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Endosymbiotic prevalence and reproductive manipulation of the spider *Mermessus fradeorum*

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ENDOSYMBIOTIC PREVALENCE AND REPRODUCTIVE MANIPULATION OF
THE SPIDER *MERMESSUS FRADEORUM*

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
College of Agriculture at the University of Kentucky

By

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2013

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ABSTRACT OF THESIS

ENDOSYMBIOTIC PREVALENCE AND REPRODUCTIVE MANIPULATION OF THE SPIDER *MERMESSUS FRADEORUM*

Spiders are host to a plethora of heritable endosymbiotic bacteria. Broad-taxa screening studies indicate that endosymbionts are particularly common among spiders, however, little is known about how these bacteria affect their spider hosts. In insects, these bacteria ensure vertical transmission by either conveying a benefit to the host or manipulating host reproduction to eliminate males that serve as evolutionary dead-ends for maternally-inherited bacteria. Common modes of reproductive manipulation include parthenogenesis, male killing, feminization, and cytoplasmic incompatibility. Screening an assemblage of *Mermessus* genus spiders, I detected a high frequency and diversity of endosymbiont infection. Within a single species, *M. fradeorum*, I detected three endosymbionts in multiple combinations. Rearing two natural infection types of *M. fradeorum* demonstrated two distinct endosymbiotic reproductive manipulations. Mothers infected with *Rickettsia* and *Wolbachia* produced extremely female-biased offspring, and antibiotic elimination of the symbionts successfully restored the sex ratio to the expected 1:1 in subsequent generations. A two-way factorial mating assay detected strong cytoplasmic incompatibility induced by a different strain of *Wolbachia*: cured females mated with infected males produced 73% fewer offspring than all other pairings. These results show that *M. fradeorum* is subject to multiple layers of reproductive manipulation that likely drive host evolution and ecology.

Key words: Araneae, concurrent reproductive manipulation, cytoplasmic incompatibility, endosymbiont frequency, female bias

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December 18th, 2013

ENDOSYMBIOTIC PREVALENCE AND REPRODUCTIVE MANIPULATION OF
THE SPIDER *MERMESSUS FRADEORUM*

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DEDICATION

I dedicate this thesis to Tyson Thomas Brown. ZP.

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Chapter 1

Review of endosymbiont ecology and function

Spiders are widely distributed generalist predators capable of reducing pest populations in agroecosystems through direct predation, disruption of herbivory, or entanglement in the web (Sunderland 1999). Generalist predators have significant top-down effects on ecological communities because of their ability to control pest populations at levels too low to support more specialized predators. In disturbed habitats such as agroecosystems, small (2-6mm) sheet-weaving spiders in the family Linyphiidae are particularly common, feeding on a multitude of pest species (Romero and Harwood 2010, Chapman et al. 2013). Linyphiid spiders have been shown to decrease cereal aphid pest populations by trapping mobile aphids in their webs, which may cover up to half of a wheat field's surface area (Sunderland et al. 1986). Despite the importance of linyphiid spiders as generalist predators in agroecosystems, the biology of these ground-dwelling spiders is often overlooked. Conserving these biological control agents requires a thorough understanding of their ecology and life history.

Among the many underexplored arenas of spider biology is their relationship with bacterial endosymbionts. Recent endosymbiont screening surveys have revealed that spiders, like the majority of arthropod taxa, host a variety of maternally inherited endosymbiotic bacteria including *Cardinium*, *Rickettsia*, *Wolbachia*, and *Spiroplasma* (Goodacre et al. 2006, Duron et al. 2008a, Martin and Goodacre 2009). In particular, spiders appear to be a *Cardinium* hotspot; 22 percent of surveyed spiders were found to be infected with *Cardinium* (Duron et al. 2008a) compared to only six percent in insect taxa (Zchori-Fein and Perlman 2004). Though the prevalence of endosymbionts detected in spiders has piqued interest, few studies have addressed their functional role. Our understanding of arthropod endosymbiont biology has primarily originated from study of symbiont infection in insects.

Over the last 15 years our awareness of non-pathogenic bacterial relationships with arthropods has gained resolution with improved molecular techniques (Moran 2006). Bacteria residing within arthropods form a symbiosis with their host, and can infect a variety of arthropod tissues. Many such bacteria are vertically transmitted, passing from mother to offspring. These arthropod bacterial endosymbionts can be either facultative or obligate for the host; however, the association is usually necessary for these bacteria, which are rarely found outside of a host. Obligate endosymbionts commonly provide a nutritional supplement for hosts with nutrient-poor diets (Moran et al. 2008) and typically have reduced genomes, exhibiting close coevolution with hosts. In contrast, facultative symbiont and host phylogenies are rarely congruent, suggesting these symbionts may have a more dynamic impact on host evolution (White et al. 2013). Facultative bacteria that are maternally inherited ensure their vertical transmission either by acting as mutualists that convey a benefit to the host, or by manipulating reproduction to increase transmission through the production of infected daughters. These two modes are not mutually exclusive. The selfish interests of endosymbionts may be in concert or in conflict with that of the host, depending upon prevailing selection pressures (Toft and Andersson 2010). Endosymbionts are an important genetic presence within the cell, affecting host evolution and ecology in ways we have only recently begun to understand.

Endosymbiotic bacteria are well known for their ability to manipulate host reproduction to increase transmission through the female germline (White 2011). This propensity to manipulate host reproduction has evolved multiple times in independent bacterial lineages, including bacteria in the genera *Wolbachia*, *Rickettsia*, *Arsenophonus*, *Spiroplasma*, *Cardinium* and *Flavobacterium* (Moran et al. 2008). Symbionts promote the production of infected female progeny because sperm represent an evolutionary dead-end for bacteria that are only transmitted in the cytoplasm of oocytes (Engelstadter and Hurst 2009). Endosymbiont infection has been shown to directly eliminate males through three mechanisms: parthenogenesis, feminization, or male killing (Stouthamer et al. 1999). Symbiont-driven thelytokous parthenogenesis causes virgin females to produce all-female offspring, and thus far has only been documented in haplodiploid arthropods (Huigens and Stouthamer 2003, Hagimori et al. 2006). In other hosts,

endosymbionts induce feminization of genetic males. In some isopods, which have a hormonal sex determination mechanism, the bacteria interfere with the production of androgenic hormones (Bouchon et al. 2008). Feminization in insects and mites has also been demonstrated; however, the mechanism in these hosts is suspected to be more complex than in isopods, as symbionts must interact with the host genome to alter chromosomal sex determination in all somatic cells (Kageyama et al. 2012). Finally, male killing endosymbionts have been described in numerous insect and several arachnid orders. A female infected with a male killing symbiont will produce male and female embryos, but few to no male offspring will develop, thereby reducing fecundity by ~50% in an animal with a 1:1 sex ratio. Female offspring may then enjoy improved fitness from either the direct consumption of their dead brothers (Elnagdy et al. 2011) or indirectly from reduced sibling competition if food resources are limited (Jaenike et al. 2003).

Alternatively, symbionts can alter reproduction without distorting the primary sex ratio by inducing a mechanism of cytoplasmic incompatibility (CI). CI is the most common form of reproductive manipulation and has been described in multiple insect, mite, and isopod taxa (Werren et al. 2008). This postzygotic mechanism of incompatibility is thought to work via a sperm modification and rescue system. Endosymbionts modify the sperm of infected males such that only females that also bear the same infection type are able to rescue the modified sperm. Females without the same infection produce few viable offspring (Poinsot et al. 2003). Infection can then spread through the population because uninfected females' fecundity is depressed relative to infected females, who enjoy the selective advantage of being able to successfully mate with either infected or uninfected males (Weeks et al. 2002). If multiple CI-inducing symbionts occur in a population, bidirectional CI can arise when females bearing either strain are unable to rescue sperm modified by the other strain (O'Neill and Karr 1990).

In some cases, different strains of the same endosymbiont taxon can elicit divergent phenotypes in different host backgrounds, ranging from reproductive manipulation to providing a facultative benefit for the host (Dobson et al. 2002).

Genomes of facultative endosymbionts have been found to carry more mobile genetic elements and are therefore considered to be more genetically flexible than those of obligate symbionts (Newton and Bordenstein 2011). *Wolbachia*, a notorious reproductive manipulator, has been shown to induce resistance to RNA viral infections in *Drosophila melanogaster* (Teixeira et al. 2008). Another α -Proteobacteria, *Rickettsia*, which is also capable of inducing multiple modes of reproductive manipulation, was recently shown to also induce the expression of genes associated with stress responses in the whitefly *Bemisia tabaci*, resulting in an increased tolerance to heat shock (Brumin et al. 2011). In a different whitefly population, a population sweep to near fixation was attributed to increased survival, faster development, and a female bias in offspring of *Rickettsia* infected whiteflies (Himler et al. 2011). Symbiont infection also can provide a suite of practical applications. Recent field (Hoffmann et al. 2011) and laboratory (Walker et al. 2011) studies have found a strain of *Wolbachia* capable of suppressing dengue fever transmission in experimentally inoculated mosquitoes. Like sterile insect techniques, male arthropods infected with endosymbionts inducing CI can be mass released to control pests (O'Connor et al. 2012).

The real potential for pest and disease control through the use of endosymbiont infection speaks to the strength of symbiont relationships with their arthropod hosts. Bacterial symbionts residing within a host represent a source of genetic novelty capable of generating phenotype variation within a population subject to natural selection. Though the list of known symbiont phenotypes is still gaining dimension, known bacterial effects thus far indicate that endosymbionts can act as powerful drivers of evolution (White et al. 2013).

Despite our improving understanding of the ways in which symbionts affect insects, our knowledge of symbiont effects on spiders lags behind. To date only three bacterial phenotypes have been experimentally characterized in spider populations. In the first study, *Rickettsia* infection in *Erigone atra* (Linyphiidae) was shown to decrease females' long distance dispersal activity (ballooning), such that spiders cured of *Rickettsia* infection showed an increased tendency to disperse (Goodacre et al. 2009).

