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Understanding the distribution of the Spiroplasma heritable bacterial endosymbiont in Drosophila

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UNDERSTANDING THE DISTRIBUTION OF THE SPIROPLASMA HERITABLE BACTERIAL ENDOSYMBIONT IN DROSOPHILA

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biology

by

Tamara S. Haselkorn

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2010
The Dissertation of Tamara S. Haselkorn is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2010
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The dissertation author was the primary investigator and author.

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VITA

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PUBLICATIONS


Symbiosis can be a major source of evolutionary innovation and a driver of ecological diversification. Insects, in particular, partner with a diversity of heritable bacterial endosymbionts that affect them in myriad ways, ranging from mutualistic to parasitic. Species of the genus *Drosophila*, however, harbor only *Wolbachia* and *Spiroplasma*. While *Wolbachia* infections in *Drosophila* are well characterized, much less is known about the consequences of *Spiroplasma* infections. *Spiroplasma* is a male-killer in some species of *Drosophila*, and recent work has demonstrated that this bacterium can also confer protection against a nematode parasite in one *Drosophila* species. Many other species of *Drosophila*, however, are infected with *Spiroplasma* that
do not cause male-killing, and their fitness effects are unclear. To discern the impacts of *Spiroplasma* infections in *Drosophila*, my dissertation work seeks to characterize the distribution of *Spiroplasma* among *Drosophila* by investigating the genetic diversity of *Spiroplasma* infecting *Drosophila*, examining the bacterial density dynamics of diverse *Spiroplasma* strains infecting different *Drosophila* species and exploring the fitness consequences of *Spiroplasma* infection in a cactophilic *Drosophila* species. Using a multilocus phylogenetic analysis, I show that there have been at least five introductions of four very different types of *Spiroplasma* into *Drosophila*, with a single origin of the male-killing *Spiroplasma* in *Drosophila*. Horizontal transmission has played an important role in driving the distribution of *Spiroplasma* among *Drosophila*, and I provide some evidence that it may be occurring within populations of certain *Drosophila* species. I detected no recombination, however, among *Spiroplasma* from different phylogenetic clades, suggesting that *Spiroplasma* may be similar to beneficial bacterial endosymbionts trapped in their host with no opportunity for recombination. Using quantitative PCR, I assessed the bacterial density dynamics of *Spiroplasma*, which vary among bacterial strain and *Drosophila* species. Although a number of facultative endosymbionts play a role in host plant specialization, this does not seem to be the case for the *Spiroplasma* strain infecting one population of *Drosophila* with high infection prevalence. This characterization of the *Drosophila/Spiroplasma* symbiosis lays much of the groundwork necessary to effectively explore the consequences of *Spiroplasma* infections in *Drosophila*. 
INTRODUCTION

Symbiosis, "the living together of unlike organisms" (de Bary 1879), is a potent force of evolutionary innovation. Like horizontal gene transfer, symbiosis allows for a host organism to acquire novel capabilities either lost, or never evolved, from a microbial partner. Such dramatic partnerships have resulted in the formation of the mitochondria and chloroplast organelles of the eukaryotic cell. Mycorrhizal associations provided plants with the nutrients necessary to colonize land, and symbioses with chemoautotrophic bacteria allowed animals to inhabit deep-sea hydrothermal vents (Cavanaugh et al., 2006; Malloch et al., 1980; Moran, 2006; Sapp, 2004). Symbioses have played a major role in evolution of many lineages, allowing for ecological diversification speciation and adaptive radiations (Moran, 2006).

Insects are arguably one of the most successful groups of animals due to their ability to occupy a variety of ecological niches, and they often do so with the help of bacterial endosymbionts that provide nutritional supplementation (Buchner, 1965). For example, Buchnera aphidicola, the obligate bacterial endosymbiont of aphids, supplies essential amino acids lacking its host's diet of plant sap (Douglas, 1998). Similarly, tsetse flies feed on blood and derive nutritional supplementation from Wigglesworthia glossinidia, and sharpshooters feed on the nutrient poor xylem sap and have two bacterial endosymbionts providing essential nutrients (Chen et al., 1999; Moran et al., 2005). In these cases, strict vertical transmission, from mother to offspring, over millions of years has led to co-diversification and coevolution, with profound effects on both host and symbiont evolution (Moran, Baumann, 1994; Moran et al., 2005). While such long-term
associations are often obligate for both the host and symbiont, symbiosis is an ongoing process and more recent associations are often facultative, or not required for the host's survival or reproduction.

Such facultative vertically transmitted symbionts invade host populations by increasing the frequency of infected females relative to uninfected ones, utilizing a variety of strategies to do so (Bull, 1983; Werren, O'Neill 1997). As evidenced by the above examples, a symbiont can increase its own fitness by conferring a fitness benefit to the host. Like obligate endosymbionts, facultative endosymbionts can provide nutritional supplementation, such as Wolbachia's provisioning of B vitamins to their bedbug host (Hosokawa et al., 2010), or increasing the fitness of Drosophila on iron-limited diets (Brownlie et al., 2009). Alternatively, many facultative endosymbionts confer protection against parasites. The facultative endosymbiont Hamiltonella defensa defends its aphid host against parasitoid wasps, and Wolbachia can protect Drosophila against RNA viruses (Brownlie, Johnson, 2009; Haine, 2008; Oliver et al., 2009). In these cases, the ability to rapidly acquire ecologically important traits allows for an expansion of host range.

Other strategies for invading host populations do not involve conferring fitness benefits to hosts. Endosymbionts can manipulate the host's reproduction to favor the production of infected females, sometimes at the cost of host fitness (Werren, O'Neill 1997). Wolbachia is the most famous reproductive manipulator, causing such phenotypes as parthenogenesis, feminization, male-killing and cytoplasmic incompatibility in a variety of hosts (Werren et al., 2008). Such reproductive manipulators can affect host mating systems and cause reproductive isolation.
Alternatively, facultative endosymbionts could be commensal partners, with high enough levels of horizontal transmission to counterbalance loss due to vertical transmission (Anderson, May 1981). For many facultative endosymbionts whose fitness effects are unknown, this is the general assumption. Detection of these bacterial partners has outpaced the study of their phenotypic effects, and often removal of such endosymbionts results in no obvious detrimental effects to their host (Oliver et al., 2009). Many fitness effects, though, are only apparent when investigated under the appropriate conditions.

Exploring the mechanisms of symbiosis is a key next step in understanding the evolutionary implications of such partnerships. Drosophila, as a model organism, provides an unprecedented great opportunity to do just that. Drosophila is infected with only two heritable bacterial endosymbionts; Wolbachia and Spiroplasma (Mateos et al., 2006). While Wolbachia infections are very well characterized, little is know about the consequences of harboring Spiroplasma. Spiroplasma infections have thus far been documented in 17 Drosophila species (Haselkorn et al., 2009; Jaenike et al., 2010a; Mateos et al., 2006; Montenegro et al., 2006; Montenegro et al., 2005; Ota et al., 1979; Watts et al., 2009; Williamson, Poulson, 1979), and yet the forces driving the distribution of Spiroplasma among Drosophila are only beginning to be understood. Spiroplasma are male-killers in some species of Drosophila, however, recent screening of natural populations has shown that Drosophila species with some of the highest prevalences of infection harbor Spiroplasma that do not cause male-killing (Watts et al., 2009), and in most cases their fitness effects are unclear. Although these Spiroplasma could be commensal bacteria, it is also possible that like many facultative endosymbionts, Spiroplasma could confer a fitness benefit to its the host in the form of nutritional
supplementation or parasite protection. In fact, recent work has demonstrated that *Spiroplasma* acts as defensive endosymbiont in *D. neotestacea*, a mushroom-feeding fly parasitized by a nematode that causes sterility (Jaenike *et al.*, 2010b). *Spiroplasma*-infected parasitized flies have restored fertility and smaller nematodes, and * Spiroplasma* infection has swept through *D. neotestacea* populations to high prevalence in the last 20 years.

To discern the impacts of *Spiroplasma* infection in *Drosophila*, and to further develop the *Drosophila/Spiroplasma* relationship as a model for studying symbiosis, the goal of my dissertation is to understand the distribution of *Spiroplasma* among its *Drosophila* host. Although dramatic effects of *Spiroplasma* have been documented, the consequences of harboring *Spiroplasma* are still unknown in many species of *Drosophila*. First, in Chapter 1 I review what is currently known about *Spiroplasma* infection in *Drosophila*. Then, to begin to determine the consequences of *Spiroplasma* infection in *Drosophila*. Fitness effects, both positive and negative, can vary based on the particular endosymbiont strain (Degnan, Moran, 2008; Pfarr, Hoerauf, 2005; Werren, 1997). Evolutionary relationships can provide insight into whether *Spiroplasma* is an ancient infection followed by co-divergence between host and bacteria, as is common for beneficial endosymbionts (Akman *et al.*, 2002; Shigenobu *et al.*, 2000; Tamas *et al.*, 2002), or whether multiple introductions have occurred via horizontal transmission as seen for reproductive parasites such as *Wolbachia* (Baldo *et al.*, 2006; Werren, Bartos, 2001). I find that there have been at least five introductions of four very different types of *Spiroplasma* in *Drosophila*. 
In my remaining work I further characterize these distinct Spiroplasma strains. In Chapter 3 I investigate the density dynamics of these diverse Spiroplasma strains infecting different species of Drosophila. Within-host bacterial endosymbiont density plays a critical role in maintenance of a symbiotic relationship, as it can affect levels of vertical transmission and expression of phenotypic effects, both of which can influence the infection prevalence in host populations (Jaenike, 2009). I find that the density dynamics vary greatly among Spiroplasma strains and Drosophila hosts, and that there does seem to be a correlation between population infection prevalence and bacterial titer for one species of Drosophila. Finally, in Chapter 4, I directly evaluate the fitness consequences of a newly discovered Spiroplasma type infecting the cactophilic D. mojavensis. Since other facultative endosymbionts have been demonstrated to supplement nutrition and/or play a role in host plant specialization (Brownlie et al., 2009; Hosokawa et al., 2010; Oliver et al., 2009), I explore this possibility in one population of D. mojavensis that has a high Spiroplasma infection prevalence.

REFERENCES


CHAPTER 1

The Spiroplasma heritable bacterial endosymbiont of Drosophila

ABSTRACT

Since the discovery of the small, gram-positive bacterium, Spiroplasma, as a sex-ratio distorting agent in Drosophila over 50 years ago, substantial progress has been made in understanding the relationship of this bacteria with its insect host. Thus far, Spiroplasma have been found as heritable endosymbionts in sixteen different species of Drosophila. In some species these bacteria cause a male-killing phenotype, where the males die during embryogenesis. In other species, however, Spiroplasma does not cause male-killing, and its fitness effects are unclear. Though recent research has identified multiple factors that affect the prevalence and transmission of Spiroplasma in Drosophila populations, much work remains to fully characterize this symbiosis. Spiroplasma is the only identified heritable bacterial endosymbiont of Drosophila, other than Wolbachia, and can serve as a useful as model for elucidating the nature of insect/bacterial interactions.

REVIEW

Spiroplasma are one of only two heritable bacterial endosymbionts to infect Drosophila (Mateos et al., 2006). Less studied than the other well-known endosymbiont, Wolbachia (Werren et al., 2008), the evolutionary consequences of harboring Spiroplasma are unclear. In general, heritable endosymbionts can have myriad effects on
their insect hosts ranging from mutualistic to parasitic, with the potential to dramatically affect their hosts' evolution. These bacteria are maternally transmitted, and as such their survival is intimately coupled to that of their hosts. Thus theory predicts that they be beneficial partners; however, these bacteria can also increase their own fitness at the cost of their host by manipulating the host's reproduction to favor the production of infected females (Anderson, May, 1982; Bull et al., 1991; Ewald, 1983; O'Neill et al., 1997).

In *Drosophila*, the effects of such endosymbionts are only beginning to be understood, though considerable progress has been made characterizing *Wolbachia* infections. According to a recent survey of GenBank, *Wolbachia* infects at least 36 species of *Drosophila*. Historically famous as a reproductive manipulator, *Wolbachia* causes such phenotypic effects as cytoplasmic incompatibility, whereby an uninfected female produces no offspring when mated to an infected male, consequently increasing the number of infected females (Werren et al., 2008). *Wolbachia* can also cause male-killing, where the males die during embryogenesis, presumably to the advantage of their infected sisters. Recently, however, several beneficial aspects of *Wolbachia* infection in *Drosophila* are under investigation: *Wolbachia* has been shown to confer protection against certain RNA viruses (Hedges et al., 2008; Teixeira et al., 2008), as well as provide nutritional supplementation in some species of *Drosophila* (Brownlie et al., 2009).

Similar to *Wolbachia*, *Spiroplasma* is a heritable bacterial endosymbiont, transmitted transovarially from mother to offspring, which causes male-killing in some species of *Drosophila*; however, other species of *Drosophila* harbor *Spiroplasma* that do not cause male-killing, and no obvious phenotypic effects of this infection have yet been
found (Kageyama et al., 2006; Ota et al., 1979; Williamson, Poulson, 1979). As male-killers, Spiroplasma infections in Drosophila have been known for over 50 years (Williamson, Poulson, 1979); though they were originally referred to as an SRO (Sex Ratio Organism) until technological advances allowed for their correct identification. The non-male-killing Spiroplasma, with a lack of obvious phenotype, were discovered much later (Ota et al., 1979), and only recently has full extent of Spiroplasma infections in Drosophila started to be recognized. Recent screening has revealed at least sixteen Drosophila species infected with genetically diverse strains of this bacterium. Within natural populations prevalence varies greatly, with an infection rate of up to 85% of non-male-killing Spiroplasma in both males and females in one Drosophila population (Watts et al. 2009). Substantial progress has been made in understanding this symbiosis by exploring the incidence and prevalence of infection, the genetic diversity of Drosophila Spiroplasma, the mechanisms of Spiroplasma infection, modes of transmission and fitness effects. However, further investigation of many aspects of this relationship is necessary to appreciate the forces driving the distribution of Spiroplasma among Drosophila as well as the evolutionary consequences of this symbiosis.

**Spiroplasma are prevalent arthropod-associated bacteria**

* Spiroplasma are obligate host-associated bacteria and may be one of the most diverse taxa of bacteria due to their extensive host range (Gasparich et al., 2004). They are small, helical, motile bacteria lacking a cell wall, phylogenetically gram-positive (Woese et al., 1980). Besides Drosophila, Spiroplasma are also male-killing heritable endosymbionts in several species of ladybird beetle (Duron et al., 2008; Hurst et al.,
1999; Majerus et al., 1999; Tinsley, Majerus, 2006) and a butterfly (Danaus chrysippus) (Jiggins et al., 2000). Spiroplasma have also been found in several species of spiders (Duron et al., 2008; Goodacre et al., 2006), where they may play a role in sex-ratio distortion. More commonly, Spiroplasma have been found as gut bacteria in many species of arthropods and assumed to be commensals, attaching to gut epithelial cells with no adverse effects (Bove, 1997).

Several are pathogenic, however, and the transition to pathogenicity may be tied to the ability to cross the insect midgut barrier and multiply in the host hemolymph and other tissues. As pathogens, they have been well-characterized in shrimp (Nunan et al., 2005), crabs (Wang et al., 2005; Wang et al., 2003) and bees (Clark, 1977; Mouches et al., 1983) causing high levels of mortality with rapid onset of symptoms. Routes of pathogen transmission are under current investigation, though it is speculated that the Spiroplasma are ingested (Heres, Lightner, 2009), horizontally rather than vertically transmitted. Bees may acquire Spiroplasma infection while foraging, as flower surfaces serve a reservoir (Clark, 1982).

Furthermore, as agronomically important plant pathogens causing citrus stubborn disease (S. citri) (Bove et al., 2003; Markham et al., 1974), and corn stunt (S. kunkelii) (Davis et al., 1972; Hackett, Clark, 1989), their small AT-rich genomes (~1-2 Mb) have been sequenced and their mechanisms of pathogenicity are being elucidated (Bove et al., 2003; Calavan, Bove, 1989). Plants infected with the diseases, transmitted via a leafhopper insect vector, suffer leaf yellowing, stunted growth and other deformations. Spiroplasma mutagenesis studies reveal that inactivation of the fructose utilization operon ameliorates these pathogenic effects, while inactivation of other key proteins
limits the transmissibility of the disease. *Spiroplasma* replication does not appear to harm its insect vector; in fact, *Spiroplasma* can be leafhopper mutualists, conferring cold tolerance to their host (Ebbert, Nault, 2001).

