RESEARCH ARTICLE

Rapid spread of the defensive endosymbiont Spiroplasma in Drosophila hydei under high parasitoid wasp pressure

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ABSTRACT

Maternally transmitted endosymbionts of insects are ubiquitous in nature and play diverse roles in the ecology and evolution of their hosts. To persist in host lineages, many symbionts manipulate host reproduction to their advantage (e.g. cytoplasmic incompatibility and male-killing), or confer fitness benefits to their hosts (e.g. metabolic provisioning and defense against natural enemies). Recent studies suggest that strains of the bacterial genus Spiroplasma protect their host (flies in the genus Drosophila) against parasitoid attack. The Spiroplasma-conferred protection is partial and flies surviving a wasp attack have reduced adult longevity and fecundity. Therefore, it is unclear whether protection against wasps alone can counter Spiroplasma loss by imperfect maternal transmission and any possible fitness costs to harboring Spiroplasma. To address this question, we conducted a population cage study comparing Spiroplasma frequencies over time (host generations) under conditions of high wasp pressure and no wasp pressure. 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 host populations despite imperfect vertical transmission.

Key words: Leptopilina heterotoma; female realized fecundity; Drosophila hydei; vertical transmission

INTRODUCTION

Numerous and diverse insects associate with maternally transmitted endosymbiotic bacteria (Moran, McCutcheon and Nakabachi 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 poses challenges to symbiont persistence. To counter loss by imperfect transmission, many of these facultative heritable endosymbionts manipulate host reproduction in one of the following ways to enhance the relative frequency of symbiont-infected to symbiont-uninfected females: cytoplasmic incompatibility, male-killing, parthenogenesis induction and male feminization (O’Neill, Hoffmann and Werren 1997).

Not all facultative heritable endosymbionts manipulate host reproduction, however. Therefore, persistence of such
symbionts despite imperfect vertical transmission must be the result of horizontal transmission and/or enhanced host fitness. A growing body of literature suggests that fitness benefits to the host are common, but typically context-dependent, including enhanced ability to utilize a particular resource (Brownlie et al., 2009), enhanced fitness in the face of abiotic stressors (Burke, Fiehn and Moran 2010; Burke et al., 2010, Brumin, Kontsedalov and Ghanim 2011) and enhanced tolerance of or resistance against natural enemies (reviewed in Haine 2008; Jaenike 2012; Hamilton and Perlman 2013; Oliver, Smith and Russell 2014). 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., 2003; Xie, Vilchez and Mateos 2010; Xie et al., 2014), parasitic nematodes (Jaenike et al., 2010), RNA viruses (Teixeira, Ferreira and Ashburner 2008) and fungi (Scarborough, Ferrari and Godfray 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 Acrithosiphon 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 sterilizing nematode Hovardula aoronymphium (Jaenike and Brekke 2011). A recent spread of this Spiroplasma strain in natural populations of D. neotestacea appears to have occurred as a result of invasion by the nematode (Jaenike et al., 2010), and is currently spreading geographically (Cockburn et al., 2013). Female D. neotestacea parasitized by the nematode and harboring Spiroplasma exhibit >10 times the fecundity of their Spiroplasma-free counterparts (Jaenike et al., 2010), implying a large fitness advantage to harboring Spiroplasma.

Spiroplasma strain hy1 (belonging to the poulsonii clade; Watts et al., 2009), a facultative endosymbiont of D. 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 poulsonii clade also includes the non-male-killing defensive strain neo of D. neotestacea, as well as the male-killing strains of D. nebulosa (known as nebulosa sex ratio Organism; NSRO), D. willistoni (i.e. Spiroplasma poulsonii, a.k.a. WSRO) and D. melanogaster (known as MSRO). This latter male-killing strain also confers protection against wasps (Xie et al., 2014). The vertical transmission rate of Spiroplasma hy1 varies widely among individuals and environmental conditions; for example, low temperatures can drastically reduce transmission efficiency (Osaka et al., 2008, 2013). 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 (Eucalii-nae, Figitidae; hereafter also referred to as Lh). Lh is a solitary endoparasitoid that oviposits into the hemocoel of first- and second-instar larvae of many Drosophila species. 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 sur-vival 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 an ~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 to date (Xie et al., 2010, 2011; Osaka et al., 2013). The aforementioned studies, however, 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 multigeneration study, in which infected and uninfected host lines are reared together, may enable better assessment of fitness consequences of infection and of 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 10 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 pressures, and thus provide a more reliable estimate of the overall fitness advantage or cost associated with Spiroplasma infection in D. hydei.

