MATERNALLY INHERITED ENDOSYMBIOTIC BACTERIA OF DROSOPHILA: *SPIROPLASMA* AND *WOLBACHIA*

A Dissertation

by

PINNADUWAGE NADISHA OSHANI SILVA

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Chair of Committee,	Mariana Mateos
Co-Chair of Committee,	James Erickson
Committee Members,	Gil Rosenthal
	Rodolfo Aramayo
Head of Department,	Tom McKnight

December 2013

Major Subject: Zoology

ABSTRACT

Maternally inherited endosymbionts are ubiquitous among insects and are known to influence the ecology and evolution of a host species. As such, burgeoning evidence exemplifies their effect on host biology through reproduction, nutrition, resistance to pathogens, and heat tolerance. Many inherited symbionts are not guaranteed passage to a new host generation, and therefore can be lost from a host population. To circumvent loss, certain inherited symbionts have evolved the ability to manipulate host reproduction to enhance their transmission. Two common strategies used to do so are: male-killing, where sons of infected females die during embryogenesis; and cytoplasmic incompatibility (CI), which leads to conditional male sterility that can be reversed by mating with a female of the same infection type. Both phenotypes selectively favor female lineages, as heritable symbionts are only transmitted through them. Many heritable symbionts from the bacterial genera *Spiroplasma* and *Wolbachia* infect Drosophila. The model organism *D. melanogaster,* in particular, is naturally infected by a male-killing *Spiroplasma* strain and weak CI-inducing *Wolbachia* strain.

Unlike other bacteria, infections of inherited symbionts remain during the life span of the host. This is energetically costly to the host, and yet these well adapted microbes persist in host populations. Many inherited symbionts provide condition dependent benefits (i.e., resistance to pathogens) to the host, to alleviate the cost of infection. Heritable symbionts typically transmit through the egg cytoplasm, and therefore rely on the female host to replicate and enter an egg. I performed quantitative PCR on *Spiroplasma* strains with and without the male-killing phenotype, to trace bacterial replication in reproductive females. My results suggest, in contrast to previous studies, that there is no evidence for the correlation between the male-killing phenotype and densities. I also compared maternal mRNA found in eggs of *Spiroplasma* and *Wolbachia* infected females to determine whether these symbionts alter gene expression of the female during oogenesis. I used RNA-sequencing and bioinformatics tools to determine differential maternal gene expression due to infection. The results suggest

that *Spiroplasma* causes expression changes in genes, that code for a protein incorporated into the vitelline membrane of the oocyte, involved in pre-mRNA splicing, and a candidate gene for the mechanism of male-killing, involved in the sex determination pathway of Drosophila. *Wolbachia* infection had a minimal effect on maternal gene expression.

DEDICATION

To my loving parents Gihan and Vajira Silva for molding me into the person I am. To my brother Dimitri whose support and love has kept me on course. And to my grandparents Dr. J.C. de Silva, Mrs. Sara Stella de Silva, Mr. P. A. Silva and Mrs. Kusumalatha Silva.

ACKNOWLEDGEMENTS

I would like to thank my committee chairs, Dr. Mariana Mateos and Dr. James Erickson, and my committee members, Dr. Rodolfo Aramayo, and Dr. Gil Rosenthal, for their guidance and support throughout the course of this research.

I would also like to thank my talented and dedicated undergraduates Lacie Guenther and Valeria Galvan for taking on the burdens of my projects. I thank Sehresh Saleem for being my friend throughout the years and Daisy Gualberto for enabling me to complete the additional experiments I took on during the last few months at Texas A & M University. A heartfelt thanks goes to Kay Goldman of the Biology Department for keeping me on course through the last seven years and Dr. Arne Lekven for sending me on my way.

Finally, I wish to thank my best friend and partner Christoph Schwedes for hanging in there during the tough times, and the rest of my family (Anna, Chuti, P, Kiki, Indi, Gani, Shali and Shan) for all the big and small gestures of encouragement. I especially thank my grandmother for all her spiritual support.

NOMENCLATURE

CI	Cytoplasmic Incompatibility
CS	Canton S
MSRO	Melanogaster Sex Ratio Organism
NSRO	Nebulosa Sex Ratio Organism
PCR	Polymerase Chain Reaction
DE	Differentially Expressed

.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

CHAPTER I
INTRODUCTION

1.1 Symbiosis

Symbiotic relationships between prokaryotes and eukaryotes are common in many taxa. This is prevalent in insects, which are tolerant hosts to a diversity of microorganisms, and provide good systems to study the ecological and evolutionary effects of symbiosis. Together the insect-microbe interactions span commensalism, mutualism, and parasitism (O'Neill SL and Werren 1998). Associations between microorganisms and their hosts are usually internal (endosymbiosis), where the microbes thrive in body cavities such as the gut, hemolymph, or in specialized cells called bacteriocytes (Baumann 2005). In many cases, the symbionts are passed vertically from mother to offspring through the cytoplasm of the eggs, and are dependent on the female host for survival and propagation (Poulson and Sakaguchi 1961). This group of microbes, referred to as heritable symbionts, are estimated to be present in ~ 65% insect species (Hilgenboecker et al. 2008). Heritable symbionts are well adapted to environmental conditions within their hosts, and therefore are fastidious to culture in vitro due to their inability to survive outside a host (Williamson and Poulson 1979).

Heritable symbionts are known to be "influential passengers" because they can have a significant impact on host biology (O'Neill SL and Werren 1998); most commonly by affecting aspects of physiology and development, nutrition, reproduction and speciation, and defense against natural enemies (reviewed in Moran et al. 2008). One well studied symbiotic association is that of aphids and their intracellular bacteria, *Buchnera aphidicola* (Buchner 1965). The phloem sap on which aphids feed is high in carbohydrates but deficient in amino acids. It has been shown that coordinated gene expression of the host and symbiont is required to synthesize essential amino acids (Hansen and Moran 2011). The aphid–*Buchnera* association exemplifies an obligate,

nutritional mutualism, between a heritable endosymbiont and an arthropod host (Baumann et al. 1995).

Many inherited endosymbionts have secondary or facultative associations that are not vital for host survival but are beneficial under certain conditions (reviewed in Duron and Hurst 2013). Because transmission of most facultative endosymbionts is imperfect, some have evolved to manipulate host reproduction to enhance infection of in host population (Werren et al. 1995). Reproductive manipulation has evolved independently in many lineages, and in general these symbionts are referred to as reproductive parasites (Duron et al. 2008). The strategies that symbionts have evolved to exploit host reproduction are intriguing (reviewed in Werren et al. 2008). In Lepidopterans, Hemipterans and Isopods, feminization causes genetic males to become functional females. In hosts with haplo-diploid sex determination, symbionts induce parthenogensis by converting haploid males into diploid females, eliminating the need for sexual reproduction. In Dipterans, Lepidopterans, Pseudoscorpians and Coleopterans, male progeny of infected females die when infected with 'male-killing' symbionts, increasing transmission of the microbe via female siblings. The most common mechanism of reproductive parasitism is cytoplasmic incompatibility; hereafter CI, where the sperm of infected males cannot produce viable offspring if a female is not of the same infection status (Figure 1). Although, CI requires males for the system to work, it does so by ultimately favoring infected females over uninfected ones and this consequently increases the frequency of the symbiont in subsequent generations. As such, this mechanism has the most potential to lead to rapid fixation of the symbiont in a host population (Turelli and Hoffmann 1995, Weeks et al. 2007). Reproductive parasitism increases the fitness of the bacteria by favoring female hosts, as they transfer the infection to future generations. Theory predicts that host populations infected by sex ratio distorters (i.e., male-killers) will suffer reduced genetic diversity and gene flow of beneficial alleles, while deleterious mutations are likely to get fixed faster (Engelstädter and Hurst 2007). Recent studies have found that facultative symbionts form conditional mutualisms with their hosts, commonly through defense against pathogens, parasites,