**Incidence and Prevalence of Spiroplasma infection in Drosophila**

Sex Ratio Organisms, later identified as *Spiroplasma*, were first observed in four species of neotropical *Drosophila* in the willistoni group of the subgenus Sophophora: *D. willistoni* (infected with the willistoni sex ratio organism [WSRO]), *D. nebulosa* (NSRO), *D. equinoxalis*, and *D. paulistorum* (Williamson, Poulson, 1979) (Fig. 1). More recently, a male-killing *Spiroplasma* was found in a *D. melanogaster* from Brazil (Montenegro *et al.*, 2005) as well as a *D. melanogaster* from Africa (Pool *et al.*, 2006). Prevalences of *Spiroplasma* infection in natural populations of *D. nebulosa* and *D. melanogaster* find a range of 1-6%, varying seasonally (Marques, de Magalhaes, 1973; Williamson, Poulson, 1979). Male-killing *Spiroplasma* infections also likely occur in the *tripunctata* radiation of *Drosophila*, in *D. neocardini*, *D. ornatifrons*, and *D. paraguayensis* (Montenegro *et al.*, 2006a).

Infection incidence and prevalence of the non-male killing *Spiroplasma* in *Drosophila* are higher. In addition to the early discovery of non-male-killing *Spiroplasma* infecting 46% of a *D. hydei* population (Ota *et al.*, 1979), recent screening of five other *D. hydei* populations in Japan has found infection prevalences ranging from 23-66% (Kageyama *et al.*, 2006). Subsequent screening of over 200 species of *Drosophila* in the *Drosophila* Species Stock Center and in more than 19 natural populations throughout southwestern North America have found seven additional species infected with
Spiroplasma that do not seem to cause male-killing. This is likely an underestimate, as
screens of Stock Center lines miss infections that have been lost over time due to
imperfect vertical transmission, as well as any Drosophila with male-killers, which could
not have been established as an isofemale line. Within the repleta species group of the
subgenus Drosophila there are infected D. aldrichi, D. wheeleri, and D. mojavensis
(Mateos et al., 2006; Watts et al., 2009). The mushroom-feeding D. tenebrosa of the
quinaria species group is infected (Watts et al., 2009) as well as D. atripex, D.
anassae, D. simulans, and D. melanogaster (Haselkorn et al., 2009; Montenegro et al.,
2005; Pool et al., 2006; Watts et al., 2009) of the melanogaster species group in the
subgenus Sophophora. Infection prevalences of the non-male-killing Spiroplasma vary
greatly, with a low prevalence in D. melanogaster and D. simulans (<1%), up to a
prevalence of 85% for one population of D. mojavensis (Watts et al., 2009). Sixteen
species of Drosophila have been found to harbor Spiroplasma so far, and it is likely that
with screening of additional Drosophila from other geographic locations more infected
species will be found.

The diversity of Spiroplasma infecting Drosophila

Drosophila Spiroplasma were first characterized using serological methods.
Though difficult to culture, the Spiroplasma infecting D. willistoni was grown in an
insect cell line media and named S. poulsonii. Cultivation, however, is difficult, and
Spiroplasma lose the ability to be vertically transmitted when reintroduced into
Drosophila (Williamson et al., 1999), preventing further characterization by traditional
microbiological methods. Later, with the advent of molecular methods to identify
bacteria using 16S rRNA, *S. poulsonii* was found to fall in the *Spiroplasma* citripoulsonii clade, closely related to *Spiroplasma* infecting plants. The *Spiroplasma* infecting two of the species of the *D. willistoni* group and *D. melanogaster* (Bentley et al., 2002; Montenegro et al., 2005; Pool et al., 2006) are very closely related. Later sequencing of the non-male-killing *Spiroplasma* infecting the *D. hydei* in Japan found that this strain was nearly identical at the 16S rRNA locus to the male-killing *Spiroplasma* infecting *D. nebulosa* (Kageyama et al., 2006). Early screening hinted at a greater diversity of *Spiroplasma* infecting *Drosophila* (Mateos et al., 2006), and a more complete sampling exposed the extent of this diversity (Haselkorn et al., 2009; Watts et al., 2009).

A multilocus phylogenetic analysis of *Spiroplasma* infecting 65 individuals of nine species of *Drosophila* revealed that these *Spiroplasma* fall into four distinct clades (Haselkorn et al., 2009) (Fig. 2). The male-killing *Spiroplasma* of the *willistoni* group and the non-male-killing *Spiroplasma* infecting *D. hydei* and *D. simulans* fall into the poulsonii clade, with identical haplotypes at the 16S ribosomal RNA gene (rRNA) locus. More rapidly evolving loci provided greater resolution, with the male-killing *Spiroplasma* grouping together with strong bootstrap support, separate from the non-male-killing *Spiroplasma*. These poulsonii *Spiroplasma* group most closely with the *Spiroplasma* found on flower surfaces (*S. insolitum*) and shrimp pathogens (*S. penaei*). The *Spiroplasma* infecting *D. wheeleri*, *D. aldrichi*, *D. mojavensis*, and four individuals of *D. hydei* fall into the citri clade of *Spiroplasma*, showing approximately 3% sequence divergence at the 16S rRNA locus from the poulsonii clade, and most closely related to the plant pathogenic *Spiroplasma* (*S. citri* and *S. kunkeli*). The *Spiroplasma* infecting *D.
*atripex* and *D. ananassae* fall into the ixodetis clade, most closely related to *S. ixodetis* infecting ticks, and the male-killing *Spiroplasma* infecting a ladybird beetle (*Anisosticta novemdecimpunctata*) and butterfly (*Danaus chrysippus*). These ixodetis *Spiroplasma* show close to 10% sequence divergence at the 16S rRNA locus to the citri-poulsonii clade. Finally, the *Spiroplasma* infecting *D. tenebrosa* were most closely related to the ixodetis clade, but exhibiting 3% sequence divergence at the 16S rRNA locus and distinct from any other *Spiroplasma* sequenced thus far.

**Modes and and routes of *Spiroplasma* transmission**

As a heritable endosymbiont, vertical transmission of *Spiroplasma* is a defining feature of this symbiosis. *Spiroplasma* are incorporated into *Drosophila* oocytes early during vitellogenesis, resulting in transovarial transmission (Niki, 1988). For non-male-killing *Spiroplasma*, males as well as females are infected. The fidelity of vertical transmission of *Spiroplasma* can be quite high, near 100%, though it can be affected by female age, host genotype, *Spiroplasma* strain and temperature. When infected female *D. nebulosa* are mated to males of different genetic backgrounds *Spiroplasma* infections were lost at different rates (Williamson, Poulson, 1979). Different lines of *D. willistoni* infected with dissimilar *Spiroplasma* strains showed an increase in infection loss with female age in one host line but not others. Rates of loss varied depending on the *Spiroplasma* strain, suggesting a complex interaction-phenotype between host genetic background and *Spiroplasma* strain (Ebbert, 1991). In several cases *Spiroplasma* infections in *D. melanogaster*, *D. nebulosa* and *D. hydei* were lost at low temperatures (18°C) (Anbutsu et al., 2008; Montenegro, Klaczko, 2003; Osaka et al., 2008), while in
others loss was associated with higher temperatures (Anbutsu et al., 2008). In the case of low temperatures, the Spiroplasma density within the fly decreases, which may be the cause of loss. At higher temperatures, however, density does not decrease and the reason for loss under these conditions is unclear. Interestingly, populations of D. hydei that maintain Spiroplasma at relatively high prevalences routinely experience extreme temperature fluctuations, implying the existence of a conditional fitness benefit and/or the occurrence of horizontal transmission to explain the maintenance of infection (Anbutsu et al., 2008).

Similar Spiroplasma haplotypes infect very divergent hosts, and closely related Drosophila are infected with very divergent Spiroplasma, indicating that horizontal transmission likely plays an important role in this symbiosis in establishing infections (Haselkorn et al., 2009). The existence of Drosophila Spiroplasma falling into four distinct clades, each of which is most closely related to Spiroplasma infecting other organisms, suggests at least four separate introductions of Spiroplasma into Drosophila. Drosophila hydei is infected with two very different Spiroplasma, likely representing an additional introduction. Furthermore, Spiroplasma with similar or identical haplotypes infect D. melanogaster from both Brazil and Africa (Pool et al., 2006), D. simulans, D. nebulosa, and D. hydei (Haselkorn et al., 2009). As D. melanogaster and D. hydei are at least 50 million years diverged (Tamura et al., 2004), it is unlikely that infection with such similar Spiroplasma resulted from an ancient infection followed by co-divergence.

The frequency of horizontal transmission has yet to be determined. Possible mechanisms for intra- and inter-specific horizontal transmission may involve mites or ingestion. Generalist ectoparasitic mites have been shown to take up Spiroplasma from
D. nebulosa and transfer it at a low frequency to D. willistoni under laboratory conditions (Jaenike et al., 2007). There is also one report of horizontal transmission of Spiroplasma by ingestion in Drosophila (Carvalho, da Cruz, 1962), though several attempts to repeat the experiment failed to find the same result (Ebbert, 1991; Williamson, 1984; Williamson, Poulson, 1979). If horizontal transmission is common, then co-infections of different Spiroplasma strains in populations could lead to recombination in the Spiroplasma genomes, as is commonly seen in Wolbachia (Baldo et al., 2006). The multi-locus phylogenetic analysis of Drosophila Spiroplasma, however, revealed no evidence for recombination within or between loci (Haselkorn et al., 2009). This may not indicate a lack of co-infection, since Drosophila Spiroplasma, like some other Spiroplasma, may lack the recA gene, which is necessary for recombination. Evidence for recombination occurring via other mechanisms, however, exists in these recA deficient Spiroplasma (Marais et al., 1996a; Marais et al., 1996b).

While horizontal transmission is important in establishing interspecific infections, it is unclear what role, if any, horizontal transmission plays in maintaining infection within a species. It is possible that Spiroplasma may be existing as commensals, and that the infections imperfect vertical transmission may be counterbalanced by some horizontal transmission (Anderson, May, 1981). A lack of Spiroplasma genetic variation within most Drosophila species makes it difficult to answer this question, since co-infections and/or recombination would not be detected. However, within populations of D. hydei and of D. mojavensis, there is not a tight correlation between Spiroplasma infection and mitochondrial haplotype, as expected with strict vertical transmission, suggesting that horizontal transmission may be occurring at the population level (Haselkorn et al., 2009).
Mechanisms of Spiroplasma infection

The maintenance and effects of male-killing Spiroplasma infections have been studied extensively. In the 1960's, the Spiroplasma infecting D. nebulosa (NSRO) and D. willistoni (WSRO) were injected in the several different D. melanogaster lines to be studied in the laboratory in an attempt to understand the process of male-killing (Sakaguchi, Poulson, 1963). While maintaining male-killing Spiroplasma infections in the lab, a non-male-killing variant arose (NSRO-A) (Yamada et al., 1982), which provided an opportunity for comparison. Anbutsu and Fukatsu (Anbutsu, Fukatsu, 2003) measured the bacterial titer using quantitative PCR, of both NSRO and NSRO-A over the course of development of D. melanogaster. The titer of NSRO increased exponentially from eclosion to three-week old adults, while the titer of NSRO-A remained the same during this same time period. This significant titer difference, along with the observation that young NSRO infected female flies produce males when their Spiroplasma titers are similar to that of NSRO-A females, led them to propose the bacterial threshold density hypothesis for the occurrence of male-killing. This hypothesis is in concordance with early studies on the dynamics of Spiroplasma infections, as observed increases in Spiroplasma presence in the hemolymph correlated with decreases in the production of male offspring (Williamson, 1965), Spiroplasma titers in the naturally occurring non-male killing D. hydei were also shown to be lower than the male-killing infections in adults one week after eclosion (Kageyama et al., 2006).

Though the precise male-killing mechanism is unknown, many techniques have been employed to gain a better understanding. Early studies using electron microscopy
established that male death occurred during early embryogenesis before gastrulation, noting irregular movement of chromosomes during meiosis in infected embryos (Counce, Poulson, 1962), with a lack of cell differentiation in the primordial nervous system tissues (Koana, Miyake, 1983; Tsuchiyama et al., 1978). Recent studies have identified the initial target as the sex determining pathway, since when any of the five interacting genes of the dosage compensation complex are inactivated, male-killing does not occur (Veneti et al., 2005). In naturally-infected D. nebulosa, male-killing occurs during a narrow developmental time period following the formation of the dosage compensation complex, with widespread apoptosis and arrested development 10-12 hours into embryonic development (Bentley et al., 2007). The NSRO Spiroplasma strain in D. melanogaster, however, caused both the early male-killing, as described above, as well as late male killing (death during the late larval stages) (Kageyama et al., 2007). The occurrence of early versus late male killing depended on maternal host age, and may be related to the density of Spiroplasma as well as whether or not the Spiroplasma strain is studied in its natural host.

Host genetic background also affects the Spiroplasma male-killing phenotype. In many instances, Drosophila harboring male-killing infections have been maintained in the laboratory using males from an uninfected line, and the efficiency of male-killing was maintained near 100% for many generations. However, this efficiency of male-killing varied, depending on the male strain used (Williamson, Poulson, 1979). To further explore this effect, Kageyama et al. (Kageyama et al., 2009) crossed Spiroplasma infected D. melanogaster females to males of ten different genetic backgrounds, and found male-killing suppressed in two lines. Spiroplasma infection, though, was
maintained at high bacterial densities, suggesting the existence of suppressors acting on the male-killing effectors directly.

Phage, or other bacterial extrachromosomal elements, may play an important role in this symbiosis (Oishi, 1971; Williamson, 1969; Williamson et al., 1999). Some evidence suggests they may be involved in the male-killing mechanism itself. In one artificial experimenal transfer of WSRO to *D. robusta*, levels of *Spiroplasma* in the hemolymph decreased to non-detectable levels, while male-killing still occurred, implying that the male-lethal agent may be separate from the bacteria (Williamson, 1966; Williamson, 1969). The different *Spiroplasma* strains, both male-killing and non-male killing, harbor different combinations of two to four different phage. There are, however, no obvious correlations between presence of certain phage and the male-killing phenotype (Cohen et al., 1987; Oishi, Poulson, 1970; Oishi et al., 1984; Ota et al., 1979). Phage may also affect the distribution of *Spiroplasma* among *Drosophila*, as these viruses lyse *Spiroplasma* from different *Drosophila* species or geographically distinct *Spiroplasma* haplotypes of the same species (Cohen et al., 1987; Williamson, Poulson, 1979). For different combinations of male-killing (*S. poulsonii*) haplotypes, artificial super-infection resulted in various amounts of bacterial lysing, observable using a dark field microscope and mixing hemolymph samples on a slide (Sakaguchi et al., 1965; Williamson, Poulson, 1979). Co-infection of two different *Spiroplasma* in vivo sometimes resulted in clearance of the original infection followed by replacement with a different infection (Oishi, Poulson, 1970).

The factors underlying the host specificity of *Spiroplasma* are not well understood. Many male-killing *Spiroplasma* infections were transferred to new
Drosophila hosts, including D. melanogaster and D. pseudoobscura among others, and the male-killing phenotype was often still expressed (Williamson, Poulson, 1979). In some instances, however, the Spiroplasma infection reduced the viability, fecundity, and fertility of their artificial D. melanogaster host (Counce, Poulson, 1962). In many cases, such as for the D. melanogaster NSRO, these newly established infections could be maintained easily in the lab. Other times such transfections were not so easily established, as when the non-male killing Spiroplasma of D. hydei was transferred to D. melanogaster. No male-killing was observed, but the flies suffered early mortality (Kageyama et al., 2006). Bacterial strain also plays a role, as the WSRO Spiroplasma transfections into different species appear much less stable than NSRO transfections (Williamson, Poulson, 1979).