MATERIALS AND METHODS

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 isolate 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 S1, Supporting Information). 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).

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.

Experimental setting

To track Spiroplasma prevalence over 10 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 ~80 ml 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 (Fig. S1 and Table S2, Supporting Information), 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 and O’Grady 2005). Seven of these replicate populations (hereafter S+Lh−) were subjected to parasitism by Lh (strain 14 used in previous studies; Schlenke et al., 2007; Kacsoh and Schlenke 2012), whereas seven were not subjected to wasps (hereafter S−Lh−).

In addition, two Spiroplasma-free (S−) control treatments were included. To establish these treatments, 10 females and 10 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 fly survival in the S−Lh− was too low to sustain subsequent generations (see the section ‘Results’). The 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 2 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. I0), 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 starting at G3 they were directly derived from the S−Lh− treatment, which was screened from Spiroplasma contamination every generation (Fig. S1, Supporting Information).

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 (PS8IV, expected amplicon length 362bp; Table S1. Supporting Information) and host-specific primers (COI, expected amplicon length 709bp; Table S1, Supporting Information). We used the EmeraldAmp® MAX PCR Master Mix (Takara) and an annealing temperature of 54°C. 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 10 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 10 generations.

Wasp treatments

In the two wasp parasitism treatments (S+Lh+ and S−Lh−), 20 Lh wasp females were introduced to each population bottle immediately after adult flies were removed. At this time, the bottle contained < 2 days-old fly larvae (first- and second-instar G1 flies). Wasps used throughout the experiment were < 5 days old and derived from a D. melanogaster Canton-S culture, where they had been allowed to mate. Flies used to maintain wasps were Spiroplasma-free and derived from lab cultures that have never been exposed to wasps, to avoid inadvertent selection for resistance against wasps. Wasps were allowed to oviposit for 2 days, after which they were removed and discarded. To assess wasp oviposition rate, immediately after wasps were removed, 10 fly larvae per replicate were collected and dissected to determine the 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 Fig. S1, Supporting Information).

Establishment of subsequent generations

Cocooning 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 flies 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 the section ‘Results’). Thus, G2 flies for these replicates were derived from a smaller number of G1 flies. The above procedures were repeated for the four treatments (S+Lh+, S−Lh−, S+Lh− and S−Lh+) during 10 consecutive generations (every other generation for S−Lh−). To examine the effects of removing wasp parasitism on Spiroplasma prevalence, we then continued the above procedures for population replicates derived from the S+Lh+ treatment for three subsequent generations (not shown in Fig. S1, Supporting Information), but in the absence of wasp parasitism (i.e. effectively treatment S−Lh−).

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 (l) 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).

**Statistical analyses**

To assess selection for or against *Spiroplasma* in *D. hydei* as a function of parasitoid attack, we used a general linear model (GLM, in SAS) on *Spiroplasma* prevalence (l) against generation (discrete, repeated), wasp treatment (fixed) and their interaction. Because all variables including the interaction term were significant (see the section ‘Results’), we included the S*+Lh*+ and S*−Lh*− treatments separately within the GLM. This model was also used to conduct the following analyses. First, we tested whether wasp oviposition frequency (arc sine square root transformed, treated as a covariate) varied between *Spiroplasma* treatments (S*+Lh*− vs S*−Lh*+; fixed) and generations (fixed and repeated). 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 of generation (repeated), including all generations, on wasp oviposition frequency (arc sine square root transformed). Second, we tested the effect of generation (fixed and repeated) and wasp treatment (fixed) on the vertical transmission efficiency of *Spiroplasma* (treatments S*+Lh*+ and S*−Lh*−).

