefore, host populations likely experience fluctuations in selection pressure from parasitoid wasps in nature. For instance, in southern France, the prevalence of *L.*

heterotoma is much higher in May than later in the summer (Fleury *et al.*, 2009). One way in which *Spiroplasma* may be able to persist is if cooler periods, during which vertical transmission is low, were accompanied by high wasp parasitism. This possibility remains to be tested. Horizontal transmission of *Spiroplasma* by mites in *D. hydei* may also contribute to the maintenance of *Spiroplasma* in host populations (Jaenike *et al.*, 2007; Osaka *et al.*, 2013b). Finally, increased *Spiroplasma* prevalence in *D. neotestacea* is reported to reduce abundance of the parasitic nematode *Howardula* (Jaenike & Brekke, 2011). Whether *Spiroplasma* prevalence in *D. hydei* affects abundance *L. heterotoma* is unclear, because this wasp is a "generalist" capable of utilizing multiple *Drosophila* species (Schlenke *et al.*, 2007).

3. MALE KILLING *SPIROPLASMA* PROTECTS *DROSOPHILA MELANOGASTER* AGAINST TWO PARASITOID WASPS

3.1 Introduction

Associations between maternally transmitted endosymbiotic bacteria and insect hosts are pervasive and exert strong influence on their ecological and evolutionary dynamics (Moran *et al.*, 2008). Some of these heritable symbioses are obligate, with host and symbiont completely dependent on each other for persistence (e.g. nutritional mutualisms; Douglas, 1998). Many other heritable symbionts are facultative, and thus, not absolutely required by the host for survival and reproduction (White *et al.*, 2013). Approximately 40–66% of arthropod species are estimated to be infected with heritable facultative symbionts from a single bacterial genus (*Wolbachia*) (Hilgenboecker *et al.*, 2008; Zug & Hammerstein, 2012), but many more bacterial groups engage in such associations with insects (Moran *et al.*, 2008). Vertical transmission of facultative symbionts is typically imperfect, and harboring the symbiont can be physiologically costly to the host. Consequently, heritable facultative symbionts can only persist in host populations, if they increase either the survival or production of infected female hosts (O'Neill *et al.*, 1997). To ensure persistence, heritable facultative symbionts have adopted various strategies; namely, reproductive manipulation of their host (e.g. male-killing and cytoplasmic incompatibility; Werren *et al.*, 2008; Engelstadter & Hurst, 2009), and/or enhancement of host fitness through a diversity of mechanisms (Brownlie & Johnson, 2009; Ferrari & Vavre, 2011; Jaenike, 2012).

Several recent studies have reported facultative symbionts that confer protection to their host against parasites and pathogens (Hurst & Hutchence, 2010). Several bacterial symbionts of aphids confer protection against parasitoid wasps (Oliver *et al.*, 2003a; Oliver *et al.*, 2005; Vorburger *et al.*, 2009) and fungi (Scarborough *et al.*, 2005; Lukasik *et al.*, 2012). *Spiroplasma* bacteria confer protection against fungi in the pea aphid (Lukasik *et al.*, 2012), against a nematode in *Drosophila neotestacea* (Jaenike *et al.*, 2010b), and against a parasitoid wasp in *Drosophila hydei* (Xie *et al.*, 2010). *Wolbachia* has been shown to increase resistance or tolerance of *Drosophila* and mosquitoes against RNA viruses and against the protozoan parasite *Plasmodium* (Hedges *et al.*, 2008; Teixeira *et al.*, 2008; Moreira *et al.*, 2009; Osborne *et al.*, 2009; Bian *et al.*, 2010; Frentiu *et al.*, 2010; Zele *et al.*, 2012).

There is growing evidence that endosymbionts can employ more than one strategy to enhance their persistence. Indeed, the use of cytoplasmic incompatibility (CI) and protection against RNA viruses, by *Wolbachia* in dipterans (Hedges *et al.*, 2008; Teixeira *et al.*, 2008; Moreira *et al.*, 2009; Osborne *et al.*, 2009; Glaser & Meola, 2010; Walker *et al.*, 2011), may explain the recent spread of *Wolbachia* in natural populations of *D. melanogaster* (Riegler *et al.*, 2005; Nunes *et al.*, 2008; Richardson *et al.*, 2012), and makes *Wolbachia* a promising agent for the control of dengue (Iturbe-Ormaetxe *et al.*, 2011; Walker *et al.*, 2011), a human pathogen transmitted by mosquitoes. Similarly, *Rickettsia* bacteria associated with whiteflies (order Hemiptera) directly enhance host fitness and also bias sex ratio towards female offspring (Himler *et al.*, 2011). The fitness of *Drosophila innubila* infected with a male-killing *Wolbachia*

strain is enhanced by both, male-killing dependent (i.e., resource reallocation due to death of male siblings) and male-killing independent mechanisms (i.e., enhanced fecundity of nutrient-deprived hosts and increased survival to RNA virus infection; Unckless & Jaenike, 2012). Not all reproductive parasites examined to date, however, confer protection against natural enemies (e.g. the male-killing *Wolbachia* strain of *Drosophila bisfasciata* does not confer protection against RNA viruses (Longdon *et al.*, 2012).

In *Drosophila*, the two defensive *Spiroplasma* strains known do not appear to engage in reproductive manipulation (Ota *et al.*, 1979; Jaenike *et al.*, 2010b), but several of their close relatives are male-killers. One of these male-killing strains is the Melanogaster Sex Ratio Organism (hereafter MSRO), which can co-occur with *Wolbachia* in certain populations of *D. melanogaster*. When present, infection frequencies of *Spiroplasma* MSRO in wild populations of *D. melanogaster* range within 1.1–17% (Montenegro *et al.*, 2005; Ventura *et al.*, 2012). It is unclear whether direct or indirect fitness effects of male-killing are sufficient to maintain such infection frequencies, particularly those at the higher end. Martins *et al.* (2010) found that MSRO-infected wild females have a higher fecundity (at least over four consecutive days), and their progeny develop faster. In contrast, Montenegro *et al.* (2006) reported no effect of MSRO on larval competitive ability or adult fecundity of *D. melanogaster* Canton-S strain. It is possible that other fitness effects unrelated to its male-killing ability contribute to the prevalence of MSRO in nature. The work presented herein examines whether the male-killing *Spiroplasma* strain of *D. melanogaster* (MSRO),

confers protection against parasitoid wasps. We also examine whether *w*Mel, the *Wolbachia* strain known to cause CI and protect against RNA viruses, influences the outcome of the fly-parasitoid interaction.

Co-occurrence of two cytoplasmically-transmitted symbionts may lead to cooperation or antagonism between them. Based on the non-random positive association of *Wolbachia* and *Spiroplasma* observed in *D. neotestacea* populations, Jaenike *et al.* (2010a) suggest that mutualism between the two symbionts might have evolved, but evidence for a cooperation mechanism itself has not been found. In contrast to *D. neotestacea*, no evidence for significant associations between the two symbionts has been observed in natural populations of *D. melanogaster* (Ventura *et al.*, 2012). In addition, Montenegro *et al.* (2006) found no evidence of cooperation between the two endosymbionts in *D. melanogaster*, based on several lab-based fitness measures. Two instances of antagonism between make-killing *Spiroplasma* and *Wolbachia* have been observed. *Spiroplasma* densities negatively affect *Wolbachia* densities in *D. melanogaster* (Goto *et al.*, 2006), but not vice versa (Goto *et al.*, 2006; Silva *et al.*, 2012). In addition, Silva *et al.* (2012) found that the male-killing ability of *Spiroplasma* MSRO was stronger in the absence of *Wolbachia*. Other cases of conflict or cooperation, however, may be revealed under conditions not tested to date, such as in the defense against natural enemies. Therefore, our study also examines if the outcome of a parasitoid wasp attack is influenced by co-occurrence of *Wolbachia* and *Spiroplasma*.

The specificity of the symbiont-mediated protection against natural enemies will influence the ecological and evolutionary dynamics of the host and its protective

symbiont. Numerous species of parasitoid wasps attack *Drosophila* flies (Fleury *et al.*, 2009). *D. melanogaster* alone is an adequate host to at least 14 species from four families of parasitic Hymenoptera that employ diverse strategies to circumvent host defenses (Kacsoh & Schlenke, 2012). Our study examined whether *Spiroplasma* and *Wolbachia* influence the outcome of parasitism by two cosmopolitan congeneric wasps that differ in their host range and attack strategies: the *Drosophila* generalist *Leptopilina heterotoma* (Lh) and the melanogaster-group specialist *L. boulardi* (Lb). Although Lb causes partial suppression of host defenses, it tends to passively evade host immunity by embedding its eggs within host tissues, thereby avoiding encapsulation by host lamellocytes. In contrast, the eggs of Lh, which float freely in the host hemocoel, avoid encapsulation via a more aggressive suppression of host defenses, including the destruction of lamellocytes (Lee *et al.*, 2009). Therefore, knowledge on the parasitoid species against which *Spiroplasma* confers protection, will provide insight into the generality of the protection and the possible defensive mechanism(s).

This work expands our knowledge on defensive associations of *Drosophila* in general, and of the model organism *D. melanogaster* in particular, by revealing that: (a) as reported for its non-male-killing counterpart, a male-killing *Spiroplasma* strain is capable of protecting its host against wasp-induced mortality, by slowing down wasp larval growth and preventing successful wasp development; (b) although the observed degree of protection alone might not guarantee *Spiroplasma* prevalence in nature, it may be relevant to persistence in combination with the fitness advantages derived from its male-killing ability; (c) this protection is conferred against two species of wasps with

contrasting strategies, suggesting a general defensive mechanism; and (d) the positive additive effect of *Wolbachia* and *Spiroplasma* on fly survival against attack by at least one species of wasp, provides empirical evidence of a mechanism by which two cytoplasmically-transmitted endosymbionts could become mutualists.

3.2 Materials and Methods

3.2.1 Insect sources

Seven isofemale-lines (hereafter fly isolines) were established from mated wild-caught *D. melanogaster* females collected with orange baits in Tapachula, Chiapas, Mexico, in January 2011. To identify potential heritable endosymbionts of these flies, at least three females per isoline were subjected to sterile ovary dissection and DNA extraction as described in Mateos *et al.* (2006). Three sets of universal PCR primer screenings were then conducted on the DNA extracts: 1) Primers for bacterial 16S rRNA gene (10F–1507R); 2) primers for bacterial 16S rRNA gene (27F–1495R); and 3) primers for 16S-23S rRNA gene fragment (559F–35R). In addition, screening with *Wolbachia*- and *Spiroplasma*-specific PCR primers was conducted (primers and conditions described in Xie *et al.*, 2010). These results indicated that all seven isofemale lines were infected with *Wolbachia w*Mel, but not with any other heritable endosymbionts.

For the generalist wasp *L. heterotoma*, we used the highly virulent inbred strain Lh14, which is infected with *Wolbachia* (Schlenke *et al.*, 2007). For the specialist wasp *L. boulardi*, we used the highly virulent inbred strain Lb17. This wasp strain lacks infection by *Wolbachia* (Schlenke *et al.*, 2007) and by the *Leptopilina boulardi*

Filamentous Virus (LbFv; Gueguen *et al.*, 2011), a virus linked to superparasitism behavior in this species (Varaldi *et al.*, 2003; Varaldi *et al.*, 2006). Wasps were maintained in *Drosophila melanogaster* Canton-S with standard cornmeal food.

3.2.2 Generation of endosymbiont treatments

For each of the seven original fly isolines, we generated four endosymbiont treatments: uninfected (S^-W^-); infected with *Wolbachia* *w*Mel only (S^-W^+); infected with *Spiroplasma* MSRO only (S^+W^-); and doubly infected (S^+W^+) (see Supplementary Figure S1). To generate the *Wolbachia*-free (W^-) treatments, a subset of each isoline was treated for three consecutive generations with a combination of Tetracycline and Erythromycin (added to the food at a final concentration of 0.2 and 0.16 mg/ml; respectively). The *Wolbachia*-specific PCR screening described above confirmed removal of *Wolbachia*. In an effort to restore their regular microbiota, flies eclosing from the antibiotic treatment were temporarily placed in vials that had previously housed un-treated flies, and maintained on antibiotic-free food for three consecutive generations. A subset of the resulting 14 fly lines, seven lacking *Wolbachia* (W^-) and seven infected with *Wolbachia* (W^+), were then artificially infected with *Spiroplasma* MSRO via adult-to-adult hemolymph transfer as described in Xie *et al.* (2010). The donor flies were naturally-infected with *Spiroplasma* MSRO, and were originally collected in Campinas, São Paulo State, Brazil (1997) and maintained in the lab by crossing to Canton-S males (Montenegro *et al.*, 2000). Success of artificial infection and establishment of vertical

transmission of *Spiroplasma* was confirmed by all-female progeny and PCR screenings with *Spiroplasma*-specific primers over at least three subsequent generations.