It is unclear what role the Drosophila immune system plays in maintenance of this symbiosis. Transfer of several different types of non-Drosophila Spiroplasma into D. pseudoobscura led to various results, including clearance of Spiroplasma infection, replication within host with no adverse effects, and replication within host and pathogenic effects, depending on the Spiroplasma type (Williamson, 1984). Only one study has examined immune response to Spiroplasma infection, and while Spiroplasma infection did not elicit an immune response, bacterial titer was suppressed by ectopic activation of antimicrobial genes (Hurst et al., 2003). It is unknown if Spiroplasma merely escapes detection because it lacks typical immune elicitors such as a bacterial cell wall, or if it interacts in any way with the host immune system.

Spiroplasma appear to be most prevalent in the Drosophila hemolymph. Early studies determined the presence of infection by observing the Spiroplasma in the
hemolymph under dark field microscopy (Williamson, Poulson, 1979). When the
Spiroplasma titers of different Drosophila host tissues, including the hemolymph, fat
body, intestine, and ovary were measured using quantitative PCR, Spiroplasma
proliferation in the extracellular environment of the hemolymph most closely matched
that of the whole body Spiroplasma dynamics. The bacteria were present, though, at
lower levels in the other host tissues (Anbutsu, Fukatsu, 2006). These infection dynamics
are in contrast to those of Wolbachia infection, which is primarily intracellular and not
found in the insect hemolymph. When Wolbachia and Spiroplasma coinfect flies, the
density of Spiroplasma is unaffected by Wolbachia, whereas the density of Wolbachia is
lowered by the presence of Spiroplasma (Goto et al., 2006). Thus, the extent to which
Wolbachia and Spiroplasma coinfections occur in natural populations may be low.
Montenegro et al. (Montenegro et al., 2005) found four individuals of D. melanogaster
from Brazil that were co-infected; however, coinfection of the non-male-killing
Spiroplasma and Wolbachia has not been observed in natural populations screened to
date.

Other fitness effects and evolutionary implications

Male-killing infections in insects have a range of effects and evolutionary
implications. Infections at high prevalences causing population level sex ratio distortions
can lead to changes in host sexual selection and mating behavior, as well as select for
male-killing resistance genes. They can decrease population density, lowering the
effective population size and reducing genetic variation by cutting offspring production
of infected females in half. At the extreme, these infections could cause population
extinction (Hatcher et al., 1999). Yet this is a phenotype caused by multiple bacterial taxa (Hurst, Jiggins, 2000) and such male-killing infections will spread if they increase fitness of infected females. This occurs if daughters benefit from the death of their brothers, either by gaining more access to limited resources (reduced larval competition), or through the prevention of inbreeding (Engelstadter, Hurst, 2007; Hurst, Jiggins, 2000).

Given that current sampling indicates that male-killing Spiroplasma prevalence in Drosophila is low, the evolutionary implications are difficult to discern. The low prevalence may reflect the ecology and life history of the infected Drosophila species, in which infected females may not receive fitness benefits from male-killing and suggests that male-killing may not be the driving force maintaining this symbiosis.

Attempts to measure the fitness effects of the male-killing Spiroplasma in Drosophila in laboratory settings have produced ambiguous results. D. nebulosa females, infected with their natural male-killing Spiroplasma strains mate two days sooner than uninfected females (Malogolowkin-Cohen, Rodrigues-Pereira, 1975). Studies by Ebbert (Ebbert, 1991) showed a similar shift in the timing of reproduction, the effect of which varied with Drosophila line and Spiroplasma strain, but no overall increase in the production of offspring. In fact, there was an overall decrease in fertility and lifespan in infected females. The possible benefit of early reproduction may occur under crowded conditions, though laboratory experiments exploring this situation found infected flies to be at a disadvantage (Ebbert, 1995). Early reproduction, however, may be advantageous during periods of population expansion. In D. melanogaster there was no measurable fitness effect of Spiroplasma infection on larval competitiveness and adult fecundity, at both low and high larval density conditions (Montenegro et al., 2006b). No early
reproduction in these Spiroplasma-infected lines was observed. Similarly, no apparent differences in survival or reproduction were detected in D. hydei infected with non-male-killing Spiroplasma, and curing D. hydei of Spiroplasma infection resulted in no fitness decrease (Kageyama et al., 2006).

Given that the fidelity of vertical transmission is imperfect, and that evident fitness effects on the host under laboratory conditions are limited or negative, the persistence of Spiroplasma infections in natural populations remains a mystery. It is possible, as mentioned previously, that Spiroplasma may be existing as commensals, and that the infections imperfect vertical transmission may be counterbalanced by some horizontal transmission (Anderson, May, 1981). Further exploration of the role and mechanisms of horizontal transmission in natural populations could elucidate this possibility. For example, if acquisition by ingestion were possible, then rotting fruits or cacti, which serve as both feeding and larval development sites for many arthropods, could serve as an infection reservoir for Drosophila. Incidence and prevalence in some Drosophila species and not others may reflect either chance acquisition or differences in species immune systems, such that some species are resistant to infection. Species-specific ecology may play a role, possibly preventing Spiroplasma replication due to habitat temperature or other constraints. Additionally, there may be costs to harboring Spiroplasma that are not yet apparent, as has been demonstrated for other facultative endosymbionts (Oliver et al., 2008).

Alternatively, many of the above studies imply the existence of one or several conditional fitness benefits that are not obvious under standard laboratory conditions. Such conditional fitness benefits observed in other bacterial endosymbionts include
nutritional supplementation (e.g. Chen et al., 2000), resistance to parasitoid wasps (e.g. Oliver et al., 2003) or predators (e.g. Piel et al., 2004), resistance to RNA viruses (e.g. Hedges et al., 2008), and thermal tolerance (e.g. Chen et al., 2000). Such fitness benefits could have large ecological and evolutionary consequences, allowing Drosophila to successfully explore new habitats and extend their geographic range. Fitness effects due to interactions with other bacteria, such as Wolbachia, with known fitness effects, may also be biologically significant. For Spiroplasma, their effects on their hosts need to be explored under such stresses and conditions to determine if any such fitness benefits exist.

To understand the effects of Spiroplasma on Drosophila, it is apparent that this symbiosis must be studied in the context of the natural ecology of both organisms. The ecology of many of the infected Drosophila species is well known (e.g. Markow, O'Grady, 2007), and the dynamics of Spiroplasma infection may become obvious when studied in their natural habitat. Moreover, the interactions may vary depending on Spiroplasma strain and Drosophila host, given the diversity of Drosophila species infected and the variety of Spiroplasma strains. Furthermore, the genetic and genomic tools of both Drosophila and Spiroplasma provide a great opportunity to make major advances in the understanding of the interactions of an insect host and its heritable endosymbiont; to further understand how these bacteria can evade the host immune system and survive in the hemolymph, as well as explore how such bacteria control their replication and enter different host tissues. With the advent of new sequencing technologies, comparative genomic analysis of Spiroplasma infecting Drosophila, as well as Spiroplasma of other hosts will become more feasible. Genomic analysis can
elucidate the factors affecting the evolution and adaptation of these bacteria to their diverse hosts, including the identification of genes that may be involved in their mutualistic or pathogenic lifestyles as well as those involved in their various modes of transmission. Such advances, in combination with previous research, will help resolve the mysteries surrounding Spiroplasma infection and further elucidate its evolutionary importance in Drosophila.

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REFERENCES


Hurst GDD, von der Schulenburg JHG, Majerus TMO, et al. (1999) Invasion of one insect species, Adalia bipunctata, by two different male-killing bacteria. Insect Molecular Biology 8, 133-139.


**Figure 1.1 Drosophila species infected with Spiroplasma.** Species are listed by species group, and the type of Spiroplasma infection is noted. Prevalence of infection is listed for each species where multiple individuals per population were sampled. The Drosophila phylogeny of species groups is modified from Mateos et al. 2006, originally based on Markow and O’Grady 2006. References: 1. Montenegro et al. 2005, 2. Watts et al. 2009, 3. Williamson, Poulsom 1979, 4. Marques EJ, de Magalhaes 1973, 5. Kageyama et al. 2006.

<table>
<thead>
<tr>
<th>Drosophila species groups</th>
<th>Drosophila species infected</th>
<th>Prevalence (ref.)</th>
<th>Spiroplasma type Male-killing (MK)</th>
</tr>
</thead>
<tbody>
<tr>
<td>melanogaster group:</td>
<td>D. melanogaster</td>
<td>0.5-3% (1,2)</td>
<td>poulsonii (MK &amp; non-MK)</td>
</tr>
<tr>
<td></td>
<td>D. simulans</td>
<td>0.4% (2)</td>
<td>poulsonii</td>
</tr>
<tr>
<td></td>
<td>D. ananassae</td>
<td></td>
<td>ixodetis</td>
</tr>
<tr>
<td></td>
<td>D. atripex</td>
<td></td>
<td>ixodetis</td>
</tr>
<tr>
<td>willistoni group:</td>
<td>D. willistoni</td>
<td>&lt; 1% (3)</td>
<td>poulsonii (MK)</td>
</tr>
<tr>
<td></td>
<td>D. paulistorum</td>
<td>0.4-13% (3)</td>
<td>unknown (MK)</td>
</tr>
<tr>
<td></td>
<td>D. equinoxialis</td>
<td>1-14% (4)</td>
<td>unknown (MK)</td>
</tr>
<tr>
<td></td>
<td>D. nebulosa</td>
<td>3-8% (3,5)</td>
<td>poulsonii (MK)</td>
</tr>
<tr>
<td>repleta group:</td>
<td>D. hydei</td>
<td>22-66% (2,8)</td>
<td>poulsonii &amp; citri</td>
</tr>
<tr>
<td></td>
<td>D. aldrichi</td>
<td>4% (1)</td>
<td>citri</td>
</tr>
<tr>
<td></td>
<td>D. mojavensis</td>
<td>15-85% (2)</td>
<td>citri</td>
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<tr>
<td></td>
<td>D. wheeleri</td>
<td>53% (2)</td>
<td>citri</td>
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<td>guarani group:</td>
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<td>D. neocardini</td>
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<td></td>
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<td>quinaria group:</td>
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<td>13% (2)</td>
<td>tenebrosa</td>
</tr>
<tr>
<td>tripunctata group:</td>
<td>D. paraguayensis</td>
<td></td>
<td>unknown (MK)</td>
</tr>
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</table>
Figure 1.2 Phylogeny of *Spiroplasma* infecting *Drosophila* and other organisms. This 16s rRNA phylogeny was reconstructed using the NJ distance-based methods as described in Haselkorn et al. 2009. Bootstrap support (1000 replicates) is noted. The four major clades into which *Drosophila Spiroplasma* (in bold) fall are denoted with vertical bars.
CHAPTER 2

Multiple introductions of the Spiroplasma bacterial endosymbiont into Drosophila

ABSTRACT

Bacterial endosymbionts are common in insects and can have dramatic effects on their host's evolution. So far, the only heritable symbionts found in Drosophila have been Wolbachia and Spiroplasma. While the incidence and effects of Wolbachia have been studied extensively, the prevalence and significance of Spiroplasma infections in Drosophila are less clear. These small, gram-positive, helical bacteria infect a diverse array of plant and arthropod hosts, conferring a variety of fitness effects. Male-killing Spiroplasma are known from certain Drosophila species; however, in others, Spiroplasma appear not to affect sex ratio. Previous studies have identified different Spiroplasma haplotypes in Drosophila populations, though no extensive surveys have yet been reported. We used a multi-locus sequence analysis to reconstruct a robust Spiroplasma endosymbiont phylogeny, assess genetic diversity, and look for evidence of recombination. Six loci were sequenced from over 65 Spiroplasma-infected individuals from nine different Drosophila species. Analysis of these sequences reveals at least five separate introductions of four phylogenetically distinct Spiroplasma haplotypes, indicating that more extensive sampling will likely reveal an even greater Spiroplasma endosymbiont diversity. Patterns of variation in Drosophila mitochondrial haplotypes in Spiroplasma-infected and uninfected flies imply imperfect vertical transmission in host populations and possible horizontal transmission.
INTRODUCTION

Microorganisms that live in close association with animals, plants and other taxa have a diverse array of effects on their partners, ranging from mutualistic to parasitic. Insects, in particular, form relationships with a variety of bacterial endosymbionts (Buchner 1965). Species of the genus *Drosophila*, despite serving as important model organisms in evolutionary biology, only recently have been screened for heritable bacterial endosymbionts. A large-scale survey across the genus revealed that *Drosophila*, unlike many other insects, harbor only *Wolbachia* and *Spiroplasma* as heritable endosymbionts (Mateos et al. 2006). While the incidence and effects of *Wolbachia* in *Drosophila* have been studied extensively (McGraw & O'Neill, 2004; Werren 1997), the prevalence and significance of *Spiroplasma* infections in *Drosophila* are far less clear.

*Spiroplasma* are small, gram-positive, wall-less, helical bacteria (Whitcomb & Tully 1982; Williamson et al. 1998). A few *Spiroplasma* are agronomically important plant pathogens causing corn stunt (*Spiroplasma kunkelii*) and citrus stubborn disease (*Spiroplasma citri*) (Bove 1997). However, *Spiroplasma* also infect a wide array of arthropod hosts (Gasparich et al. 2004) in which they have diverse effects: they can be mutualistic (Ebbert & Nault, 2001), pathogenic (Bove 1997), or sex-ratio distorters (Goodacre et al. 2006; Tinsley & Majerus 2006; Williamson & Poulson 1979). Initial reports of *Spiroplasma* in *Drosophila* species involved male-killing, in which male offspring die during embryogenesis (Williamson & Poulson, 1979). Numerous other *Drosophila* species, however, are infected with *Spiroplasma* that do not cause male-
killing, and their fitness effects are unknown (Kageyama et al. 2006; Mateos et al. 2006; Watts et al. 2009).

Knowledge of the diversity of *Spiroplasma* infecting *Drosophila* is key to fully understanding the consequences of harboring this endosymbiont. Fitness effects, positive or negative, can vary depending on the particular bacterial strain (e.g. Pfarr & Hoerauf 2005; Werren 1997; Degnan & Moran 2008). Elucidation of evolutionary relationships also will provide insight into whether *Spiroplasma* is an ancient infection followed by co-divergence between host and bacteria, as is common for beneficial endosymbionts (Akman et al. 2002; Shigenobu et al. 2000; Tamas et al. 2002; van Ham et al. 2003), or whether multiple introductions have occurred via horizontal transmission as seen for reproductive parasites such as *Wolbachia* (Baldo et al. 2006; Werren & Bartos 2001). Thus far, *Spiroplasma* infections have been observed in sixteen *Drosophila* species. Male-killing *Spiroplasma* are known to infect *D. willistoni, D. nebulosa, D. paulistorum*, and *D. equinoxialis* of the willistoni species group (Williamson & Poulson, 1979), likely *D. ornatifrons, D. neocardini*, and *D. paraguayensis* of the tripunctata group (Montenegro et al. 2006a), as well as *D. melanogaster* (Montenegro et al. 2005; Pool et al. 2006). Non-male-killing *Spiroplasma* infect *D. hydei* (Mateos et al. 2006; Ota et al. 1979) *D. aldrichi, D. mojavensis* (Mateos et al. 2006), *D. wheeleri, D. tenebrosa* (Watts et al. 2008), *D. simulans, D. atripex*, and *D. ananassae* (Markow unpublished). Previous phylogenetic analyses have revealed close relationships among several male-killing *Spiroplasma* and the non-male-killing *Spiroplasma* infecting some *D. hydei* (Kageyama et al. 2006; Montenegro et al. 2005) while Mateos et al. (2006) explored the relationships of the non-male-killing *Spiroplasma* infecting other *D. hydei, D. aldrichi* and *D.*
*mojavensis*. The evolutionary relationships, however, of other newly discovered *Spiroplasma* remain poorly understood, as do the relationships of the male-killing to other non-male-killing *Drosophila Spiroplasma*.

Population processes, such as horizontal transmission and recombination, also are little known for *Spiroplasma* in *Drosophila* and other arthropod species where it is a vertically transmitted endosymbiont (Majerus *et al.* 1999). Recombination could obscure true infection histories for phylogenetic relationships determined by a single locus (Feil & Spratt 2001; Holmes *et al.* 1999), could affect the adaptive potential of the *Spiroplasma* genome, and lend insight into the dynamics of the *Drosophila/Spiroplasma* symbiosis. We used a multi-locus sequencing approach to address the following questions: 1. What are the evolutionary relationships of the *Spiroplasma* infecting *Drosophila*? 2. How many introductions of *Spiroplasma* have occurred in *Drosophila*? 3. Is there any recombination? 4. What is the association between host mitochondrial haplotype and *Spiroplasma* infection, and what are implications for the relative roles of vertical and horizontal transmission within *Drosophila* populations?