Though the fitness consequences associated with decreased dispersal tendencies are unknown, *Rickettsia* infection did not negatively affect longevity or fecundity in laboratory-reared spiders within this study. However, decreased dispersal should ultimately restrict gene flow. In the second study, another linyphiid spider, *Oedotharax gibbous*, exhibited a female-biased sex ratio that was shown to be the result of *Wolbachia* infection. The *Wolbachia*-associated female bias was suspected to result from a male killing mechanism, because an overall trend of decreased fecundity was observed in infected clutches. However, clutch size varied too greatly to establish a significant effect of infection on fecundity. *Oedotharax gibbous* also carries *Rickettsia* and *Cardinium*, but their function was not investigated (Vanthournout et al. 2011). In the third study, a complex interaction between *Wolbachia* and sex ratio distortion was detected in the linyphiid *Pityohyphantes phrygianus*, which exhibits a primary female bias in natural populations. Maternal size, maternal *Wolbachia* infection, and paternal *Wolbachia* infection were all found to influence sex ratio, which was controlled by maternal post-copulatory body position. Analysis of fecundity and chromosomal sex determination pathways in *P. phrygianus* indicated that neither male killing nor feminization of genetic males could explain the female bias (Gunnarsson et al. 2009). This species' spermatheca is twisted and the authors suggest that some method of cryptic choice or differential storage may explain the sex ratio skew (Gunnarsson et al. 2004). Finally, unpublished data by Sleaford and Goodacre (Goodacre 2011) suggest that endosymbiont infection in a linyphiid spider might influence sensitivity to deltamethrin, a common pyrethroid suggestively echoing associations between endosymbionts and pesticide sensitivity in other arthropods (Kikuchi et al. 2012). Of the three fully described bacterial phenotypes in spiders, two are novel behavioral modifications not observed in insects, potentially indicating that symbionts may interact with spiders in different ways than they do with insects (Goodacre and Martin 2012).

In spiders, the majority of symbiont phenotypes and infection frequencies detected thus far have been found in linyphiid spiders. Demonstration of bacterial phenotypes generally requires antibiotic curing so that infected and uninfected individuals of the same genetic background can be compared in a controlled laboratory

environment. Many taxa of spiders have a reputation for being difficult to rear. However, some linyphiids have a well documented history of successful laboratory rearing (Dinter 2004), a short generation time, and require less rearing space than larger spiders. These characteristics make select linyphiid species good model systems to study the bacterial endosymbionts of spiders. In linyphiid spiders, many modes of conflict between male and female spider reproductive strategies have been described (Huber 2005). Within the Araneae order, multiple instances of antagonistic reproductive strategies such as sexual dimorphisms, sexual cannibalism, male palpal dimorphisms, mating plugs, cryptic female choice, sperm competition have been detected (Eberhard 2004). The relationship between endosymbionts and sexual selection may prove to be interesting in spider taxa rife with reproductive oddities known to be infected with a high incidence of notorious bacterial reproductive manipulators.

Objectives

- 1) Detect symbiont frequency in natural Linyphiid populations, within the *Mermessus* genus and within a single species, *Mermessus fradeorum*, using diagnostic PCR, Sanger sequencing and 454-Pyrosequencing.

- 2) Examine the effects of endosymbiont infection on basic life history parameters of *M. fradeorum*. Specifically, to compare the fecundity and offspring sex ratio among mothers that are infected with both *Rickettsia* and *Wolbachia*, only *Wolbachia*, or were antibioticly cured.

- 3) Determine if *Wolbachia*-infected *M. fradeorum* males induce cytoplasmic incompatibility when mated with experimentally cured females.

Chapter 2

Multiple reproductive manipulations within a population of the spider *Mermessus fradeorum*

Introduction

Maternally inherited bacterial endosymbionts can manipulate their arthropod and nematode hosts through a plethora of mechanisms to ensure their own transmission. Because males are typically evolutionary dead ends, facultative endosymbionts bolster the number of infected females by either improving host fitness or altering host reproduction, thereby influencing host evolution (White et al. 2013). Sweeps of beneficial symbionts through a population have been documented in which infection frequency reaches near fixation in less than six years (Himler et al. 2011). An endosymbiont is an independent genetic presence within the cell that has interests which may or may not align with that of the host, depending upon selection pressures (Toft and Andersson 2010). The most common form of endosymbiotic reproductive manipulation is cytoplasmic incompatibility (CI), which modifies the sperm of infected males such that it cannot produce a viable embryo unless the same endosymbiont infection is also present in the egg, thus enabling rescue of sperm functionality (Werren et al. 2008). CI-inducing bacteria have also been observed to sweep through a population (Hoffmann and Turelli 1997). Additionally, symbionts can directly skew the sex ratio of hosts by inducing thelytokous parthenogenesis in haplodiploid arthropods (Huigens and Stouthamer 2003, Koivisto and Braig 2003), killing males (Hurst and Jiggins 2000), or feminizing genetic males (Negri et al. 2006).

Bacterial endosymbionts are very common among arthropods; more than 30% of arthropods are infected with *Wolbachia* and other endosymbionts known to cause reproductive anomalies (Duron et al. 2008a). Though most screening studies traditionally have focused on insects, recent surveys demonstrate that spiders also harbor a wealth of endosymbionts (Duron et al. 2008b), with multiple endosymbionts often co-occurring with the same host population, or even co-infecting the same host individual

(Goodacre et al. 2006, Perlman et al. 2010, Vanthournout et al. 2011). Though endosymbiont transmission is primarily vertical, from mother to offspring, incongruencies between host and symbiont phylogenies are thought to result from horizontal transfer of symbionts between host taxa occurring rarely on an evolutionary timescale. Spiders in particular have a high incidence of diverse symbionts, which is suspected to result from a predatory lifestyle that increases exposure to horizontal transmission of endosymbionts from infected prey (Duron et al. 2008b). Furthermore, the order Araneae is rife with reproductive anomalies like sexual cannibalism or extreme sexual size dimorphisms thought to result from antagonistic sexual selection between male and female reproductive interests (Eberhard 2004). Coupling a high incidence of notorious reproductive manipulators with the curious nuances of spider reproduction creates a rich context to examine the relationship between maternally inherited symbionts and antagonistic sexual selection (Goodacre 2011).

Despite our improving knowledge of endosymbionts in insects, very little is known about the function of facultative symbionts in spiders. To date, only three bacterial phenotypes have been described in spiders: *Rickettsia*-induced dispersal alteration in *Erigone atra* (Goodacre et al. 2009), *Wolbachia*-associated female bias in *Oedothorax gibbosus*, likely the result of male killing (Vanthournout et al. 2011), and *Wolbachia*-linked maternal post-copulatory behavior causing a female biased sex ratio in the spider *Pityohyphantes phrygianus* (Gunnarsson et al. 2004, Gunnarsson et al. 2009). Most of the well-documented phenotypes induced by endosymbionts in insects have not yet been demonstrated in spiders. Of the three described endosymbiont phenotypes in spiders, two are novel behavioral alterations not previously observed in insects, implying that symbionts may have a different or expanded suite of manipulative tools in spiders than their insect relatives. Understanding the potentially different range of phenotypes that may manifest in spider populations laden with endosymbionts becomes imperative in light of the current (Hoffmann et al. 2011, Bourtzis et al. 2013) and future use of endosymbionts in biological control. The promising potential uses of endosymbionts as drivers of biological control necessitates a broader taxonomic comprehension of endosymbiotic effect upon individual hosts and within host population prior to mass release of infected hosts.

To improve understanding of the functional significance of endosymbionts in spiders, we investigated the prevalence and effects of endosymbionts with *Mermessus fradeorum*. This spider is a small sheet-weaving member of the Linyphiidae, common to Kentucky agroecosystems (Chapman et al. 2012, Welch 2013) and has been successfully reared in the laboratory (Welch et al. 2013). We investigated the natural frequency of endosymbiont infection in natural populations of *Mermessus* spp., then specifically in *M. fradeorum*. After characterizing the symbiont strain in two distinct matriline of *M. fradeorum*, a *Rickettsia-Wolbachia* infected line and a *Wolbachia* infected line, we determined symbiont effects on sex ratio and fecundity by comparing each infected matriline with antibiotically cured uninfected lines. We then conducted mating assays to test for *Wolbachia*-induced cytoplasmic incompatibility between infected males and uninfected females.

Materials and Methods

Symbiont infection frequency in natural *Mermessus* populations

To gain a better understanding of endosymbiont prevalence in natural linyphiid populations, we screened 60 previously collected specimens of the *Mermessus* genus (Chapman et al. 2012) for common bacterial endosymbionts. These spiders were collected in 2009 from winter wheat at the Spindletop Research Farm in Lexington, KY (coordinate: 38.1272°N, 84.5081°W) as part of a study of Linyphiid predation patterns. Spiders were morphologically identified to genus prior to whole body DNA extraction as described in Chapman et al. (2012). Diagnostic PCR for *Wolbachia*, *Rickettsia*, and *Cardinium* was performed on field collected spiders using primers and protocols described in Table 2.1. PCR products were visualized under UV light on a 1.5% agarose gel treated with Gel-Red (Biotium, Hayward, California). Endosymbiont identity for all positive PCR results was confirmed via Sanger Sequencing at the University of Kentucky's AGTC sequencing facility.

We conducted a subsequent field survey focusing solely only on *Mermessus fradeorum*, because we specifically investigated the endosymbionts of this spider in the laboratory. In August 2013, 45 live *M. fradeorum* adults were collected from alfalfa and winter wheat fields at the Spindletop research farm in Lexington, KY (coordinate: 38.1276°N, 84.5102°W). Live specimens were reared in the laboratory for a minimum of three days on symbiont-free *Proisotoma minuta* Collembola prey, and then starved for at least two days prior to extraction, allowing at least five days for decay of field-caught prey in the gut to eliminate potential detection of symbionts in prey (Greenstone et al. 2013). Upon morphological species identification, specimens were killed and dissected to remove epigynal and palpal vouchers. Adult spiders were surface sterilized prior to extraction with sequential 10 s rinses of ethanol, deionized water, 5% bleach solution, and deionized water again using a protocol adapted from Medina et al. (2011). DNA was extracted using a Chelex extraction protocol adapted from White et al. (2009); abdomens of adults were pulverized in 4μL of Proteinase K, incubated in a 20% Chelex bead solution for 60 m at 37°C, then heat shocked for 10 m at 95°C to deactivate Proteinase K. Each individual was screened for *Rickettsia*, *Wolbachia*, and *Cardinium* using methods identical to the previous screen of field collected *Mermessus* spp. spiders. Extraction quality was assessed by measuring DNA concentration using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, Delaware) and by PCR using universal eukaryotic primers NSF 18/19 or CO1 (Table 2.1). Poor quality extractions were excluded from analysis. All specimens that were scored as positive for a symbiont were validated by Sanger sequencing as described previously.

Establishment of laboratory matrilines

Upon detecting a diverse range of symbionts in the *Mermessus* genus, we hand-collected gravid *M. fradeorum* females in the winter of 2011 from Spindletop Research Farm. Field collected individuals were initially reared on a live culture of the collembolan *Sinella curviseta*, potting soil, and potato. Subsequent generations were reared in individual rearing cups constructed of 4.6cm tall by 7.3cm diameter plastic cups with a plaster base and two imbedded plastic straws for web attachment. Juveniles were

fed only *P. minuta* Collembola, whereas adults were fed a combination of *P. minuta*, *S. curviseta*, and *Drosophila melanogaster* flies. Juveniles and adults were fed twice per week and all experimental feeding was standardized across treatments. Collembola were aspirated and flies were stunned before being placed directly on the web. Prey items were screened for common endosymbionts and no endosymbiotic bacteria were detected in laboratory stocks. All generations were reared in a 24h dark incubator with a 16h:8h thermal cycle of 28°C: 18°C, and an average relative humidity of 98 percent. Spiders were identified morphologically by Dr. Kelton Welch and confirmatory COI sequences for *M. fradeorum* will be added to Genbank. The previously described methods of Chelex DNA extraction and diagnostic PCR screening methods were used to detect endosymbionts in laboratory populations. Initial detection and sequencing of *Rickettsia* and *Wolbachia* was made using previously published diagnostic primers, then new primers (Ricklong and mfwsp) were designed using the Primer3 tool (Rozen et al. 1998) in Geneious 5.6.4 (Drummond 2012) to specifically amplify the *Rickettsia* and *Wolbachia* detected in *M. fradeorum* and used for all subsequent symbiont screening (Table 2.1).