We then used a GLM for the S*−Lh*− treatment, to test whether wasp success rate changed over time (i.e. generation; fixed and repeated), 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 the S*+Lh*+ treatment, we tested whether wasp success was correlated with *Spiroplasma* prevalence. This analysis, however, was restricted to wasp success in G1 versus *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 effects of several variables (i.e. *Spiroplasma* and wasp treatment, generation and wasp oviposition) on female realized fecundity. To examine whether wasp and/or *Spiroplasma* treatment had an effect of fly sex ratio, we used a GLM to test the effect of treatment (i.e. S*+Lh*−, S*−Lh*+, S*+Lh*+ and S*−Lh*−; fixed) and generation (fixed and repeated; first seven generations only) on the transformed (arc sine square root) proportion of males.

### RESULTS

#### Effect of high wasp parasitism on *Spiroplasma* frequency

The *Spiroplasma* infection frequencies in the seven *D. hydei* lab populations subjected to parasitism by *Lh* (treatment S*+Lh*+) increased from a mean ± SE of 59.1 ± 6.5% (G0) to 93.4 ± 3.4% (G1) in a single generation (Table 1, Fig. 1A). *Spiroplasma* prevalence reached 100% in all seven replicates by G6, and remained stable thereafter, including the additional three generations of no-wasp treatment to which treatment S*+Lh*− was subjected (G11–G13, data not shown). In contrast, mean *Spiroplasma* prevalence in the populations not exposed to wasps (S*−Lh*−) exhibited a slight decrease from 66.6 ± 4.4% (G0) to 55.7 ± 15.2% (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 (Fig. 2).

The effect of wasp parasitism on *Spiroplasma* prevalence was highly significant ($F_{(1,18)} = 25.14, P < 0.0001$, Table 2). The trends of *Spiroplasma* change over time in the two treatments also differed significantly (as indicated by the significant wasp treatment X generation interaction: $F_{(0,117)} = 4.14, P < 0.0001$, Table 2). We further analyzed the generation effect separately on the S*+Lh*+ and S*−Lh*− treatments due to the significant interaction. In the treatment subjected to wasps, generation had a significant effect on *Spiroplasma* frequency ($F_{(4,151)} = 20.02, P < 0.0001$, Table 2). In contrast, for the replicates not exposed to
Figure 1. Spiroplasma frequency and fly fitness measures throughout the experimental period (S = Spiroplasma; Lh = Leptopilina heterotoma treatment). (A) Solid line: Spiroplasma frequency in treatment S+Lh+ across 10 generations. Dashed line: fly success rate (number of eclosed fly adults/ (fly adults + wasp adults)) across 10 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+, S+Lh− (dashed black line), and S−Lh− (solid black 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.

Table 2. Effect of wasp treatment and generation on Spiroplasma frequency. P-values < 0.05 are indicated with boldface.

<table>
<thead>
<tr>
<th>Effects</th>
<th>F-ratio(deg)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLM on all data of S+Lh+ and S−Lh− treatments</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wasp treatment (fixed)</td>
<td>25.14(1,18.1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Generation (fixed and repeated)</td>
<td>4.39(10,117)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Wasp X Generation (fixed)</td>
<td>4.14(10,117)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>GLM for the S+Lh+ and S−Lh− treatments separately</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S+Lh+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Generation (fixed and repeated)</td>
<td>20.02(10,51.7)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>S−Lh−</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Generation (fixed and repeated)</td>
<td>1.03(10,57.9)</td>
<td>0.4267</td>
</tr>
</tbody>
</table>

wasps, generation did not have a significant effect on the Spiroplasma frequency (F(10,57.9) = 1.03, P = 0.4267; Table 2). Thus, no evidence of selection for or against harboring Spiroplasma was observed in populations lacking wasps (Table 2), where the pattern of Spiroplasma prevalence change over time appears to be random (Fig. 2).