and parasitoids (Teixeira et al. 2008, Jaenike et al. 2010, Xie et al. 2010, Parker et al. 2013). The coupling of a reproductive phenotype with a benefit could provide a plausible means for the maintenance of costly reproductive parasitism; furthermore, it provides support for the existence and maintenance of facultative symbionts that lack reproductive manipulation, as imperfect transmission would lead to the eventual loss of infection from host populations (Brownlie and Johnson 2009).

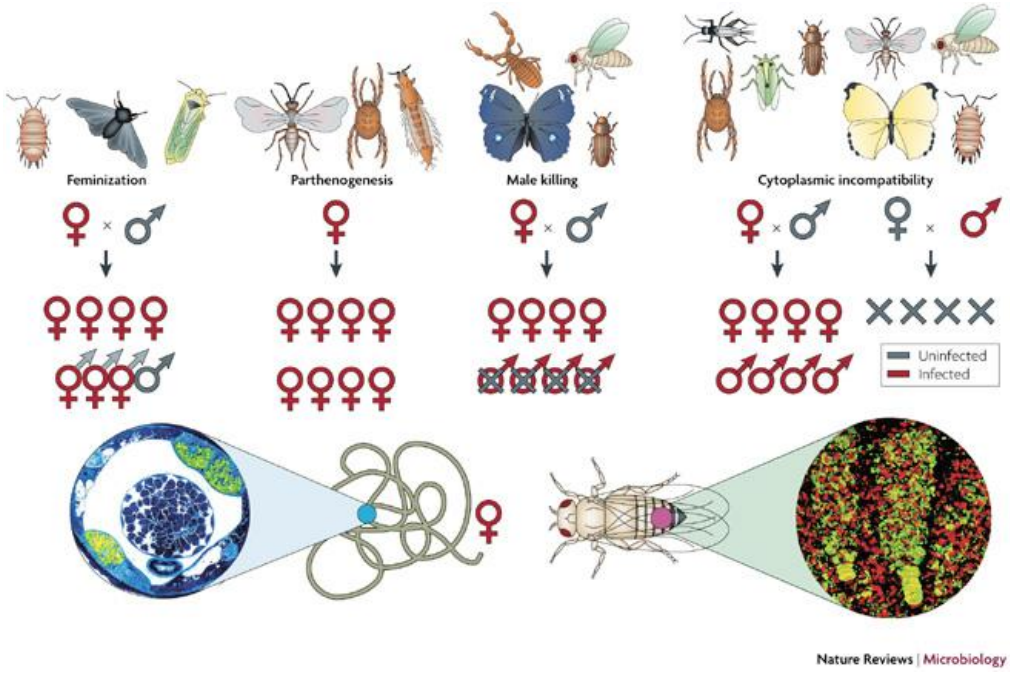

Figure 1. Graphical representation of reproductive phenotypes. Feminization, parthenogenesis and male-killing phenotypes cause a skew in the sex ration towards females (from Werren et al. 2008).

1.2 Heritable endosymbionts of Drosophila

Spiroplasma are small wall-less helical bacteria within the Gram + lineage, that are maternally transmitted among some Drosophila species, aphids, coccinelid beetles,

and butterflies (Hurst et al. 1999, Jiggins et al. 2000, Fukatsu et al. 2001, Mateos et al. 2006, Haselkorn 2010). Several *Spiroplasma* strains cause male-killing (Hurst and Jiggins 2000). The different strains that infect Drosophila fall into four phylogenetic groups. Male-killing *Spiroplasma* are grouped in the poulsonii clade, whereas the strains in the other clades (citri, tenebrosa, and ixodetis) do not express a reproductive phenotype in their host (Mateos et al. 2006, Haselkorn 2010) (Figure 2). Male-killing *Spiroplasma* strains associated with butterflies and coccinelid beetles however, occur in the ixodetis clade (Hurst et al. 1999, Jiggins et al. 2000). Recently a male-killing strain of *Spiroplasma* infecting *D. melanogaster* (Melanogaster Sex Ratio Organism, MSRO) was found in Brazil (Montenegro et al. 2005). This fortuitous discovery has expanded the tools and opportunity to study the effects of facultative symbionts on host biology. Furthermore, this provides one of the few opportunities to study the mechanism of reproductive parasitism (male-killing) in a model organism, as most reproductive phenotypes recorded to date, occur in hosts lacking genetic tools. Little is known, however, about this male-killing strain of *Spiroplasma* and its effects on the host, due to its recent discovery. The poulsonii clade also includes three strains that are closely related to the male-killing strains, but that lack a reproductive phenotype (non-male-killing strains). One of them is native to *D. hydei* (strain Hyd1), which has been documented to have moderate to high infection frequencies in this host (Kageyama et al. 2006).

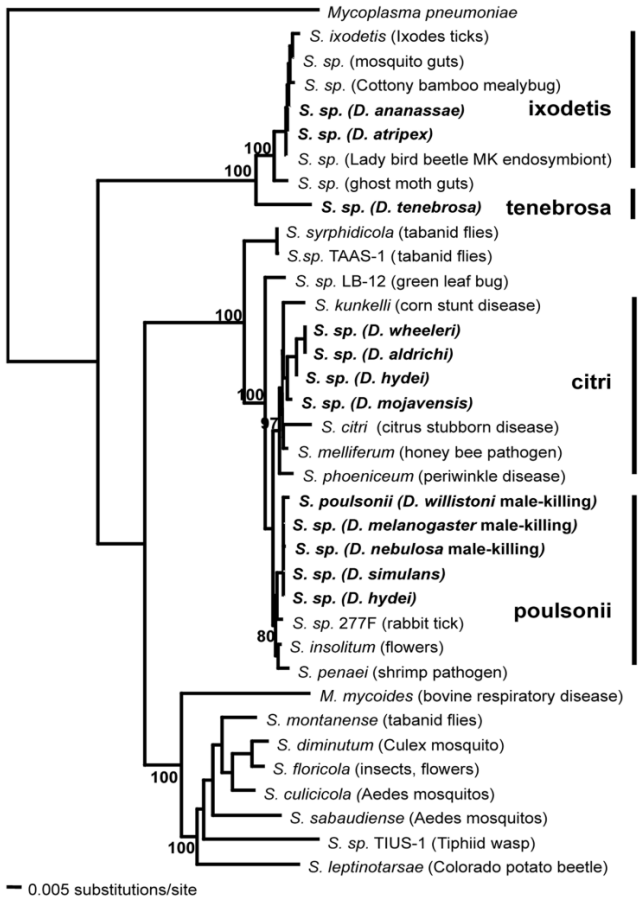

Figure 2. The phylogeny of *Spiroplasma* infecting Drosophila and other organisms. The male-killing strains in Drosophila are within the poulsonii clade (from Haselkorn 2010).