Offspring of two field collected females were used to establish two genetically homogenous matriline of differential endosymbiont infection types; “R₁W₁” bearing a double *Rickettsia*-*Wolbachia* infection and “W₂” infected with only *Wolbachia*. *Rickettsia* and *Wolbachia* infection status of each generation and vertical symbiont transmission were confirmed with diagnostic PCR. Because only female offspring were produced in the R₁W₁ line, this line was initially propagated by matings with males from the W₂ line. To prevent inbreeding depression, additional *M. fradeorum* infected with W₂ were collected from a different Lexington, KY population in spring of 2011 and reared in the lab for one generation. Males from three different mothers were introgressed equally into third generation R₁W₁ and W₂ matriline prior to antibiotic treatment.

Experimentally cured lines were generated for both R₁W₁ and W₂ matriline to investigate endosymbiotic effects on the host. To eliminate endosymbiotic bacteria in *M. fradeorum*, second and third instar spiderlings from both the R₁W₁ matriline and the W₂

matrilines were treated with antibiotics previously shown to eliminate these symbionts from spiders (Goodacre et al. 2009, Vanthournout et al. 2011). For five consecutive days, 38 spiderlings (13 R₁W₁ and 25 W₂) were sprayed with a fine mist of 0.1% Ampicillin solution once per day. After one week of no treatment the same spiderlings (now third and fourth instar) were sprayed with a 0.1% Tetracycline solution one time per day, for five days consecutively. After the ultimate molt, surviving spiders were mated, and curing was confirmed in the subsequent generation using diagnostic PCR. Antibiotic treatment successfully eliminated all symbiotic bacteria from R₁W₁ and W₂ lines, but did not result in differential elimination of only *Rickettsia* or only *Wolbachia* from the R₁W₁ line. A later curing attempt using 0.05% antibiotic concentration applied only three times also fully eliminated both symbionts for the R₁W₁ line. Cured lines (R₁-W₁- and W₂-) were established using the descendants of treated individuals. Experiments were conducted with spiders at least two generations removed from antibiotic treatment to alleviate any direct negative fitness effects of the antibiotics.

Pyrosequencing

To explore whether other potential endosymbiotic bacteria were present in laboratory lines, one female from each matriline, R₁W₁ and W₂, was selected for 16S 454-Pyrosequencing. DNA from abdomens of sterilized spiders was extracted using DNeasyblood and tissue kits (Qiagen, Valencia, California) according to manufacturer's recommended protocol and sent to Research and Testing Laboratory (Lubbock, Texas) for bacterial diversity analysis. 454-Pyrosequencing and data analysis were conducted as described in Brady and White (Brady and White 2013). Representative sequences will be deposited in GenBank and the full data set will be deposited in the NCBI sequence reads archive.

Wolbachia strain typing and *Rickettsia* sequencing

A Basic Local Alignment Search Tool (BLAST) search (NCBI, <http://blast.ncbi.nlm.nih.gov>) was performed for R₁W₁ and W₂ *Wolbachia* surface protein

(*wsp*) genes. In addition to *wsp* gene sequencing we also used multi locus sequence typing (MLST) to characterize *Wolbachia* from each laboratory-reared matriline. M13-tagged *Wolbachia*-specific MLST primers for five housekeeping genes were amplified, sequenced, and analyzed according to standardized PCR protocols (Baldo et al. 2006). Virtually all alleles from both strains were unique and will be submitted to the MLST database (<http://pubmlst.org/wolbachia/>) for new allele number assignment. MLST genes were concatenated and aligned with 27 strains from the database, including all strains originating from spiders within the database, as well members from different representative *Wolbachia* super groups. A neighbor-joining tree was constructed comparing these concatenated MLST genes to the W_1 and W_2 *Wolbachia* strains in *M. fradeorum*. *Rickettsia* 16S rRNA genes for all *Rickettsia* strains detected in the *Mermessus* spp. spiders and the *Rickettsia* strain detected in *M. fradeorum* were sequenced and percent identity to the most closely related endosymbiont strains were determined using a BLAST search. A neighbor joining tree was also constructed comparing all *Rickettsia* strain types found in *Mermessus* sp., *M. fradeorum*, and representative *Rickettsia* sequences. All phylogenetic analyses were conducted in Geneious version 5.6.4 using default settings (Drummond 2012).

Symbiont effects on sex ratio and fecundity

The effects of *Rickettsia* and *Wolbachia* symbionts in the doubly infected R_1W_1 line and the singly infected W_2 line were quantified by comparing life history parameters of naturally infected R_1W_1 and W_2 lines with antibiotically cured R_1-W_1- and W_2- lines. Females of the four different lines were mated with males from antibiotically cured lineages, to eliminate the influence of paternal symbiont type (see table 2.8 for sample sizes). Both R_1-W_1- and W_2- males were used. Males were allowed to rappel into the female's container, mating was confirmed by visual observation, then males were removed one day after mating. Females were culled 10 d after initial egg sac deposition to prevent cannibalism. All parents were preserved in 95% ethanol and stored at -20°C . All mothers and 11 randomly selected fathers were screened for *Rickettsia* and *Wolbachia* to verify appropriate infection status. We found no difference in the total

production of offspring (One-way ANOVA, $F=0.043$, d.f.= 1, 42, $p=0.836$), sex ratio (logistic regression, $\chi^2=1.556$, d.f.=1, $p=0.212$), or survivorship (logistic regression, $\chi^2=0.525$, d.f. =1, $p=0.217$) between R_1 - W_1 - and W_2 - fathers, therefore cured males of both matriline were lumped for further analysis and methods of statistical analysis are described in the following section.

During the 10 d egg sac deposition period, females typically produced two to three egg sacs. One egg sac from each mating pair was randomly selected to hatch; all other egg sacs were removed 10 d post mating and stored in 95% alcohol for embryo counting. Remaining egg sacs were monitored daily. Upon hatching, spiderlings were immediately transferred to individual rearing cups and each egg sac was checked for undeveloped embryos. Maternal patella-tibia length, a common method of size estimation that is often strongly correlated with fecundity (Stefanini and Duron, 2012, Skow and Jakob, 2003) was determined using a micrometer in a Leica S8AP0 stereoscope at 80X magnification. However, no relationship between maternal size and total fecundity was found ($R^2=0.002$, d.f.=42, $p=0.7903$), therefore, maternal size was not included in subsequent analyses. Total fecundity was the sum of all embryos produced during the 10 d egg sac deposition period; it was calculated by adding the number of hatched spiderlings, the number of undeveloped embryos found in hatched egg sacs, and the number of embryos in culled egg sacs for each mother. Total fecundity per mother was compared among matriline categories (R_1W_1 , $R_1W_1^-$, W_2 , W_2^-) using one-way ANOVA and the separation of means for each category was compared using Tukey's honest significant difference test (HSD). All ANOVA analyses were conducted in JMP 10 (SAS Institute Inc. 2012). Symbiont effect on sex ratio was determined using an exact binomial two-tailed test for goodness of fit, testing each group against the expected 1:1 sex ratio (McDonald and Delaware 2009) because quasi-separation of the data precluding the use of logistic regression. Differences in embryo development (hatched versus unhatched eggs) and survivorship of hatched spiderlings to adulthood among lines were analyzed with logistic regression. All logistic regressions were made using Arc 1.06 (Cook and Weisberg 2009). Overall change in deviance of the model was

evaluated via a chi squared statistic, and if overall significance of the model was established, Wald statistics were used to make comparisons among types.

CI Experiments

To determine if W_2 *Wolbachia* induces CI, we conducted a two-way factorial cross, mating W_2 and W_2^- females and males in all four combinations ($W_2^- \times W_2^-$, $W_2^- \times W_2$, $W_2 \times W_2^-$, $W_2 \times W_2$; see table 2.8 for sample sizes). Matings, husbandry methods, and fitness parameters for the CI cross were the same as those used in the previously described mating assay with one exception. In the CI cross, egg sacs were not removed and all egg sacs were allowed to hatch. Prior to egg sac hatching, one egg sac from each pair was randomly selected for full individual rearing of offspring to adulthood. Emerging spiderlings from the other egg sacs were immediately culled and preserved in alcohol. All egg sacs were allowed 10 d post deposition to hatch and the total number of spiderlings to emerge from each egg sac was recorded. All egg sacs were dissected post-hatching to check for undeveloped embryos and any spiderlings remaining within the egg sac were scored as undeveloped. Embryo development, spiderling survivorship to adulthood, and sex ratio were compared among the cross types using logistic regression in Arc 1.06 with Williams' correction (Williams 1982) to address moderate overdispersion in the data when necessary. Embryo development was quantified by comparing number spiderlings that successfully hatched with the total number of embryos produced (the sum of hatched spiderlings and undeveloped embryos). Linear regression once again indicated that maternal size did not influence fecundity ($R^2 < 0.001$, d.f.=48, $p=0.882$) so maternal size was not considered as a covariate of fecundity. Following mating, mothers and fathers of the CI cross were screened for *Wolbachia*; all fathers reflected the expected infection status, but one female did not carry her maternal *Wolbachia* and was recategorized as W_2^- for statistical analysis.

We also investigated compatibility in matings between R_1W_1 females and W_2 males, because the *Wolbachia* strains present in the R_1W_1 and W_2 lines are different. Using analogous mating conditions to the previous CI cross, four R_1W_1 females were

mated with W_2 males and four R_1W_1 females were mated with W_2 - males. For each mating pair, all conditions were identical to the previous CI cross, with the exception that no spiderlings were reared to adulthood. Hatching success was again analyzed using logistic regression in Arc 1.06.