3.2.3 Fly survival assay

This experiment was carried out at least four generations after *Spiroplasma* artificial infection. Prior to experiments, all the flies were maintained at low-density larval conditions. For each isoline and endosymbiont treatment (7 isolines x 4 endosymbiont treatments = 28), we conducted approximately three replicates (28 x 3 = 84 replicates). Each replicate consisted of a mating/oviposition group (3 females plus 6 males). Females were < 15d old; males were from the same isoline and *Wolbachia* infection status as females, but free of *Spiroplasma*. Mating groups were allowed to mate and oviposit on standard cornmeal vials for two days, after which they were transferred to a fresh food vial. Approximately 30 first/second instar larvae (2d old) per vial were collected and transferred into a fresh vial. Three larvae vials were generated per replicate (approximately 84 x 3 = 252 larvae vials; see Supplementary Figure S1). Each vial per replicate was subjected to one of the following wasp treatments: (1) no wasp control; (2) *L. heterotoma* (Lh); or (3) *L. boulardi* (Lb). Five ~3d old wasps were added per vial and allowed to oviposit for 2d. For each vial, we recorded the number of starting fly larvae, puparia, emerging flies, and emerging wasps. Endosymbiont infection status of the three mothers used in each replicate was examined by the *Wolbachia*- and *Spiroplasma*-specific PCR assays described above. Only replicates for which all three mothers had the expected infection status were used in the analyses. In

addition, to assess *Spiroplasma* MSRO vertical transmission rate in the presence and absence of *Wolbachia*, we used PCR to examine the *Spiroplasma* infection status of 10 female flies per replicate per isoline emerging from the treatments lacking wasps (approximately = 140 total).

We used SAS Enterprise Guide version 4.2 statistical package to fit a Generalized Linear Mixed Model (GzLMM) with a binomial distribution of the raw data for: (a) number of emerging adult flies/initial number of fly larvae (i.e., fly larva-to-adult survival rate); (b) number of emerging adult flies/total number of puparia (i.e., fly pupa-to-adult survival rate); (c) number of pupae/initial number of fly larvae (i.e., fly larva-to-pupa survival rate); (d) number of emerging adult wasps/initial number of fly larvae; and (e) number of emerging adult wasp/total number of puparia. The independent variables were *Spiroplasma* infection status (fixed), *Wolbachia* infection status (fixed) and their interaction term (fixed), fly strain (isoline, random). The random interactions (i.e., isoline X *Wolbachia*, isoline X *Spiroplasma*, isoline X *Wolbachia* X *Spiroplasma*) were excluded from final model due to lack of significance. Significance tests of random effects were based on the ratio of pseudo-likelihoods (Covtest in SAS).

3.2.4 Differential oviposition and development of parasitoids in *D. melanogaster*

To examine whether wasps lay different number of eggs in fly larvae with different endosymbiont infections, we compared the number of wasp eggs or larvae per fly larva among the four endosymbiont infection treatments. In addition, to examine whether *Spiroplasma* MSRO and/or *Wolbachia w*Mel affect the larval growth rate of Lh and Lb

in *D. melanogaster*, we measured wasp body length in the four endosymbiont infection treatments at several time points. These assays were conducted separately from the fly fitness experiments on three out of the seven isolines. We followed the same protocol described above to set up mating groups, collect larvae, and apply the wasp treatments, except that the no-wasp control was omitted. Immediately after wasp removal (hereafter time point 0 h), ten fly larvae were collected per vial, and dissected under a microscope to count and measure wasp eggs/larvae. To examine wasp growth, we measured body length of the dominant wasp larva in each of five fly larvae per vial at one subsequent time point (72 h) for Lh, and at two subsequent time points (72 h and 144 h) for Lb (only one subsequent time point was necessary to detect differences between endosymbiont treatments in Lh; see Results). The dominant wasp larva in each fly larva was fixed in ~96% ethanol and immediately digitally photographed with a stage micrometer. The software Spot Basic (version 4.7; Diagnostic Instruments, Inc., Sterling Heights, MI) was used to measure body length as the straight-line distance between the tip of the mouth and caudal end.

For the differential oviposition assay, we examined 20–40 fly larvae (10 larvae per vial) per treatment per fly isoline; each fly larva was treated as a replicate. We used SAS Enterprise Guide version 4.2 statistical package to fit a Generalized Linear Mixed Model (GzLMM) with: (a) a binary distribution of the raw data for at least one vs. zero wasp eggs or larvae per fly larva; and (b) a Poisson distribution of the raw data for the number of the wasp eggs or larvae per fly larva. The independent variables were *Spiroplasma* infection status (fixed), *Wolbachia* infection status (fixed), and their

interaction term (fixed), fly strain (isoline, random), and vial (random, nested within isoline). Significance tests of random effects were based on the ratio of pseudo-likelihoods (Covtest in SAS).

For the wasp development assay, we performed at least three replicates per treatment per fly isoline; each replicate corresponded to a measurement of the dominant wasp egg/larva in a single fly larva. We used SAS Enterprise Guide version 4.2 statistical package to fit a General Linear Mixed Model (GLMM) with the raw measurement of wasp body length. The independent variables were *Spiroplasma* infection status (fixed), *Wolbachia* infection status (fixed), hours-post wasp attack (fixed), and all of their interaction terms (fixed); and fly strain (isoline, random). Non-significant interactions were excluded from the final analysis. Significance tests of random effects were based on the ratio of pseudo-likelihoods (Covtest in SAS).

3.3 Results

3.3.1 Fly survival and wasp success

In the absence of parasitoid wasps, mean fly larva-to-adult survival was > 87.85% in all the endosymbiont infection treatments (Figure 3. 1A). Neither *Spiroplasma* nor *Wolbachia* infection states were significant for any of the fly survival measures. The effect of fly isoline, however, was significant for both larva-to-pupa survival ($\chi^2 = 5.72$, $P = 0.0084$; Figure 3. 1A and Table S1) and pupa-to-adult survival ($\chi^2 = 2.87$, $P = 0.0451$; Figure 3. 1A and Table S1), but not for larva-to-adult survival ($\chi^2 = 0.59$, $P =$

0.221; Table S1). The effect of isoline was not significant for any of the survival measures in any of the wasp treatments (Table S1), and is thus not discussed any further.

In the presence of the generalist wasp *Leptopilina heterotoma* (Lh), *Spiroplasma* infection had a significantly positive effect on fly larva-to-adult survival and on pupa-to-adult survival (respectively, $F_{1,84} = 6.72$, $P = 0.0041$ in Figure 3. 1B and $F_{1,84} = 9.34$, $P = 0.003$ in Table S1). Similarly, *Wolbachia* infection also had a significantly positive effect on these two measures ($F_{1,84} = 5.16$, $P = 0.0256$ in Figure 3. 1B; $F_{1,84} = 4.58$, $P = 0.0353$, Table S1). The interaction between *Spiroplasma* and *Wolbachia* was not significant. The positive effect of each symbiont on fly survival was small and appears to be additive or slightly synergistic; mean larva-to-adult survivorship of the four endosymbiont treatments was: endosymbiont-free (S^-W^-) = 0.86%; *Wolbachia*-infected (S^-W^+) = 2.59%; *Spiroplasma*-infected (S^+W^-) = 3.28%; and doubly-infected (S^+W^+) = 7.78% (Table S1).

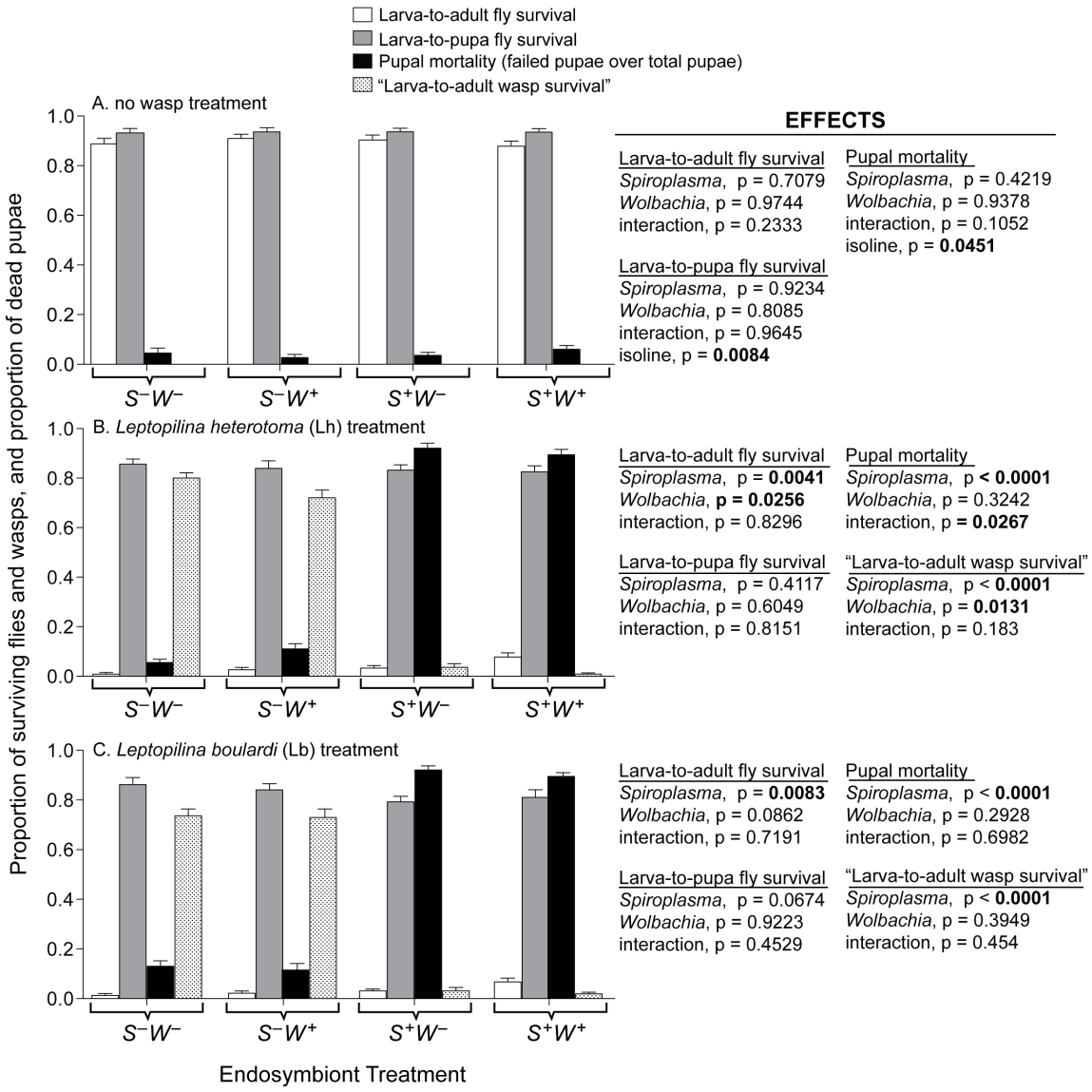

Figure 3. 1 Fly larva-to-adult survival, larva-to-pupa survival, pupal mortality, and wasp success in the four endosymbiont infection treatments (S = *Spiroplasma*; W = *Wolbachia*) and in the three wasp treatments. A. No wasp control. B. *Leptopilina heterotoma* (Lh) treatment. C. *Leptopilina boulardi* (Lb) treatment. P-values shown for each effect: *Spiroplasma*-infection state; *Wolbachia*-infection state; their interaction; and fly strain (isoline). For isoline, only significant *P*-values are shown (see Table S1). Bars: white, proportion of fly larvae that survived to adulthood; grey, proportion of fly larvae that survived to pupation; black, proportion of total pupae that failed (neither fly nor wasp emerged); dotted, exposed fly larvae that gave rise to eclosing wasps. Error bars: standard error.