Wolbachia is a gram negative alphaproteobacteria that was first discovered in the gonads of the mosquito *Culex pipiens* (Hertig and Wolbach 1924). A recent large scale survey implicates *Wolbachia* to be one of the most abundant bacteria of its kind (Hilgenboecker et al. 2008). The phylogeny of *Wolbachia* has seven supergroups (A-F and H). Strains belonging to supergroups D and C have mutualistic associations with nematodes, whereas strains in groups A and B tend to be reproductive parasites of arthropod hosts (Figure 3). The nature of the associations involving strains within the other groups remains unknown. *Wolbachia* is the only bacterial genus that employs all four reproductive manipulations.

Currently, 16 of 52 tested species in the genus Drosophila, harbor *Wolbachia* (Giordano et al. 1995, Bourtzis et al. 1996, Charlat et al. 2004, Dyer et al. 2005, Mateos et al. 2006, Miller and Riegler 2006). The predominant phenotype found in the Drosophila genus is CI. In diploid insects, CI results in embryonic mortality immediately after fertilization as a result of a delay in paternal chromatin condensation and delayed nuclear envelope breakdown during the 1st mitotic division post fertilization (Callaini et al. 1997, Tram and Sullivan 2002). The model suggests that *Wolbachia* induces karyogamy failure by modifying sperm during early spermatogenesis (Breeuwer and Werren 1993, Reed and Werren 1995, Lassy and Karr 1996), and that the same strain of *Wolbachia* has a rescue function that is expressed in the egg. The expression of CI varies between and within host species (Bourtzis et al. 1996). Studies on Drosophila and other insects indicate that the expression of CI is dependent on the intrinsic nature of the bacterium as well the host genetic background (reviewed in Serbus et al. 2008). *D. melanogaster* is naturally infected with a *Wolbachia* strain (*w*Mel) that causes low levels of CI (Hoffmann 1988). Although, current understanding of the molecular mechanism of induction and rescue of the CI is extensive, information about global gene expression changes that take place in the host as a cause or consequence of the phenotype remains limited.

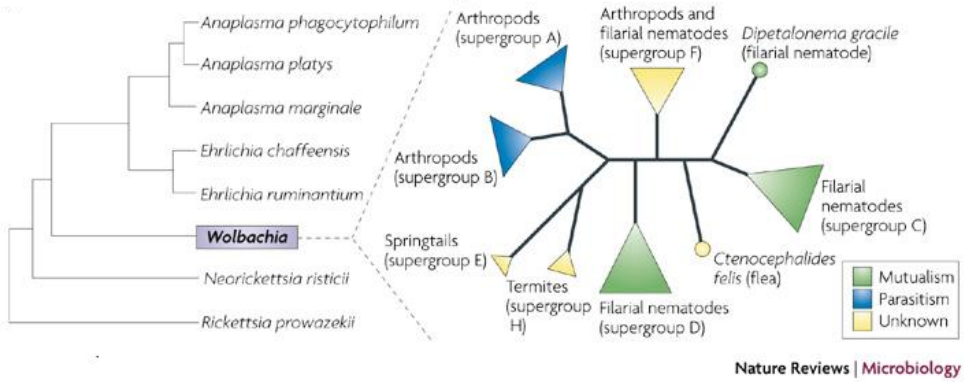

Figure 3. The phylogeny of *Wolbachia*. Strains infecting Drosophila belong to supergroups A and B, (from Werren et al. 2008).

The purpose of my dissertation work was to examine the effects of male-killing by *Spiroplasma,* and cytoplasmic incompatibility by *Wolbachia,* on the female host. The results of the experiments discussed herein, describe the nature of these infections in the model organism *D. melanogaster*, which can be naturally infected by both strains of symbionts (Montenegro et al. 2006). In addition, this work compares infection of the same host by a non-native *Spiroplasma* (Hyd1) that lacks a reproductive phenotype. The following paragraphs outline the rationale for the experiments conducted in chapters one and two of this document.

Hosts of inherited bacteria, remain infected throughout their life span. This should be physiologically demanding and yet, the bacteria is maintained by the host system (Dale and Moran 2006). As such, it is expected that aspects of the host immune system, longevity, and fecundity may be altered as a result. While the bacteria have to multiply efficiently in order to infect a majority of the host eggs produced in a lifetime, densities must be balanced so as to not become deleterious to the host. Some host organs can be more suitable microenvironments for successful proliferation. *Wolbachia* is an intracellular bacterium that exhibits tissue tropism; i.e., ovaries (Dobson et al. 1999), whereas *Spiroplasma* is found predominantly outside cells in the hemolymph (Anbutsu and Fukatsu 2006).

As mentioned above, the male-killing *Spiroplasma* infection of *D. melanogaster* was a recent discovery (Montenegro et al. 2005). Prior to this, studies had determined infection patterns of non-native *Spiroplasma* by artificially introducing them to genetically tractable *D. melanogaster* (Anbutsu and Fukatsu 2003, Anbutsu and Fukatsu 2006). The results of these studies found a correlation between the densities of *Spiroplasma*, and the ability to induce the male-lethal phenotype. As a necessary step towards understanding the infection levels and patterns in the female host, I tested the only male-killing strain in its natural host background (*D. melanogaster*) and compared it to strains described in former studies. As mentioned above, *D. melanogaster* is also host to a weak CI-causing *Wolbachia* (Hoffmann 1988). Wild strains of this host are doubly infected with both symbionts (Montenegro et al. 2006). This allowed the

examination of infection dynamics of *Spiroplasma,* in the presence of an antagonistic symbiont, as it would be under natural conditions.

Most maternally inherited symbionts are transmitted to a new host through the egg cytoplasm. As such, they manipulate the female host's reproductive system and usurp her cellular machinery in order to invade developing oocytes (Ferree et al. 2005, Fast et al. 2011). Infection-induced changes during oogenesis therefore, may have effects on the composition of an egg. Eggs contain maternal RNAs that are exclusively expressed during early development. Zygotic transcription is silent during this period and therefore maternal mRNAs play a crucial role in early embryonic development (Tadros and Lipshitz 2009). The egg, in a sense, is a point of convergence between the existing host, symbiont, and new host, and thus could also involve changes that lay the foundation for the occupation of the symbiont within the new host. Furthermore, it is likely that infection by a reproductive parasite could cause changes in maternal genes that are necessary to induce a reproductive phenotype. This aspect of the host-symbiont interaction has not previously been researched, and therefore, was the objective of my second experiment. Chapter three of this document characterizes the transcriptional state of fertilized eggs that were infected by *Spiroplasma* and *Wolbachia,* by examining maternal mRNAs.