Results

Symbiont infection frequency in natural *Mermessus* populations

Screening of the 60 *Mermessus* spp. specimens from 2009 revealed a broad diversity of endosymbionts (Table 2.2), with 75% of the samples testing positive for one or more of the screened bacterial symbionts (Figure 2.1). The most common endosymbiont, *Rickettsia*, was detected in 36% of all individuals. Sequencing revealed that five strains were present (R_2, R_3, R_4, R_5, R_6), four of which were closely related to R_1 laboratory strain (Table 2.3). The second most common symbiont was *Wolbachia*; four strains (W_3, W_4, W_5, W_6) were found in 33% of individuals. Sequencing of the *wsp* gene indicated that none of these four *Wolbachia* strains were closely related to W_1 or W_2 strain types of laboratory stock *M. fradeorum* (Table 2.4). The most common *Wolbachia* strain, W_3 , was a 92.9 % match to W_2 (Table 2.4) for the *wsp* gene. *Cardinium* (C_1, C_2 , and C_3) were found in only four individuals (7%) representing three strains that differed from one another by 1.2-2.4% for a 394bp region of 16S bacterial DNA (Table 2.5). In 11% of individuals we detected multiple infections with symbionts of different genera, however, our protocol did not allow detection of more than one strain of the same bacterial genus. Morphological or molecular delineation of spiders to the species level was not possible, but the short CO1 sequences that were examined for five individuals indicated that at least three different *Mermessus* species were present, none of which was a match to the laboratory lineages of *M. fradeorum*. These samples included partially digested prey within the spiders, which may also have been infected with symbionts.

In the second field collection, which focused solely on *M. fradeorum*, 42% of all individuals bore an endosymbiont infection (Figure 2.2). Diagnostic PCR screening

indicated that 18% of individuals were positive for only *Rickettsia*, 18% of individuals were positive for only *Wolbachia*, 6% were doubly infected with both *Rickettsia* and *Wolbachia*, and no *Cardinium* was found (Figure 2.2). Of the 11 individuals that were positive for *Rickettsia*, we successfully sequenced *Rickettsia* from seven spiders, and found that all were a 100% match for the laboratory strain R₁. Of the 11 individuals that were positive for *Wolbachia*, we successfully sequenced *Wolbachia* from nine spiders, and found that six were a 100% match for the laboratory strain W₂ and three were a 100% match to W₁. All three symbiont strains present in laboratory populations (R₁, W₁, W₂) were detected in the field collected *M. fradeorum* in four different combinations: W₁ only, W₂ only, R₁ only, and R₁W₂. Additional *Rickettsia*-*Wolbachia* infected spiders were present in the population but not strain typed and likely included some R₁W₁ individuals. The overall sex ratio in the sample was 75.6% female (11 males: 34 females). Three of the males were *Rickettsia* positive, but overall male prevalence was too low to test for statistical associations between symbiont infection and sex ratio. A single R₁ female was allowed to reproduce in the lab and produced multiple male offspring.

Symbiont strain characterization in laboratory matrilines

Diagnostic PCR and Sanger sequencing confirmed that both laboratory-reared matrilines were positive for *Wolbachia* and offspring of one matriline were also positive for *Rickettsia*. R₁W₁ and W₂ matrilines were reared in the laboratory for nine generations and vertical transmission rates for the symbionts were near perfect. Transmission of *Rickettsia* and *Wolbachia* in the R₁W₁ line from mothers to offspring was never observed to fail, exhibiting 100% fidelity (19 mothers, 51 offspring). In the W₂ line, we failed to detect maternal *Wolbachia* infection in two offspring, resulting in 98% transmission efficiency (17 mothers, 86 offspring). Sequencing of a 673 bp region of 16S rRNA indicated that the *Rickettsia* present in the R₁W₁ line of *M. fradeorum* most closely matches endosymbiotic *Rickettsia* in the leaf hopper *Nephotettix cincticeps*, AB702995 (99% similarity; Figure 2.3). Sequencing of the *wsp* gene revealed a 30/561bp difference between the *Wolbachia* present in the R₁W₁ and W₂ lines. However, the *Wolbachia* surface protein (*wsp*) is hypervariable (Baldo and Werren 2007) and divergence between

the strains as measured by other more conserved genes was considerably less (82/2079 = 4% divergence). For both the W_1 and W_2 *wsp* gene, the closest genetic match was *Wolbachia* from the beetle *Acrogonia virescens* (DQ450162) at 92% and 95% respectively. Only one MLST *M. fradeorum* gene was a 100% match to a previously described allele (Table 2.6). Phylogenetic comparison of all five concatenated MLST alleles with representative *Wolbachia* strains places W_1 basally within *Wolbachia* supergroup A, although not sister to W_2 , nor closely related to other supergroup A *Wolbachia* from spiders in the genera *Hylyphantes* and *Agelenopsis* (Figure 2.4). W_2 , however, placed as sister taxa to a *Wolbachia* isolated from the linyphiid spider *Hylyphantes graminicola*, both likely sharing a recent common ancestor with other members of the supergroup A (Figure 2.4).

Pyrosequencing of 16S rRNA to characterize bacterial diversity from single specimens per lineage was marginally informative. Although nearly 7000 16S bacterial reads were generated for the W_2 female, none of these were *Wolbachia*, despite diagnostic confirmation of the presence of *Wolbachia* in this specimen. Virtually all reads came from members of the Coxiellaceae family, and most closely aligned with the genus *Rickettsiella* (Table 2.7). *Rickettsiella* is a genus that is largely composed of entomopathogens, but also contains some mutualistic endosymbionts. *Rickettsiella* was also detected from the R_1W_1 female, along with *Rickettsia* and *Wolbachia*. However, subsequent diagnostic results using both published MLST *Rickettsiella* primers, and novel primers designed from the pyrosequencing reads (Table 2.1) failed to detect *Rickettsiella* from either matriline. Our tentative hypothesis is that a pathogenic strain of *Rickettsiella* was likely present in early non-experimental generations, but dissipated from subsequent generations under individual rearing conditions that curtailed horizontal spread. No other known endosymbionts were detected, but the possibility of other endosymbionts cannot be excluded, due to the strong presence of *Rickettsiella* in the pyrosequenced samples, which decreased sensitivity to detect other symbiotic species. Inspection of the *Wolbachia* and *Rickettsia* sequences in R_1W_1 did not indicate the presence of more than one strain of either symbiont.

Symbiont effects on sex ratio and fecundity

Whereas the R_1W_1 line was entirely female and significantly differed from a 1:1 sex ratio ($p < 0.001$), the antibiotically cured R_1-W_1- line produced male offspring and showed a restoration to the expected Fischerian 1:1 sex ratio when both bacteria were eliminated ($p = 0.064$; Figure 2.5). Neither the *Wolbachia* infected W_2 line ($p = 0.777$) nor its cured W_2- counterpart ($p = 0.618$) had sex ratios that differed from 1:1, indicating that this strain of *Wolbachia* does not affect sex ratio (Figure 2.5). The extreme female bias in the R_1W_1 line was observed not only in this experiment, but also in nine generations of rearing. Only four males in total were produced out of 328 non-experimental R_1W_1 line spiders individually reared in the lab. Of the four R_1W_1 males produced, all four were reproductively viable and successfully mated with females. Three of these males were screened for endosymbionts, and all three were positive for *Rickettsia* and *Wolbachia*.

No significant difference in total fecundity was detected among the four infection types ($F = 0.618$, d.f. = 3, 39, $p = 0.608$; Fig. 2.6) and few undeveloped embryos were found in hatched egg sacs, yielding no significant differences in proportion of undeveloped embryos among groups ($\chi^2 = 2.98$, d.f. = 3, $p = 0.395$). Therefore, we found no evidence that endosymbiotic bacteria in the R_1W_1 and W_2 mothers affect fecundity or embryo development. Furthermore, survivorship to adulthood for hatched offspring did not significantly differ among infection categories ($\chi^2 = 2.98$, d.f. = 3, $p = 0.395$).

CI experiments

Wolbachia infected W_2 fathers induced cytoplasmic incompatibility when mated with cured W_2- mothers. We found that matings between uninfected mothers and infected fathers ($W_2-\times W_2$) produced a mean of 73% fewer hatched spiderlings than any other mating combination ($\chi^2 = 38.4$, d.f. = 3, $p < 0.001$, Figure 2.7) with no significant differences among the other cross types ($W_2-\times W_2-$, $W_2 \times W_2-$, $W_2 \times W_2$). Cross type

did not influence the sex ratio of offspring ($\chi^2 = 4.35$, d.f. = 3, $p = 0.22$, Table 2.8). Survivorship of hatched offspring to adulthood differed significantly among the four different cross types (Wald $\chi^2 = 10.12$, d.f. = 3, $p = 0.017$), with greater survival of offspring in the incompatible cross type ($W_2^- \times W_2$) than when infected mothers mated with infected fathers ($W_2 \times W_2$). Survivorship of hatched offspring in the other two crosses was intermediate, and did not differ significantly from any other crosses.

Though W_2 exhibited strong CI when W_2^- females were crossed with W_2 males, the same was not true when R_1W_1 females were crossed with W_2 males. The proportion of egg hatch in $R_1W_1 \times W_2$ matings was lower than $R_1W_1 \times W_2^-$ matings relative to the total number of eggs deposited ($\chi^2 = 5.06$, d.f. = 1, $p = 0.0245$, Figure 2.8), but the reduction in hatch was relatively modest. Comparing across the independent CI experiments, W_2 *Wolbachia* reduced hatching proportion by 35% in R_1W_1 females whereas it reduced hatching proportion by 73% in W_2^- uninfected females (Table 2.8). This cross does not support the strong level of CI observed with the W_2^- mothers, indicating that the symbionts or other cytoplasmic elements present in R_1W_1 mothers may alleviate cytoplasmic incompatibility induced by W_2 .

Discussion

Natural symbiont frequency

We detected a high prevalence and diversity of endosymbionts within the *Mermessus* genus. Our results are consistent with previous screening studies of spider taxa, which demonstrate high symbiont infection frequency in spiders (Goodacre et al. 2006, Duron et al. 2008b, Duron et al. 2008a, Stefanini and Duron 2012). We detected symbionts in most individuals, with only 28% of individuals lacking symbiont infection. The two most common endosymbionts, *Wolbachia* and *Rickettsia*, were detected in 33% and 36% of *Mermessus* spp. spiders respectively. Within the present study we only detected *Cardinium* in 7% of individuals, in contrast with other surveys that found a particularly high incidence of *Cardinium* across spider taxa (Duron et al. 2008a, Perlman

et al. 2010). Because the original purpose of this set of *Mermessus* specimens was to disentangle spider food web relationships (Chapman et al 2012), all specimens were extracted with gut contents intact. It is therefore possible that some of the symbionts detected originated from prey, rather than the spiders themselves. Nevertheless, these results indicate that the *Mermessus* genus is exposed to and likely inhabited by a wide diversity of symbionts. We detected 11 symbionts strains (five *Rickettsia* strains, four *Wolbachia* strains, and three *Cardinium* strains) in 12 different combinations.