Spiroplasma had a strong and highly significant ($F_{1,84} = 196.39$, $P < 0.0001$; Figure 3. 1B and Table S1) negative effect on the success of Lh, measured as the proportion of exposed fly larvae that gave rise to eclosing wasps: *Spiroplasma*-infected means were 3.63% (S^+W^-) and 0.9% (S^+W^+), whereas *Spiroplasma*-free means were 80.03% (S^-W^-) and 72.09% (S^-W^+). *Wolbachia* appears to reduce Lh wasp success slightly, albeit significantly ($F_{1,84} = 6.42$, $P = 0.013$, Figure 3. 1B and Table S1). In essence, a large proportion (~89–92%) of pupae failed to complete development in the *Spiroplasma*-infected Lh-attacked treatments, but not in the absence of *Spiroplasma* (~5–13%) or in the absence of wasps (~3–6%; Figure 3. 1 and Table S1). These results suggest that although *Spiroplasma* may not be highly efficient at rescuing the flies from a wasp attack, it is efficient at preventing wasp success. The effects of either symbiont were only detectable in measures encompassing the pupa-to-adult stage. In contrast, larva-to-pupa survival was relatively high and not significantly different among endosymbiont treatments (range = 82.51–85.66%; Figure 3. 1B and Table S1).

In the presence of the specialist parasitoid wasp *Leptopilina boulardi* (Lb), the effect of *Spiroplasma*, but not of *Wolbachia*, on fly survival and wasp success was similar to that observed in the presence of Lh. *Spiroplasma* significantly enhanced fly larva-to-adult survival ($F_{1,87} = 7.29$, $P = 0.0083$ in Figure 3. 1C) and pupa-to-adult survival ($F_{1,87} = 9.26$, $P = 0.0031$; Table S1). In contrast, although the means suggest a potentially positive effect of *Wolbachia* on fly survival (Figure 3. 1C), this effect was not significant for any of the fly survival measures. As in the Lh treatment, the effect of *Spiroplasma* on fly fitness in the Lb treatment was only detectable in measures involving

the pupa-to-adult stage. Despite the significant effect of *Spiroplasma* on fly fitness, the fitness benefit from *Spiroplasma* infection is small (mean larva-to-adult survival: $S^{-}W^{-}$ = 1.26%; $S^{-}W^{+}$ = 2.16%; $S^{+}W^{-}$ = 3.13%; and $S^{+}W^{+}$ = 6.91%; Table S1). Nevertheless, wasp success in the presence of *Spiroplasma* was extremely low (mean $S^{+}W^{+}$ = 1.89%; mean $S^{+}W^{-}$ = 3.15%) and significantly different from the treatments lacking *Spiroplasma* (mean $S^{-}W^{-}$ = 73.6%; mean $S^{-}W^{+}$ = 72.95%). As with Lh, the main outcome of *Spiroplasma*-infection in the Lb treatments was failed pupae, which contrasts with the relatively high success of both wasp species in the absence of *Spiroplasma*.

The higher fly survival observed in S^{+} treatments (which were all-female) could be due to a higher host-encoded resistance of female flies against *Leptopilina* wasps, rather than *Spiroplasma*-encoded protection. Indeed, a study by Kraaijeveld *et al.* (2008) found that *Drosophila* males are less likely than females to encapsulate an egg from the braconid wasp *Asobara tabida*. We therefore tested for an effect of *Leptopilina* treatment on host sex ratio in treatments lacking male-killing *Spiroplasma*: *D. melanogaster* with and without *Wolbachia* ($S^{-}W^{+}$ and $S^{-}W^{-}$; respectively); and *D. hydei* with and without a non-male-killing strain of *Spiroplasma* ($S^{+}W^{-}$ and $S^{-}W^{-}$; respectively) that confers protection against Lh (Xie *et al.*, 2010). The effect of *Leptopilina* on host sex ratio (proportion of surviving male flies) was not significant (see Table S4 for results and details). These results are consistent with a protective effect of *Spiroplasma* against *Leptopilina* wasps, rather than superior female resistance or tolerance.

The overall vertical transmission rate of *Spiroplasma* MSRO was 97% in this experiment. *Spiroplasma* MSRO vertical transmission rate was not significantly

different between *Wolbachia* infected and uninfected flies (95% and 99%, respectively; $F_{1,14} = 1.62$, $P = 0.2244$).

3.3.2 Differential oviposition

Several observations suggest that the presence of *Spiroplasma* prevents successful development of the two wasp species upon oviposition: (a) extremely low wasp emergence in the presence of *Spiroplasma*; (b) large proportion of failed pupae not observed in the absence of wasps; and (c) the presence of a detectable effect of *Spiroplasma* on fly survival only at the pupa-to-adult stage, which is consistent with the stage at which protection by *Spiroplasma* hy1 is detectable in *Drosophila hydei* attacked by Lh (Xie *et al.*, 2010). Nevertheless, a pre-oviposition mechanism may have contributed to the low degree of wasp emergence observed (e.g. if female wasps were able to detect *Spiroplasma* infection and preferred to oviposit on *Spiroplasma*-free fly larvae). We therefore examined whether the two species of wasps lay different numbers of eggs according to the endosymbiont infection status of the fly larvae, under equivalent conditions to the fitness assays described above. Wasps were not given a choice of infected and uninfected fly larvae. The number of wasp eggs found per fly larva did not differ significantly among different *Spiroplasma* and *Wolbachia* infection states for either the GzLMM with Poisson distribution or the GzLMM with a binary distribution (i.e., one or more wasp eggs grouped into a single category; Figure 3. 2; Table 3. 1). A significant difference was observed however, between the two wasp species, regarding the exact number of wasp eggs per fly larva. Lb females tended to lay more eggs per

host larva (mean ± SE = 3.69 ± 0.2 wasp eggs, among all the parasitized fly larvae and pooled across endosymbiont treatments) than Lh females (mean ± SE = 2.10 ± 0.12), regardless of the fly endosymbiont infection states ($F_{1,287}$= 16.35, $P < 0.0001$). The superparasitism rate (i.e., number of fly larvae with 2 or more wasp eggs/number of parasitized fly larvae) was 83.47% in Lb and 52.76% in Lh treatment. Although this observation contrasts with Gueguen *et al.*'s (2011) report that the same wasp strain (Lb17) does not superparasitize, the difference may be explained by the higher parasitism pressure of our assay; five female wasps competing for ~30 fly larvae over 48 h in this study vs. one female wasp competing for 10 fly larvae over 17 h in Patot *et al.* (2009) and Gueguen *et al.* (2011). The average oviposition rate (i.e., proportion of fly larvae with at least one wasp egg or larva) was 87.17% for Lh and 90.98% for Lb. These results suggest that although a pre-oviposition mechanism does not appear to explain the differential survival of flies with and without *Spiroplasma*, the few flies emerging from the wasp treatments might have not been attacked.

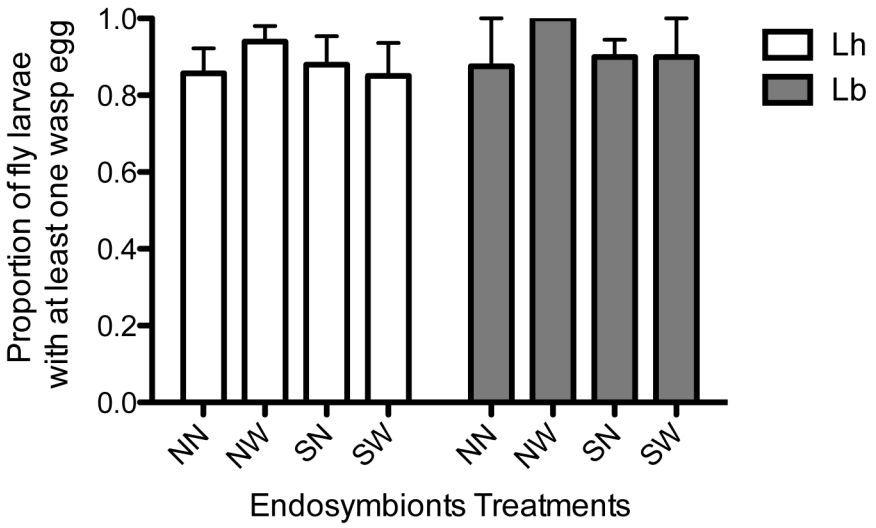

Figure 3. 2 Comparison of wasp oviposition frequency among fly larvae from the four endosymbiont treatments (S = *Spiroplasma*; W = *Wolbachia*). Lh = flies subjected to *L. heterotoma*; Lb = flies subjected to *L. boulardi*. Error bars: standard error.

Table 3. 1 Effect of wasp species, fly *Spiroplasma*-infection state, *Wolbachia*-infection state, and fly strain on wasp oviposition preference in two models: Poisson model for raw numbers of eggs; and binary model for 0 vs. ≥ 1 eggs.

Effects (reduced model) *	*F*-ratio/*Z* value$_{(df)}$	*P*-value†
Poisson Distribution		
Wasp (fixed)	$16.35_{(1,287)}$	**<0.0001**
Wolbachia infection (fixed)	$1.10_{(1,287)}$	0.2962
Spiroplasma infection (fixed)	$0.15_{(1,287)}$	0.6992
Spiroplasma X *Wolbachia* (fixed)	$0.39_{(1,287)}$	0.5306
Fly strain (random)	$0.00_{(n/a)}$	1.0000
Vial (random)	$27.00_{(n/a)}$	**<0.0001**
Binary Distribution		
Wasp (fixed)	$1.71_{(1,287)}$	0.1926
Wolbachia infection (fixed)	$3.56_{(1,287)}$	0.0601
Spiroplasma infection (fixed)	$0.70_{(1,287)}$	0.4034
Spiroplasma X *Wolbachia* (fixed)	$1.09_{(1,287)}$	0.2974
Fly strain (random)	$2.62_{(n/a)}$	0.0528
Vial (random)	$15.83_{(n/a)}$	**<0.0001**

* After removing non-significant random interaction terms
† Boldface: *P*-values significant at $\alpha = 0.05$

3.3.3 Wasp growth rate

The presence of *Spiroplasma*, but not of *Wolbachia*, interfered with normal larval growth of both wasp species. The two species of wasps started out at similar body lengths (~0.33 mm; 0h), hatched successfully (at least the dominant wasp larva when more than one wasp egg was present), and achieved some initial growth (Figure 3. 3). *Spiroplasma* infection state, hours post-attack, and their interaction had a highly significant effect on the body length of both wasp species (see Table 3. 2). The significant *Spiroplasma* infection state and hours post-attack interaction indicates that wasp growth rate differs between the *Spiroplasma*-infected and uninfected treatments (Figure 3. 3A and B). Lb and Lh differed however, in the time point and wasp length at which a significant decrease in wasp growth rate was detectable: 72 h for Lh, and 144 h for Lb (Table 3. 2).

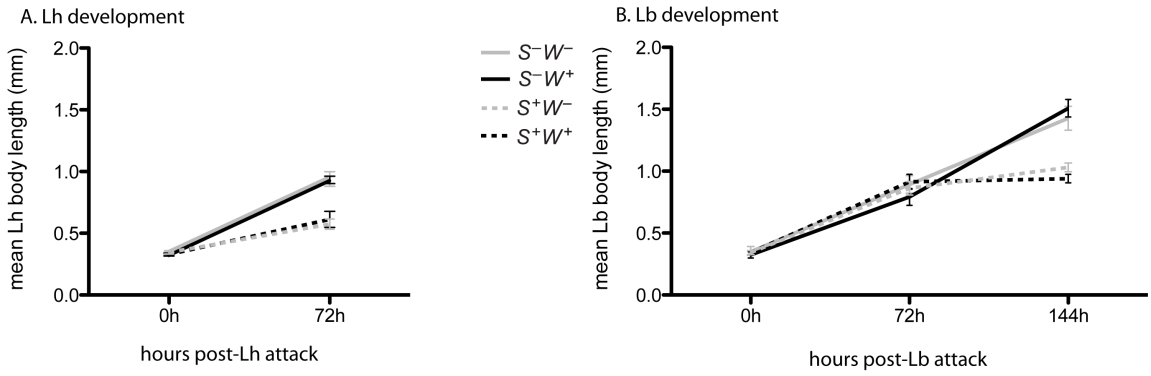

Figure 3. 3 Comparison of wasp growth rate within fly larvae from the four endosymbiont infection treatments (S = *Spiroplasma*; W = *Wolbachia*). A. *L. heterotoma* (Lh) egg/larvae body length (mean ± SE) through 72h post wasp attack. B. *L. boulardi* (Lb) egg/larvae body length through 144h post wasp attack.

Table 3. 2 Effect of hours, *Spiroplasma*-infection state, *Wolbachia*-infection state, fly strain, and the corresponding interactions on the length of developing wasp (Lh = *L. heterotoma*; Lb = *L. boulardi*).