CHAPTER II

INFECTION DENSITIES OF THREE STRAINS OF *SPIROPLASMA* IN *Drosophila melanogaster**

2.1 Introduction

Symbiotic relationships between prokaryotes and eukaryotes are widespread. This is especially true of arthropods, which are common hosts to a broad diversity of endosymbiotic microorganisms (Buchner 1965, Jeyaprakash and Hoy 2000, Bandi et al. 2001). Many of these endosymbionts are maternally-transmitted (i.e., heritable) and thus, are completely dependent on their female hosts for survival and propagation. Endosymbionts can be classified according to the nature of the association as obligate (primary) or facultative (secondary) (Baumann et al. 1995). Maternal transmission of most facultative endosymbionts is imperfect. To account for the resulting loss, many endosymbionts have evolved to manipulate their host's reproduction to their own benefit (Werren et al. 1995).

Reproductive parasitism has evolved in multiple divergent bacterial lineages (e.g., Alphaproteobacteria, Gammaproteobacteria, Mollicutes, and Bacteriodetes; Moran et al. 2008). One example is the genus *Spiroplasma* (class Mollicutes), a group of wall-less bacteria that infect a broad range of hosts such as plants, crustaceans, and insects (Gasparich 2002). In some insect hosts (e.g., Drosophila, aphids, butterflies, and coccinelid beetles; Moran et al. 2008), *Spiroplasma* are maternally-inherited facultative endosymbionts. As such, several of these heritable strains of *Spiroplasma* have evolved the reproductive manipulation strategy of male-killing (reviewed in Haselkorn 2010),

---

*Reprinted with permission from " Infection densities of three *Spiroplasma* strains in the host *Drosophila melanogaster*" by N.O. Silva, L.L Guenther, J. Xie and M. Mateos, 2012. *Symbiosis*, 57, 83-93, Copyright 2012 by Springer.

which is expected to enhance endosymbiont transmission into subsequent host generations at the cost of males (Hurst and Majerus 1993).

In the genus Drosophila, 18 species are reported to harbor *Spiroplasma*. Of these, eight harbor male-killing strains, eight harbor non-male-killing strains, and one species harbors both types of strains (Williamson and Poulson 1979, Mateos et al. 2006, Montenegro et al. 2006, Haselkorn et al. 2009, Watts et al. 2009, Jaenike et al. 2010, Chandler et al. 2011). Phylogenetic analyses of 11 of these Drosophila-associated *Spiroplasma* strains indicate that they fall into four clades (i.e., poulsonii; citri; tenebrosa; and ixodetis; Haselkorn et al. 2009), whose closest known relatives are strains associated with other arthropods and plants; some of which are horizontally transmitted. Of these, the only clade known to contain Drosophila-associated male killers is poulsonii, but male-killing *Spiroplasma* strains associated with butterflies and coccinelid beetles occur in the ixodetis clade (Hurst et al. 1999, Jiggins et al. 2000). Furthermore, the phylogenetic position of the male-killing strains associated with *D. paulistorum*, *D. equinoxialis*, *D. ornatifrons*, *D. neocardini*, and *D. paraguayensis* has not been determined. Although the poulsonii clade includes the male-killers that infect Drosophila, it also includes non-male-killing strains native to *D. hydei*, *D. melanogaster*, and *D. simulans* (Haselkorn et al. 2009).

The *Spiroplasma* male-killing phenotype typically results in death of male progeny of infected females early in development (but see Kageyama et al. 2007). The cause of male mortality is not well understood, but several studies have improved our understanding of this phenotype (Counce and Poulson 1962, Sakaguchi and Poulson 1963, Bentley et al. 2007). These studies show that most embryos die before gastrulation due to abnormal cleavage patterns during mitosis. In addition, *Spiroplasma* does not target the somatic sex, because it fails to kill somatic males with two X chromosomes; and is likely to interact with components of the sex determination pathway. A study by Veneti et al. (2005) suggests that the dosage compensation complex, which is required for the hypertranscription of the single X chromosome in males (Cline 1986, Lucchesi and Manning 1987), is used by *Spiroplasma* to discriminate

between males and females. A few studies have also examined *Spiroplasma* titers within adult Drosophila hosts and reported a correlation between the male-killing phenotype and infection density in the host female (Anbutsu and Fukatsu 2003, Anbutsu and Fukatsu 2011). In the first study, a male-killing strain derived from *Drosophila nebulosa* (Nebulosa Sex Ratio Organism; NSRO) was compared to a variant of it that spontaneously lost the male-killing phenotype in the lab (NSRO-A). The male-killing strain was found at higher infection densities within the female hosts than the non-male-killing strain. Observation of low infection densities was also reported by a subsequent study on a different non-male-killing *Spiroplasma* strain from *D. hydei* (Kageyama et al. 2006). These findings have led to a general assumption that *Spiroplasma* strains capable of male-killing maintain higher densities in hosts than strains that lack the phenotype. Nevertheless, all studies of *Spiroplasma* densities in *D. melanogaster* to date have been conducted on the *D. melanogaster* Oregon-R (OR) background (Anbutsu and Fukatsu 2003, Kageyama et al. 2006, Anbutsu and Fukatsu 2011, Herren and Lemaitre 2011). Thus, it is unclear whether patterns of infection densities in the OR background are observed in other *D. melanogaster* backgrounds.

Here I expanded previous studies by examining the densities of three closely related strains of *Spiroplasma* (poulsonii clade) that differ in their male-killing phenotype and/or native host, over three consecutive host generations following artificial transfer into the *D. melanogaster* Canton-S (CS) background. The strains examined were the male-killing strain NSRO (native to *D. nebulosa*), the male-killing strain MSRO (Melanogaster Sex Ratio Organism; native to *D. melanogaster*), and the non-male-killing Hyd1 (native to D. hydei; Mateos et al. 2006). The CS strain of *D. melanogaster* is naturally infected with the cytoplasmic incompatibility (CI)-inducing *Wolbachia* strain *w*Mel (Bourtzis et al. 1996), and therefore titers of the three *Spiroplasma* strains were examined in the presence of *Wolbachia*. In addition, for two *Spiroplasma* strains (MSRO and Hyd1), I also examined densities in the absence of *Wolbachia*. In this study I address the following questions: (1) does the observation of higher titers of male-killing *Spiroplasma* compared to non-male-killing strains hold

across other *Spiroplasma* strains and in a different host background?; (2) does the presence of *Wolbachia* affect *Spiroplasma* densities in doubly-infected hosts?; and (3) do *Spiroplasma* densities differ between generations following artificial infection?

2.2 Materials and Methods

2.2.1 Origin of flies and symbionts

Flies used for the experiments were derived from a CS strain of *D. melanogaster* naturally free of *Spiroplasma* and naturally infected with *Wolbachia* strain *w*Mel (multi-locus sequence typing of strain described below). To generate *Wolbachia*-free (W^-) fly strains, a group of CS flies was treated with tetracycline (final concentration of 0.02g/ml) for two consecutive generations, followed by three consecutive generations on antibiotic-free media. The *Wolbachia*-infected (W^+) fly strain was derived from a group of CS flies not subjected to antibiotic treatment.