Our second field screen, which focused solely on *M. fradeorum* and controlled for false positives from symbionts of undigested prey items, confirmed that endosymbiotic *Rickettsia* and *Wolbachia* infect *M. fradeorum*, although at somewhat lower frequency than in the generic screen of *Mermessus*, with 42% of *M. fradeorum* individuals infected by one or both symbionts (Figure 2.2). Uninfected spiders were more common (58%) than infected spiders. We detected three different symbionts (one *Rickettsia* and two *Wolbachia* strains) in four combinations (R_1 , W_1 , W_2 , and $R_1 W_2$) with a fifth combination ($R_1 W_1$) likely present in other RW individuals that were not fully strain-typed. Among infected spiders, the most prevalent infection type was a *Rickettsia*-only infection (18%). This strain of *Rickettsia* (R_1) was distinct from the five strains of *Rickettsia* (R_2 , R_3 , R_4 , R_5 , R_6) detected in the general *Mermessus* screen. Sequencing one *Rickettsia* gene indicated that four of the *Rickettsia* strain types R_3 , R_4 , R_5 and R_6 of the *Mermessus* genus were a 98.9%-99.5% match to the R_1 strain of *M. fradeorum* (Figure 2.6). The relatively close phylogenetic relationship among four of the five *Rickettsia* strains found in the *Mermessus* genus spiders suggests that these infections may be endemic to *Mermessus*, not false positives from prey contamination, because closely related endosymbionts are sometimes found clustered in closely related host taxa (Yun et al. 2011).

In *M. fradeorum*, *Wolbachia* infections were composed of two distinct strains, W_1 and W_2 , coexisting within the same geographic population. Two previous broad geographic surveys of *Wolbachia* in spiders did not detect multiple *Wolbachia* strains in the same population. Extensive screening of one species of Linyphiid spider, *Hylyphantes graminicola*, found 10 different *Wolbachia* strain types in 11 populations with no single population bearing more than one strain (Yun et al. 2011). The spider

genus *Agelenopsis* was also found to host many (11) *Wolbachia* strains across 45 populations, however, multiple strain types were not ever found in the same population (Baldo et al. 2008).

In *M. fradeorum* we detected single *Wolbachia* or *Rickettsia* infection in 18% and *Rickettsia-Wolbachia* double infections in 7% of individuals. This rate of double infections is somewhat higher than the 3% we would expect by chance when 18% of individuals are infected with *Rickettsia* and 18% are infected with *Wolbachia*. We also cannot rule out the possibility that W_1W_2 superinfected individual occur, as no cloning or pyrosequencing was used to detect the presence multiple strains of the same symbiont in individual field collected *M. fradeorum*.

Female bias in *M. fradeorum*

Our data indicates that endosymbionts induced a radical female bias in *M. fradeorum* infected with *Rickettsia* and *Wolbachia*. Elimination of both symbionts from the R_1W_1 matriline successfully restored the sex ratio of offspring to the expected 1:1 ratio. In insects, endosymbiotic mechanisms that have been documented to result in male elimination are male killing, parthenogenesis, or feminization (Engelstädter and Hurst 2009). Only two reproductive manipulations have been described in spiders, both of which were *Wolbachia*-associated female biases of linyphiid species. While the female bias detected in *Oedothorax gibbosus* was likely the result of male killing (Vanthournout et al. 2011), the female bias detected in *Pityohyphantes phrygianus* appeared to result from a more complicated relationship in which female size, maternal *Wolbachia* infection, and paternal *Wolbachia* infection were all found to influence sex ratio, which in turn was controlled by maternal post-copulatory body position (Gunnarsson et al. 2009). In *M. fradeorum*, neither male killing nor parthenogenesis were supported as mechanisms for symbiotically induced female bias. Fecundity in R_1W_1 versus R_1-W_1 - spiders was similar (Figure 2.6), whereas one would expect a 50% reduction in fecundity if male killing were at play. Furthermore, we did not observe an increase in undeveloped R_1W_1 embryos relative to R_1-W_1 - embryos. Parthenogenetic reproduction also seems an unlikely explanation for the observed female bias in *M. fradeorum*, because we never observed

virgin females to produce offspring during nine generations of laboratory rearing. However, it is possible that some more complicated form of parthenogenesis occurs. For example, gynogenesis is a possibility, in which insemination is required to trigger reproduction, but the paternal genome is subsequently excluded from offspring (Schlupp, 2005).

A more likely explanation for the extreme female bias of the R_1W_1 matriline is feminization. Chromosomal observations of R_1W_1 line embryos indicate that approximately half of the offspring have $n=22$ chromosomes and approximately half have $n=24$ (Paliulis et al. manuscript in preparation). Previous studies of male and female spiders have demonstrated that sex determination is often mediated by chromosome number $X0,XX$ (Kořínková and Král 2013). Therefore, bimodal chromosome numbers in the R_1W_1 line of *M. fradeorum* would be consistent with a mixture of genetic females ($n=24$ chromosomes) and feminized genetic males ($n=22$ chromosomes). Relative to other forms of reproductive manipulation, endosymbiont-driven feminization is less common and has only been reported in five arthropod taxa: *Cardinium* induced feminization in Hymenoptera (Giorgini et al. 2009) and Acari (Weeks et al. 2003) and *Wolbachia* induced feminization in Isopoda (Bouchon et al. 2008), Lepidoptera (Hiroki et al. 2002), and Hemiptera (Negri et al. 2006). The only other described feminization of an arthropod with an XO/XX sex determination mechanism was a *Wolbachia*-induced feminization of the leafhopper *Zyginidia pullula*, in which the strength of feminization was also near 100% until antibiotic treatment triggered the production of both males and females (Negri et al. 2006). Like feminized leaf hoppers, R_1W_1 female *M. fradeorum* are all reproductively functional, however, we found no sex-linked morphological distinctions among R_1W_1 females whereas male morphological traits can be detected on feminized leaf hoppers.

The roles that *Rickettsia* versus *Wolbachia* play in inducing female bias in the R_1W_1 matriline are not entirely clear, but it appears likely that *Wolbachia* is the responsible symbiont. Following initial establishment of the R_1W_1 matriline from the field, antibiotic curing efforts always resulted in elimination of both symbionts in tandem, precluding experimental disentanglement of the individual effects of each symbiont.

However, subsequent observations of field-collected individuals infected only with R_1 indicate that infection with this symbiont does not appear to prohibit the production of males: three out of 11 field collected males appeared to be infected with the same *Rickettsia* strain as the R_1W_1 lab line. Additionally, the egg sac of one field collected *Rickettsia* infected mother was reared out, and produced three sons and one daughter in the lab. Furthermore, W_1 *Wolbachia* was not detected in any of the 11 males screened; one male bore a *Wolbachia* infection, however, the strain type was unclear. Although it is possible that *Rickettsia* and *Wolbachia* are both required to induce a female bias, the most parsimonious explanation is that the W_1 *Wolbachia* strain in the R_1W_1 line is responsible. Though examples of multiple symbiont infections thought to be cooperative or mutualistic have been documented (McCutcheon and Moran 2007, Jaenike et al. 2010), thus far no reproductive manipulation has been attributed to more than one symbiont acting within the same host. *Rickettsia*'s functional role in *M. fradeorum* remains unclear. In *Erigone atra* dispersal tendency is modified by *Rickettsia* (Goodacre et al. 2009). A background *Rickettsia* infection with an unknown function was also detected in the spider *Oedothorax gibbosus*, whereas the *Wolbachia* present in this spider was associated with a female bias (Vanthournout et al. 2011). Additionally, laboratory rearing and broad survey of *Cardinium* infection in the cellar spider *Holocnemus pluchei* showed no bacterial influence on reproductive biology (Stefanini and Duron 2012).

Cytoplasmic incompatibility in *Mermessus fradeorum*

Our results demonstrate that W_2 *Wolbachia* induces strong CI. Fecundity was reduced by ~70% when W_2 males were mated with W_2 - females relative to all other mating combinations. To the best of our knowledge, this is the first demonstration of CI in a spider. Upon reaching a threshold infection frequency, strong CI is predicted to reach fixation quickly in a population (Hoffmann et al. 2011), however, the CI inducing W_2 was only detected in 11% of the screened *M. fradeorum* individuals, not including three *Wolbachia* positive samples of unknown strain type. This relatively low infection frequency could result from a dilution of CI strength if survivors of CI crosses experience improved fitness from the consumption of undeveloped siblings. CI penetrance was not complete; 27% of offspring from cured females and W_2 males ($W_2 \times W_2$) successfully

developed. W_2 - \times W_2 offspring enjoyed higher chance of survival to adulthood than offspring from the W_2 \times W_2 cross. Visual observation of hatching spiderlings indicated that these spiderlings often emerged later than other crosses, appeared larger, and were observed consuming undeveloped embryos. Spiders surviving W_2 - \times W_2 matings may enjoy a fitness benefit relative to other mating combinations. This concomitant benefit for the surviving uninfected offspring of CI crosses in combination with incomplete penetrance, and imperfect vertical transmission would likely slow the spread of a CI symbiont through a population.

A second explanation for the low prevalence of the CI-inducing W_2 strain could be the presence of other endosymbionts in the population able to fully or partially rescue the W_2 modified sperm, thereby depressing W_2 infiltration. When W_2 males were crossed with R_1W_1 females, we found that offspring production was only slightly reduced, indicating that W_2 is not able to induce CI as effectively in R_1W_1 females as W_2 - females. This disruption of CI could result from two different mechanisms. First, we cannot completely rule out the possibility that a low titer of W_2 *Wolbachia* also infects R_1W_1 females. Pyrosequencing of a R_1W_1 female detected 1857 16S bacterial reads of *Wolbachia* and Anaplasmataceae sequences (family containing *Wolbachia*). Sequence analysis did not reveal an obvious presence of second *Wolbachia* strain. However, noise associated with the 454 sequencing technology resulting from homopolymers may have hindered detection of additional *Wolbachia* strains (Quinlan et al. 2008). Second, one or both endosymbionts present in the R_1W_1 line may reduce W_2 's ability to inflict strong CI by partially rescuing W_2 modified sperm.

One alluring aspect of examining endosymbionts in spiders is the potentially unique relationships that may form between reproductive manipulators and spider hosts notoriously subject to a wide array of reproductive oddities. Across spider taxa, antagonistic reproductive strategies between males and females, such as sexual dimorphisms, sexual cannibalism, male palpal dimorphisms, mating plugs, cryptic female choice, and sperm competition are often observed, making spiders a particularly interesting taxon in which to study sexual selection (Eberhard 2004). In *M. fradeorum*, only one antagonistic reproductive trait, mating plugs, were observed in about half of

gravid females from field collection and in the lab. Mating plugs are glue-like secretions deposited by the male in the female genital opening post mating that if effective, should prohibit polyandry and thereby reduce sperm competition (Uhl et al. 2010). *M. fradeorum* does not display extreme sexual size dimorphism and adult female *M. fradeorum* typically outweighed males, but male patella-tibia length on average 7% longer than female's. Laboratory observation revealed that cannibalism of male *M. fradeorum* was generally rare. Even a spider displaying few extreme sexual traits can be subject to intense endosymbiotic reproductive manipulation, indicating that endosymbionts may be a cryptic, but common force of spider biology.