Wasp oviposition rate and wasp success rate

Wasp oviposition rate was high throughout the experiment (overall mean 96.8 ± 0.9%), ranging from 88.6% in G10 to 100% in G2 and G4; Table 1), and did not differ significantly among treatments (S+Lh+ vs S−Lh+; all generations) or among
Figure 2. The random distribution of the Spiroplasma frequency in the treatment S+Lh− over 10 generations. Each color indicates a single replicate bottle.

generations (Table 3, Fig. 3). Wasp success rate (number of emerged wasps/total number of emerged flies and wasps) in the treatment lacking Spiroplasma (S−Lh+) ranged between 78.3 ± 4.4% (G9) and 97.4 ± 0.7% (G1, Table 1, Fig. 4), with an overall mean across generations of 90.2 ± 1.8%. No correlation was observed between wasp oviposition and wasp success rate (S−Lh+ treatment; Table 3), which could be attributable to a lack of variance in oviposition rate. Interestingly, however, in the treatment lacking Spiroplasma (S−Lh+), wasp success rate varied significantly among generations ($F_{(4,20.9)} = 4.71; P = 0.0072; Table 3$). A post-hoc test indicated that the only significant difference was between G1 and G9 (Fig. 4).

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 45.7 ± 4.8% (G1) to 6.8 ± 3.1% (G2) to less than 1.5% in subsequent generations (Table 1). Wasp success rate in G1 was significantly negatively correlated with the Spiroplasma frequency of the preceding generation G0 (Table 3). This relationship could not be tested in subsequent generations due to insufficient variation among replicates for both variables.

Table 3. Effects of multiple variables on wasp oviposition, wasp success and vertical transmission of Spiroplasma. P-values < 0.05 are indicated with boldface.

<table>
<thead>
<tr>
<th>Effects</th>
<th>F-ratio ($df$)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLM of wasp oviposition frequency (arc sine square root transformed, S+Lh+ and S−Lh+ in G1, 3, 5, 7, 9)</td>
<td>Spiroplasma treatment (fixed)</td>
<td>0.06_{(1,23.9)}</td>
</tr>
<tr>
<td>GLM of wasp oviposition frequency (arc sine square root transformed, S+Lh+ in all generations)</td>
<td>Generation (fixed and repeated)</td>
<td>0.48_{(4,46)}</td>
</tr>
<tr>
<td>GLM of wasp oviposition frequency (arc sine square root transformed, S+Lh+ in all generations)</td>
<td>Treatments X generation</td>
<td>0.85_{(4,46)}</td>
</tr>
<tr>
<td>GLM of wasp success rate (arc sine square root transformed; S−Lh+ in G1, 3, 5, 7, 9)</td>
<td>Oviposition (continuous, arc sine square root transformed)</td>
<td>1.63_{(8,42.9)}</td>
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<tr>
<td>GLM of wasp success rate (arc sine square root transformed; S−Lh+ in G1)</td>
<td>Generation (fixed and repeated)</td>
<td>0.47_{(4,20.9)}</td>
</tr>
<tr>
<td>GLM of wasp success rate (arc sine square root transformed; S−Lh+ in G1)</td>
<td>Spiroplasma frequency G0 (fixed)</td>
<td>7.80_{(1,1)}</td>
</tr>
<tr>
<td>GLM of Spiroplasma vertical transmission (S−Lh+ and S−Lh− in all generations)</td>
<td>Generation (fixed and repeated)</td>
<td>1.41_{(9,66.4)}</td>
</tr>
<tr>
<td>GLM of Spiroplasma vertical transmission (S−Lh+ and S−Lh− in all generations)</td>
<td>Wasp treatment (fixed)</td>
<td>0.03_{(1,41.3)}</td>
</tr>
</tbody>
</table>

Figure 3. 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.