Spiroplasma strains used for artificial infection, hereafter "transfection", were maintained in their original host strain: (a) MSRO in *D. melanogaster* Red 42 (Montenegro et al. 2005) females maintained by backcrossing to *Spiroplasma*-free CS males; (b) NSRO (Williamson and Poulson 1979) in *D. nebulosa* maintained by backcrossing to *Spiroplasma*-free *D. nebulosa* males; and (c) Hyd1 in *D. hydei* isofemale line TEN104-106 reported in Mateos et al. (2006).

2.2.2 Generation of Spiroplasma *infection treatments*

All *Spiroplasma* transfections were performed via adult-to-adult hemolymph transfer from the native host fly strain (the donor) to the recipient flies—i.e., *Spiroplasma*-free *D. melanogaster* CS flies with *Wolbachia* (W^+) or without *Wolbachia* (W^-). Five infection treatments were generated: (a) three *Wolbachia*-infected (W^+) *Spiroplasma* treatments (MSRO^{W+}, NSRO^{W+}, and Hyd1^{W+}); and (b) two *Wolbachia*-free (W^-) *Spiroplasma* treatments (MSRO^{W-} and Hyd1^{W-}). The NSRO^{W-} treatment was omitted because our NSRO donor line lost the *Spiroplasma* infection before we could transfer it to *Wolbachia*-cured (W^-) flies, and because the effect of *Wolbachia* on NSRO

densities in *D. melanogaster*, was previously examined by Goto et al. (2006), albeit in the OR background. For each of the five infection treatments, 6–8 recipient females were artificially infected through the thorax with ~0.2µl hemolymph from an infected donor fly with a manual microinjector.

2.2.3 Sample collection

Each of the 6–8 transfected females per infection treatment was individually housed in a vial with three *Spiroplasma*-free CS males of the same *Wolbachia*-infection state, and reared on standard cornmeal food at 25°C under controlled conditions of 12h dark and 12h light cycles. Each transfected female (Generation 0 or G0) and her descendants, in the subsequent generations (G1, G2, and G3), represent a treatment replicate. Newly emerged adult females derived from G0 females were harvested at 12h (0 days), 7, 14 and 21 days and immediately stored at –80ºC until DNA extraction. Five females per treatment replicate per time point were sampled. Each treatment replicate was followed for three consecutive generations (G1, G2, and G3), sampled at the four time points (i.e., adult host ages 0, 7, 14, and 21 days).

To generate each subsequent generation (e.g. G2), five *Spiroplasma*-infected virgin females (e.g. G1) per treatment replicate were aged for six days. Each of these females was individually housed with three *Spiroplasma*-uninfected CS males (same *Wolbachia*-infection status as female) for 24 h, and allowed to oviposit beginning 7 days post-eclosion. Females were then killed and their *Spiroplasma* infection status was determined via diagnostic PCR (described below). One of these five females was selected as the founder of the subsequent generation (e.g. G2) of its respective treatment replicate, on the basis of positive *Spiroplasma* infection and sufficient progeny for the necessary procedures (i.e., real-time PCR, male-killing assays, and founding of subsequent generation).

2.2.4 DNA extraction, diagnostic PCR, and real-time PCR

I followed the manufacturer's recommendations to extract DNA from individual female flies (final elution volume = 200µl) with the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA). Extracted samples were stored at –80ºC until the real-time PCR assay. The success of *Spiroplasma* transfections and the maintenance of infection every generation were evaluated with a *Spiroplasma*-specific PCR reaction on the G0 transfected females (10–15 days after hemolymph transfer), and on the founder females (G1 and G2) of each subsequent generation. The PCR was targeted at a portion of the *Spiroplasma* 16S rDNA gene with primers Spoul F/R and conditions reported in Montenegro et al. (2006).

Real-time PCR (iQ™ SYBR® Green Supermix and Bio-Rad CFX96 detection system) was used to perform a relative quantification of the copy numbers of a *Spiroplasma* gene (*DnaA*) to a single-copy gene of *D. melanogaster* (ribosomal protein S15a; *Rps15A*). Each DNA extract was assayed in triplicate for *DnaA* and in duplicate for *Rps15A*. For the *Spiroplasma DnaA* gene, we used primers *DnaA*109F 5'-TTAAGAGCAGTTTCAAAATCGGG-3'), and *DnaA*246R (5'-TGAAAAAAACAAACAAATTGTTATTACTTC-3') from Anbutsu and Fukatsu (2003). We were not able to use the host gene *Ef1α* used by Anbutsu and Fukatsu (2003), because it did not have equivalent amplification efficiencies to the *DnaA* fragment under our experimental conditions (results not shown). Therefore, we designed the following primers that target a 109 bp fragment of the *D. melanogaster Rps15A* gene: Forward 5'-GTTCCTGACCGTGATGATG-3'; Reverse 5'-GCACTTGTTTAGCCTACCG-3'. Each primer had a final concentration of 1µM in 10µ total reaction volume. The PCR cycling conditions were as follows: 40 cycles of 30 sec at 95°C; 1 min at 55°C; 30 sec at 72°C and 10 sec at 95°C. To rule out non-specific amplification, I examined the melting curve of all Real-time PCR reactions. Amplification efficiencies for both primer sets were measured by performing Real-time PCR on serial dilutions of 54 samples (42 and 12 for the *Spiroplasma* and host primers, respectively), representing different treatments and host ages. The slope of the relationship between threshold cycle number (CT) and initial

concentration indicated that both primer sets had equivalent amplification efficiencies (mean = 107.5%). At this efficiency, each PCR cycle is estimated to increase the number of gene copies by a factor of ~2.15 (i.e., 1.075 x 2). Thus, the relative *Spiroplasma* densities were measured as $2.15^{\Delta CT}$, instead of $2^{\Delta CT}$, which assumes a 100% amplification efficiency (Giulietti et al. 2001), where ΔCT is the number of PCR cycles required to reach the threshold fluorescence by the *Spiroplasma* gene (*DnaA*) minus the corresponding threshold for the host gene (*Rps15A*).

2.2.5 Statistical analyses of infection densities

For each generation and host age separately (i.e., three generations x four host ages = 12), a General Linear Mixed Model (GLMM, SAS Enterprise Guide) was used to examine the effect of infection treatment (fixed; five levels) and transfection-replicate (random; nested within infection treatments) on ΔCT (i.e., $\log_{2.15}$-transformed relative *Spiroplasma* copies). In addition, to examine whether *Spiroplasma* densities increased over the host's adult life, for each infection treatment and generation separately (i.e., five infection treatments x three generations = 15 tests), I compared ΔCT at the host age with the maximum mean density vs. ΔCT on Day 0. To determine whether the maximum densities achieved by *Spiroplasma* differed between generations, for each infection treatment separately (i.e., five tests), I compared ΔCT at the host age with the maximum mean density among generations (i.e., one time point per infection treatment per generation). Finally, to determine whether the ability of *Spiroplasma* to proliferate within the host differed among infection treatments, I compared ΔCT at the day with the maximum mean density of all three generations (i.e., one time point per infection treatment) among the five infection treatments (i.e., one test).