Conclusions

In *M. fradeorum*, we have delineated two distinct reproductive manipulations occurring in the same population of spiders, a near complete female bias and strong CI. Multiple competing reproductive manipulations within the same host population have only been described in two other systems. A double reproductive manipulation was found in the butterfly *Eurema hecabe* in which the wHecCI *Wolbachia* strain induced CI and a double infection of wHecCI and wHecFem *Wolbachia* strains induced feminization (Hiroki et al. 2002, Hiroki et al. 2004). The *Eurema hecabe* sex determination mechanism was heterogametic (XX,XY) and curing of mothers produced all male offspring. The mechanism of feminization would likely be different in *M. fradeorum*, as antibiotic curing restored the sex ratio to 1:1 and sex determination is likely not heterogametic. In another heterogametic Lepidopteran, *Hypolimnas bolina*, two *Wolbachia* strains inducing two different reproductive manipulations were detected in island populations; one strain induced male killing and a second strain induced CI. Multi-island surveys of *Wolbachia* infection in *H. bolina* showed little within population coexistence of either *Wolbachia* strain, likely indicating a symbiont-mediated competitive exclusion (Charlat et al. 2006). Though we only examined one population of *M. fradeorum*, we detected the co-occurrence of two strong reproductive manipulations, an extreme female bias and CI, which may either indicate coexistence at equilibrium, or dynamic symbiont replacement in progress.

Studies of endosymbiont infection dynamics in *H. bolina* and *E. hecabe* have provided our first empirical insights into the competitive interactions among symbionts within a population (Charlat et al. 2006, McGraw and O'Neill 2007, Charlat et al. 2009) and within a host (Narita et al. 2007). However, both of these examples are limited in scope to island populations of Lepidopterans with presumed limited migration rates. High migration rates are expected to increase the threshold for symbionts inducing different reproductive manipulations (Engelstädter et al. 2004). Linyphiid spiders like *M. fradeorum* are often subject to frequent disturbance in agroecosystems, necessitating high rates of dispersal (Weyman et al. 2002). Therefore, elucidating infection dynamics of the five infection types detected in *M. fradeorum* may offer the opportunity to examine symbiont frequencies subject to high migration rates. Our survey of the *Mermessus* genus detected a high prevalence and diversity of endosymbionts, indicating that these interactions among differentially manipulated hosts may be common within these spider populations. Increasing evidence for widespread multiple symbiont infection in spiders (Duron et al. 2008a, Perlman et al. 2010, Vanthournout et al. 2011) indicate that infections are dynamic, making these spiders an interesting system to investigate the competitive interactions among symbionts within the same population.

Table 2.1 Primers, PCR cycling conditions, and references used for diagnostic PCR detection of endosymbionts and DNA quality control.

Target Symbiont or DNA	Gene	Primer name	Primer sequence 5' to 3'	References	PCR cycling conditions
<i>Rickettsiella</i>	16S	MFRKLF MFRKLR	TATGGGTGGCGAGTGGCGGA GGCACAGAGTTAGCCGGTGCTT	this study	95°C for 3 m, followed by 34 cycles consisting of 95°C for 30 s, 64°C for 30 s, and 72°C for 1 m
<i>Rickettsiella</i>	16S	rrsF rrsR	TGAAGAGTTTGATCCTGGCTCAG CCTACGGCTACCTGTGTACGACTT	Leclerque and Kleepies 2008	94°C for 3 m, followed by 35 cycles consisting of 94°C for 30 s, 53°C for 30 s, and 72°C for 1 m
<i>Rickettsia</i>	16S	RKlongF RKlong R	ACGTGGGAATCTACCCATCA TAGCCTAGATGACCGCCTTC	this study	95°C for 2 m, followed by 35 cycles consisting of 92°C for 30 s, 60°C for 30 s, and 72°C for 30 s
<i>Rickettsia</i>	16S	RICS741F RICT1197R	CATCCGGAGCTAATGGTTTGC CATTTCTTTCCATTGTGCCATC	Davis et al. 1998	95°C for 2 m, followed by 35 cycles consisting of 92°C for 30 s, 60°C for 30 s, and 72°C for 30 s
<i>Wolbachia</i>	wsp	MFwspF MFwspR	TGGCTGGTGGTGGTGC GTTT CGCTACTCCAGCTTCTGCACCA	this study	95°C for 2 m, followed by 35 cycles consisting of 92°C for 30 s, 66°C for 30 s, and 72°C for 30 s
<i>Wolbachia</i>	wsp	wsp81F wsp691R	TGGTCCATAAGTGATGAAGAACTAGCTA AAAAATTAACGCTACTCCAGCTTCTGCAC	Zhou et al. 1998	95°C for 2 m, followed by 35 cycles consisting of 92°C for 30 s, 66°C for 30 s, and 72°C for 30 s
<i>Cardinium</i>	16S	ChF ChR	TACTGTAAGAATAAGCACCGGC GTGGATCACTTAACGCTTTTCG	Zchori-Fein and Perlman 2004	94°C for 3 m, followed by 40 cycles consisting of 94°C for 30 s, 56°C for 45 s, and 72°C for 1 m
<i>COI</i>	COI	lco1490 hco700	GGTCAACAAATCATAAAGATATTGG TCAGGGTGACCAAAAAATCA	Folmer et al. 1994	94°C for 3 m, followed by 35 cycles consisting of 94°C for 30 s, 53°C for 30 s, and 72°C for 1 m
Eukaryote	18S rDNA	NSF 18 NSF 19	CTGTTGATYCTGCCAGT TCTCAGGCTCCYCTCCGG	Duron et al. 2008a	94°C for 4 m, followed by 35 cycles consisting of 94°C for 1 m, 55°C for 1 m, and 72°C for 1 m

Table 2.2 Frequency of detection and percent infection of different endosymbiont strains successfully sequenced in both the *Mermessus* genus survey and the *M. fradeorum* survey of natural infection frequency.

Collection	Wolbachia and <i>Cardinium</i> Strains types	<i>Rickettsia</i> strain type						
		1 (R ₁ W ₁)	2	3	4	5	6	none
<i>Mermessus</i> spp.								
	Wol. 3				2 (3%) ^a	1 (2%)		16 (27%)
	Wol. 5							1 (2%)
	Wol. 6						1 (2%)	
	Wol. 4 & Card. 1							1 (2%)
	Card. 2		1 (2%)					1 (2%)
	Card 3				1 (2%)			
	none			2 (3%)	16 (27%)			17 (28%)
<i>Mermessus fradeorum</i>								
	Wol 1 (R ₁ W ₁)							3 (7%) ^b
	Wol 2 (W ₂)	1 (2%)						5 (11%)
	none	7 (16%)						26 (58%)

^aPercentage out of N = 60 *Mermessus* spp. specimens for which bacterial strain type was determined.

^bPercentage out of N=45 *Mermessus fradeorum* specimens for which bacterial strain type was determined, which is lower than the total number of symbiont positive individuals described in Fig 2.2

Table 2.3 Distance matrix of genetic similarity among *Rickettsia* strains in *Mermessus* spp. as determined by sequencing of (673bp) of bacterial 16S rRNA

		<i>M. fradeorum</i>	<i>Mermessus</i> spp.				
		R ₁	R ₂	R ₃	R ₄	R ₅	R ₆
<i>M. fradeorum</i>	R ₁	100	95.9	99.5	99.1	99.3	98.9
<i>Mermessus spp.</i>	R ₂		100	95.8	95.6	95.9	95.6
	R ₃			100	99.3	99.5	99.1
	R ₄				100	99.1	98.8
	R ₅					100	99.6
	R ₆						100

Table 2.4 Distance matrix for the genetic similarity among *Wolbachia* strains in *Mermessus* spp. as determined by sequencing (561bp) of the *Wolbachia wsp* gene

		<i>M. fradeorum</i>		<i>Mermessus</i> spp.		
		W ₁	W ₂	W ₃	W ₄	W ₅
<i>M. fradeorum</i>	W ₁	100	91.2	90.5	77.8	86.1
	W ₂		100	92.9	78.7	90.0
<i>Mermessus</i> spp.	W ₃			100	79.9	86.1
	W ₄				100	75.9
	W ₅					100

Table 2.5 Distance matrix for the genetic similarity among *Cardinium* strains in *Mermessus* spp. as determined by sequencing (333bp) of bacterial 16S rRNA

	C ₁	C ₂	C ₃
C ₁	100	98.8	97.6
C ₂		100	98.5
C ₃			100

Table 2.6 Closest MLST^a and *wsp*^b allele numbers^c and percent match for each MLST gene of both W₁ and W₂ *Wolbachia* strain types

Matriline	genes					
	<i>gatB</i>	<i>coxA</i>	<i>hcpA</i>	<i>ftsZ</i>	<i>fbpA</i>	<i>wsp</i>
W ₁ ^d	34 (97%)	47 (99.5%)	42 (97%)	158 (93%)	36 (100%)	588 (92.4%)
W ₂	33 (98.5%)	2 (99.5%)	123 (95%)	184 (97.4%)	179 (99%)	43 (92.3%)

^a Multi Locus Strain Typing genes: *gatB*, *coxA*, *hcpA*, *ftsZ*, and *fbpA*

^b *Wolbachia* surface protein gene: *wsp*

^c allele numbers from the online MLST database: <http://pubmlst.org/wolbachia/>

^d W₁ corresponds to R₁W₁ lab line

Table 2.7 Number of reads and % bacterial community composition obtained from 454-pyrosequencing of bacterial 16S diversity.

Bacterial Taxon ^a	W ₂ Female	R ₁ W ₁ Female
<i>Coxiellaceae</i> (unknown genus)	6345 (91.73)	381(16.3)
<i>Rickettsiella</i>	494 (7.14)	35 (1.5)
<i>Anaplasmataceae</i> (unknown genus)	0	52 (2.22)
<i>Wolbachia</i>	0	1805 (77.20)
<i>Rickettsia</i>	0	18 (0.77)
<i>Mycobacterium</i>	0	41 (1.75)
<i>Salinivibrio</i>	15 (0.22)	0
<i>Lactococcus</i>	8 (0.12)	0
<i>Legionellales</i> (unknown family)	6 (0.09)	0
<i>Porphyrobacter</i>	5 (0.072)	0
<i>Diaphorobacter</i>	4 (0.058)	0
<i>Pseudomonas</i>	4 (0.058)	
<i>Acidobacterium</i>	3 (0.043)	0
<i>Faecalibacterium</i>	3 (0.043)	0
<i>Nocardioideaceae</i> (unknown genus)	3 (0.043)	0
Total	6890 (99.6) ^b	2713 (99.74)

^a Generic classifications include both genus (95–97% identity) and species (>97% identity) matches.

^b bacterial taxa that were amplified only once were not included in the table.