Vertical transmission rate

The overall vertical transmission rate of Spiroplasma hy1 was 99% throughout the experiment (range 97–100%; Table 1, Fig. S2, Supporting Information), and did not differ significantly between the treatment exposed to wasps and the treatment lacking wasps (99.18 and 98.75%, respectively). Vertical transmission rates also did not differ significantly among generations (Table 3).

Realized female fly fecundity

As a measure of realized female fecundity, we recorded the number of flies emerging per bottle each generation for the first ~12 days of fly emergence and standardized this by the number of potential mothers (typically 50). Realized female fecundity varied significantly among treatments and generations. In the absence of Spiroplasma, this measure was significantly larger in the treatment lacking wasps than in the treatment exposed to wasps (Table 4), consistent with the high wasp oviposition and...
wasp success described above. A significant effect of generation and generation X wasp treatment was observed (Table 4). A closer look indicates that realized female fecundity in the treatment lacking wasps (S’ Lh’) varied significantly among generations (Fig. 5), oscillating within ∼3–11 progeny/female (Fig. 1B). 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; Fig. 1B), 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. A decrease in fecundity of adult Drosophila exposed to high densities as larvae has been reported in several studies (reviewed in Rodriguez 1988). Female fecundity in the treatment exposed to wasps (S’ Lh’) appeared to increase slightly over time from 0.15 ± 0.1 in G1 to 0.64 ± 0.1 in G9, with the last two generations examined (G7 and G9) significantly higher than the first three (G1, G3 and G5; Fig. 5). 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.

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 4). 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; Fig. 1B), 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 realized female fecundity in the S’ Lh- treatment 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 1, Fig. 1A). Given the high transmission rate (~99%), eggs laid by G1 females must have had a comparable Spiroplasma

**Table 4.** Effects of multiple 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. P-values < 0.05 are indicated with boldface.

<table>
<thead>
<tr>
<th>Effects</th>
<th>F-ratio(40)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLM of realized female fecundity (S’ Lh+ and S’ Lh- in G1, 3, 5, 7, 9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wasp treatment (fixed)</td>
<td>205.21(1,184)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Generation (fixed)</td>
<td>15.83(4,75)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Treatment X generation</td>
<td>17.97(4,75)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>GLM of realized female fecundity (S’ Lh+, S’ Lh- , and S’ Lh- in all ten generations)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spiroplasma and wasp treatment (fixed)</td>
<td>3.16(2,556)</td>
<td>0.0498</td>
</tr>
<tr>
<td>Generation (fixed)</td>
<td>15.01(9,154)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Treatment X Generation</td>
<td>3.99(18,157)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>GLM of realized female fecundity (S’ Lh+ in G1, 3, 5, 7, 9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wasp oviposition frequency</td>
<td>0.22(1,281)</td>
<td>0.6406</td>
</tr>
<tr>
<td>Generation (fixed and repeated)</td>
<td>1.91(4,218)</td>
<td>0.1449</td>
</tr>
</tbody>
</table>
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 5). The proportion of males averaged over treatments and generations was 47.3 ± 0.9%. These results indicate that infection by Spiroplasma does not affect 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 Lh parasitism.

**Estimation of the selection coefficients (s)**

Under the assumption of constant and high (100%) parasitoid attack, 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 (e.g. from generation 1 to its next generation $I'$) by applying the following equation:

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

where $\beta$ is the vertical transmission efficiency (Xie et al., 2014). 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 that both vertical transmission ($\beta$) and wasp oviposition were 100% during this generation (Table 6).