Wasp Treatment	Effects (reduced model)*	*F*-ratio/*Z*-value$_{(df)}$	*P* value†
Lh	Hours (fixed)	$391.12_{(1,171)}$	**<0.0001**
	Wolbachia infection (fixed)	$0.07_{(1,172)}$	0.7967
	Spiroplasma infection (fixed)	$59,41_{(1,171)}$	**<0.0001**
	Spiroplasma X *Wolbachia* (fixed)	$0.19_{(1,172)}$	0.6631
	Hours X *Spiroplasma* (fixed)	$64.61_{(1,171)}$	**<0.0001**
	Fly strain (random)	$0.74_{(n/a)}$	0.229
Lb	Hours (fixed)	$282.95_{(2,165)}$	**<0.0001**
	Wolbachia infection (fixed)	$0.25_{(1,165)}$	0.619
	Spiroplasma infection (fixed)	$22.79_{(1,165)}$	**<0.0001**
	Spiroplasma X *Wolbachia* (fixed)	$0.01_{(1,165)}$	0.9381
	Hours X *Spiroplasma* (fixed)	$33.32_{(2,165)}$	**<0.0001**
	Fly strain (random)	$0.91_{(n/a)}$	0.1821

* After removing non-significant random interaction terms
† Boldface: *P*-values significant at $\alpha = 0.05$

3.3.4 Conditions under which defense against wasps may contribute to *Spiroplasma* MSRO persistence

The equilibrium prevalence of a male-killing endosymbiont depends on the advantage that females gain by the infection, the viability and fertility cost of infection to females, and the transmission efficiency (Dyer & Jaenike, 2004). Dyer and Jaenike (2004) developed a model in which the fitness of female progeny produced by an infected female is equal regardless of their infection status (i.e., uninfected females benefit just as much as their infected sisters from the symbiont-induced death of their infected brothers). To assess the conditions under which the *Spiroplasma*-induced defense observed in our study might contribute to persistence, we modified the model of Dyer and Jaenike (2004) to account for the unequal fitness of uninfected and infected progeny produced by the same infected mother.

Under the assumption of constant parasitoid attack, let the fitness of a *Spiroplasma*-infected female be 1 and that of an uninfected female be $1 - s$, where s is the fitness difference due to the *Spiroplasma* infection. β is the proportion of infected daughters produced by the infected mother (vertical transmission efficiency). If I is the prevalence of infection among females in one generation, then their daughter's generation infection prevalence (I') is:

$$I' = \frac{I\beta}{I\beta + \left[I(1-\beta) + (1-I)\right](1-s)}$$
$$= \frac{I\beta}{1 - s(1 - I\beta)} \qquad (1)$$

The equation (1) has two equilibria. When $I = 0$, there is no *Spiroplasma* infection in the host population. Hurst (1991) modeled the invasion of a male killer under the resource release hypothesis, thus this equilibrium will not be discussed further here. The other equilibrium is reached when $I = I'$,

$$I' = \frac{I\beta}{1 - s(1 - I\beta)} = I$$

At this internal equilibrium for equation (1), the fitness difference between *Spiroplasma* infected and uninfected flies is:

$$s = \frac{\beta - 1}{I\beta - 1}$$

When $\beta = 0.97$ and I ranges between ~1–17.7% (i.e., the range of *Spiroplasma* prevalence observed in *D. melanogaster* natural populations), s must range between ~0.0303–0.03622 to maintain the equilibrium frequency I (eq. 1).

Now, assuming that *Spiroplasma*-infected and uninfected females undergo equal wasp attack rates (as suggested by our oviposition assay), as well as equal mortality rates in the absence of wasps (as suggested by the survival assay), the relative fitness of *Spiroplasma*-infected to uninfected flies according to the survival assay of the present study is:

$$\frac{Fitness_{In}}{Fitness_{Un}} = \frac{1}{1 - s}$$
$$s = 1 - \frac{Fitness_{Un}}{Fitness_{In}} \qquad (2)$$

According to the *Spiroplasma*-enhanced larva-to-adult survival observed in our experiments, $s = 0.33–0.94$ in the presence of Lh and $s = 0.14–0.87$ in the presence of Lb

($Fitness_{Un} = S^{-}W^{-}$ and $Fitness_{In} = S^{+}W^{-}$ values from mean ± SE of larva-to-adult fly survival from Table S1; details for calculation of *s* ranges in Table S2). These values are largely above those required to observe equilibrium frequencies of ~1–17.7%. These findings suggest that, in the context of high wasp parasitism (100%), defense against wasps could play a major role in the persistence of the male-killing *Spiroplasma* strain of *D. melanogaster*.

Nevertheless, although wasp parasitism rates can be high in nature, they are unlikely to be 100%, and they vary over time and space (reviewed in Fleury *et al.*, 2009). If we take into account imperfect parasitism rate (*P*), and define the fitness of unattacked flies as 1 (regardless of the *Spiroplasma* infection), and the post-wasp attack fitness of *Spiroplasma*-infected and uninfected flies as *k* and *h*, respectively, then, at equilibrium *I'*:

$$I' = \frac{I\beta\left[Pk + (1-P)1\right]}{I\beta\left[Pk + (1-P)1\right] + \left[I(1-\beta) + (1-I)\right]\left[Ph + (1-P)1\right]} = \frac{I\beta(Pk + 1 - P)}{P(I\beta k + h - I\beta h - 1) + 1} \quad (3)$$

As above, the equilibrium $I = 0$ will not be discussed. For the internal equilibrium $I' = I$, $\beta > 0$; $0 < P \leq 1$ and $0 < k < 1$, thus $Pk + 1 - P \neq 0$, and:

$$P = \frac{1-\beta}{\beta(k - Ik + Ih - 1) + 1 - h}$$

Here, the fly survival rate observed in the absence of wasps (mean of all four endosymbiont treatments = 89.5%) is assumed to represent the fitness of unattacked flies and used to standardize the *k* and *h* observed in this study for each wasp species assay.

We also assume that most of the surviving flies within the wasp treatments were indeed attacked by wasps (i.e., ~87% for Lh and ~91% for Lb treatment, based on our observed oviposition rates). The relationship of wasp parasitism rate (*P*) to *Spiroplasma* prevalence (*I*) for both wasp species is shown in Figure 3. 4A. Under these conditions, Lh parasitism rate *P* must be > 53.92% and > 58.31% to maintain a *Spiroplasma* equilibrium frequency (*I*) of 1% and 17.7%, respectively (solid line; Figure 3. 4A; Table S3). For Lb, *P* must be > 60.43% and > 64.65%, respectively to maintain comparable *Spiroplasma* equilibrium frequencies (solid line; Fig. 4B; Table S3).

The post-wasp attack reproductive fitness of *Spiroplasma*-infected flies (*k*) however, may be lower than that observed in this study, as Xie *et al.* (2011) showed that *Spiroplasma*-infected flies (*D. hydei*) surviving a wasp attack (Lh) suffer detrimental fitness effects after eclosion (i.e., ~34% reduction in adult 0–10 day longevity and ~30% reduction in fecundity). To account for a potentially equivalent fitness decrease after eclosion in *Spiroplasma*-infected *D. melanogaster*, we also examined the relationship between *Spiroplasma* prevalence (*I*) and wasp parasitism rate (*P*), under a more conservative value for *k* (i.e., observed *k* x 0.66 x 0.7). Under this lower *k*, Lh parasitism rate *P* must be > 81.69% and > 84.30% respectively to maintain a *Spiroplasma* equilibrium frequency of 1% and 17.7% (dashed line; Figure 3. 4A). Even higher levels of Lb parasitism are required to maintain comparable *Spiroplasma* equilibrium frequencies; *P* must be > 94.96% and 95.95% for *I* = 1% and 17.7%, respectively (dashed line; Figure 3. 4B).

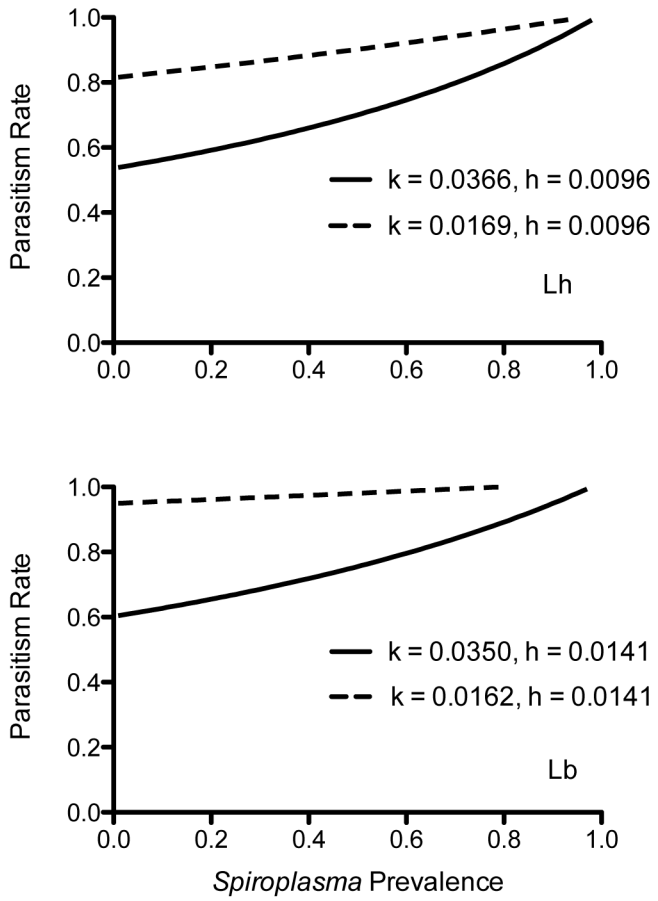

Figure 3. 4 Relationship of wasp parasitism rate (P) to *Spiroplasma* prevalence (I) in the fly population for A. *Leptopilina heterotoma* and B. *Leptopilina boulardi*, according to the larva-to-adult survival advantage conferred by *Spiroplasma* MSRO, as estimated directly from our experiments (solid line), and an adjusted fitness advantage accounting for reduced longevity and fecundity of adult flies surviving a wasp attack (dashed line; see text for details). Grey areas indicate the range of prevalences (1–17.7%) reported for *Spiroplasma* MSRO in natural populations of *D. melanogaster*.

3.4 Discussion

The present work indicates that *Spiroplasma* MSRO, a maternally transmitted reproductive parasite of *D. melanogaster*, prevents successful development of two parasitoid wasps (*L. heterotoma* and *L. boulardi*). These results expand the taxonomic diversity of *Spiroplasma*-mediated parasitoid killing from *D. hydei* to *D. melanogaster* (two species that diverged up to ~63 million years ago; Tamura *et al.*, 2004), from the non-male-killer strain hy1 (Xie *et al.*, 2010) to its male-killing relative MSRO (divergent by ~1.8% at the *fru* locus; uncorrected p-distance; GenBank Acc. Nos. AJ628444 and FJ657017), and from *L. heterotoma* to its congeneric, but distant relative *L. boulardi* (Allemand *et al.*, 2002; ~14 % divergent at the Cytochrome Oxidase I gene; uncorrected p-distance; GenBank Acc. Nos. JQ808444 and JQ808436).

3.4.1 Can the defense against wasps contribute to the persistence of male-killing *Spiroplasma*?

The results suggest that *Spiroplasma* MSRO confers a small, albeit significant, survival advantage to flies that have been attacked by either species of wasp. Fly survival against Lh was approximately 3.8 times higher in the $S^{+}W^{-}$ treatment (mean = 3.28%) than in $S^{-}W^{-}$ treatment (mean = 0.86%). Similarly, fly survival against Lb was approximately 2.5 times higher in the $S^{+}W^{-}$ treatment (mean = 2.15%) than in $S^{-}W^{-}$ treatment (mean = 0.86%). The above advantage conferred by *Spiroplasma* contrasts with that reported for *D. hydei* attacked by Lh, where *Spiroplasma* hy1 increases larva-to-adult survival approximately 9.25 times; from ~4% in the S^{-} treatment to ~37% in the S^{+} treatment

(Xie *et al.*, 2010). The small selective advantage conferred by *Spiroplasma* MSRO in the present study, raises the question as to whether this protective mechanism is relevant to *Spiroplasma* persistence.