**Materials and Methods**

**Samples of Drosophila**

Sixty-nine infected individuals from nine *Drosophila* species were examined (Table 1). Most individuals were sampled in 2005-2007 from natural populations in western North America. Others were obtained from the Tucson *Drosophila* Stock Center or recently collected in other parts of the world. For some individuals, isofemale lines were established to assay for the male-killing phenotype.
DNA extractions were performed as in Mateos et al. (2006) or Gloor & Engels (1992), and 2µl was used as template in a 25µl PCR reaction, using PCR methods as in Mateos et al. (2006). PCR cycling conditions were an initial denature of 3 minutes 94°C, followed by 30 seconds 94°C, 45 seconds 68°C, 45 seconds 72°C; annealing temperature was lowered 1.0°C per cycle for 15 cycles, then kept for 20 cycles at 48°C. Variations in cycling conditions as well as primer sequences for the various loci are listed in Table 2. PCR products were directly sequenced in both directions using amplification primers and an ABI3730 sequencer at the Genomics and Analysis Technology Core Facility at the University of Arizona.

**Sequencing**

*Spiroplasma* multi-locus sequencing

Six loci were chosen to compare *Drosophila Spiroplasma* to other sequenced *Spiroplasma*, to detect phylogenetic incongruence among loci, and to increase phylogenetic resolution. The 16S ribosomal RNA (rRNA) and ITS (internal transcribed spacer) loci were selected because these conserved loci have been sequenced for numerous other *Spiroplasma*. The remaining genes, *rpoB* (RNA polymerase B), *ftsZ* (cell-division protein), *parE* (DNA topoisomerase), and *fruR* (partial fructose operon) are more rapidly evolving bacterial housekeeping genes that are good phylogenetic markers because they are unlikely to be under positive selection and are likely to be orthologous among all *Spiroplasma* (Dunning-Hotopp et al. 2006; Welch et al. 2002).

The partially assembled *S. citri* genome was used to locate several of the genes and confirm that they are in different chromosomal regions. Additionally, *rpoB* and *parE*
have been sequenced for other *Spiroplasma* species, allowing for elucidation at multiple loci of the relationships of *Drosophila Spiroplasma* to those infecting other organisms. Finally, we sequenced a small portion (~400bp) of the fructose operon (*fru*), previously found to be a variable locus in other *Drosophila Spiroplasma* studies (Montenegro *et al.* 2005).

Amplification of each locus was attempted for all infected *Drosophila*, followed by sequencing. For those not amplifying after two attempts, primers were redesigned for re-amplification. A complete listing of *Drosophila* samples used and their amplification success is provided in Supporting Information Table 1. GenBank accession numbers are FJ656998-FJ657372.

**Drosophila mitochondrial DNA sequencing**

To detect variable mitochondrial sequences within populations of *D. hydei*, the partial *cytochrome oxidase II (COII)* locus was sequenced (600bp) (PCR conditions were as in Folmer *et al.* (1994)) as well as a 600bp of the AT-rich region (primers and PCR conditions as in Brehm *et al.* 2004). Twenty infected and thirty uninfected flies, roughly reflecting the proportion of infected individuals in this species (Watts *et al.* 2009), were sequenced for these regions. These flies were from five localities throughout the Sonoran desert and southern Arizona (Table 1). For *D. mojavensis*, the *cytochrome oxidase I (COI)* locus was sufficiently variable and was sequenced for thirty infected and forty uninfected individuals from three localities (Table 1). GenBank accession numbers are FJ656811-FJ656997.
Sequence analysis and phylogenetics

The sequences were cleaned in Sequencher 4.5 (Gene Codes, Ann Arbor, MI), aligned using Muscle (Edgar 2004), and adjusted in the SeAl manual alignment program (Rambaut 1996). Additional Spiroplasma sequences were downloaded from NCBI. These sequences included the highest blast hits for the different haplotypes at each locus and other related Spiroplasma species based on published Spiroplasma phylogenies (Gasparich et al. 2004; Regassa & Gasparich 2006). The outgroup species for the more conserved 16S rRNA, ITS, and rpoB was the most closely related species with a full genome sequenced, Mycoplasma mycoides. For the more rapidly evolving ftsZ, parE, and fruR, the M. mycoides sequences were too divergent to reasonably align, and the most closely related Spiroplasma species outside of the groups of interest were used. Where none were available the tree was mid-point rooted.

Phylogenetic analyses were performed individually on each locus as well as on combinations of loci. Distance-based (neighbor-joining) phylogenetic reconstructions with 1000 bootstrap replicates were performed using PAUP* 4.0b10 (Swofford 1998). Shimodaira-Hasegawa (SH) tests were run to compare the likelihood score of the best tree for the dataset of each locus against the likelihood of the topology of every other locus. The SH tests were run using full optimization and 1000 bootstrap replicates in PAUP. Bayesian phylogenetic analyses were performed using Mr. Bayes version 3.1.2 (Huelsenbeck & Ronquist, 2001). Bayesian analyses were run for 10,000,000 generations on four simultaneous Monte Carlo Markov chains using the general time reversible model, collecting trees every 100 generations. The first 5000 trees were discarded as "burn-in".
DNAsp (Rozas et al. 2003) was used to calculate population genetic parameters such as nucleotide diversity, GC content, average Ka/Ks, and recombination. Additionally, recombination within the alignments of each individual locus was detected with Genconv (Sawyer 1989). Haplotype networks were constructed using the TCS program (Clement et al. 2000) while Arlequin (Schneider et al. 2000) was used to build minimal spanning trees.

RESULTS

Genetic diversity of *Spiroplasma* infecting *Drosophila*

* Spiroplasma* from all nine *Drosophila* species amplified for the 16S rRNA, ITS, and rpoB loci. For parE, ftsZ, and fruR, the *Spiroplasma* infecting *D. atripex*, *D. ananassae*, and *D. tenebrosa* did not amplify after multiple attempts. The inability to amplify these loci after several attempts with multiple primer sets likely reflects the large sequence divergence at these more rapidly evolving loci. *Spiroplasma* infecting *D. simulans* amplified only for 16S rRNA, rpoB, and ftsZ.

A basic description of the genetic diversity indices is given in Table 3. Amplified loci ranged from 327-1252 base pairs in length with an average of 35% G+C content. Levels of nucleotide diversity and sequence divergence were different at each of the six loci, with the 16S rRNA locus being the most conserved and the fruR locus having the highest nucleotide diversity. The average pairwise Ka/Ks for protein-coding loci ranged from 0.038-0.253, reflecting purifying selection. Only one *Spiroplasma* haplotype was found to infect each *Drosophila* species except for the case of *D. hydei*, which contained two. The same *Spiroplasma* haplotype infects both *D. aldrichi* and *D. wheeleri*. 
**Same phylogenetic pattern seen across loci indicates a lack of recombination**

Similar evolutionary relationships are seen among the *Drosophila Spiroplasma* at each locus (Figures 1, 2, and 3) indicating an absence of intergenic recombination. No statistically significant phylogenetic incongruence was found at any pairwise comparison between loci (Supporting Information Table 2). Furthermore, no intragenic recombination was detected within any locus, with the exception of a possible recombinant in *S. chrysicola*. Given that recombination was not detected, the loci were concatenated, and the resultant tree with only unique *Spiroplasma* haplotypes is shown in Figure 4.

**Drosophila Spiroplasma fall into four distinct phylogenetic clades**

A Bayesian phylogenetic tree based on 1252 bp of 16S rRNA from all 69 individuals (Figure 1) is representative of the evolutionary relationships at each locus. The *Spiroplasma* infecting *Drosophila* (denoted *S. sp. Drosophila*) fall into four distinct clades with high bootstrap support. The clade containing the *S. poulsonii* of *D. willistoni* also contains the *Spiroplasma* infecting 32 *D. hydei* individuals from various locales North America, as well as one from Japan. Additionally, the same *Spiroplasma* haplotype infects *D. simulans*. Within the citri clade, about 2% sequence divergent from the those of the poulsonii clade, is another group of *Spiroplasma* infecting four *D. hydei* individuals as well as *D. aldrichi, D. wheeleri, and D. mojavensis*. Contained within this citri clade are three well-supported *Spiroplasma* groups: *S. sp. D. mojavensis, S. sp D. hydei, and S. sp. D. wheeleri/D. aldrichi*. The remaining two clades in which
*Drosophila Spiroplasma* are found, the ixodetis and tenebrosa clades, show about 12% sequence divergence from the poulsonii and citri clades. Falling into the ixodetis clade are the *Spiroplasma* infecting *D. atripex* and *D. ananassae* from Africa as well as a *D. ananassae* from Hawaii. Finally, the *Spiroplasma* infecting *D. tenebrosa* fall into a distinct clade, most closely related to the ixodetis clade, but nonetheless separated by an average 3% sequence divergence.

**At least five separate introductions of *Spiroplasma* into *Drosophila***

*Spiroplasma* found to infect *Drosophila* are not monophyletic. The *Drosophila* *Spiroplasma* in each clade are more closely related to those infecting other organisms than they are to those infecting other *Drosophila*. For example, the *Drosophila* *Spiroplasma* in the poulsonii clade are most closely related to *S. phoencium*, prevalent on flower and plant surfaces (Bove 1997), as well as *S. penaei*, a pathogen of shrimp (Figure 1). Another major group of *Drosophila Spiroplasma* haplotypes is more closely related to *S. citri* and *S. kunkelii*, plant pathogens, than to the *Drosophila Spiroplasma* in the poulsonii clade. A third group is most closely related to the *Spiroplasma* of the ixodetis tick, several species of spider and ladybird beetles. Finally, the *Spiroplasma* infecting *D. tenebrosa* are different from any *Spiroplasma* species represented thus far in GenBank. At the ITS locus, where additional sequences are available, the *D. tenebrosa Spiroplasma* appears to be most closely related to that infecting spiders, though it is still > 4% sequence different from any previously sequenced *Spiroplasma*. Thus, each clade represents a separate introduction into *Drosophila* hosts. Furthermore, two very different *Spiroplasma* infect *D. hydei*; those in the poulsonii clade that infect the majority of *D.*
individuals, and those in the citri clade, found in only four *D. hydei* individuals.

The phylogenetic relationships of the *Spiroplasma*-infected *Drosophila* species used in this study are denoted in Figure 5.

**Relationships between the male-killing and non-male-killing *Spiroplasma***

Only one male-killing *Spiroplasma*, that infecting *D. melanogaster*, was available for sequencing at all loci, though others were represented by 16S rRNA and *fruR* sequences in GenBank. Male-killing *Spiroplasma* infecting *D. melanogaster* and *D. nebulosa* have 16S rRNA sequences identical to that of the non-male-killers infecting *D. hydei*. At the five other loci, however, the male-killer from *D. melanogaster* has a haplotype different from the *D. hydei* *Spiroplasma*. At the *fruR* locus (Figure 3.), the male-killing *Spiroplasma* all group together with strong bootstrap support and are clearly separate from the non-male-killers in *D. hydei*, with a 2% sequence divergence between the haplotypes.

**Spiroplasma infections within populations**

We looked for associations between *Drosophila* mitochondrial haplotype and *Spiroplasma* infection. If an infection has occurred recently and is maintained in the population due to a high fidelity of vertical transmission within descendant matrilines, we expect a particular *Spiroplasma* infection to be associated with one or only a few mitochondrial haplotypes within a population. Alternatively, *Spiroplasma* infection affecting all or most mitochondrial haplotypes would suggest an older infection followed by loss in some lineages and/or frequent horizontal transmission of *Spiroplasma* among
individuals in the populations. We were able to test these predictions in two *Drosophila* species.

For *D. mojavensis*, 81 individuals from three populations (Organ Pipe National Monument (OPNM), San Carlos (SC) and Catalina Island (CI)) belong to 14 total haplotypes forming three distinct clusters (Figure 6a). The CI flies form a separate cluster with only two mitochondrial haplotypes. Flies with both haplotypes were both *Spiroplasma*-infected and *Spiroplasma*-uninfected. The *D. mojavensis* mitochondrial haplotypes from SC and OPNM of mainland Sonora are intermixed in the two remaining groups. One cluster contains a prevalent mitochondrial haplotype (containing more than 20 individuals) that belongs to both infected and uninfected flies. Other mitochondrial haplotypes in this clade also contain both infected and uninfected flies. The other cluster, however, contains mitochondrial haplotypes consisting of mostly uninfected flies. Only one individual in this group of haplotypes is infected. In total, *Spiroplasma* is associated with seven of the 14 mitochondrial haplotypes in the population. A majority of the sampled individuals, both infected and uninfected, fall into two haplotypes. This lack of a strong association of infection status with mitochondrial haplotype is consistent with either an older infection followed by loss or horizontal transmission.

The 53 *D. hydei* sampled contain two types of *Spiroplasma*, the poulsonii clade and the citri clade. For this species, both the *COII* and AT-rich region of the mitochondrial genome had limited sequence variation, despite the wide geographical sampling. Only 12 closely related haplotypes are shown in the haplotype network (Figure 6b), and both infected and uninfected *D. hydei* have these haplotypes. The citri clade *Spiroplasma* is associated with only two connected *Drosophila* haplotypes. The
poulsonii clade Spiroplasma infects most of the other haplotypes. Similar to the pattern seen in D. mojavensis, this distribution of infection is consistent with horizontal transmission, or an older infection with subsequent loss.

**DISCUSSION**

Phylogenetic analyses show at least five separate introductions of four distinct clades of Spiroplasma into Drosophila. This surprising amount of Spiroplasma diversity was discovered despite limited sampling. The majority of samples in this study were collected from only the western part of North America, yet, in addition to finding citri and poulsonii Spiroplasma, we identified a very divergent Spiroplasma infecting D. tenebrosa. Its most closely related Spiroplasma species is S. ixodetis, though it is still 3-15% divergent from S. ixodetis at various loci. Our limited sampling of Drosophila outside of western North America identified Drosophila infected with ixodetis-type Spiroplasma from Africa. Recent screening of arthropods for a few Spiroplasma strains uncovered additional Spiroplasma hosts (Duron et al. 2008), and it is likely that a wider geographical and taxonomic sampling of Drosophila will reveal an even greater diversity of this prevalent bacterium.

The four divergent clades of Drosophila Spiroplasma represent four separate introductions, as the closest relatives for each clade are Spiroplasma infecting other organisms. Furthermore, D. hydei appears to have been infected at least twice, by Spiroplasma from two different clades. Five separate introductions is a minimum estimate, and more horizontal transmission events have likely occurred. For example, D. hydei and D. simulans Spiroplasma display identical haplotypes at every locus. This low
divergence is inconsistent with a single ancient infection pre-dating the split of these two species, estimated at over 50 million years (Tamura et al. 2004). For some of the other more closely related *Drosophila* species it is unclear whether shared infections are ancient or recent introductions. For example, *D. mojavensis*, *D. aldrichi*, and *D. wheeleri* all are in the repleta species group, and *D. aldrichi* and *D. wheeleri* are closely-related sister species, so an older infection of the three is possible. More extensive sampling of related species would resolve the pattern, though other evidence, such as the lack of genetic variation in the *Spiroplasma* infecting each *Drosophila* species, suggests that horizontal transmission is more likely. This lack of variation, sometimes extending over a large geographical region, suggests that each infection is recent and has rapidly spread.

A potential mechanism for horizontal transmission, mites, has been demonstrated in a laboratory setting (Jaenike et al. 2007). In addition, *Spiroplasma* are common gut bacteria in many insects, and plant surfaces, with deposited fecal matter, have been found to act as a reservoir for *Spiroplasma* (Bove 1997). Both *S. citri* and *S. kunkelli* are vectored by leafhoppers, and thus these *Spiroplasma* have the ability to be picked up by insects and horizontally transmitted. Furthermore, the *D. mojavensis*, *D. aldrichi*, *D. wheeleri*, and *D. hydei* sampled have sympatric ranges at many of the collection sites and breed in similar cactus rots (Ruiz & Heed 1982). Many arthropods use cactus rots as breeding sites, and consequently these rots may also serve as reservoirs for *Spiroplasma*.