Table 2.8 Mean \pm standard error for all life history parameters observed in both maternal symbiont effects and cytoplasmic incompatibility experiments with *M. fradeorum*.

n mothers	mate type: (female \times male)	hatched	unhatched	total fecundity	ratio female	proportion undeveloped	survivorship of hatched spiderlings
Experiment 1: Maternal symbiont effects							
12	R ₁ W ₁ \times cured male ^a	10.67 \pm 1.14	1.00 \pm 0.78	20.69 \pm 2.44 ^b	1 \pm 0	0.16 \pm 0.11	0.97 \pm 0.01
10	R ₁ -W ₁ - \times cured male	11.71 \pm 1.41	0.14 \pm 0.12	21.78 \pm 2.16	0.62 \pm 0.06	0.02 \pm 0.02	0.97 \pm 0.02
7	W ₂ \times cured male	11.90 \pm 0.84	1.20 \pm 0.85	18.62 \pm 3.38	0.54 \pm 0.07	0.12 \pm 0.08	0.97 \pm 0.01
4	W ₂ - \times cured male	12.00 \pm 1.82	0 \pm 0	16.40 \pm 3.58	0.42 \pm 0.17	0 \pm 0	1 \pm 0
Experiment 2: CI							
12	W ₂ - \times W ₂ +	7.25 \pm 2.37	14.00 \pm 3.28	21.25 \pm 2.51 ^c	0.53 \pm 0.08	0.63 \pm 0.063	1 \pm 0
15	W ₂ + \times W ₂ +	24.93 \pm 1.85	1.50 \pm 1.20	26.43 \pm 1.64	0.54 \pm 0.03	0.05 \pm 0.052	0.87 \pm 0.04
11	W ₂ - \times W ₂ -	28.09 \pm 1.87	1.00 \pm 0.66	29.09 \pm 1.49	0.40 \pm 0.03	0.04 \pm 0.063	0.94 \pm 0.03
11	W ₂ + \times W ₂ -	27.55 \pm 2.31	0.72 \pm 0.63	28.27 \pm 1.77	0.54 \pm 0.07	0.05 \pm 0.063	0.91 \pm 0.03
Experiment 3: CI							
4	R ₁ W ₁ \times W ₂	25.50 \pm 5.44	8.00 \pm 4.32	33.50 \pm 1.26 ^d	na	0.25 \pm 0.15	na
4	R ₁ W ₁ \times W ₂ -	39.50 \pm 3.84	0.50 \pm 0.29	40.00 \pm 3.67	na	0.01 \pm 0.01	na

^a Cured males are composed of R₁-W₁- and W₂-.

^b Total fecundity for Experiment 1 as embryo counts from egg sacs not allowed to hatch

^c Total fecundity are the sum of hatched and unhatched embryos over 10 d .

^d Total fecundity are the sum of hatched and unhatched embryos over 10 d .

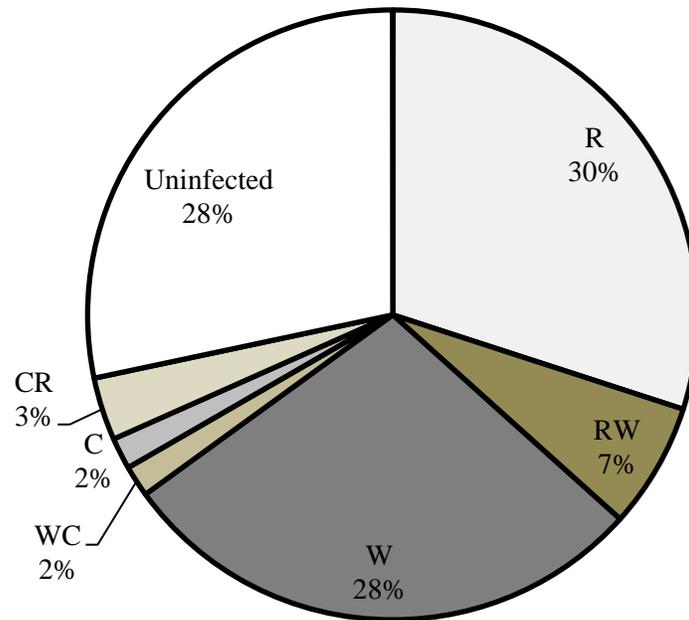


Figure 2.1 Endosymbiont infection frequency as detected with diagnostic PCR of the 60 *Mermessus* spp. spiders, not differentiating strain types within each bacterial genus. W = *Wolbachia*, R= *Rickettsia*, and C=*Cardinium*

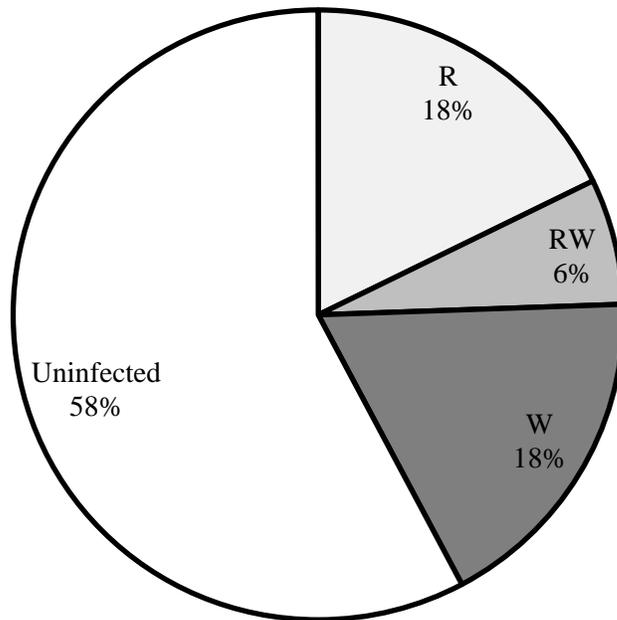


Figure 2.2 Endosymbiont infection frequency of the 45 *M. fradeorum* screened via diagnostic PCR, not differentiating among strains within the same bacterial genus. W = *Wolbachia*, R= *Rickettsia*, and C=*Cardinium*

Figure 2.3 Neighbor joining tree comparing *Rickettsia* strains R₁ through R₆ from *Mermessus* with each other and representative *Rickettsia* strains. *Rickettsia* strains from *Mermessus* are indicated by asterisks. The National Center for Biotechnology Information genInfo identifier number is listed in parentheses for each taxa.

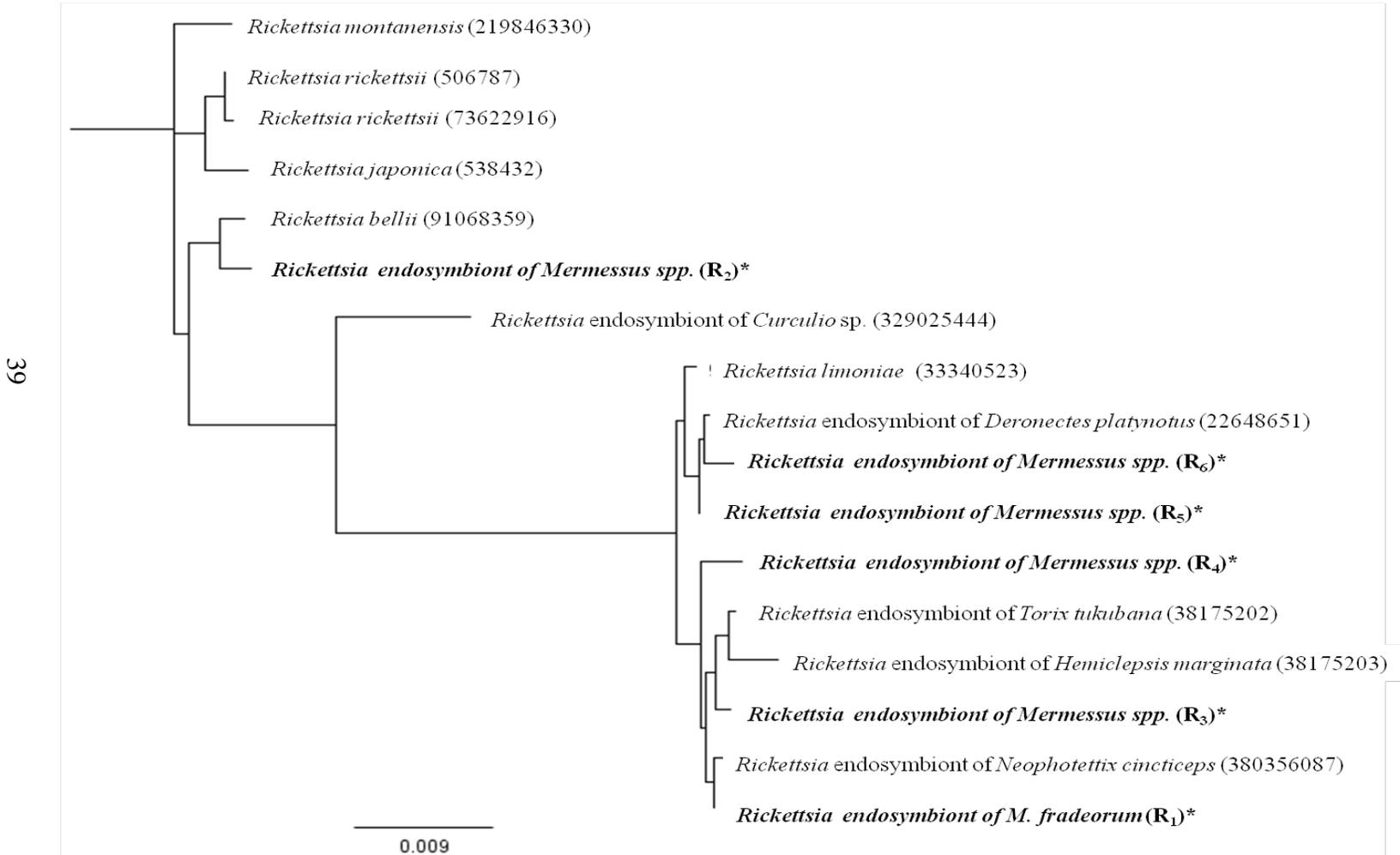
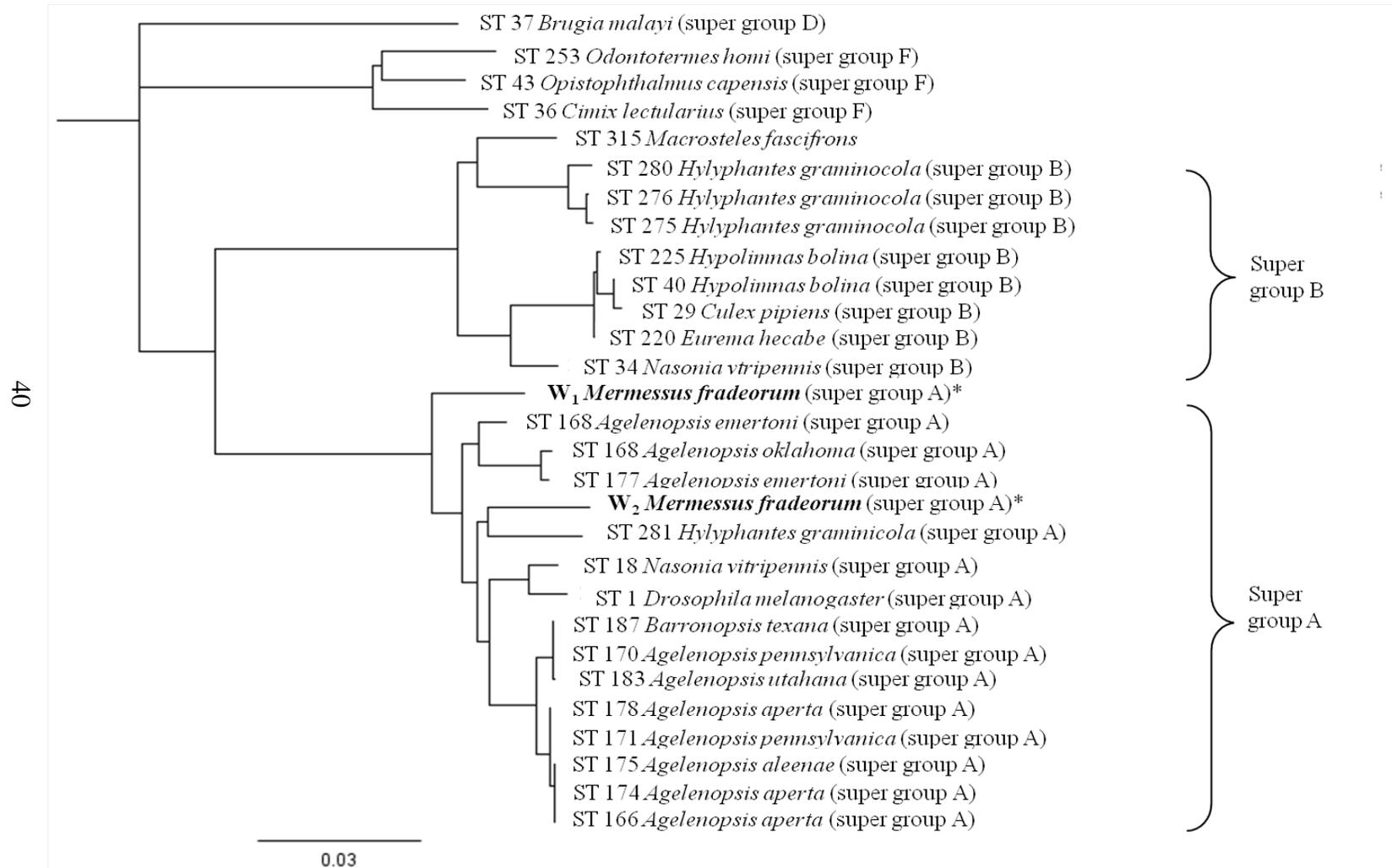