To address the above question, we developed a model that takes into account vertical transmission efficiency and the selective advantage of infection (*s*) under conditions of high wasp parasitism (see Results). Under such conditions, and based on our experimentally determined vertical transmission rates and larva-to-adult survival advantage, *Spiroplasma* MSRO is expected to persist at the range of infection frequencies observed in nature (~1–17.7%). We then modified the model to account for lower and more realistic wasp parasitism rates. In addition, we assumed a lower post-wasp attack fitness of *Spiroplasma*-infected flies (*k*) to account for the reported reduction in adult fecundity and longevity experienced by *D. hydei* surviving a parasitoid attack (Xie *et al.*, 2011). These results suggest that maintenance of *Spiroplasma* at infection frequencies observed in nature can only be achieved at wasp parasitism rates > 82% for Lh and > 95% for Lb. Although up to 80% parasitized *Drosophila* larvae have been reported in several regions, an average parasitism range of 5–40% is more common, which fluctuates geographically and seasonally (reviewed in Fleury *et al.*, 2009). Therefore, it appears that the selective advantage conferred by defense alone does not guarantee *Spiroplasma* persistence. Nevertheless, it is possible that a combination of defense and other net fitness benefits conferred by this male-killing strain (i.e., higher fecundity of wild-caught flies and faster development; Martins *et al.*, 2010), ensure its persistence. Furthermore, our experiment was limited to a few host

backgrounds (seven isofemale lines not known to harbor *Spiroplasma* naturally), and two highly virulent wasp strains. It is possible that combinations of other host and wasp backgrounds present in nature result in more (or less) efficient rescue by *Spiroplasma*.

3.4.2 Effect of *Wolbachia* wMel and its co-occurrence with *Spiroplasma* MSRO on the outcome of wasp parasitism

*Wolbachia w*Mel had a weak positive, but non-significant, effect on survival of flies subjected to Lb attack. Lack of a significant effect of *w*Mel on the interaction of *L. boulardi* with *D. melanogaster* (two backgrounds) was also reported by Martinez *et al.* (2012). Other strains of *Wolbachia* are reported to have negative and positive effects on the interaction of *D. simulans* with *L. boulardi* (Fytrou *et al.*, 2006; Martinez *et al.*, 2012), but these effects are dependent on whether or not *L. boulardi* carries the virus LbFV (Martinez *et al.*, 2012), which does not occur in the Lb strain used in our study (Gueguen *et al.*, 2011). Thus, it appears that in *D. melanogaster,* at least, *Wolbachia w*Mel does not significantly influence the outcome of oviposition by *L. boulardi*.

Infection with *Wolbachia w*Mel significantly reduced parasitism success of Lh, but its effect was much smaller than that of *Spiroplasma* MSRO. Fly survival against Lh attack was also significantly enhanced by *w*Mel at a similar rate as *Spiroplasma* MSRO ($S^{-}W^{+}$ mean = 2.6% vs. $S^{+}W^{-}$ mean = 3.3 %). The effect of the two symbionts on fly survival appears to be additive ($S^{+}W^{+}$ mean = 7.8%). These observations provide empirical evidence for a mechanism by which two cytoplasmically-transmitted endosymbionts may evolve cooperation. If the observed additive benefits of co-

infection by *Spiroplasma* and *Wolbachia* against Lh are ecologically relevant, we expect a non-random positive association of the two symbionts in natural populations of *D. melanogaster*, such as that observed in *D. neotestacea* (Jaenike *et al.*, 2010b). Nonetheless, Ventura *et al.* (2012) failed to detect a significant association between the two symbionts in natural populations of *D. melanogaster* in Brazil. Therefore, it is possible that the additive effects observed in our lab experiments are too weak to counter potential disadvantages of co-infection in nature, including the antagonistic reproductive manipulation strategies of the two symbionts: the cytoplasmic incompatibility of *Wolbachia*, which relies on infected males vs. the male-killing effect of *Spiroplasma*.

3.4.3 Wasp-killing mechanism

The extremely low success of wasps in the presence of *Spiroplasma* MSRO could be the result of reduced oviposition rates (i.e., a pre-oviposition mechanism), or reduced survival of developing wasps in *Spiroplasma*-infected flies (i.e., a post-oviposition mechanism). Our wasp oviposition results indicate that wasps do not lay significantly different numbers of eggs in any of the four endosymbiont treatments, ruling out a pre-oviposition mechanism. Furthermore, the high proportion of dead pupae observed only when both *Spiroplasma* and wasps were present provides additional evidence that wasp failure associated with the presence of *Spiroplasma* is exerted mostly at the pupa-to-adult stage, and thus, after oviposition.

The mechanism by which *Wolbachia w*Mel appears to enhance fly survival of Lh-attacked flies is unclear. The wasp oviposition results suggest that it occurs post-

oviposition, but wasp growth rates are not affected by *w*Mel. *Wolbachia w*Mel has been reported to increase hemolymph melanization in *D. melanogaster* (Thomas et al. 2011), but evidence for melanization was not observed in Lh-attacked flies (discussed below).

Both wasp species exhibited slower larval growth rates in *D. melanogaster* infected with *Spiroplasma* MSRO, but *w*Mel had no effect on wasp growth rate. Slower growth was also reported in Lh developing within *D. hydei* infected with *Spiroplasma* hy1 (Xie *et al.*, 2011). Within *D. melanogaster*, although the growth trajectory of the two wasps in the hosts lacking *Spiroplasma* is similar, the growth inhibition mediated by *Spiroplasma* MSRO is detectable earlier in Lh than in Lb. The differences between the two wasps may reflect different interactions between the fly, wasp, and endosymbiont, including the possible effect of Lb superparasitism (e.g. injection of larger venom amounts through repeated oviposition may counter the effects of *Spiroplasma*). For example, the parasitoid wasp *Aphidius ervi* intentionally superparasitizes endosymbiont-infected aphids, presumably to overcome the symbiont-encoded defense (Oliver *et al.*, 2012). In our study however, the higher superparasitism of Lb compared to Lh does not seem to result in higher wasp survival.

One of the mechanisms by which *Spiroplasma* could cause wasp death is by enhancing host immunity (e.g. encapsulation with or without melanization). Lh counters host defenses by destroying lamellocytes, one of the essential cell types responsible for encapsulation (Morales *et al.*, 2005; Lee *et al.*, 2009). Our results with Lh suggest that *Spiroplasma* does not enhance this aspect of fly immunity, as we observed no melanized tissues in any Lh-attacked flies at the time point examined (i.e., 72 h post-attack; not

shown), and all the wasp embryos hatched successfully. Lack of melanization was also reported in *D. hydei* attacked by Lh, regardless of *Spiroplasma* infection state (Xie *et al.*, 2011).

In contrast to Lh, the strategy of Lb includes embedding embryos within host tissues and altering lamellocyte shape without causing lamellocyte lysis (Lee *et al.*, 2009). As a result, encapsulation is thwarted, but subsequent melanization and systemic production of antimicrobial peptide production continue (Lee *et al.*, 2009). In this study, some of the fly larvae in the Lb treatment exhibited melanized tissues at 72h and 144 h post-attack. To be effective, however, melanotic encapsulation should kill the wasp before egg hatching, and it is typically completed by 24–40 h post-attack (equivalent to ~0 h in our study) (Russo *et al.*, 1996; Williams *et al.*, 2006). These observations suggest that *Spiroplasma* does not enhance the fly's ability to encapsulate wasp embryos, but improvement of other aspects of immunity cannot be ruled out (e.g. enhancement of cytotoxic products such as Reactive Oxygen Species or intermediates of the melanization cascade; Lemaitre & Hoffmann, 2007).

Two mechanisms unrelated to host-encoded immunity by which *Spiroplasma* may prevent wasp success include: the presence of a substance toxic to the developing wasp, and the absence (or reduction) of a substance necessary for wasp development. Although our results do not allow us to distinguish between these, observation of similar effects of two *Spiroplasma* strains (MSRO and hy1; poulsonii clade), in two distantly related *Drosophila* hosts (*D. hydei* and *D. melanogaster*) against two congeneric but distantly related parasitoid wasps (*L. heterotoma* and *L. boulardi*), suggests that the

mechanism might be quite general. Furthermore, the mycophagous fly *D. neotestacea* harbors a non-male-killing *Spiroplasma* strain (also within the poulsonii clade) that inhibits growth of *Howardula aoronymphium*, a parasitic nematode of adult hemocoel (Jaenike *et al.*, 2010b). Thus, assuming the same mechanism is responsible for growth inhibition of the two types of endo-macroparasites (i.e., wasps and nematodes), this trait may have been present in the ancestor of the poulsonii clade, which includes male-killing and non-male-killing strains associated with several other species of *Drosophila* (e.g. D. nebulosa, D. willistoni, and D. simulans; Haselkorn *et al.*, 2009).

The present study indicates that *Spiroplasma*-mediated defense against parasitoid wasps occurs in both male-killing and non-male-killing strains of *Spiroplasma* associated with *Drosophila*, and reveals another example of a symbiont that likely employs more than one strategy to ensure persistence. The similar wasp growth inhibitory effects exerted by two different *Spiroplasma* strains on two wasps with distinct host avoidance/suppression strategies and within two divergent *Drosophila* hosts, suggests that the defensive mechanism is quite general, and probably not associated with enhanced cellular immunity of the host. Furthermore, discovery of symbiont-mediated protection against wasps in a model organism offers a tractable system in which to further explore the defensive mechanism. Finally, the additive positive effect of *Spiroplasma* and *Wolbachia* on fly survival against attack by one parasitoid (*L. heterotoma*) constitutes a mechanism by which two, otherwise antagonistic maternally-transmitted symbionts, may behave as mutualists.

4. CONCLUSION

Symbiotic interactions between microbes and their insect hosts vary in abundance, diversity, and function among species, populations, and environmental conditions (Hilgenboecker *et al.*, 2008; Moran *et al.*, 2008; Toju & Fukatsu, 2011; Haselkorn *et al.*, 2013; Osaka *et al.*, 2013a; Russell *et al.*, 2013). The recent emergence of pyrosequencing technologies has exponentially increased the ability to discover new associations between insects and heritable bacteria that are typically fastidious to culture (Kautz *et al.*, 2013; Russell *et al.*, 2013). Nevertheless, comprehensive understanding of the ecological and evolutionary implications of such associations will require lab and field studies that examine their fitness consequences and population dynamics (Moran *et al.*, 2008; Ishak *et al.*, 2011; Russell *et al.*, 2013).

This dissertation used experimental lab studies to investigate the intimate relationship between heritable facultative endosymbionts of flies in the diverse genus *Drosophila.* Building upon our previous findings that *Spiroplasma* hy1 confers protection to its natural host *D. hydei* against the parasitoid wasp *Leptopilina heterotoma*, we demonstrated that *Spiroplasma* can spread rapidly in fly populations exposed to high parasitism pressure. Furthermore, our results indicate that in the absence of wasp parasitism, *Spiroplasma* infection is effectively neutral. Given that parasitism pressure and maternal transmission efficiency (which is strongly influenced by temperature; Osaka *et al.*, 2008) are likely variable over time and space, the dynamics of this system in nature are likely complex. In addition, horizontal transmission via

ectoparasitic mites might be relevant in this system (Osaka *et al.*, 2013b). To better understand these dynamics, future theoretical and experimental (both lab and field) studies should consider the interaction of variable abiotic and biotic factors. The implications of this dynamic at the community level might also be relevant (e.g. Jaenike & Brekke, 2011), as *L. heterotoma* utilizes other *Drosophila* hosts, which could in turn influence competitive interactions of *D. hydei*.

The taxonomic diversity of both, *Spiroplasma* and insect lineages, involved in heritable associations is broad. Of these, three *Spiroplasma*-insect associations are known to confer protection against natural enemies, including two closely related *Spiroplasma* strains that protect their respective *Drosophila* hosts against parasitism by one nematode species and by one wasp species (*L. heterotoma*), and a distantly related *Spiroplasma* lineage that protects aphids against a fungal pathogen. Therefore, defensive systems involving *Spiroplasma* are recorded in two distant *Spiroplasma* clades, two insect orders (i.e., the hosts), and natural enemies representing two kingdoms, and two phyla within a kingdom. Whether symbiont-mediated defense is a common theme in insect-*Spiroplasma* associations is unknown.

In this dissertation, we tested whether a male-killing strain of *Spiroplasma* (MSRO) that is closely related to the two known protective strains of *Drosophila*, and that is naturally associated with the model organism *Drosophila melanogaster*, protects against two species of parasitoid wasps that employ different strategies to overcome host immune response. High *Spiroplasma*-induced wasp mortality against both wasp species was observed, implying that the defensive mechanism is general enough to function in

two different hosts against two different natural enemies. Future studies should examine additional combinations of hosts, symbionts, and natural enemies (including ectoparasitoid wasps). Discovery of this phenomenon in the model organism *D. melanogaster* provides a tractable system for a comprehensive investigation of the protection mechanism. At the same time, with 24 ongoing or completed *Spiroplasma* genome projects, and recent advances in high throughput transcritptomics and proteomics tools, use of comparative and functional bacterial genomics approaches to investigate the mechanism is now feasible (e.g. Hansen *et al.*, 2011; Mortimer *et al.*, 2013).