To investigate *Spiroplasma* transmission within *Drosophila* populations we assessed patterns of variation in *Drosophila* mitochondrial haplotypes in *Spiroplasma*-infected and uninfected flies. We expected to find strong associations between *Spiroplasma* infection and a particular *Drosophila* mitochondrial haplotype, suggestive of
a recent infection maintained in the population by strict vertical transmission. We did not find this pattern for either *D. hydei* or *D. mojavensis* populations. In exploring the association of *Drosophila* haplotype and infection, however, we were only able to look at populations infected with the non-male-killing *Spiroplasma*. We would expect an even stronger association with a male-killing *Spiroplasma* infection and mitochondrial haplotype, as this mechanism increases the chance of vertical transmission.

For *D. mojavensis*, *Spiroplasma* is associated with seven of fourteen total haplotypes in three sampled populations. A majority of the sampled individuals, both infected and uninfected, had two of these haplotypes. For the CI population, the prevalence of infection is 60% (*Watts et al.* 2008), and the diversity of mitochondrial haplotypes is low, with only two sampled. Given that this small, isolated population likely underwent a bottleneck (*Reed et al.*, 2007), the prevalence may reflect infection status of the few flies colonizing the island. *Machado et al.* (2007), however, found higher levels of genetic diversity in the CI *Drosophila* at nuclear loci, and postulated that the lack of mitochondrial diversity may be due to a mitochondrial sweep. Reproductive parasites such as *Wolbachia* often cause mitochondrial sweeps (*Engelstadter & Hurst 2007; Jiggins 2003*) and such sweeps suggest the presence of some kind of reproductive manipulation or strong fitness advantages for infected females. For the Sonoran *D. mojavensis*, the diversity of mitochondrial haplotypes was higher. Given that infection is associated with only a subset of Sonoran haplotypes, but that those haplotypes are two of the three total haplotype groups, *Spiroplasma* may be an older infection in *D. mojavensis* that was subsequently lost in the third group before its diversification. In this case, *Spiroplasma* may be being maintained solely by a high fidelity of vertical transmission,
with some loss. Alternatively, horizontal transmission may be spreading the infection among susceptible *Drosophila*, with those individuals in the uninfected group *Spiroplasma*-resistant.

In the *D. hydei* populations, the citri-type *Spiroplasma* appears to be a relatively recent infection maintained by vertical transmission, as the four individuals that have this *Spiroplasma* type have two very similar mitochondrial haplotypes. The uninfected individuals with this haplotype may have lost the *Spiroplasma* infection, or the mitochondrial loci may lack sufficient resolution to fully distinguish matrilines. The four individuals infected with the citri-type *Spiroplasma* each were collected from different geographic regions. As *D. hydei* is a cosmopolitan species (Markow & O'Grady 2005), the spread of this infection throughout the range of collection is not unexpected. For the *D. hydei* infected with the poulsonii-type *Spiroplasma* there may have been an ancient infection, prior to the diversification of haplotypes, followed by loss of the infection from many individuals of each haplotype. If *Spiroplasma* only were vertically maintained, all the while undergoing loss from all haplotypes, the infection is likely to have been lost completely in some cases in the absence of some fitness benefit. Populations of *D. hydei* in Japan, however, have been documented to maintain high population prevalence levels (25-46%) over the course of 30 years (Kageyama et al. 2006), even though the fidelity of vertical transmission of this *Spiroplasma* is low at the colder temperatures these populations experience (Osaka et al. 2008). Thus it also is possible that some horizontal transmission is maintaining *Spiroplasma* in *D. hydei* populations.

We found no evidence for recombination among *Drosophila Spiroplasma* from different clades. Any recombination among *Spiroplasma* strains infecting a single
species may have been undetected because of the lack of intraspecific genetic diversity. Alternatively, recombination may not be possible in these bacteria. Several *S. citri* strains contain a truncated, non-functional, *recA* gene. In *Escherichia coli* and other bacteria, *recA* is responsible for promoting homologous recombination and recombinatorial DNA repair (Kowalczykowski 2000). In fact, *S. citri* has been shown to be more sensitive to UV damage than other closely related bacterial taxa with a functional *recA* gene (Marais *et al.* 1996). Other pathways exist, however, such as recombination involving extrachromosomal DNA such as plasmids and bacteriophage known to occur in various *S. citri* strains (Barroso & Labarere 1988; Marais, *et al.* 1996a). A lack of recombination may suggest that horizontal transmission rarely causes coinfection or that coinfections are not stable. Both the citri and poulsonii haplotypes are circulating in the *D. hydei* populations of San Carlos, Magdalena, and OPNM, so if *Spiroplasma* is horizontally transmitted, co-infection is possible.

The strains of *Spiroplasma* that cause male-killing group together, separated from the non-male-killing *Spiroplasma* infecting *D. hydei and D. simulans* with high bootstrap support. This is consistent with suggestions made for a single origin for male-killing *Spiroplasma* in *Drosophila* (Montenegro *et al.* 2005; Pool *et al.* 2006). This phylogenetic pattern is seen at 16S rRNA, *fruR* and *spoT* (data not shown), the loci for which sequences from the male-killing *Spiroplasma* infecting willistoni group *Drosophila* were available for comparison. Interestingly, a different species of *Spiroplasma*, *S. ixodetis*, is known to cause male-killing in the ladybird beetle (Tinsley & Majerus 2006) and the butterfly (Jiggins *et al.* 2000). This strain of *Spiroplasma* is most closely related to the
Spiroplasma infecting D. ananassae and D. atripex, which have been stably maintained in the lab with no evidence of male-killing.

**Conclusions**

*Drosophila* are infected with four very different types of *Spiroplasma*, the majority of which do not cause male-killing. Given that our sampling was limited to western North America, a wider geographical and taxonomic sampling of *Drosophila* will undoubtedly reveal still other types of *Spiroplasma*, each of which could potentially have different fitness consequences for their *Drosophila* hosts. The existence of multiple introductions implies that horizontal transmission has played an important role in the distribution of *Spiroplasma* in *Drosophila*. Additionally, patterns of variation in *Drosophila* mitochondrial haplotypes in *Spiroplasma*-infected and uninfected flies imply imperfect vertical transmission in host populations and possible horizontal transmission. Further exploration of the roles and mechanisms of vertical and horizontal transmission of the different *Spiroplasma* strains can also help determine conditions under which this endosymbiont persists in *Drosophila* populations. Finally, our multilocus analysis supports clonality in *Spiroplasma* infecting *Drosophila*, despite evidence for horizontal transmission. Thus *Spiroplasma* may be more similar to beneficial bacteria trapped in their hosts with no opportunity for recombination. Though previous studies have not found strong fitness consequences of *Spiroplasma* infection in the laboratory (Ebert 1991; Kageyama et al., 2006; Montenegro et al., 2006b), conditionally beneficial fitness effects may help to explain its distribution in host populations.
ACKNOWLEDGEMENTS

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The text of Chapter 2, in full, is a reprint of the material as it appears in Haselkorn, T.S., T.A. Markow, and N.A. Moran. 2009. Multiple introductions of the Spiroplasma bacterial endosymbiont into Drosophila. Molecular Ecology 18: 1294-1305. The dissertation author was the primary investigator and author.

REFERENCES


Table 2.1 *Spiroplasma*-infected *Drosophila* used in this study

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### Table 2.2 Primers and annealing conditions for each locus

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<td>partial fructose operon</td>
<td>FruF</td>
<td>Montenegro et al. 2000</td>
<td>58-48°C touchdown</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FruR</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 2.3 Features of the six loci used in this study

<table>
<thead>
<tr>
<th>Locus</th>
<th>Number of alleles</th>
<th>#sites</th>
<th>#polymorphic sites</th>
<th>Nucleotide diversity per site</th>
<th>GC content</th>
<th>Ka/Ks</th>
<th>Recombination</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>7</td>
<td>1252</td>
<td>205</td>
<td>0.034</td>
<td>49%</td>
<td>N/A</td>
<td>None</td>
</tr>
<tr>
<td>ITS</td>
<td>7</td>
<td>202</td>
<td>51</td>
<td>0.034</td>
<td>30%</td>
<td>N/A</td>
<td>None</td>
</tr>
<tr>
<td>RpoB</td>
<td>8</td>
<td>1292</td>
<td>182</td>
<td>0.094</td>
<td>34%</td>
<td>0.038</td>
<td>Outgroup</td>
</tr>
<tr>
<td>ParE</td>
<td>5</td>
<td>933</td>
<td>155</td>
<td>0.082</td>
<td>32%</td>
<td>0.096</td>
<td>None</td>
</tr>
<tr>
<td>FtsZ</td>
<td>5</td>
<td>886</td>
<td>140</td>
<td>0.077</td>
<td>38%</td>
<td>0.085</td>
<td>None</td>
</tr>
<tr>
<td>FruR</td>
<td>5</td>
<td>327</td>
<td>72</td>
<td>0.108</td>
<td>32%</td>
<td>0.253</td>
<td>None</td>
</tr>
</tbody>
</table>
Figure 2.1 Bayesian phylogeny based on Spiroplasma 16S rRNA gene. Spiroplasma infecting different Drosophila species in different colors. Support for clades given as Bayesian posterior probabilities. The Spiroplasma infecting Drosophila fall into four distinct clades, which are labeled in bold type with black bars.
Figure 2.2 Bayesian phylogenies based on *Spiroplasma* loci ITS, RpoB, FtsZ, and ParE. Identical *Spiroplasma* haplotypes condensed at each locus. ITS and RpoB are rooted with *M. mycoides*, while FtsZ and ParE are mid-point rooted. Support for clades given as Bayesian posterior probabilities. The major clades into which the *Drosophila* *Spiroplasma* fall are labeled in bold type. Abbreviations: D. whe. (D. wheeleri), D. ald. (D. aldrichi), D. moj. (D. mojavensis), D. sim. (D. simulans), D. melUGA (male-killing spiroplasma infecting *D. melanogaster* from Uganda). The same phylogenetic pattern is seen across all loci.
Figure 2.3 Bayesian phylogeny based on the FruR locus. Spiroplasma infecting different Drosophila species in different colors. Support for clades given as Bayesian posterior probabilities. The male-killing Spiroplasma fall into a single well-supported clade and are a small proportion of the Spiroplasma diversity sampled thus far.
Figure 2.4 Bayesian phylogeny based on concatenated sequences of multiple Spiroplasma loci. Identical Spiroplasma haplotypes are condensed, and the number of individuals with each haplotype is given in parenthesis following the haplotype name. Drosophila Spiroplasma colored in red. Support for clades given as Bayesian posterior probabilities.
Figure 2.5 Cladogram of *Spiroplasma*-infected *Drosophila* species used in this study. *Drosophila* species relationships based on Markow and O’Grady (2005). The clade of *Spiroplasma* infecting each *Drosophila* species, as well as its male-killing phenotype, is denoted.
Figure 2.6 Minimal spanning haplotype network of *Drosophila* mitochondrial loci. The size of the open circles reflects the number of individuals with each haplotype. Each dot connecting haplotypes represent a single mutational step. The proportion of infected *Drosophila* for each haplotype is shaded. a. Cytochrome oxidase I network for *D. mojavensis*. b. Combined COII and AT-rich region network for *D. hydei*. Individuals infected with the poulsonii-type Spiroplasma are shaded in green, while individuals infected with the citri-type Spiroplasma are shaded in purple.
CHAPTER 3

Density dynamics of diverse *Spiroplasma* strains naturally infecting different species of *Drosophila*

**ABSTRACT**

Facultative heritable bacterial endosymbionts can have dramatic effects on their hosts, ranging from mutualistic to parasitic, and the magnitude of these effects can depend on the infection prevalence in a host population. Within-host bacterial endosymbiont density plays a critical role in maintenance of a symbiotic relationship, as it can affect levels of vertical transmission and expression of phenotypic effects, both of which can influence the infection prevalence in host populations. Species of genus *Drosophila* are infected with *Spiroplasma*, historically known as a male-killing reproductive parasite. Recent screening, however, has uncovered a diversity of *Spiroplasma* strains infecting at least 17 species of *Drosophila*, most of which do not cause male-killing and their fitness effects are only beginning to be revealed. The infection prevalence of these *Spiroplasma* vary within and among *Drosophila* species, and little is known about the within-host density dynamics of these diverse strains. To characterize the patterns of *Spiroplasma* density variation among *Drosophila* we used quantitative PCR to assess bacterial titer at various life stages of three species of *Drosophila* naturally infected with two different types of *Spiroplasma*. We find for
naturally infected *Drosophila* species that non-male-killing infections had consistently lower densities than the male-killing infection. The patterns of *Spiroplasma* titer change during aging varied among *Drosophila* species infected with different *Spiroplasma* strains. Bacterial density varied within and among populations of *Drosophila*, with individuals from the population with the highest prevalence of infection having the highest density. This density variation suggests a complex interaction of *Spiroplasma* strain and host genetic background in determining endosymbiont density and lends insight into *Drosophila* infection prevalence variation.

**INTRODUCTION**

Numerous organisms harbor maternally transmitted bacterial endosymbionts that can have dramatic effects on their host. Dependent on their host for their own survival, these endosymbionts can act as mutualists increasing their own fitness by increasing that of the host. Alternatively, these bacteria can manipulate their host's reproduction to enhance their own transmission by increasing the proportion of infected females. Such facultative endosymbionts are not required for host survival, and as such their incidence can vary greatly among host species and populations. Consequently, the population level impacts of these endosymbionts are dependent on their prevalence. In turn, the prevalence of an endosymbiont in a population is greatly affected by its within-host density dynamics (Jaenike 2009), as infection density affects both the fidelity of vertical transmission as well as the strength of expression of fitness effects, two key parameters maintaining bacteria in host populations.
Species of the genus *Drosophila* are infected with only two heritable bacterial endosymbionts: *Wolbachia* and *Spiroplasma* (Mateos et al., 2006). These bacteria have an array of effects on their drosophilid hosts. The well-characterized *Wolbachia*'s effects range from the reproductive parasitism phenotypes of male-killing and cytoplasmic incompatibility (Werren et al., 2008) to mutualistic effects such as protection against RNA viruses (Hedges et al., 2008; Teixeira et al., 2008) and possible nutritional supplementation (Brownlie et al., 2009). Less is known about the phenotypic effects of *Spiroplasma*. Similar to *Wolbachia*, *Spiroplasma* can act as a reproductive manipulator, causing male-killing in certain species of *Drosophila* (Williamson and Poulson 1979). Additional screening, however, has uncovered a diversity of *Spiroplasma* infecting *Drosophila* (Haselkorn et al., 2009; Kageyama et al., 2006; Mateos et al., 2006; Ota et al., 1979; Watts et al., 2009), most of which do not cause the male-killing phenotype and their fitness effects are largely unknown. Recent discovery of *Spiroplasma* that afford *D. neotestacea* protection against a nematode parasite (Jaenike et al., 2010) indicates that such fitness benefits, along with the fidelity of vertical transmission, play an important role in determining the distribution of these bacteria among *Drosophila*.

Bacterial titer has been empirically correlated with both vertical transmission and fitness effects in many endosymbionts. *Wolbachia* strains with higher bacterial densities have a higher vertical transmission fidelity in flies and mosquitoes (Dutton, Sinkins, 2004; Dyer et al., 2005; Kittayapong et al., 2002). As a reproductive parasite, the strength of its male-killing and cytoplasmic incompatibility phenotypes increases with bacterial titer (Clancy, Hoffmann, 1998; Clark, Karr, 2002; Dyer et al., 2005; Hurst et al., 2000a; Sinkins et al., 1995). Even recently demonstrated fitness benefits, such as
resistance to RNA viruses, have been shown to vary by the particular *Wolbachia* strain, with the strains having the highest density showing the greatest protective effects (Osborne *et al*., 2009). In *Spiroplasma*, it has been hypothesized that a certain bacterial density is necessary for expression of its male-killing phenotype, as studies have shown that a male-killing *Spiroplasma* had a higher density non-male-killing *Spiroplasma* (Anbutsu, Fukatsu, 2003; Kageyama *et al*., 2006). *Spiroplasma* density also affects the developmental stage at which male-killing occurs, with a higher infection density causing male-killing at an earlier stage (Kageyama *et al*., 2007).