2.2.6 Sex ratio

For each infection treatment, I examined offspring sex ratios over three generations. Because it takes time for the infection to achieve vertical transmission in a previously uninfected host, we also recorded the time before 100% male-killing was

achieved (i.e., for the NSRO^{W+}, MSRO^{W+} and MSRO^{W-} treatments). Individually-mated transfected females (G0) were transferred to fresh vials daily for ten days, whereas G1 and G2 mated females were transferred daily for four days, as they were able to produce all-female offspring earlier (in most cases, by the 1st or 2nd day after mating). A Chi-squared test was used to examine the effect of the three infection treatments on proportion of males for G2 and G3 separately, and Fisher's exact test for pairwise comparisons between infection treatments.

2.2.7 Multi-locus sequence typing of Wolbachia *strain*

To identify the *Wolbachia* strain in *D. melanogaster* CS fly strain, multi-locus sequence typing was used as described in Baldo et al. (2006). This involved PCR amplification and sequencing of the following *Wolbachia* genes in the fly DNA extract: *gatB, coxA, hcpA, ftsZ,* and *fbpA*. The *Wolbachia* strain in our *D. melanogaster* CS strain is identical to *w*Mel, which is known to cause cytoplasmic incompatibility (Bourtzis et al. 1996), but not male killing.

2.3 Results

The relative number of copies of the *Spiroplasma DnaA* gene to the host gene *Rps15A* ($2.15^{\Delta CT}$) was used as a proxy for *Spiroplasma* titers; hereafter also referred to as *Spiroplasma* relative copies or *Spiroplasma* densities. We examined *Spiroplasma* densities in five treatments: *Wolbachia*-present MSRO^{W+}, NSRO^{W+}, and Hyd1^{W+}; and *Wolbachia*-free MSRO^{W-} and Hyd1^{W-}. Upon host emergence, *Spiroplasma* titers ranged within 0–6 relative copies in all treatments and generations (Fig. 4 and Table 1). The maximum average number of *Spiroplasma* relative copies was ~31–33 (MSRO^{W+} G3-Day 21; and Hyd1^{W-} G3-Day 21; Fig. 1 and Table 1). With few exceptions, mean *Spiroplasma* titers tended to increase between Day 0 and 14. After Day 14, mean *Spiroplasma* titers increased, decreased, or remained stable.

Table 1 Mean relative *Spiroplasma* densities. Infection was measured as $2.15^{\Delta CT}$ at each time point (Day) and generation**.** Boldfaced values indicate highest mean *Spiroplasma* densities within each generation for each infection treatment. Underlined values indicate the highest mean densities among all generations within each infection treatment. Values in parentheses indicate standard deviations.

	Infection Treatment				
	MSRO^{W+}	**MSRO^{W-}**	**NSRO^{W+}**	**Hyd1^{W+}**	**Hyd1^{W-}**
Generation 1					
Day 0	1.32 (1.71)	5.0 (2.93)	4.56 (1.80)	0.04 (0.06)	0.33 (0.49)
Day 7	3.26 (4.31)	14.62 (8.43)	**10.40 (4.70)**	2.33 (3.24)	6.14 (3.85)
Day 14	**9.82 (11.73)**	**20.92 (11.05)**	9.67(5.39)	7.86 (10.92)	20.89 (9.2)
Day 21	1.65 (5.17)	17.29 (17.34)	2.26 (4.63)	**9.50 (14.53)**	**23.30 (18.35)**
Fold change[a]	7.4	4.2	2.3	237.5	70.5
Generation 2					
Day 0	3.20 (0.92)	4.66 (2.06)	5.85 (3.17)	0.16 (0.15)	0.45 (0.73)
Day 7	12.08 (7.61)	14.44 (6.29)	3.32 (1.63)	4.44 (4.67)	6.96 (6.14)
Day 14	12.87 (7.70)	23.94 (8.30)	7.78 (7.82)	14.60 (10.49)	**22.26 (10.2)**
Day 21	**20.76 (13.89)**	**27.16 (10.97)**	**15.85 (9.67)**	**19.58 (23.25)**	17.35 (19.45)
Fold change[a]	6.5	5.8	2.7	122.4	49.5
Generation 3					
Day 0	2.74 (1.78)	3.90 (2.25)	4.05 (1.85)	0.15 (0.15)	0.42 (0.34)
Day 7	16.99 (9.23)	10.87 (5.13)	**9.40 (4.91)**	7.07 (6.02)	8.04 (3.24)
Day 14	26.79 (12.77)	15.89 (7.58)	9.12 (6.0)	19.55 (12.53)	20.67 (6.64)
Day 21	**33.19 (11.34)**	**19.35 (7.53)**	7.60 (2.48)	**28.07 (12.53)**	**31.76 (13.34)**
Fold change[a]	12.1	4.9	2.3	187.1	75.6

[a] Fold change between mean densities at Day 0 and maximum mean densities (i.e., boldfaced)