In addition to contributing to understanding interactions between hosts and endosymbionts, this dissertation addressed the interaction between two endosymbionts that co-occur within the same host, *Spiroplasma* and *Wolbachia*, which are the only two known heritable endosymbiont lineages of *Drosophila* (Mateos *et al.*, 2006). The observed additive positive effect of *Spiroplasma* and *Wolbachia* on fly survival against attack by *L. heterotoma* constitutes a mechanism by which two, otherwise antagonistic maternally-transmitted symbionts, may behave as mutualists. Similar phenomena may occur in other systems where a positive association between two facultative endosymbionts is reported, but no obvious mechanism is evident (e.g. Jaenike *et al.*, 2010a).

REFERENCES

Allemand R, Lemaitre C, Frey F, Bouletreau M, Vavre F, Nordlander G *et al* (2002). Phylogeny of six African *Leptopilina* species (Hymenoptera : Cynipoidea, Figitidae), parasitoids of *Drosophila*, with description of three new species. *Ann Soc Entomol Fr* **38:** 319-332.

Anbutsu H, Goto S, Fukatsu T (2008). High and low temperatures differently affect infection density and vertical transmission of male-killing *Spiroplasma* symbionts in *Drosophila* hosts. *Appl Environ Microbiol* **74:** 6053-6059.

Ballard JWO, James AC (2004). Differential fitness of mitochondrial DNA in perturbation cage studies correlates with global abundance and population history in *Drosophila simulans*. *Proc R Soc Lond B Biol Sci* **271:** 1197-1201.

Baumann P (2005). Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. *Annu Rev Microbiol* **59:** 155-189.

Baumann P, Baumann L, Thao ML (1999). Detection of messenger RNA transcribed from genes encoding enzymes of amino acid biosynthesis in *Buchnera aphidicola* (endosymbiont of aphids). *Curr Microbiol* **38:** 135-136.

Bian GW, Xu Y, Lu P, Xie Y, Xi ZY (2010). The endosymbiotic bacterium *Wolbachia* induces resistance to Dengue virus in *Aedes aegypti*. *PLoS Pathog* **6:** e1000833.

Brownlie JC, Cass BN, Riegler M, Witsenburg JJ, Iturbe-Ormaetxe I, McGraw EA *et al* (2009). Evidence for metabolic provisioning by a common invertebrate endosymbiont, *Wolbachia* pipientis, during periods of nutritional stress. *PLoS Pathog* **5:** e1000368.

Brownlie JC, Johnson KN (2009). Symbiont-mediated protection in insect hosts. *Trends Microbiol* **17:** 348-354.

Brumin M, Kontsedalov S, Ghanim M (2011). *Rickettsia* influences thermotolerance in the whitefly *Bemisia tabaci* B biotype. *Insect Sci* **18:** 57-66.

Bull JJ, Molineux IJ, Werren JH (1992). Selfish genes. *Science* **256:** 65.

Burke G, Fiehn O, Moran N (2010a). Effects of facultative symbionts and heat stress on the metabolome of pea aphids. *ISME J* **4:** 242-252.

Burke GR, McLaughlin HJ, Simon JC, Moran NA (2010b). Dynamics of a recurrent *Buchnera* mutation that affects thermal tolerance of pea aphid hosts. *Genetics* **186:** 367-U577.

Carton Y, Boulétreau M, Van Alphen JJM, Van Lenteren JC (1986). The *Drosophila* parasitic wasps. In: Ashburner M, Carson HL and Thompson JN (eds) The Genetics and Biology of *Drosophila*. Academic Press: London, pp 347–394.

Currie CR, Wong B, Stuart AE, Schultz TR, Rehner SA, Mueller UG *et al* (2003). Ancient tripartite coevolution in the attine ant-microbe symbiosis. *Science* **299:** 386-388.

Douglas AE (1998). Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. *Annu Rev Entomol* **43:** 17-37.

Dyer KA, Jaenike J (2004). Evolutionarily stable infection by a male-killing endosymbiont in *Drosophila innubila*: Molecular evidence from the host and parasite genomes. *Genetics* **168:** 1443-1455.

Ebbert MA (1991). The interaction phenotype in the *Drosophila* w*illistoni*-*Spiroplasma* symbiosis. *Evolution* **45:** 971-988.

Engelstadter J, Hurst GDD (2009). The ecology and evolution of microbes that manipulate host reproduction. *Annu Rev Ecol Evol Syst* **40:** 127-149.

Ferrari J, Vavre F (2011). Bacterial symbionts in insects or the story of communities affecting communities. *Philos T Roy Soc B* **366:** 1389-1400.

Fleury F, Gibert P, Ris N, Allemand R (2009). Ecology and life history evolution of frugivorous *Drosophila* parasitoids. *Adv Parasitol* **70:** 3-44.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol Mar Biol Biotechnol* **3:** 294-299.

Frentiu FD, Robinson J, Young PR, McGraw EA, O'Neill SL (2010). *Wolbachia*-mediated resistance to Dengue virus infection and death at the cellular level. *PLoS ONE* **5:** e13398.

Fytrou A, Schofield PG, Kraaijeveld AR, Hubbard SF (2006). *Wolbachia* infection suppresses both host defence and parasitoid counter-defence. *Proc R Soc Lond B Biol Sci* **273:** 791-796.

Glaser RL, Meola MA (2010). The native *Wolbachia* endosymbionts of *Drosophila melanogaster* and *Culex quinquefasciatus* increase host resistance to West Nile Virus infection. *PLoS ONE* **5:** e11977.

Gloor GB, Preston CR, Johnson-Schlitz DM, Nassif NA, Phillis RW, Benz WK *et al* (1993). Type I repressors of P element mobility. *Genetics* **135:** 81-95.

Goto S, Anbutsu H, Fukatsu T (2006). Asymmetrical interactions between *Wolbachia* and *Spiroplasma* endosymbionts coexisting in the same insect host. *Appl Environ Microbiol* **72:** 4805-4810.

Gueguen G, Rajwani R, Paddibhatla I, Morales J, Govind S (2011). VLPs of *Leptopilina boulardi* share biogenesis and overall stellate morphology with VLPs of the heterotoma clade. *Virus Res* **160:** 159-165.

Haine ER (2008). Symbiont-mediated protection. *Proc R Soc Lond B Biol Sci* **275:** 353-361.

Hansen AK, Vorburger C, Moran NA (2011). Genomic basis of endosymbiont-conferred protection against an insect parasitoid. *Genome Res* **22:** 106-114.

Haselkorn TS, Markow TA, Moran NA (2009). Multiple introductions of the *Spiroplasma* bacterial endosymbiont into *Drosophila*. *Mol Ecol* **18:** 1294-1305.

Haselkorn TS, Watts TD, Markow TA (2013). Density dynamics of diverse *Spiroplasma* strains naturally infecting different species of *Drosophila*. *Fly (Austin)* **7**.

Hedges LM, Brownlie JC, O'Neill SL, Johnson KN (2008). *Wolbachia* and virus protection in insects. *Science* **322:** 702-702.

Herren JK, Lemaitre B (2011). *Spiroplasma* and host immunity: activation of humoral immune responses increases endosymbiont load and susceptibility to certain Gram-negative bacterial pathogens in *Drosophila melanogaster*. *Cell Microbiol* **13:** 1385-1396.

Hilgenboecker K, Hammerstein P, Schlattmann P, Telschow A, Werren JH (2008). How many species are infected with *Wolbachia*? - A statistical analysis of current data. *FEMS Microbiol Lett* **281:** 215-220.

Himler AG, Adachi-Hagimori T, Bergen JE, Kozuch A, Kelly SE, Tabashnik BE *et al* (2011). Rapid spread of a bacterial symbiont in an invasive whitefly is driven by fitness benefits and female bias. *Science* **332:** 254-256.

Hosokawa T, Koga R, Kikuchi Y, Meng XY, Fukatsu T (2010). *Wolbachia* as a bacteriocyte-associated nutritional mutualist. *Proc Natl Acad Sci USA* **107:** 769-774.

Hurst GDD, Hutchence KJ (2010). Host defence: getting by with a little help from our friends. *Curr Biol* **20:** R806-R808.

Hurst GDD, Jiggins FM, von der Schulenburg JHG, Bertrand D, West SA, Goriacheva II *et al* (1999). Male-killing *Wolbachia* in two species of insect. *Proc R Soc Lond B Biol Sci* **266:** 735-740.

Hurst LD (1991). The incidences and evolution of cytoplasmic male killers. *Proc R Soc Lond B Biol Sci* **244:** 91-99.

Ishak HD, Plowes R, Sen R, Kellner K, Meyer E, Estrada DA *et al* (2011). Bacterial diversity in *Solenopsis invicta* and *Solenopsis* geminata ant colonies characterized by 16S amplicon 454 pyrosequencing. *Microb Ecol* **61:** 821-831.

Iturbe-Ormaetxe I, Walker T, Neill SLO (2011). *Wolbachia* and the biological control of mosquito-borne disease. *EMBO Rep* **12:** 508-518.

Jaenike J (2012). Population genetics of beneficial heritable symbionts. *Trends Ecol Evol* **27:** 226-232.

Jaenike J, Brekke TD (2011). Defensive endosymbionts: a cryptic trophic level in community ecology. *Ecol Lett* **14:** 150-155.

Jaenike J, Polak M, Fiskin A, Helou M, Minhas M (2007). Interspecific transmission of endosymbiotic *Spiroplasma* by mites. *Biol Lett* **3:** 23-25.

Jaenike J, Stahlhut JK, Boelio LM, Unckless RL (2010a). Association between *Wolbachia* and *Spiroplasma* within *Drosophila neotestacea*: an emerging symbiotic mutualism. *Mol Ecol* **19:** 414-425.

Jaenike J, Unckless R, Cockburn S, Boelio L, Perlman S (2010b). Adaptation via symbiosis: recent spread of a *Drosophila* defensive symbiont. *Science* **329:** 212-215.

Jeyaprakash A, Hoy MA (2000). Long PCR improves *Wolbachia* DNA amplification: *wsp* sequences found in 76% of sixty-three arthropod species. *Insect Mol Biol* **9:** 393–405.

Kacsoh BZ, Schlenke TA (2012). High hemocyte load is associated with increased resistance against parasitoids in *Drosophila suzukii*, a relative of *D. melanogaster*. *PLoS ONE* **7:** e34721.

Kageyama D, Anbutsu H, Watada M, Hosokawa T, Shimada M, Fukatsu T (2006). Prevalence of a non-male-killing *Spiroplasma* in natural populations of *Drosophila hydei*. *Appl Environ Microbiol* **72:** 6667-6673.

Kautz S, Rubin BER, Russell JA, Moreau CS (2013). Surveying the microbiome of ants: comparing 454 pyrosequencing with traditional methods to uncover bacterial diversity. *Appl Environ Microbiol* **79:** 525-534.

Kraaijeveld AR, Barker CL, Godfray HCJ (2008). Stage-specific sex differences in *Drosophila* immunity to parasites and pathogens. *Evol Ecol* **22:** 217-228.

Lane DJ (1991). 16S/23S rRNA sequencing. In: Stackebrandt E and Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. John Wiley and Sons: New York, NY, pp 115-175.

Lee MJ, Kalamarz ME, Paddibhatla I, Small C, Rajwani R, Govind S (2009). Virulence factors and strategies of *Leptopilina* spp.: selective responses in *Drosophila* hosts. *Adv Parasitol* **70:** 123-145.

Lemaitre B, Hoffmann J (2007). The host defense of *Drosophila melanogaster*. *Annu Rev Immunol* **25:** 697-743.

Longdon B, Fabian D, Hurst G, Jiggins F (2012). Male-killing *Wolbachia* do not protect *Drosophila bifasciata* against viral infection. *BMC Microbiol* **12:** S8.

Lukasik P, van Asch M, Guo H, Ferrari J, Charles JGH, van der Putten W (2012). Unrelated facultative endosymbionts protect aphids against a fungal pathogen. *Ecol Lett* **16:** 214-218.

Malogolowkin-Cohen, Rodriguespereira MAQ (1975). Sexual drive of normal and sr flies of *Drosophila nebulosa*. *Evolution* **29:** 579-580.

Markow TA, and P. M. O'Grady. (2005). *Drosophila: A guide to species identification and use*. Academic Press Elsevier: London.

Martinez J, Duplouy A, Woolfit M, Vavre F, O'Neill SL, Varaldi J (2012). Influence of the virus LbFV and of *Wolbachia* in a host-parasitoid interaction. *PLoS ONE* **7:** e35081.

Martins AB, Ventura I, Klaczko L (2010). *Spiroplasma* infection in *Drosophila melanogaster*: what is the advantage of killing males? *J Invertebr Pathol* **105:** 145–150.