Given the dramatic consequences of endosymbiont titer variation, understanding the factors that affect endosymbiont density is critical. Such factors include host genotype, bacterial strain, host age and temperature. In *Wolbachia*, host genotype can affect bacterial titer (Kondo *et al*., 2005; McGraw *et al*., 2002), as well as bacterial strain (Duron *et al*., 2007; Dutton, Sinkins, 2004; Mouton *et al*., 2003). Other factors, such as temperature (Hurst *et al*., 2000b; Mouton *et al*., 2006; Mouton *et al*., 2007) and host age (Duron *et al*., 2007; Tortosa *et al*., 2010), affect *Wolbachia* titer, which in turn affects population prevalence depending on the environment and age structure of the population (Unckless *et al*., 2009). Similarly, *Spiroplasma* density in certain *Drosophila* species increases with age (Anbutsu, Fukatsu, 2003; Kageyama *et al*., 2006), and decreases at lower temperatures (Anbutsu *et al*., 2008; Osaka *et al*., 2008). Lower density, either at young ages or lower temperatures, is correlated with either loss of the male-killing phenotype or loss of the *Spiroplasma* altogether.

* Spiroplasma* density dynamics have been explored in only a few strains in a limited number of *Drosophila* species, namely in relation to the male-killing phenotype.
These strains are identical or closely related to the first characterized *Spiroplasma* endosymbiont infecting *D. nebulosa*, *S. poulsonii* (Kageyama et al., 2006; Williamson et al., 1999). Many of these strains are artificial infections; *Spiroplasma* strains from different *Drosophila* species transferred to *D. melanogaster*. While useful for exploring the mechanisms of male-killing, these artificial infections give limited insight into density variation in natural populations. Furthermore, these poulsonii-type *Spiroplasma* strains are only a small subset of the diversity of *Spiroplasma* infecting *Drosophila* (Haselkorn et al., 2009; Watts et al., 2009). At least seventeen species of *Drosophila* are infected with four genetically distinct types of *Spiroplasma*, most of which do not cause the male-killing phenotype and, for many, their fitness effects are unknown. Screening of natural populations of *Drosophila* revealed that *Spiroplasma* infection prevalence varies not only among, but within species, with infection prevalence ranging from 15% to 85% in *D. mojavensis* (Watts et al., 2009). *Drosophila mojavensis* is infected with one of the newly discovered strains of *Spiroplasma* that are more closely related to *S. citri*, a well-known plant pathogen (Bove 1997), than to the poulsonii-type *Spiroplasma*. These citri-type *Spiroplasma* infect seven species in the *Drosophila* repleta group, which have some of the highest infection prevalence of *Spiroplasma* screened to date (Watts et al., 2009).

The forces driving the distribution of these different types of *Spiroplasma* among and within *Drosophila* species are largely unknown, though certainly both the fitness effects and fidelity of vertical transmission are involved. Given that bacterial titer can affect both parameters, characterizing the density dynamics of various *Spiroplasma* strains among different *Drosophila* species is critical to understanding this symbiosis. In this study we characterize the variation in *Spiroplasma* density among different
Spiroplasma strains infecting species of Drosophila. The flies were measured under controlled laboratory conditions to minimize the effect of temperature and other environmental factors that may affect bacterial density. We ask the following questions: Do the non-male-killing Spiroplasma have lower densities than male-killing Spiroplasma? Do densities of natural poulsonii-type and citri-type Spiroplasma infections change over the lifetime of the host? Do the poulsonii-type and citri-type Spiroplasma have similar densities? How does Spiroplasma density vary within and between D. mojavensis populations?

**Materials and Methods**

**Fly lines and symbionts**

Thirteen naturally infected fly lines were measured in this experiment, five of which were measured across different life stages. The Drosophila species, Spiroplasma strain, and origin of isofemale line are shown in Table 1.

**Fly rearing conditions**

Spiroplasma-infected D. melanogaster (UGA), D. melanogaster (SC), D. hydei (TEN104-102), and D. mojavensis (OPNM-10) were reared on standard banana food at room temperature in summer 2008. D. hydei (ABH5) and the D. mojavensis (CI and OPNM) isolines were reared in spring 2010. All fly lines were in the laboratory for at least 10 generations prior to collection. Approximately one hundred females were placed in an egg-laying chamber, and allowed to oviposit for three hours to control for larval density and age of offspring. Flies were collected at various time points over the course
of development, including 3rd instar larvae, day of eclosion (D0), second day after eclosion (D2), at one week (D6) and two weeks (D13). *D. mojavensis* isolines were collected only at one week of age. After eclosion, males and female flies were separated and held as virgins for the later collections. At each time point, for each species, six flies were frozen at -80°C for quantitative PCR analysis, and another six flies were frozen to obtain dry weight measurements. Flies were dried at 55°C for 72 hours and weighed individually to obtain average dry weights.

**DNA extraction**

Each biological replicate for each species at each time point was extracted individually using a Qiagen DNA extraction kit. DNA was eluted in 100-200µl of buffer AE, and quantified using a Nanodrop. DNA samples were diluted to 25ng/µl for quantitative PCR analysis.

**Quantitative PCR standard curve construction**

We measured bacterial density using quantitative real-time PCR with the bacterial *dnaA* gene. To estimate *Spiroplasma* titer, absolute *dnaA* copy number was determined using a standard curve. To generate this standard curve, a 500 base pair region containing the quantitative PCR DNA amplicon was amplified using primers SRdnaAF1 and SRdnaAR1 (Anbutsu, Fukatsu, 2003) (Table 2) from *Spiroplasma* from each *Drosophila* species, sequenced, and cloned using Invitrogen's Topo-TA pCR 2.1 topo vector cloning kit. Quantitative PCR primers were verified and redesigned as necessary for the different *Spiroplasma* strains (Table 2). Plasmids containing the larger cloned
fragment were used to construct two separate standard curves, one for poulsonii-type *Spiroplasma* and one for citri-type *Spiroplasma*, using dilutions of $10^9$, $10^8$, $10^7$, $10^6$, $10^5$, $10^4$, $10^3$, and $10^2$ *dnaA* copies per four microliters. For several of the *Spiroplasma* density measurements, an internal DNA standard, designed in the *Drosophila* single copy nuclear gene elongation factor 1 alpha (*ef1-alpha*), was constructed for each *Drosophila* species in a similar manner. Specific *ef1-alpha* primers for each *Drosophila* species are listed in Table 2.

**Quantitative PCR**

Quantitative PCR was run using Aplied Biosystems (ABI) Power Sybrgreen PCR mix on an ABI 7000 machine. Twenty-five microliter PCR reactions, using 4µl of DNA, were run on a program of 95°C for 10 minutes, then 95°C for 15 seconds, 55°C for 45 seconds, 60°C for 45 seconds for 45 cycles. Primer concentrations were 300nm each per reaction. Technical replicates were run for each biological replicate, and if there was a discrepancy of greater than 0.5 amplification cycles, then the sample was run again. Otherwise, the two amplification cycle values were averaged and used for copy number calculations. Dissociation curves were run for verification of the target amplicon.

**Statistical analyses**

*Spiroplasma* titer was calculated at all *Drosophila* life stages as *dnaA* *Spiroplasma* copy equivalents per milligram fly weight. For a subset of life stages (D0, D6, and D13), the internal standard *ef1-alpha* was also amplified, and *Spiroplasma* titer was calculated as number of *dnaA* *Spiroplasma* copy equivalents per number of *ef1-alpha*
copies. *DnaA* copy numbers were square root transformed to fit a normal distribution. T-tests and a one-way analysis of variance were used to compare densities among species at different life stages.

**RESULTS**

**Male-killing vs. non-male-killing strains**

The male-killing *Spiroplasma* infecting *D. melanogaster* (UGA) had the highest density at all life stages (p < 0.05), followed by the non-male killing poulsonii-type *Spiroplasma* infecting *D. hydei* (p < 0.05). Lowest densities were found in the non-male-killing *Spiroplasma* of the citri-type infecting *D. mojavensis* and *D. hydei* (Figures 1).

This observation was consistent when calculating *Spiroplasma* density either as number of copies per milligram dry weight or number of *Spiroplasma* copies per *ef1-alpha* gene. The citri-type *Spiroplasma* in both *D. hydei* and *D. mojavensis* had lower densities at all life stages.

The non-male-killing *Spiroplasma* infecting *D. melanogaster* had the lowest titers, undetectable using these quantitative PCR methods at eclosion and at two weeks in males. Low titers, around 1000 copies, of *Spiroplasma* were detected only in one female at eclosion, and one male at day six. Infection levels could consistently be detected in one-week-old females (5/6 biological replicates amplified), though titers were quite low. While *Spiroplasma* titer, on average, was higher in two-week-old females, density measurements were highly variable and infections were not detected in three females. Even at the highest titer levels (around $2 \times 10^4$ *Spiroplasma* copies per milligram fly), these non-male-killing *D. melanogaster* infections were still several orders of magnitude
below that of the male-killing *Spiroplasma* infecting *D. melanogaster* (around $4 \times 10^8$ *Spiroplasma* copies per milligram fly). Thus, for all *Spiroplasma* strains measured, the non-male-killing *Spiroplasma* strains had lower densities, at all life stages, than the male-killing strain.

**Bacterial titer change with age**

In general, the bacterial titers increased as the flies aged, although not at the same rate among *Drosophila* species or *Spiroplasma* types. The female *D. melanogaster* infected with the male-killing *Spiroplasma* showed a pattern of increasing *Spiroplasma* density from the 3rd instar larval stage (around $2.8 \times 10^7$ *Spiroplasma* copies per mg fly) to two-week-old females (around $4 \times 10^8$ *Spiroplasma* copies per mg fly). A similar trend was seen for the female *D. hydei* infected with the non-male-killing poulsonii *Spiroplasma* strain. Titers in male *D. hydei* infected with this *Spiroplasma* strain increased from the 3rd instar larval stage to eclosion, but remained the same at the one-week and two-week-old stages. In the *D. hydei* females infected with the citri-type *Spiroplasma*, the increase in bacterial titer did not occur until the two-week-old stage. Similar to the pattern observed for *D. hydei* poulsonii-type *Spiroplasma* infected males, the *D. hydei* citri-type *Spiroplasma* infected males did not increase in bacterial titer with fly age. A different pattern, however, was seen with *D. mojavensis*, infected with the citri-type *Spiroplasma*. This *Spiroplasma* had lower titers and several individuals had no detectable *Spiroplasma* by day 13. In two-week-old females, four out of six individuals had no detectable *Spiroplasma*, while in two-week-old males, three out of six had no detectable *Spiroplasma*. Individuals with measurable levels of *Spiroplasma* had titers
much lower than those at day 6 (D6 females $6.01 \times 10^7$ versus D13 females $6.18 \times 10^6$; D6 males $1.55 \times 10^7$ versus D13 males $8.81 \times 10^6$). The patterns of Spiroplasma titer change during aging varied among Drosophila species infected with different Spiroplasma strains.

**Spiroplasma citri-type density variation**

The citri-type Spiroplasma infecting *D. hydei* had a consistently lower density than did the poulsonii-type Spiroplasma across all life stages ($p < 0.05$) (Figure 2). Within the citri-type Spiroplasma infecting *D. mojavensis*, the Spiroplasma density also varied among isofemale lines (Figure 3). Three of the four isofemale lines of *D. mojavensis* from the Sonoran Desert (OP24, OP27, and OP65) had statistically significant lower densities than those from Catalina Island, though there was variation in the flies from Catalina Island as well. Despite this variation, the densities of the Spiroplasma from Catalina Island were, on average, higher than that of the Sonoran Desert Spiroplasma ($p < 0.05$). Thus, even among closely related strains within a particular type of Spiroplasma there was large density variation.

**Discussion**

This study represents the first characterization of Spiroplasma density among several naturally infected Drosophila species harboring diverse Spiroplasma strains. Much of what is known about Spiroplasma density in Drosophila is from studies on artificially infected fly strains. While a few naturally infected flies have been measured, the variation in Spiroplasma density among Drosophila has remained largely obscure.
The density dynamics of *Spiroplasma* in *Drosophila* clearly vary among *Drosophila* species and *Spiroplasma* strains. The non-male-killing *Spiroplasma* strains have lower densities than the male-killing *Spiroplasma* strains in all *Drosophila* species examined (Figure 1). Previous work has only compared an artificial male-killing infection (NSRO: the *D. nebulosa* *Spiroplasma* transferred to *D. melanogaster*), an artificial non-male-killing infection (NSRO-A: a lab variant of NSRO that lost its male-killing ability), and a single *D. hydei* non-male-killing *Spiroplasma* isolated in Japan (Anbutsu, Fukatsu, 2003; Kageyama et al., 2006). Finding similar patterns in naturally infected strains of different *Spiroplasma* types in additional *Drosophila* species provides further support for the bacterial threshold density hypothesis for the expression of the male-killing phenotype.

Furthermore, the *D. melanogaster* with the non-male-killing strain was an extremely low titer infection, with less than 1000 copies detectable in males and females under two weeks of age. This strain is genetically identical to the male-killing strain at the three loci for which it was sequenced (Haselkorn et al., 2009); however, undetected genetic variation may exist at other loci. The extremely low titer of this infection could be an inherent property of this bacterial strain, which may have lost either the ability to replicate quickly, or the ability to avoid or suppress the host immune system. Effects of host genetic background, though, cannot be ruled out. This strain could possibly cause male-killing, but may not be able to produce enough effector molecule to have any effect, due to its low titer, in accordance with the bacterial threshold density hypothesis.

The consistently lower density of *Spiroplasma* of the citri-type, along with the lower density of the citri-type *Spiroplasma* compared to the poulsonii-type *Spiroplasma*
in *D. hydei* suggest that this may be a general property of the *Spiroplasma* of this type, and that bacterial strain itself plays a role in density regulation. A similar pattern is seen in *Wolbachia*, where different *Wolbachia* strains in the same host genotype show very different bacterial titers (Ikeda *et al.*, 2003; McGraw *et al.*, 2002; Mouton *et al.*, 2004; Veneti *et al.*, 2004), indicating that the bacterial strain determines bacterial density dynamics. Even in a single host infected with multiple strains of *Wolbachia*, the different strains seem to be independently regulated (Ijichi *et al.*, 2002; Ikeda *et al.*, 2003; Kondo *et al.*, 2005; Mouton *et al.*, 2004; Mouton *et al.*, 2003), further supporting an important role for bacterial strain in density dynamics.

Though bacterial strain is likely a key player in *Spiroplasma* density regulation, host factors such as immune response also may be involved. Given that one of the primary locations of *Spiroplasma* among host tissues is in the hemolymph (Anbutsu, Fukatsu, 2006), this bacterium is exposed to antimicrobial peptides and other immune factors. One study demonstrated that *Spiroplasma* do not induce a global immune response, perhaps due to their lack of a cell wall, a common immune response elicitor. *Spiroplasma* are, however, affected by ectopic activation of the host immune system (Hurst *et al.*, 2003). There is also evidence that the male-killing *Spiroplasma*, NSRO, suppresses several antimicrobial peptides, while the non-male-killing NSRO-A variant does not. Furthermore, it appears that non-male-killing NSRO-A *Spiroplasma* may be more sensitive to host immune response, as its infection is unable to be established or maintained in immune-activated flies, whereas the male-killing NSRO is able to survive, albeit at lower densities (Anbutsu, Fukatsu, 2010).
Variation in Spiroplasma densities among *D. mojavensis* isofemale lines implies the involvement of host genotype in density regulation. There are high levels of genetic variation in both the Sonoran Desert and Catalina Island populations of *D. mojavensis* (Machado *et al.*, 2007). Sequencing of these Spiroplasma strains at six different loci revealed no genetic variation in the Spiroplasma that infect *D. mojavensis* (Haselkorn *et al.*, 2009). Though undetected variation in Spiroplasma strains cannot be ruled out, genetic variation in host immune response or another host factor that affects Spiroplasma density could be responsible for the variation in bacterial titer across isofemale lines.

There is other evidence for complex interactions between *Drosophila* host genotype and Spiroplasma strain. In *D. nebulosa*, Spiroplasma infection was lost at varying rates when infected females were crossed to males of different genetic backgrounds (Williamson, Poulson, 1979). In *D. willistoni*, when different host genotypes were infected with different strains of *Spiroplasma*, infection was lost as the females aged in one line but not another (Ebbert, 1991). In those studies Spiroplasma density was not measured, so it is unclear if the loss of infection was due to a decrease in bacterial titer.