Prevalence of Spiroplasma hy1 (a non-male-killing strain) in natural populations of D. hydei reported to date ranges between 23 and 66% (Kageyama et al., 2006; Watts et al., 2009). To maintain an equilibrium frequency of 23–66%, when the vertical transmission rate ($\beta$) is 0.99 (as estimated in the present study throughout the 10 generations), s must range within −0.0129–0.0289 (Table 7). 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 15°C (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., 2013). 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 7), and more similar to the selection coefficient estimated in the present study (i.e. 0.7882–0.9632). We note, however, that our estimation of s is based on 100% parasitism rate, which is probably not realistic (i.e. up to 80% parasitized Drosophila larvae have been reported, but an average parasitism range of 5–40% is more common; reviewed in Fleury et al., 2009), and thus, the selective advantage in nature might be lower due to this factor alone (see Xie et al., 2014 for the relationship between parasitism rate and Spiroplasma prevalence).

**DISCUSSION**

Exposure to high parasitism pressure from the parasitoid wasp Lh resulted in a rapid increase of Spiroplasma prevalence in lab populations of D. hydei. This is consistent with our previous findings that larva-to-adult survival is higher in Spiroplasma-infected than -uninfected flies exposed to Lh (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 is sustainable. Furthermore, although prior studies did not detect fitness costs associated with Spiroplasma infection (Xie et al., 2010, 2011; Osaka et al., 2013), their experimental setup based on fitness component assays might have provided limited power to detect slight differences in fitness (Oliver et al., 2008; Dykstra et al., 2014). Nonetheless, the present study corroborates previous findings that Spiroplasma infection in the absence of wasps appears to be effectively neutral (G1–G10 of treatment S$^+$-Lh$^+$ and G11–G13 of S$^-$-Lh$^+$). It is possible,
however, that other more subtle or context-dependent fitness costs to Spiroplasma infection exist that were not detectable in our study. For example, Herren and Lemaitre (2011) reported that D. melanogaster infected with Spiroplasma strain MSRO is more susceptible to Gram-negative pathogens. More recently, Herren et al. (2014) reported that under a ‘nutrient-rich’ diet, longevity of MSRO-infected D. melanogaster is significantly lower than their Spiroplasma-free counterparts, but this effect becomes evident until flies are ~21–25 days old, and is positively correlated with Spiroplasma titer. Reduced longevity associated with infection by H. defensa has also been reported in the black bean aphid, Aphis fabae (Vorburger and Gouskov 2011). Whether a similar detrimental effect of Spiroplasma hy1 occurs on D. hydei, a relatively long-lived species (~84–105 days for males; S. Pitnick, pers. comm.; Pitnick and Miller 2000; Xie et al., 2011), is not known. Our experimental design is unlikely to have allowed detection of such fitness cost because relatively young flies were used.

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., 2014). Oliver et al. (2008) reported that prevalence of H. defensa, the symbiont that confers protection to the aphid A. pismum against the parasitic wasp A. 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 H. orornymphium, is reported in both natural and lab fly populations exposed to nematodes (Jaenike et al., 2010; Jaenike and Brekke 2011). In the absence of the natural enemy, however, different patterns are observed. The prevalence of H. defensa in two species of aphid exhibits a steady decline in the absence of the parasitoid, implying a fitness cost to infection (Oliver et al., 2008; Dykstra et al., 2014). In contrast, the prevalence of Spiroplasma strain neo in lab populations of D. neotestacea not exposed to nematodes (Jaenike and 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 (i.e. individuals carrying different inherited factors; in this case harboring or not Spiroplasma). Lack of detectable fitness costs to Spiroplasma infection in D. hydei not exposed to wasps suggests that reductions in Spiroplasma frequency in natural populations are more likely 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; Kagayama et al., 2006), assuming a highly efficient vertical transmission rate (β) of 0.99 (i.e. the rate estimated in the present study). Selection coefficients in natural populations may differ from those than under our lab conditions due to differences in the rates of vertical transmission and wasp parasitism, and/or due to the occurrence of horizontal transmission. Vertical transmission of Spiroplasma in wild-caught D. hydei is highly variable among individuals, even at an optimal temperature of 25°C, at which it has been reported to be as low as 0.36 (Osaka et al., 2013). 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 and Klaczkö 2004; Anbutsu, Goto and Fukatsu 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 thereby 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 in 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 Lh 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, but should also take into consideration the potential influence of temperature on resistance to parasitoids mediated by symbionts (e.g. Fellowes, Kraaijeveld and Godfray 1999; Bensadia et al., 2006), which has not been evaluated in Drosophila. In addition, parasitoid pressure could be influenced by Spiroplasma frequencies themselves. For example, increased Spiroplasma prevalence in D. neotestacea is reported to reduce abundance of the parasitic nematode Howardula (Jaenike and Brekke 2011). Whether Spiroplasma prevalence in D. hydei affects the abundance of Lh is unclear, because this wasp is a ‘generalist’ capable of utilizing multiple Drosophila species (Schlenke et al., 2007), which commonly occur in sympatry.