Mateos M, Castrezana SJ, Nankivell BJ, Estes AM, Markow TA, Moran NA (2006). Heritable endosymbionts of *Drosophila*. *Genetics* **174:** 363-376.

Montenegro H, Klaczko LB (2004). Low temperature cure of a male killing agent in *Drosophila melanogaster*. *J Invertebr Pathol* **86:** 50-51.

Montenegro H, Petherwick A, Hurst G, Klaczko L (2006). Fitness effects of *Wolbachia* and *Spiroplasma* in *Drosophila melanogaster*. *Genetica* **127:** 207–215.

Montenegro H, Solferini VN, Klaczko LB, Hurst GDD (2005). Male-killing *Spiroplasma* naturally infecting *Drosophila melanogaster*. *Insect Mol Biol* **14:** 281-287.

Montenegro H, Souza WN, Leite DDS, Klaczko LB (2000). Male-killing selfish cytoplasmic element causes sex-ratio distortion in *Drosophila melanogaster*. *Heredity* **85:** 465–470.

Morales J, Chiu H, Oo T, Plaza R, Hoskins S, Govind S (2005). Biogenesis, structure, and immune-suppressive effects of virus-like particles of a *Drosophila* parasitoid, *Leptopilina victoriae*. *J Insect Physiol* **51:** 181-195.

Moran NA, McCutcheon JP, Nakabachi A (2008). Genomics and evolution of heritable bacterial symbionts. *Annu Rev Genet* **42:** 165-190.

Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu GJ, Pyke AT, Hedges LM *et al* (2009). A *Wolbachia* symbiont in *Aedes aegypti* limits infection with Dengue, Chikungunya, and Plasmodium. *Cell* **139:** 1268-1278.

Mortimer NT, Goecks J, Kacsoh BZ, Mobley JA, Bowersock GJ, Taylor J *et al* (2013). Parasitoid wasp venom SERCA regulates *Drosophila* calcium levels and inhibits cellular immunity. *Proc Natl Acad Sci USA* **110:** 9427-9432.

Munson MA, Baumann P, Clark MA, Baumann L, Moran NA, Voegtlin DJ *et al* (1991). Evidence for the establishment of aphid-eubacterium endosymbiosis in an ancestor of four aphid families. *J Bacteriol* **173:** 6321-6324.

Nunes MD, Nolte V, Schlotterer C (2008). Nonrandom *Wolbachia* infection status of *Drosophila melanogaster* strains with different mtDNA haplotypes. *Mol Biol Evol* **25:** 2493-2498.

O'Neill SL, Hoffmann AA, Werren JH (1997). *Influential passengers: Inherited microorganisms and arthropod reproduction*. Oxford University Press: New York.

Oliver K, Russell J, Moran N, Hunter M (2003a). Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proc Natl Acad Sci USA* **100:** 1803 - 1807.

Oliver KM, Campos J, Moran NA, Hunter MS (2008). Population dynamics of defensive symbionts in aphids. *Proc R Soc Lond B Biol Sci* **275:** 293-299.

Oliver KM, Degnan PH, Hunter MS, Moran NA (2009). Bacteriophages encode factors required for protection in a symbiotic mutualism. *Science* **325:** 992-994.

Oliver KM, Moran NA (2009). Defensive symbionts in aphids and other insects. In: White J and Torres M (eds) Defensive Mutualism in Microbial Symbiosis, 1st edn. CRC Press, Taylor & Francis Group, pp 129-148.

Oliver KM, Moran NA, Hunter MS (2005). Variation in resistance to parasitism in aphids is due to symbionts not host genotype. *Proc Natl Acad Sci USA* **102:** 12795-12800.

Oliver KM, Noge K, Huang EM, Campos JM, Becerra JX, Hunter MS (2012). Parasitic wasp responses to symbiont-based defense in aphids. *BMC Biol* **10:** 11.

Oliver KM, Russell JA, Moran NA, Hunter MS (2003b). Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proc Natl Acad Sci USA* **100:** 1803-1807.

Oliver KM, Smith AH, Russell JA (2013). Defensive symbiosis in the real world – advancing ecological studies of heritable, protective bacteria in aphids and beyond. *Funct Ecol***:** in press.

Osaka R, Ichizono T, Kageyama D, Nomura M, Watada M (2013a). Natural variation in population densities and vertical transmission rates of a *Spiroplasma* endosymbiont in *Drosophila hydei*. *Symbiosis* **60:** 73-78.

Osaka R, Nomura M, Watada M, Kageyama D (2008). Negative effects of low temperatures on the vertical transmission and infection density of a *Spiroplasma* endosymbiont in *Drosophila hydei*. *Curr Microbiol* **57:** 335-339.

Osaka R, Watada M, Kageyama D, Nomura M (2011). Population dynamics of the maternally transmitted *Spiroplasma* infection in *Drosophila hydei*. *Symbiosis* **52:** 41-45.

Osaka R, Watada M, Kageyama D, Nomura M (2013b). Detection of *Spiroplasma* from the mite *Macrocheles* sp. (Acari; Macrochelidae) ectoparasitic to the fly *Drosophila hydei* (Diptera; Drosophilidae): a possible route of horizontal transmission? *Symbiosis* **60:** 79-84.

Osborne SE, Leong YS, O'Neill SL, Johnson KN (2009). Variation in antiviral protection mediated by different *Wolbachia* strains in *Drosophila simulans*. *PLoS Pathog* **5:** e1000656.

Ota T, Kawabe M, Oishi K, Poulson DF (1979). Non-male-killing *Spiroplasma* in *Drosophila hydei*. *J Hered* **70:** 211-213.

Patot S, Lepetit D, Charif D, Varaldi J, Fleury F (2009). Molecular detection, penetrance, and transmission of an inherited virus responsible for behavioral manipulation of an insect parasitoid. *Appl Environ Microbiol* **75:** 703-710.

Richardson MF, Weinert LA, Welch JJ, Linheiro RS, Magwire MM, Jiggins FM *et al* (2012). Population genomics of the *Wolbachia* endosymbiont in *Drosophila melanogaster*. *PLoS Genet* **8:** e1003129.

Riegler M, Sidhu M, Miller WJ, O'Neill SL (2005). Evidence for a global *Wolbachia* replacement in *Drosophila melanogaster*. *Curr Biol* **15:** 1428-1433.

Russell JA, Latorre A, Sabater-Munoz B, Moya A, Moran NA (2003). Side-stepping secondary symbionts: widespread horizontal transfer across and beyond the *Aphidoidea*. *Mol Ecol* **12:** 1061-1075.

Russell JA, Weldon S, Smith AH, Kim KL, Hu Y, Lukasik P *et al* (2013). Uncovering symbiont-driven genetic diversity across North American pea aphids. *Mol Ecol* **22:** 2045-2059.

Russo J, Dupas S, Frey F, Carton Y, Brehelin M (1996). Insect immunity: Early events in the encapsulation process of parasitoid (*Leptopilina boulardi*) eggs in resistant and susceptible strains of *Drosophila*. *Parasitology* **112:** 135-142.

Sakaguchi B, Poulson DF (1963). Interspecific transfer of the "Sex-Ratio" condition from *Drosophila willistoni* to *D. melanogaster*. *Genetics* **48:** 841-861.

Scarborough CL, Ferrari J, Godfray HCJ (2005). Aphid protected from pathogen by endosymbiont. *Science* **310:** 1781-1781.

Schlenke TA, Morales J, Govind S, Clark AG (2007). Contrasting infection strategies in generalist and specialist wasp parasitoids of *Drosophila melanogaster*. *PLoS Pathog* **3:** 1486-1501.

Silva N, Guenther L, Xie J, Mateos M (2012). Infection densities of three *Spiroplasma* strains in the host *Drosophila melanogaster*. *Symbiosis* **57:** 83-93.

Tamura K, Subramanian S, Kumar S (2004). Temporal patterns of fruit fly (*Drosophila*) evolution revealed by mutation clocks. *Mol Biol Evol* **21:** 36-44.

Teixeira L, Ferreira A, Ashburner M (2008). The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. *PLoS Biol* **6:** 2753-2763.

Toju H, Fukatsu T (2011). Diversity and infection prevalence of endosymbionts in natural populations of the chestnut weevil: relevance of local climate and host plants. *Mol Ecol* **20:** 853-868.

Unckless RL, Jaenike J (2012). Maintenance of a male-killing *Wolbachia* in *Drosophila Innubila* by male-killing dependent and male-killing independent mechanisms. *Evolution* **66:** 678-689.

Varaldi J, Fouillet P, Ravallec M, Lopez-Ferber M, Bouletreau M, Fleury F (2003). Infectious behavior in a parasitoid. *Science* **302:** 1930-1930.

Varaldi J, Ravallec M, Labrosse C, Lopez-Ferber M, Bouletreau M, Fleury F (2006). Artifical transfer and morphological description of virus particles associated with superparasitism behaviour in a parasitoid wasp. *J Insect Physiol* **52:** 1202-1212.

Ventura IM, Martins AB, Lyra ML, Andrade CA, Carvalho KA, Klaczko LB (2012). *Spiroplasma* in *Drosophila melanogaster* populations: Prevalence, male-killing, molecular identification, and no association with *Wolbachia*. *Microb Ecol* **64:** 794-801.

Vorburger C, Sandrock C, Gouskov A, Castañeda LE, Ferrari J (2009). Genotypic variation and the role of defensive endosymbionts in an all-parthenogenetic host-parasitoid interaction. *Evolution* **63:** 1439–1450.

Walker T, Johnson PH, Moreira LA, Iturbe-Ormaetxe I, Frentiu FD, McMeniman CJ *et al* (2011). The *w*Mel *Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations. *Nature* **476:** 450-U101.

Watts T, Haselkorn TS, Moran NA, Markow TA (2009). Variable incidence of *Spiroplasma* infections in natural populations of *Drosophila* species. *PLoS ONE* **4**.

Werren JH, Baldo L, Clark ME (2008). *Wolbachia*: master manipulators of invertebrate biology. *Nat Rev Microbiol* **6:** 741-751.

White J, Giorgini M, Strand M, Pennacchio F (2013). Arthropod endosymbiosis and evolution. In: Minelli A, Boxshall G and Fusco G (eds) Arthropod biology and evolution. Springer Berlin Heidelberg, pp 441-477.

Williams MJ, Wiklund ML, Wikman S, Hultmark D (2006). Rac1 signalling in the *Drosophila* larval cellular immune response. *J Cell Sci* **119:** 2015-2024.

Xie J, Tiner B, Vilchez I, Mateos M (2011). Effect of the *Drosophila* endosymbiont *Spiroplasma* on parasitoid wasp development and on the reproductive fitness of wasp-attacked fly survivors. *Evol Ecol* **25:** 1065–1079.

Xie J, Vilchez I, Mateos M (2010). *Spiroplasma* bacteria enhance survival of *Drosophila hydei* attacked by the parasitic wasp *Leptopilina heterotoma*. *PLoS ONE* **5:** e12149.

Zele F, Nicot A, Duron O, Rivero A (2012). Infection with *Wolbachia* protects mosquitoes against *Plasmodium*-induced mortality in a natural system. *J Evol Biol* **25:** 1243-1252.

Zug R, Hammerstein P (2012). Still a host of hosts for *Wolbachia*: analysis of recent data suggests that 40% of terrestrial arthropod species are infected. *PLoS ONE* **7:** e38544.

APPENDIX

Table S1. Effects of *Spiroplasma* infection state (fixed), *Wolbachia* infection state (fixed), their interaction (fixed), fly strain (isoline; random), on each of the survival measures in each of the wasp treatments (No wasp control; Lh = treatment with *Leptopilina heterotoma*; Lb = treatment with *Leptopilina boulardi*). Mean ± SE (%) for each endosymbiont treatment combination. S^+ = *Spiroplasma*-infected; S^- = *Spiroplasma*-free; W^+ = *Wolbachia*-infected; W^- = *Wolbachia*-free.