The variation in Spiroplasma dynamics as the flies age may reflect differences in immune response and/or differences in the age of reproductive maturity. When Spiroplasma titer was measured as number of copies per milligram fly, the females consistently had higher Spiroplasma titers than the males from day two onwards. This pattern could reflect Spiroplasma proliferation in the ovaries, as is necessary for vertical transmission, and the timing may correlate to when flies are most likely to reproduce in the wild. In *D. mojavensis*, though, the decrease in Spiroplasma titer in two-week-old females as compared to one-week-old females is striking. Whether this is an artifact of
losing Spiroplasma due to ovaposition of Spiroplasma-laden eggs, as D. mojavensis will lay unfertilized eggs, or a general trend of decrease with aging, as bacterial titer decreases with age in males as well, is unclear. In any case, this trend indicates that infection prevalence in natural populations is underestimated, depending on the age of the flies that are sampled.

Spiroplasma infection prevalence varies not only among species, but also among populations within species. In D. mojavensis populations, Spiroplasma infection prevalence varies dramatically (Watts et al., 2009). Populations at Organ Pipe National Monument in the Sonoran Desert have an infection prevalence of ~15%, whereas on Catalina Island the infection prevalence is greater than 85%. Given that within-host symbiont densities are often correlated with factors that affect populations prevalence, namely the fidelity of vertical transmission and the strength of phenotypic effects (Jaenike, 2009), we may expect a correlation between infection prevalence and bacterial density. This expectation certainly is observed in the Spiroplasma density differences between the Sonoran Desert D. mojavensis and the Catalina Island D. mojavensis.

The higher densities of the D. mojavensis Spiroplasma from Catalina Island compared to those from the Sonoran Desert may reflect either a higher fidelity of vertical transmission, or the expression of a fitness benefit that explains the higher prevalence of infection on Catalina Island. Of course, environmental factors, such as temperature, also likely play a role in the infection prevalence. The Sonoran Desert population of D. mojavensis experiences more temperature extremes, which could lower infection prevalence if the citri-type Spiroplasma are effected by temperature in a similar manner as the poulsonii-type Spiroplasma. Measuring Spiroplasma density variation in flies
collected directly from the field will lend insight in to how environmental variation influences the bacterial density dynamics of this endosymbiont. Since numerous cactophilic Drosophila are infected with Spiroplasma from the citri-type, further exploration of the factors affecting Spiroplasma density dynamics will lend critical insight into this symbiosis.

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The text of Chapter 3, in full, is currently being prepared for submission for publication as Haselkorn, T.S., T. Watts, N.A. Moran, and T.A. Markow. Density dynamics of diverse strains of Spiroplasma infecting different species of Drosophila. The dissertation author was the primary investigator and author.

REFERENCES


Table 3.1 *Spiroplasma* infected *Drosophila* lines
Abbreviations: mk = male-killing, OPNM = Organ Pipe National Monument, AB = Anza Borrego. (1) Pool *et al.* 2005

<table>
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<tr>
<th>Fly ID</th>
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<th><em>Spiroplasma</em> type</th>
<th>Phenotype</th>
<th>Collection details</th>
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<td>D. melUGA</td>
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Table 3.2 Quantitative PCR primers

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Figure 3.1 Density of Spiroplasma in different Drosophila across life stages. Values (per milligram fly) are an average density of six biological replicates, and the error bars represent standard error. Drosophila species measured include *D. mojavensis* infected with citri-type non-male-killing *Spiroplasma*, *D. hydei* infected with citri-type non-male-killing *Spiroplasma*, *D. hydei* infected with non-male-killing poulsonii-type *Spiroplasma*, and *D. melanogaster* infected with poulsonii-type male-killing *Spiroplasma*. The male-killing *Spiroplasma* infecting *D. melanogaster* have the highest densities across all life stages, whereas the citri-type *Spiroplasma* have the lowest.
Figure 3.2 Citri-type vs. poulsonii-type *Spiroplasma* density in *D. hydei* across life stages. *Spiroplasma* density was measured as number of *Spiroplasma* copies per milligram fly in both males (M) and females (F) at day 0 (D0), day 2 (D2), day 6 (D6), and day 13 (D13). Values are an average density of six biological replicates, and the error bars represent standard error.
Figure 3.3 *D. mojavensis Spiroplasma* density variation among eight fly lines from two different populations. Density was measured as number of *Spiroplasma* copies per milligram fly. Values are an average density of six biological replicates, and the error bars represent standard error. CI: Catalina Island, OP: Organ Pipe National Monument (Sonoran Desert population).
CHAPTER 4

Small and variable fitness effects of Spiroplasma on Drosophila mojavensis

ABSTRACT

Spiroplasma is the only heritable bacterial endosymbiont other than Wolbachia found to infect Drosophila. At least four genetically distinct types of Spiroplasma have been documented in 17 species of Drosophila thus far. Although one Spiroplasma type is historically known as a male-killer in certain species of Drosophila, many more Drosophila are infected with Spiroplasma that lack this male-killing phenotype, and their fitness effects are unclear. Although recent work has identified a Spiroplasma acting as a defensive endosymbiont in one Drosophila species, for many other Drosophila no obvious fitness effects have been observed under standard laboratory conditions. Many of the fitness benefits or costs conferred by facultative endosymbionts may not be evident under with standard laboratory analyses, however, and must be examined in the context of the natural ecology of the host. A number of Drosophila species have a well-characterized ecology, such as those that feed and develop in cactus necroses. Several of these cactophilic Drosophila are infected with a non-male-killing Spiroplasma that is closely related to S. citri, a plant pathogenic Spiroplasma. One such species, D. mojavensis, utilizes prickly pear cactus as a host plant on Santa Catalina Island, and has infection prevalence greater than 85% of this citri-type Spiroplasma. To understand the effects of this unusually high Spiroplasma infection, I compared life history traits,
including survival, development time, thorax size and desiccation resistance of
Spiroplasma-infected and uninfected D. mojavensis raised on their natural prickly pear cactus hosts. No consistent effects of Spiroplasma infection found across lines and experimental replicates in this study. Fitness effects related to host plant specialization on Catalina Island thus may not, on their own, explain the prevalence of Spiroplasma in this D. mojavensis population.

INTRODUCTION

Facultative heritable bacterial endosymbionts can have numerous effects on their insect hosts ranging from mutualistic to parasitic. Although many insect groups harbor a diversity of facultative endosymbionts, species of the genus Drosophila are infected with only two; Wolbachia and Spiroplasma. Wolbachia infections in Drosophila have been well characterized, and as a reproductive manipulator Wolbachia can cause male-killing and cytoplasmic incompatibility in some species of Drosophila (Werren et al., 2008). As a mutualistic partner, Wolbachia confers protection against certain RNA viruses (Hedges et al., 2008; Teixeira et al., 2008) and provides nutritional supplementation to others (Brownlie et al., 2009). The fitness effects of Spiroplasma on Drosophila, however, are far less clear. Spiroplasma can cause male-killing in certain species of Drosophila (Williamson, Poulson, 1979), and recent work had demonstrated that Spiroplasma can confer protection against a nematode parasite in D. neotestatcea (Jaenike et al., 2010). No phenotypic effects, however, have yet been demonstrated in many other Drosophila species.
Spiroplasma are small, helical, gram-positive bacteria that infect a diverse range of hosts besides Drosophila (Gasparich et al., 2004). They are most commonly arthropod commensals (Hackett, Clark, 1989), but can also have effects ranging from mutualistic (Ebbert, Nault, 2001) to pathogenic (Bove, 1997), including reproductive parasitism (Goodacre et al., 2006; Jiggins et al., 2000; Tinsley, Majerus, 2006; Williamson, Poulson, 1979). Recent screenings of natural populations of Drosophila have uncovered 17 species infected with a diversity of Spiroplasma strains (Haselkorn et al., 2009; Jaenike et al.; Kageyama et al., 2006; Mateos et al., 2006; Montenegro et al., 2005; Watts et al., 2009; Williamson, Poulson, 1979), most of which do not cause male-killing and, for many, their effects on their hosts are unclear. Many of these non-male-killing Spiroplasma reach high prevalence in natural populations (Jaenike et al., ; Kageyama et al., 2006; Watts et al., 2009), and yet the forces maintaining them at such levels are only beginning to be discerned. The fidelity of vertical transmission of Spiroplasma, while high, is not perfect (Anbutsu et al., 2008; Ebbert, 1991; Montenegro, Klaczko, 2003; Osaka et al., 2008; Williamson, Poulson, 1979), implying that maintenance of infection in natural populations must be due to either reacquisition by horizontal transmission, or selection due to a fitness benefit. No obvious fitness effects were reported, however, in D. hydei infected with non-male-killing Spiroplasma raised under laboratory conditions (Kageyama et al., 2006). While levels of horizontal transmission are not yet fully characterized, it is also possible that Spiroplasma confer a fitness benefit apparent only when examined in the context of the natural ecology of the host.

Several of the newly discovered strains of Spiroplasma infecting Drosophila are genetically distinct from the previously characterized male-killing strains and the non-
male-killing strain infecting *D. hydei*. These unique *Spiroplasma* fall in the same phylogenetic clade as *S. citri* (Haselkorn et al., 2009), a well known plant pathogen (Bove, 1997). Citri-type *Spiroplasma* infect seven species in the *Drosophila repleta* group (Watts et al., 2009), which consists of flies that breed and forage in cactus necroses. Cactus necroses are unique habitats consisting of a diverse microbial community of yeasts and bacteria (Fogleman, 1981; Fogleman et al., 1981; Starmer, 1982; Vacek, 1979) that convert the cactus tissue into a distinctive chemical profile, often containing toxic compounds such as alcohols, alkaloids and triterpene glycosides (Kirchner, 1982), to which the resident flies have adapted (Matzkin, 2005; Matzkin, 2008; Matzkin et al., 2006; Starmer et al., 1986). Infected repleta group flies have some of the highest infection prevalences of *Spiroplasma* screened to date (Watts et al., 2009), raising questions concerning a potential relationship between cactophilic ecology and infection prevalence. While the phenotypic effects of these newly discovered *Spiroplasma* strains are completely unknown, other facultative endosymbionts have been demonstrated to play a role in host plant specialization. For example, aphids utilizing clover as a host plant have a higher prevalence of the facultative endosymbiont *Regiella insecticola* (Leonardo, Muiru, 2003; Oliver et al., 2009), which, under some conditions may increase fitness of infected females. *Wolbachia* provisions B vitamins to some species of bedbugs (Hosokawa et al., 2010), and increases fecundity in *Drosophila* on iron-limited diets (Brownlie et al., 2009). Whether or not *Spiroplasma* in *Drosophila* plays a similar role in its natural cactus environment deserves examination.

In particular, *Drosophila mojavensis* on Catalina Island has an infection prevalence of greater than 85% in both males and females (Watts et al., 2009). *D.*
*mojavensis* is divided into four geographically distinct subspecies (Pfeiler et al., 2009), each of which specializes on a different host cactus (Fellows, Heed, 1972; Reed et al., 2007; Ruiz, Heed, 1988). On Catalina Island, *D. mojavensis* breeds in necrotic prickly pear (*Opuntia demissa*) (Ruiz et al., 1990), whose chemical composition is distinct from that of the columnar cactus hosts of other *D. mojavensis* populations (Kirchner, 1982). Given the high prevalence of infection, and the well-known ecology of these flies, I asked: Are there any fitness effects of *Spiroplasma* on *D. mojavensis* when raised on their natural prickly pear cactus host? Fitness effects of many facultative endosymbionts can be affected by host genotype, environmental conditions and other factors (e.g. (Fry et al., 2004). Given this variability, I tested the fitness effects of several *D. mojavensis* genotypes and consistency of these effects over experimental replicates.

Fitness effects consistent with a role for *Spiroplasma* in cactus host plant specialization include a shorter development time, larger size, and/or increased desiccation resistance. Though development time, thorax size and dry weights are not direct measures of fitness, these traits are directly correlated with fitness in many species of *Drosophila*. Thorax size has been correlated with ovariole number in *D. mojavensis* (Mangin 1978), and, while not directly measured in *D. mojavensis*, ovariole number has been correlated with fecundity in other *Drosophila* species (Cohet, David, 1978; Lefranc, Bundgaard, 2000; Roberson, 1957; Tantawy, Vetukhiv, 1960; Watada et al., 1986). Furthermore, a shorter development time should be advantageous for *Drosophila* that breed in ephemeral resources such as cactus rots (Lewontin, 1965; Nylin, Gotthard, 1998), and provide a competitive advantage against the diverse community of organisms utilizing the same limited cactus necrosis (Castrezana, Markow, 2001; Fellows, Heed,
Desiccation is a particularly relevant fitness assay for the desert adapted *D. mojavensis*, as previous work examining desiccation resistance in several ecologically distinct *Drosophila* species has shown *D. mojavensis* to be among the most desiccation resistant (Matzkin *et al.*, 2009).

**Materials and Methods**

**Generation of fly lines**

I generated *Spiroplasma*-infected and uninfected flies of the same genotypic background for each of four isofemale lines (lines 1, 2, 4, 15). Each *Drosophila* line was originally established from a single female collected on Catalina Island in 2008. Multiple females from each of these lines were placed singly in vials, allowed to oviposit, and then were screened for *Spiroplasma* infection. Natural loss of the bacteria occurs in the lab, and the offspring whose mother's tested negative for infection were combined, while the offspring of *Spiroplasma* positive females were also combined. The procedure repeated twice for each line, and was verified three additional times using PCR. The experiment was run in triplicate, the first with isofemale line 1 in November 2009 (experimental replicate A), the second with isofemale line 4 (experiment replicate B) in December 2009, and the third time with isofemale lines 1, 4, 2, and 15 (experimental replicate C) simultaneously in April 2010.

**Generation of necrotic cactus**

Cactus was prepared for all experiments using a modified protocol of (Matzkin *et al.*, 2006). Prickly-pear cactus collected from Catalina Island (*Opuntia demissa*) was cut
into pieces, autoclaved, and then inoculated with a subset of the natural microflora (four yeast species: *Candida sonorensis, Pichia cactophila, P. amethiona*, and *P. cacticada* and one bacterium: *Pectobacterium cacticada*) as described in Starmer (1982). Though the particular community composition for *Opuntia demissa* on Catalina Island has not specifically been described, the microflora used were common to different species of prickly pear in the southwestern United States (Starmer, 1982). The inoculated cactus was incubated at 37°C for two weeks, homogenized, and then frozen in aliquots for subsequent experiments. Immediately prior to each experiment an aliquot was thawed, combined with 0.5% agar and poured into vials.

**Experimental procedures**

For each line and each treatment (*Spiroplasma*-infected or uninfected), 100 females and 100 males were placed in an egg-laying chamber for three hours. These parental flies had been raised under controlled densities in the 25°C incubator, collected shortly after emergence, and held for 5-8 days before egg-laying. Flies were placed on a yeasted 0.5% agar plate to facilitate oviposition. The yeast paste later was later removed and replaced with cactus food. Thirty-six hours later, the 1st instar larvae were transferred into cactus food vials at a density of 30 flies per vial. The set-up of infected and uninfected vials was alternated. Because generating the quantity of flies necessary for each assay was extremely labor-intensive, each line was set up separately, about one month apart for the first two experimental replicates (A and B). Forty-five vials per treatment were set up for the first two experiments (A and B), while 25 vials per treatment per line were prepared for the third (C). The vials for all experiments were
placed in a 25°C incubator on a 12 hour light-dark cycle (9am to 9pm). The parents were then frozen and a subset screened using PCR with primers TKss and 23F (as in Watts et al. 2009) to verify their infection status. A subset of the offspring also was screened after each experiment to verify infection status.

**Fitness assays**

I measured larval to adult survival, development time, thorax size, dry weights, and desiccation resistance for the first two experimental replicates (A and B), and only survival, development time and thorax size for the third (C). Dry weights were used as another measure of size.