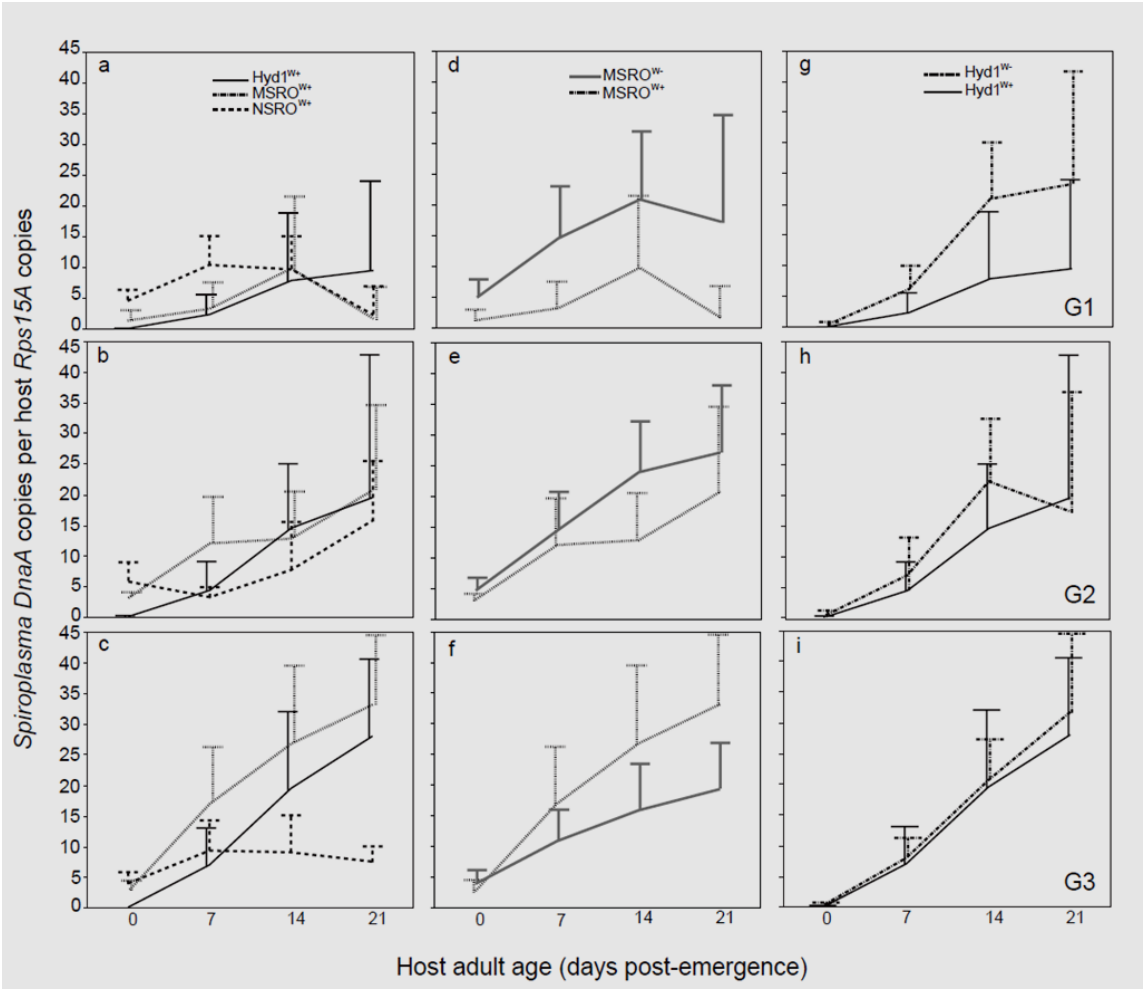

Figure 4 *Spiroplasma* infection densities in *D. melanogaster* adult female hosts. Female age ranges from 0–21 days after emergence. Infection was observed during three generations post-transfection (G1 top; G2 middle; G3; bottom). Non-transformed relative copies ($2.15^{\Delta CT}$) of the *Spiroplasma* gene to host gene (mean + S. D.). **a–c** comparison of the *Spiroplasma* strains (MSRO; NSRO; and Hyd1) in the presence of *Wolbachia* strain *w*Mel ($^{W+}$). **d–f** comparison of MSRO in the presence ($^{W+}$) and absence ($^{W-}$) of *Wolbachia*. **g–i** comparison of Hyd1 in the presence and absence of *Wolbachia*. Data points for each infection treatment at each generation and age time point had a sample size of ~30 females. Significant comparisons among infection treatments are reported in Table 2.

Table 2 Significant *Spiroplasma* titers between infection treatments within a generation and time point. "<" indicates which treatment had higher densities. Numbers shown: Tukey-Kramer adjusted *P*-value $P <$ 0.004; standard Bonferroni correction = 0.05/12 tests) ; degrees of freedom, (*t*-test).

Generation	Day 0	Day 7	Day 14	Day 21
G1	**Hyd1^{W+} < NSRO^{W+}**	**Hyd1^{W+} < MSRO^{W-}**		
	0.0005; 27.2 (-4.76)	0.0024; 26 (-4.19)		
	Hyd1^{W+} < MSRO^{W-}			
	0.0003; 27.3 (-5.02)			
	MSRO^{W+} < MSRO^{W-}			
	0.0029; 26.8 (-4.10)			
G2	**Hyd1^{W+} < NSRO^{W+}**)			
	< 0.0001; 25.1 (-7.17			
	Hyd1^{W+} < MSRO^{W+}			
	< 0.0001; 25.1 (-5.88)			
	Hyd1^{W+} < MSRO^{W-}			
	< 0.0001; 25.1 (-7.67)			
	Hyd1^{W-} < NSRO^{W+}			
	0.0001; 25 (-5.43)			
	Hyd1^{W-} < MSRO^{W+}			
	0.002; 25 (-4.3)			
	Hyd1^{W-} < MSRO^{W-}			
	< 0.0001; 25 (-5.67)			
G3	**Hyd1^{W+} < NSRO^{W+}**		**NSRO^{W+} < MSRO^{W+}**	**NSRO^{W+} < Hyd1^{W+}**
	< 0.0001; 28.1 (-9.26)		0.0026; 27.3 (-4.14)	< 0.0001; 27.5 (-6.96)
	Hyd1^{W+} < MSRO^{W+}			**NSRO^{W+} < MSRO^{W+}**
	< 0.0001; 28.2 (-7.92)			< 0.0001; 26.2 (-8.15)
	Hyd1^{W+} < MSRO^{W-}			**NSRO^{W+} < MSRO^{W-}**
	< 0.0001; 28.1 (-9.60)			<0.0001; 26.2 (-5.29)
	Hyd1^{W-} < NSRO^{W+}			**NSRO^{W+} < Hyd1^{W-}**
	< 0.0001; 28 (-6.30)			< 0.0001; 28.4 (-7.74)
G3	**Hyd1^{W-} < MSRO^{W+}**			
	< 0.0001; 28 (-6.40)			

2.3.1 Comparison of Spiroplasma *densities within each time point*

The effect of infection treatment was significant in 11 out of twelve comparisons (i.e., three generations x four time points). Several post-hoc comparisons revealed lower densities of the non-male killer strain compared to the male-killer strain (Table 2). On Generation 1 (G1) Day 0, *Spiroplasma* densities in the Hyd1^{W+} treatment were significantly lower than in the NSRO^{W+} and MSRO^{W-} treatments (Table 2), indicating higher densities of the male killers than the non-male killers in two out six possible male killer vs. non-male killer comparisons. By G2 and G3, the non-male killer strain had significantly lower Day 0 densities than the male-killer strains in all six comparisons. In general, mean densities of Hyd1 on Day 0 of all generations were close to zero (range 0.15–0.45), whereas mean densities of the male-killers NSRO and MSRO ranged within 1.32–5.85. On Day 7, only one comparison was significant: G1 Hyd1^{W+} had lower densities than MSRO^{W-}. No other comparisons revealed significantly higher densities of the male killers, but a few other comparisons were significant. On G3 Day 14, NSRO^{W+} had lower densities than MSRO^{W+}. On G3 D21, the non-native male killer (treatment NSRO^{W+}) had significantly lower densities than both, MSRO (MSRO^{W-} and MSRO^{W+}) and Hyd1 (Hyd1^{W+} and Hyd1^{W-}) strains. In summary, the results indicate that densities of the non-male killer were lower than those of the make killers shortly after emergence, but not later, and that *Wolbachia* had little to no effect on *Spiroplasma* densities.

2.3.2 Comparison of Spiroplasma *densities during host aging*

In most infection treatments, mean maximum titers (boldfaced values in Table 1) were achieved on Day 21; but a few occurred on Day 14 and, and as early as Day 7 (NSRO^{W+} only). In all infection treatments, the mean densities at Day 0 were lower than mean densities at one or more subsequent days. Nevertheless, not all Day 0 densities vs. maximum densities were statistically significant (four out of the 15 comparisons; Table 3). In general, variance tended to be higher for the highest mean densities, which probably contributed to the lack of significance in the comparisons mentioned above.

An overall growth trend between Day 0 and at least one later time point was observed, suggesting an increase in *Spiroplasma* densities during at least some period of adult life.