Horizontal transmission may also contribute to the maintenance of Spiroplasma in host populations, but rates of horizontal transmission in nature, which are inherently difficult to assess, remain unknown. One route for horizontal transmission of Spiroplasma is via ectoparasitic mites (Jaenike et al., 2007; Osaka et al., 2013). Another potential route for horizontal transmission of symbionts that has not been examined in Drosophila is via the wasps themselves (i.e. through sequential stabbing of Spiroplasma-infected and -uninfected host individuals), but has been reported in aphids (Gehrer and Vorburger 2012). Although such a phenomenon could have influenced the rate of Spiroplasma spread in our experiments, several lines of evidence suggest that wasp-mediated horizontal transfer of Spiroplasma in Drosophila might not be effective. If wasps had effectively transferred Spiroplasma to G0 Spiroplasma-free fly larvae via stabling, we would expect to have seen (1) a much higher fly success rate in G1 (due to enhanced fly survival and/or decreased wasp success stemming from a higher proportion of larvae harboring Spiroplasma) and/or (2) a much higher realized fly fecundity in G1 (see Fig. 1B). Another observation, albeit in a different Drosophila–Spiroplasma system, that argues against effective wasp-mediated horizontal transfer is the report that Spiroplasma strain MSRO in D. melanogaster achieves relatively low titers in fly larvae (Herren and Lemaitre 2011). Whether this observation holds for our host-symbiont combination is not known, however. Future experiments involving Drosophila parasitoids that are resistant to Spiroplasma (several such species have been
identified; unpublished results) might allow better assessment of the potential for wasp-mediated horizontal transmission.

Despite the increasing reports of symbiont-mediated defense in insects, it is unclear why in the face of substantial benefits to harboring the symbiont, most of these symbionts remain at intermediate frequencies in natural host populations. Although field-based approaches might ultimately be needed for a comprehensive understanding the ecological dynamics of such systems (reviewed in Oliver et al., 2014), multigenerational lab studies represent an initial and more tractable step towards this goal. Still, such studies have been limited to few host taxa—two species of aphid (Oliver et al., 2008; Dykstra et al., 2014) and two species of Drosophila (Jaenike and Brekke 2011) and this study)—revealing some initial differences among them. Whereas the protective symbiont (H. defensa) of aphids is costly in the absence of the natural enemy, the protective Spiroplasma symbionts of D. neotestacea and D. hydei exhibit no evidence of costs in the absence of the respective natural enemy (nematode and parasitic wasp). Differences in the dynamics within Drosophila are also evident. Whereas present-day frequencies of Spiroplasma in D. neotestacea are at or close to fixation, frequencies in D. hydei (a cosmopolitan species) are not, and vary substantially over space. This, coupled rapid spread in the presence of wasps and the lack of detectable costs to infection in lab populations, provides a system where the contribution of temporal and spatial variation in biotic and abiotic factors can be further explored. The results from such studies will be beneficial for the understanding of protective endosymbionts in insect population dynamics and may shed light on the future outcome of the recently applied pest control projects, e.g. the use of Wolbachia to control mosquito-borne viruses (Hoffmann et al., 2011).

SUPPLEMENTARY DATA

Supplementary data is available at FEMSEC online.

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REFERENCES