Figure 2.4 Neighbor joining tree comparing phylogenetic relationship of W_1 and W_2 *Wolbachia* from *Mermessus fradeorum* with other representative strain types (ST) using 5 concatenated MLST genes. *Wolbachia* strains from *M. fradeorum* are indicated by asterisks.



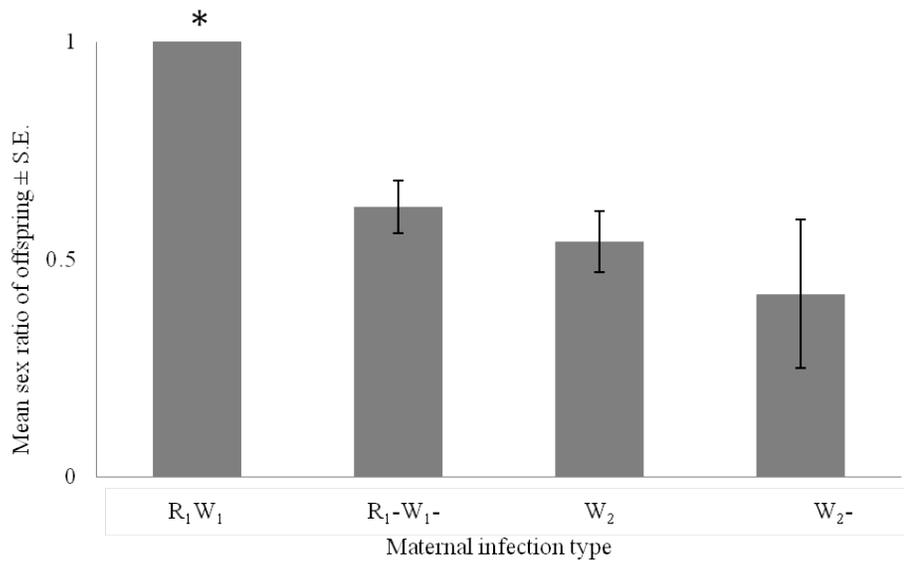


Figure 2.5 Mean \pm S.E. ratio of female to male offspring from a controlled mating assay of each maternal infection type mated with cured males. Asterisk indicates a sex ratio that is significantly different from 1:1.

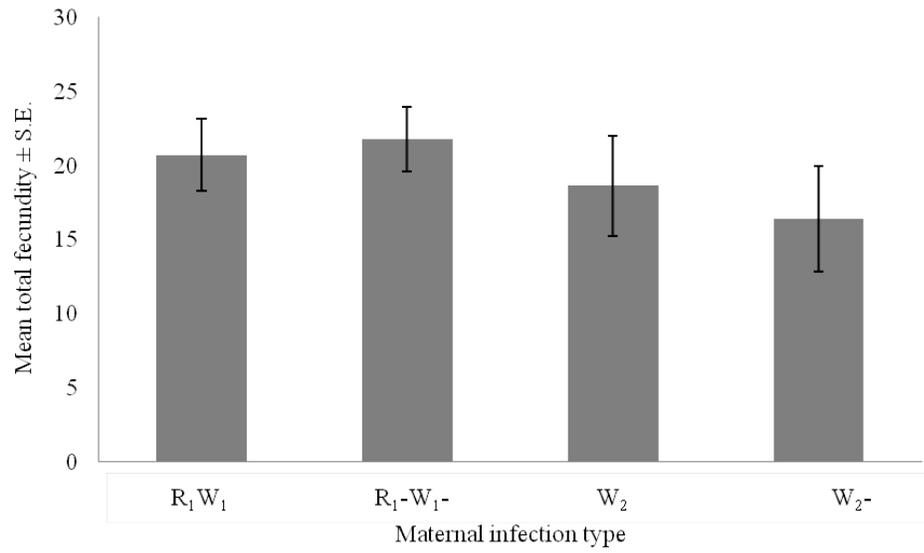


Figure 2.6 Mean \pm S.E. total 10 d fecundity (sum of all embryos produced per mother) from a controlled mating assay of each maternal infection type mated with cured males.

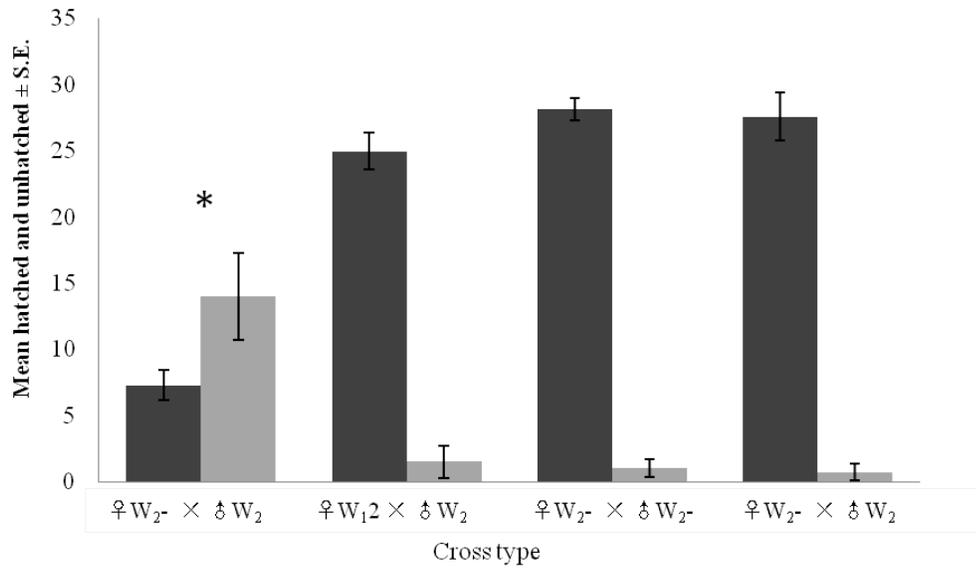


Figure 2.7 Mean \pm S.E. number of hatched spiderlings (black) and unhatched embryos (gray) from controlled matings of W_2 infected and W_2^- cured males and females. The asterisk indicates a significantly lower proportion of hatched embryos relative to the other cross types at $\alpha = 0.05$.

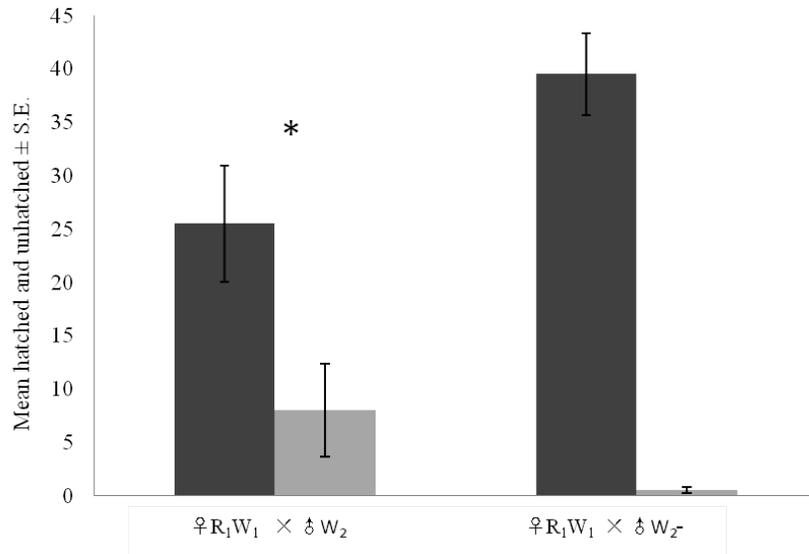


Figure 2.8 Mean \pm S.E. hatched spiderlings (black) and unhatched embryos (gray) from a controlled mating assay of R_1W_1 females with either W_2 or W_{2-} males. The asterisk indicates a significant difference in proportion of hatched embryos between cross types at $\alpha = 0.05$.

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