**Development time and survival**

Flies were monitored for eclosion every three hours (9am, 12pm, 3pm, 6pm, and 9pm) during the daytime cycle of the incubator. I initially included nighttime checks, however, very few flies eclosed during these hours and those time points were discontinued. Development time was calculated as an average over all individuals eclosed. Survival was determined by counting the number of flies eclosing per vial out of 30.

**Thorax size and dry weights**

For thorax size and dry weights, 100 flies of each sex of each treatment were measured. These flies were randomly selected from eclosed flies over the course of development, with the exception of the morning collections on the days of peak
emergence for the first two experimental replicates, as those flies were used for the desiccation resistance assay. Thorax size was measured on flies laterally positioned, and measurements were taken from the anterior margin to the end of the scutellum using an optical micrometer on a Leica MZ6 dissecting microscope (Leica Microsystems Inc., Bannockburn, IL, USA). Flies used for dry weight measurements were collected no longer than three hours after emergence to minimize adult feeding and immediately frozen at -80°C. They were later dried for 48 hours at 55°C and weighed individually.

Desiccation resistance

In the first two experimental replicates (A and B), flies for desiccation resistance were collected on the day of peak emergence and immediately transferred to agar holding vials for six hours, allowing them time to harden prior to starting the assay while preventing adult feeding. The desiccation resistance assay was performed on newly eclosed flies, which is biologically relevant as cactus rots can be short-lived. Prickly pear, in particular, dry out quickly owing to their thin pads compared to the thicker tissues of columnar cacti (Castrezana and Markow unpublished) and newly emerged flies may immediately be subjected to desiccating conditions as they locate a new food source. Five flies each were placed in the bottom half of an empty vial, with a cotton plug in the middle. Five grams of Drierite was added on top of the cotton and the vial was sealed with parafilm for each of the twenty vials per sex per treatment that were set up. Location of fly vials within four racks were randomized, and the racks were rotated in the 25°C incubator on a 12 hour light cycle. Flies were monitored every two hours until all flies had died, and the time of death was recorded per fly.
Statistical analyses

Statistical analyses were done separately for each experimental replicate using JMP version 7 (SAS, Cary, NC). Statistical analyses on development time (hours to eclosion, log transformed), survival (proportion survived per vial, arcsin transformed), thorax size (millimeters, log transformed), and desiccation resistance (hours, log transformed), were performed using a three-way analysis of variance (with line, infection status, and sex as factors). The effect of experiment was analyzed as a one-way analysis of variance comparing line 1 replicates (from A and C) and line 2 replicates (from B and C). Tukey's adjustment was used to correct all post hoc comparisons.

RESULTS

There were many small and statistically significant effects of Spiroplasma infection on survival, development time, thorax size, desiccation resistance and dry weights (Table 1). At the same time, however, significant effects of experimental replicate were detected in many assays (Table 2). Oftentimes the effects of Spiroplasma infection were reversed between experimental replicates.

Survival

Spiroplasma infection increased survival for line 1 in both experimental replicates (Tukeys; p < 0.05) (Table 1 and Figure 1a). For line 4 Spiroplasma infection did not significantly affect survival, though there was an effect of experiment (Table 2, Figure 1b). In the third experimental replicate (C), the effect of Spiroplasma infection on
survival varied across lines, as there was a significant line and line by infection status effect, but no overall effect of infection status (Table 1, Figure 1c). *Spiroplasma* infection significantly increased survival of infected flies in line 2 (Tukeys; p < 0.05), but decreased survival in line 15 (Tukeys; p < 0.05).

**Development time**

There was a significant effect of line, infection, and line by infection on development time (Table 1). These effects varied across the experimental replicates, however, as there were significant effects of experiment for both line 1 and 4, and a significant line by infection effect in line 4 (Table 2). In the first two experimental replicates (A and B) the infected flies developed approximately 5-7 hours faster (lines 1, 4 Tukeys; p < 0.05 ; Figure 2a,b) however, in the third replicate (C), the uninfected flies developed 11-14 hours faster in three of the four isofemale lines (lines 2, 4, 15 Tukeys; p < 0.05; Figure 2c).

**Thorax size and dry weights**

For thorax size there was a line effect, an infection effect in some lines, and a line by infection effect (Table 1). In the first two experiments (A and B) there was a trend for infected flies to be larger than uninfected flies (Figure 3a, b), though the effect was only statistically significant in line 4 (Tukeys; p < 0.05). In the third replicate (C), however, the trends were reversed, as indicated by the significant experiment by infection effect for both lines 1 and 4 (Table 2). In the third replicate (C) the trends were for uninfected flies to be larger than infected flies, though the differences were small and statistically
significant only in lines 1 and 15 (Tukeys; p < 0.05; Figure 3c). Dry weights were only measured in the first two experimental replicates, so no effect of experiment could be assessed. For line 1 and line 4, infected flies weighed less than uninfected flies (line 1 infected: 0.202 mg vs. uninfected: 0.209 mg, Tukeys; p < 0.05 and line 4 infected: 0.221 mg vs uninfected: 0.226 mg, Tukeys; p < 0.05)

**Desiccation resistance**

The desiccation resistance assay was performed only in the first two experiments, and thus no effect of experiment could be assessed. For both line 1 and line 4 there was a significant infection effect (Table 1); the infected flies died sooner than the uninfected flies in all cases (Tukeys; p < 0.05) except line 4 males (line 1 infected: 41 hours vs. uninfected: 45 hours; line 4 infected: 43 hours vs. uninfected: 46 hours).

**DISCUSSION**

No consistent effects of *Spiroplasma* infection were found across lines and experimental replicates in this study. The effect of *Spiroplasma* infection on survival, while fairly consistent across experimental replicates, varied across lines. *Spiroplasma* infection affected development rate and thorax size differently across experimental replicates. In the first two experimental replicates the infected flies developed faster and had larger thoraces. In the third replicate the opposite pattern was seen; in three of the four lines the infected flies developed more slowly and were smaller. The shorter development time and larger size for *Spiroplasma*-infected flies seen in the first two experimental replicates would indicate a positive fitness effect; however, given the
inconsistency of this effect it is difficult to determine its significance, if any, in natural populations. There were small but significant effects of *Spiroplasma* infection on desiccation resistance and dry weight measurements; infected flies died sooner and weighed less. Decreased desiccation resistance could indicate a cost of *Spiroplasma* infection; however, these assays were not repeated in the third experimental replicate and it is possible that their trends have been reversed as well.

Some of the variable effects of *Spiroplasma* infection on survival are likely due to different host genetic backgrounds. In two of the lines *Spiroplasma* infection increased the survival of flies developing on cactus food. In the other two lines, however, there was either no effect or a negative effect of *Spiroplasma* infection on survival. Additional lines would need to be assayed to elucidate the effect, if any, of *Spiroplasma* infection has at the population level. Other aspects of *Spiroplasma* biology are affected by interactions with a host genotype, in particular the fidelity of vertical transmission and expression of the male-killing phenotype in the male-killing *Spiroplasma* strains (Kageyama et al., 2009; Williamson, Poulson, 1979). Host genotype, however, may not be the only factor causing the observed variability. Previous studies on the fitness effects of male-killing and non-male-killing *Spiroplasma* infections revealed an effect of both host genotypic background and bacterial strain, and a complex interaction between the two (Ebbert, 1991; Ebbert, 1995). The *Spiroplasma* strains infecting the *D. mojavensis* on Catalina Island, though identical at six sequenced loci (Haselkorn et al., 2009), may have some undetected genetic variation that could affect the fitness of its host.

The effects, though variable, of *Spiroplasma* infection on *D. mojavensis* development time, thorax size, dry weight, and desiccation resistance are likely
interrelated. Development time and size are often involved in complicated life history trade-off in many insect species (Nijhout et al., 2010; Nylin, Gotthard, 1998).

Spiroplasma caused an increase in development rate under some conditions, but not others. Although the direction of the effect changed among experimental replicates, the relationship between the two remained consistent. For the two experimental replicates where dry weights were also measured, the Spiroplasma infected flies had larger thoraces, but smaller dry weights. This relationship could represent some trade-off in relation to cuticle investment or fat storage during larval development. Spiroplasma-infected flies died sooner in the desiccation resistance assay, consistent with this explanation. They would be less able to retain moisture if their cuticle were thinner; or, as the effects of desiccation and starvation are intertwined in the desiccation resistance assay, they could be less starvation resistant if they had fewer fat stores. If the desiccation resistance or dry weight measurements had been repeated in the third experimental replicate, however it is possible that the trends for those assays, like development time and thorax size, would have been reversed.

Factors underlying the inconsistent effects of Spiroplasma infection in this study are unclear. Small differences in the food or some other environmental condition may have had a strong interaction with the Spiroplasma. Undetected temporal or seasonal dynamics of Spiroplasma infection also could alter the nature of the interaction. Changes in Spiroplasma density, either the amount in the egg, or the dynamics of infection during larval development could affect development time. Variable results have been found in other studies measuring the fitness effects of male-killing Spiroplasma in Drosophila. Some, but not all studies have found an increase in development time or early
reproduction (Montenegro et al., 2006). Various negative fitness consequences of Spiroplasma infection in flies have been reported, including reduced fertility, fecundity and lifespan (Ebbert, 1991); however, a different study found no positive or negative fitness effects of Spiroplasma infection on survival or fecundity (Montenegro et al., 2006). The small and variable fitness effects found in this study indicate that fitness effects related to host plant specialization on Catalina Island do not, on their own, explain the prevalence of Spiroplasma in this D. mojavensis population.

Spiroplasma may be a commensal bacterium, and its persistence explained by a high fidelity of vertical transmission, with occasional acquisition by horizontal transmission. The plant pathogenic Spiroplasma, S. citri, is common in plants (Bove et al., 2003), including some cacti (Christiansen et al., 1980) and their insect vectors in the southwestern United states (Calavan, Bove, 1989). Thus cactus necroses could serve as a Spiroplasma reservoir for horizontal transmission of the bacteria, via an infected plant or any other infected member of the diverse arthropod community associated with cactus rots, which can vary seasonally (Castrezana, Markow, 2001). Thus any loss due to imperfect vertical transmission may be counteracted by reacquisition. The extent of horizontal transmission of Spiroplasma in Drosophila is unknown. One study found evidence for Spiroplasma acquisition by feeding (Carvalho, da Cruz, 1962), though attempts to replicate this were unsuccessful (Ebbert, 1991; Williamson, 1984; Williamson, Poulson, 1979). Horizontal transmission of Spiroplasma via mites has been demonstrated in the lab (Jaenike et al., 2007), and phylogenetic evidence suggests at least five independent introductions of Spiroplasma into Drosophila (Haselkorn et al., 2009).
Alternatively, *Spiroplasma* may confer some other conditional fitness benefit, such as defense against natural parasites. Many different facultative endosymbionts can confer protection against parasitoid wasps (Oliver et al., 2003), fungus (Scarborough et al., 2005), and RNA viruses (Hedges et al., 2008; Teixeira et al., 2008). The recent discovery of a poulsonii-type Spiroplasma defending *D. neotestacea* against its nematode parasite (Jaenike et al. 2010) makes this phenotype more likely. Although the citri-type *Spiroplasma* is genetically divergent from the poulsonii-type (Haselkorn et al. 2009), it may still be able to confer protection against a parasite that is associated with cactus necroses. Further characterization of the ecology of *D. mojavensis* on Catalina Island, such as an exploration of the natural predators and parasites, will provide insight into the effects that *Spiroplasma* has on its *D. mojavensis* host.

**ACKNOWLEDGEMENTS**

I would like to thank Luciano Matzkin for assistance in experimental design and statistics, Sarah Johnson and Chris Paight for invaluable laboratory assistance setting up the fitness experiment and Doug Hooton for helping to optimize the cactus rot protocol.

**REFERENCES**


Oliver KM, Degnan PH, Burke GR, Moran NA (2009) Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. Annu Rev Entomol 55, 247-266.


Vacek DC (1979) The microbial ecology of the host plants of *Drosophila mojavensis*. *Thesis*, University of Arizona, Tucson AZ.


Table 4.1 Effects of *Spiroplasma* infection on *D. mojavensis* in each experimental replicate. Statistically significant results are highlighted in bold.

<table>
<thead>
<tr>
<th>Experimental replicate / line</th>
<th>Source</th>
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Table 4.2 Effects of experimental replicate. Statistically significant differences are highlighted in bold.

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Figure 4.1 The effect of *Spiroplasma* infection on survival (1st instar larvae to adult) on cactus food. Error bars represent standard error, and statistically significant differences are denoted with stars. (a) First experimental replicate with line 1, (b) Second experiment replicate with line 4, and (c) Third experimental replicate with lines 1, 4, 2 and 15.
Figure 4.2 The effect of *Spiroplasma* infection on development time (egg to adult) on cactus food. Error bars represent standard error, and statistically significant differences are denoted with stars. (a) First experimental replicate with line 1, (b) Second experiment replicate with line 4, and (c) Third experimental replicate with lines 1, 4, 2 and 15.
Figure 4.3 The effect of *Spiroplasma* infection on thorax size (log transformed) on *cactus food*. Error bars represent standard error, and statistically significant differences are denoted with stars. (a) First experimental replicate with line 1, (b) Second experiment replicate with line 4, and (c) Third experimental replicate with lines 1, 4, 2 and 15.
CONCLUSIONS

This dissertation work has laid much of the groundwork necessary to effectively explore the consequences of Spiroplasma infections in Drosophila. As new phenotypic effects are discovered, we can now begin to investigate additional central questions concerning this symbiosis. Are phenotypic effects Spiroplasma strain specific, or host specific? For example, the Spiroplasma acting as a defensive endosymbiont in D. neotestacea is a poulsonii-type Spiroplasma. Do all poulsonii-type Spiroplasma have this effect on their hosts? Does the genetically divergent Spiroplasma strain infecting D. tenebrosa, a mushroom-breeding fly like D. neotestacea, also provide defense against parasites? I found no evidence for recombination, and Spiroplasma screens have detected no individuals co-infected with different types of Spiroplasma, implying that horizontal transmission is rare, co-infection cannot occur, or recombination is not possible. This leads to further questions concerning the frequency, mode, and routes of horizontal transmission. Screening for Spiroplasma in the cactus necroses, as well as other arthropods these necroses would lend insight into these questions.

Understanding the consequences of Spiroplasma infection in Drosophila is not only imperative because of the great number of Drosophila species infected and the potential dramatic effects that Spiroplasma can have on evolution in Drosophila, but also because this system can serve as a model for understanding the mechanisms of symbiosis. The genetic and genomic tools of D. melanogaster, as well as other Drosophila species whose genomes have been sequenced, provide an unparalleled opportunity to explore the interaction between Spiroplasma and the Drosophila immune system, the host specificity
of *Spiroplasma* strains, and the genetics underlying the effects of host genetic background. We can begin to understand why some *Drosophila* species are infected and not others. My work has shown that there is variation in the density dynamics of different strains of *Spiroplasma* in different *Drosophila* species, and now we can explore the relative roles of host factors and bacterial factors in regulating bacterial density. This is particularly significant if, like so many other effects caused by endosymbionts, there is correlation between strength of these phenotypic effects and *Spiroplasma* titer. We can also begin to address whether phenotypic effects caused by *Spiroplasma*, such as defense against parasites, are transferable among *Drosophila* species.

There is also great potential to develop *Spiroplasma* as a model endosymbiont using genetics and comparative genomics to explore the mechanisms of *Spiroplasma*'s phenotypic effects. Genomic sequencing of different *Spiroplasma* strains causing the same phenotype, or similar strains causing different phenotypes will lend insight into the genes involved in conferring fitness effects. Furthermore, *Spiroplasma* has the potential to be cultured, so reverse genetics could be used to assess the functions of the genes essential for this symbiosis. Finally, there are many *Spiroplasma* infecting other organisms that are not vertically transmitted endosymbionts, such as *S. citri*, the plant pathogen closely related to the *Spiroplasma* infecting the repleta group *Drosophila*. Comparative genomics has the potential to indicate genes that may be involved in such dramatic lifestyle changes. As this work suggests there have been at least five introductions of *Spiroplasma* into *Drosophila*, we can look for similar genomic patterns among the different *Drosophila Spiroplasma* phylogenetic clades, illuminating aspects of the transition to vertically transmitted endosymbiont and the mechanisms of vertical
transmission. From this system as a model for symbiosis, we can gain a greater understanding of a significant and ubiquitous evolutionary process.