Effects	Statistic $_{(degrees\ of\ freedom)}$, ($P$-value) *			Mean ± SE(%) per endosymbiont treatment			
	No wasp	Wasp=Lh	Wasp=Lb		No wasp	Wasp=Lh	Wasp=Lb
Fly larva-to-adult survival (number of emerging adult flies/initial number of fly larvae)							
Wolbachia	$F_{(1,78)}$=0 (0.9744)	$F_{(1,84)}$=5.16 (**0.0256**)	$F_{(1,87)}$=3.01 (0.0862)	S^-W^-	88.79±2.20	0.86±0.62	1.26±0.76
Spiroplasma	$F_{(1,78)}$=0.14 (0.7079)	$F_{(1,84)}$=6.72 (**0.0041**)	$F_{(1,87)}$=7.29 (**0.0083**)	S^-W^+	90.98±1.71	2.59±0.97	2.16±0.90
Interaction	$F_{(1,78)}$=1.44 (0.2333)	$F_{(1,84)}$=0.05 (0.8296)	$F_{(1,87)}$=0.13 (0.7191)	S^+W^-	90.39±1.94	3.28±1.06	3.13±0.77
Isoline	χ^2=0.59 (0.221)	χ^2=1.26 (0.131)	χ^2=0 (1)	S^+W^+	87.85±2.03	7.78±1.69	6.91±1.52
Fly pupa-to-adult survival (number of emerging adult flies/total number of puparia)							
Wolbachia	$F_{(1,78)}$=0.01 (0.9378)	$F_{(1,84)}$=4.58 (**0.0353**)	$F_{(1,87)}$=2.75 (0.1011)	S^-W^-	95.44±1.99	0.94±0.67	1.3±0.77
Spiroplasma	$F_{(1,78)}$=0.65 (0.4219)	$F_{(1,84)}$=9.34 (**0.003**)	$F_{(1,87)}$=9.26 (**0.0031**)	S^-W^+	97.34±1.31	2.8±1.03	2.33±0.96
Interaction	$F_{(1,78)}$=2.69 (0.1052)	$F_{(1,84)}$=0.03 (0.8695)	$F_{(1,87)}$=0.03 (0.8651)	S^+W^-	96.42±1.22	3.98±1.36	4±1.00
Isoline	χ^2=2.87 (**0.0451**)	χ^2=1.3 (0.1272)	χ^2= 0 (1)	S^+W^+	93.91±1.53	9.507±2.23	7.99±1.75

Table S1. Continued

Effects	Statistic $_{(degrees\ of\ freedom)}$, ($P$-value) *			Mean ± SE(%) per endosymbiont treatment			
	No wasp	Wasp=Lh	Wasp=Lb		No wasp	Wasp=Lh	Wasp=Lb
Fly larva-to-pupa survival (number of pupae/initial number of fly larvae)							
Wolbachia	$F_{(1,78)}$=0.06 (0.8085)	$F_{(1,84)}$=0.27 (0.6049)	$F_{(1,87)}$=0.01 (0.9223)	S^-W^-	93.23±1.74	85.66±2.04	86.20±2.85
Spiroplasma	$F_{(1,78)}$=0.01 (0.9234)	$F_{(1,84)}$=0.68 (0.4117)	$F_{(1,87)}$=3.43 (0.0674)	S^-W^+	93.64±1.70	83.96±3.03	84.09±2.45
Interaction	$F_{(1,78)}$=0 (0.9645)	$F_{(1,84)}$=0.06 (0.8151)	$F_{(1,87)}$=0.57 (0.4529)	S^+W^-	93.67±1.40	83.20±2.17	79.2±2.29
Isoline	χ^2= 5.72 (**0.0084**)	χ^2= 0.21 (0.3223)	χ^2= 0.08 (0.3893)	S^+W^+	93.53±1.43	82.505±2.46	81.04±3.07
Wasp “larva-to-adult survival” (number of emerging adult wasps/initial number of fly larvae)							
Wolbachia		$F_{(1,84)}$=6.42 (**0.0131**)	$F_{(1,87)}$=0.73 (0.3949)	S^-W^-		80.025±2.15	73.60±2.81
Spiroplasma		$F_{(1,84)}$=196.39 (<**0.0001**)	$F_{(1,87)}$=211.15 (<**0.0001**)	S^-W^+		72.09±3.08	72.95±3.43
Interaction		$F_{(1,84)}$=1.8 (0.183)	$F_{(1,87)}$=0.57 (0.454)	S^+W^-		3.628±1.44	3.15±1.32
Isoline		χ^2= 0 (1)	χ^2= 0(1)	S^+W^+		0.895±0.46	1.89±0.67

Table S1. Continued

Effects	Statistic $_{(degrees\ of\ freedom)}$, ($P$-value) *				Mean ± SE(%) per endosymbiont treatment		
	No wasp	Wasp=Lh	Wasp=Lb		No wasp	Wasp=Lh	Wasp=Lb
Wasp "pupa-to-adult survival" (number of emerging adult wasps/number of puparia)							
Wolbachia		$F_{(1,84)}$=8.23 (**0.0052**)	$F_{(1,87)}$=0.42 (0.52)	S^-W^-		93.51±1.46	85.65±2.27
Spiroplasma		$F_{(1,84)}$=261.1 (<**0.0001**)	$F_{(1,87)}$=264.57 (<**0.0001**)	S^-W^+		86.18±2.40	86.16±2.73
Interaction		$F_{(1,84)}$=0.41 (0.5226)	$F_{(1,87)}$=0.6 (0.4401)	S^+W^-		3.95±1.50	3.946±1.62
Isoline		χ^2= 0.04 (0.423)	χ^2= 0 (1)	S^+W^+		1.076±0.58	2.535±0.87
Pupal mortality (number of failed pupae/total pupae)							
Wolbachia	$F_{(1,78)}$=0.01 (0.9378)	$F_{(1,84)}$=0.98 (0.3242)	$F_{(1,87)}$=1.12 (0.2928)	S^-W^-	4.56±1.99	5.55±1.30	13.05±2.18
Spiroplasma	$F_{(1,78)}$=0.4219 (0.4219)	$F_{(1,84)}$=387.47 (<**0.0001**)	$F_{(1,87)}$=397.7 (<**0.0001**)	S^-W^+	2.67±1.31	11.02±2.14	11.51±2.63
Interaction	$F_{(1,78)}$=2.69 (0.1052)	$F_{(1,84)}$=5.08 (**0.0267**)	$F_{(1,87)}$=0.15 (0.6982)	S^+W^-	3.58±1.22	92.07±2.05	92.05±1.70
Isoline	c^2= 2.87 (**0.0451**)	χ^2= 2.41 (0.0603)	χ^2= 0 (1)	S^+W^+	6.09±1.53	89.42±2.20	89.47±1.52

* F-ratio and P-value given for fixed effects. χ^2, P-value given for the random effect (isoline). Boldface: P-values significant at $\alpha = 0.05$

Table S2. Estimates of *s* based on Equation (2); upper and lower estimates of *s* are boldfaced. (a) *Leptopilina heterotoma* (Lh) treatment. (b) *Leptopilina boulardi* (Lb) treatment. Lower and upper bounds for $Fitness_{Un}$ ($S^{-}W^{-}$) and $Fitness_{In}$ ($S^{+}W^{-}$) are based on mean – SE and mean + SE values, respectively, of the fly larva-to-adult survival measure (from Table S1).

(a) Lh treatment

		$Fitness_{Un}$	
		Lower (0.86 – 0.62)	Upper (0.86 + 1.062)
$Fitness_{In}$	Lower (3.28 – 1.06)	0.89	**0.33**
	Upper (3.28 + 1.06)	**0.94**	0.66

(b) Lb treatment

		$Fitness_{Un}$	
		Lower (1.26 – 0.76)	Upper (1.26 + 0.76)
$Fitness_{In}$	Lower (3.13 – 0.77)	0.79	**0.14**
	Upper (3.13 + 0.77)	**0.87**	0.48

Table S3. Details of the estimation of parasitism pressure (*P*) based on the raw and adjusted fitness advantage.

	No wasp		Lh		Lb	
Infection state	S^-W^-	S^+W^-	S^-W^-	S^+W^-	S^-W^-	S^+W^-
Parameter in Model	1		*h*	*k*	*h*	*k*
Parameter Value *	0.895		0.0086	0.0328	0.0126	0.0313
Normalized	1		0.0096	0.0366	0.0141	0.0350
When $I = 0.01$ $P =$			0.5392		0.6043	
When $I = 0.17$ $P =$			0.5831		0.6465	
Adjusted for fitness loss	1		0.0096	0.0169	0.0141	0.0162
When $I = 0.01$ $P =$			0.8169		0.9496	
When $I = 0.17$ $P =$			0.8430		0.9595	

* Parameter values for *h* and *k* taken from fly larvae-to-adult survival in Table S1; Values for no wasp treatment were averaged from the fly larvae-to-adult survival of S^-W^- and S^+W^- without wasp attack. Value for *P* were calculated from eq (3) under corresponding *I* value; ß = 0.97

Table S4. Effect of wasp treatment (no wasp, Lh, Lb) on the proportion of emerged male flies surviving a wasp attack for three separate experiments.

Effects	**Statistic$_{(\text{degrees of freedom})}$, p-value**	**Mean ± SE(%) per wasp treatment**		
		Lh	**Lb**	**No wasp**
Controlled Larval Density[a]				
Wasp	$F_{(2,12)}$=0.50 p=0.6211			
Wolbachia	$F_{(1,12)}$=0.23 p=0.6400	64.01±13.49	53.89±14.92	46.76±3.98
Interaction	$F_{(2,12)}$=1.43 p=0.2771			
En masse Canton S[b]				
Wasp	$F_{(2,12)}$=0.04 p=0.9648	41.11±18.89	45.35±6.65	41.58±2.12
En masse D. hydei[c]				
Wasp	$F_{(1,57)}$=1.79 p=0.1858	43.39±4.78	N/A	49.84±0.91

[a] "Controlled larval density" (~40 fly larvae + six female wasps per vial) was conducted on *D. melanogaster* with and without *Wolbachia* infection ($S^{-}W^{+}$ and $S^{-}W^{-}$; respectively); thus, the effect of *Wolbachia* and of the *Wolbachia* X Wasp interaction were also tested.

[b] "*En masse* Canton S" (uncontrolled high density of fly larvae and wasps) was conducted on *Wolbachia*-infected ($S^{-}W^{+}$) *D. melanogaster* Canton S strain.

[c] "*En masse D. hydei"* (uncontrolled high density of fly larvae and wasps) was conducted on *Spiroplasma*-infected ($S^{+}W^{-}$) and *Spiroplasma*-free ($S^{-}W^{-}$) flies (this strain does not kill males). *D. hydei* is not an adequate host of Lb, and does not harbor *Wolbachia*.

Figure S1

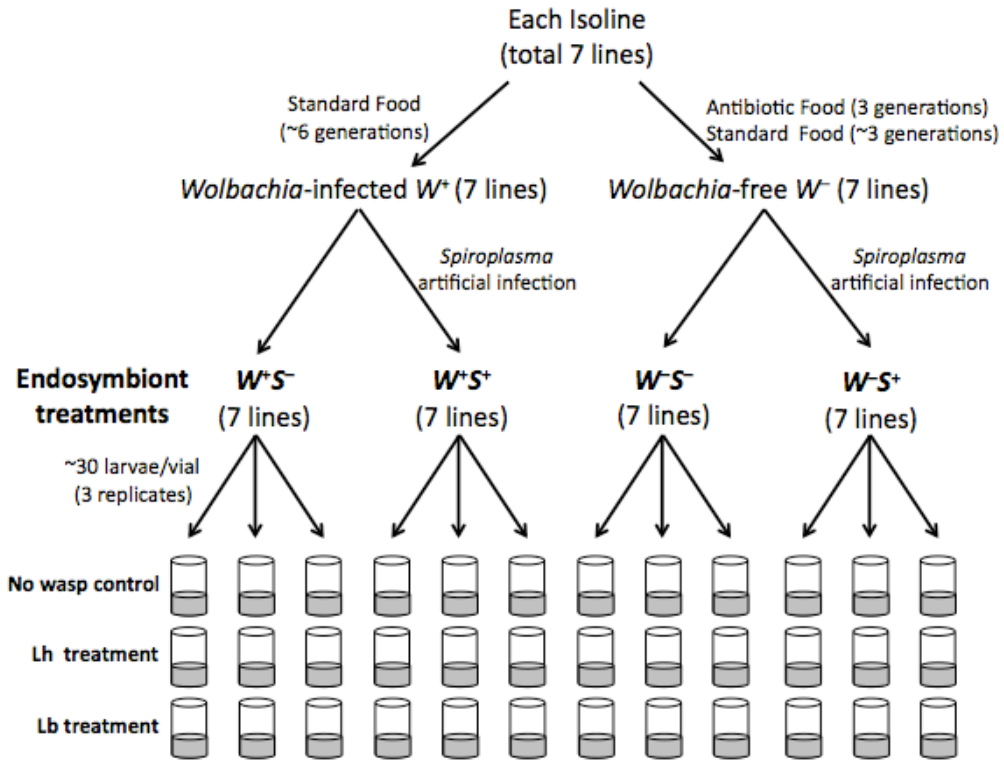

Fig S1. Experimental design. Format: png.