NSRO^{W+} consistently showed the least degree of difference between mean Day 0 densities and maximum mean densities (i.e., fold change 2.3–2.7; Table 1). Interestingly, the male-killing treatments (MSRO^{W+}, MSRO^{W-} and NSRO^{W+}) exhibited slower growth rates (fold change 2.3–12.1) than the non-male-killing treatments (Hyd1^{W+} and Hyd1^{W-}; fold change 49.5–237.5; Table 1).

2.3.3 Comparison of maximum Spiroplasma *titer*

Maximum *Spiroplasma* densities achieved within each infection treatment (boldfaced values in Table 1) were compared between generations (Table 3). MSRO^{W+} was the only treatment that exhibited significant differences in the maximum titers reached between generations ($F_{2,14.1} = 8.52$; $P = 0.0037$; non-significant results not shown). Maximum titers in G2 (20.76 ± 13.89; Table 1) were significantly higher than those in G1 (9.82 ± 11.73; $P = 0.0153$; $t = –3.24$, d.f. = 14.3). Similarly, the comparison between G1 and G3 (33.19 ± 11.34) was significant ($P = 0.0052$, $t = –3.79$, d.f. = 13.9). In general, generation post-infection did not have a strong effect on maximum *Spiroplasma* densities achieved. Comparison of the highest titers reached by each infection treatment throughout the course of the experiment (underlined values in Table 1) revealed two significant comparisons ($F_{4, 25.5} = 3.61$; $P = 0.0185$; non-significant results not shown): NSRO^{W+} maximum infection density was significantly lower than MSRO^{W+} ($P = 0.0166$; $t = –3.42$; d.f. = 25.1) and Hyd1^{W-} ($P = 0.026$; $t = –3.23$; d.f. = 26.8). Maximum densities of NSRO^{W+} were consistently lower, albeit not significantly, than the remaining treatments (MSRO^{W-} and Hyd1^{W+}).

Table 3 Comparison of minimum and maximum *Spiroplasma* densities. Densities compared at emergence (adult Day 0) vs. day at which highest mean densities were reached (within each infection treatment and generation). Maximum age for each generation refers to when mean titers were highest. Boldface values indicate $P < 0.003$ (i.e., standard Bonferroni correction; $P = 0.05/15$ tests).

Strain	Generation	Day at which maximum mean *Spiroplasma* densities were reached [a]	*P*-value	d.f. (*F*-ratio)
NSRO^{W+}	1	7	**< 0.0001**	1,51 (*F* = 49.97)
	2	21	**< 0.0001**	1,39.5 (*F* = 32.03)
	3	7	**< 0.0001**	1,51 (*F* = 49.01)
MSRO^{W+}	1	14	0.3126	1,50.2 (*F* = 1.04)
	2	21	**<0.0001**	1,35.4 (*F* = 305.09)
	3	21	**< 0.0001**	1,51.1 (*F* = 521.73)
MSRO^{W-}	1	14	**0.0009**	1,6.88 (*F* = 30.79)
	2	21	**< 0.0001**	1,67.3 *(F* = 336.59*)*
	3	21	**< 0.0001**	1,70 (*F* = 277.66)
Hyd1^{W+}	1	21	0.2936	1,53.1 (*F* = 1.13)
	2	21	0.6102	1,57.3 (*F* = 0.26)
	3	21	**< 0.0001**	1,51.3 (*F* = 1136.03)
Hyd1^{W-}	1	21	0.0913	1,42 (*F* = 2.99)
	2	14	**< 0.0001**	1, 50.1 (*F* = 323)
	3	21	**< 0.0001**	1,45.5 (*F* = 806.81)

2.3.4 Sex ratio

The offspring (G1) of the *Wolbachia*-infected females transfected with male-killing strains of *Spiroplasma* (i.e., MSRO^{W+} and NSRO^{W+} treatments) contained some males (MSRO^{W+} = 32.8% and NSRO^{W+} = 15.8%; Table 4a). The broods produced after 7 days and 5 days post-transfection; respectively, were 100% female. Similarly, the initial broods of *Wolbachia*-free females transfected with MSRO (i.e., MSRO^{W-}) produced ~33% male progeny, but by 6 days post-transfection, they produced 100% females. G2 and G3 offspring of both MSRO^{W+} and NSRO^{W+} continued to include some males during the collection period (4 days), whereas *Wolbachia*-free females (MSRO^{W-}) produced no males after G1 (Table 4a). A significant effect of infection treatment (only treatments involving male-killing strains were included) was detected in the Chi-square test for both, G2 and G3 (Table 4b). Fisher's exact tests indicated that in G2, the proportion of male offspring in both, NSRO^{W+} and MSRO^{W+}, was significantly

higher than in MSRO^{W-} (Table 4b). In G3, the percentage of male offspring in MSRO^{W+} was significantly higher than in MSRO^{W-} and NSRO^{W+}; whereas the latter two did not differ significantly from each other. These results suggest the following order of male-killing "strength" among infection treatments: MSRO^{W-} > NSRO^{W+} > MSRO^{W+}. As expected, Hyd1^{W+} showed no evidence of male killing in any generation with a sex ratio close to 50:50 in all cases (Table 4a). Although, sex ratio was not quantified in the Hyd1^{W-} treatment, numerous males were produced.

Table 4 Sex ratios for three generations of progeny. **a.** Days to complete male killing is the average time post-transfection before offspring (G1) from transfected (G0) females were all female. **b.** Chi-square test results for the effect of infection treatment on proportion of males for each G2 and G3 separately, and Fisher's exact test for pairwise comparisons

a.

Strain	Generation 1		Generation 2	Generation 3
	% Males	Days to complete male killing	% Males	% Males
NSRO^{W+}	15.8 (315)	5	8 (150)	1.3 (150)
MSRO^{W+}	32.8 (625)	7	8.9 (112)	8.2 (109)
MSRO^{W-}	33.0 (1150)	6	0 (155)	0 (150)
Hyd1^{W+}	49.6 (1372)	NA	51.4 (725)	56.3 (451)

b.

Strain	Generation 1		Generation 2	Generation 3
	% Males	Days to complete male killing	% Males	% Males
NSRO^{W+}	15.8 (315)	5	8 (150)	1.3 (150)
MSRO^{W+}	32.8 (625)	7	8.9 (112)	8.2 (109)
MSRO^{W-}	33.0 (1150)	6	0 (155)	0 (150)
Hyd1^{W+}	49.6 (1372)	NA	51.4 (725)	56.3 (451)

2.4 Discussion

One of the goals of this study was to compare infection densities of the recently discovered *Spiroplasma* strain native to *D. melanogaster* (MSRO), to those of the previously studied non-native NSRO and Hyd1 strains. In doing so, I tested whether the

pattern of higher titers of male-killing strains relative to non-male-killing strains, previously reported in *D. melanogaster* OR background, is observed in another *D. melanogaster* background. Comparison of male-killing vs. non-male-killing strains infecting *D. melanogaster* CS background did not demonstrate a correlation between the type of strain and bacterial titers. In contrast, Anbutsu and Fukatsu (2003, 2006) reported significantly higher titers of the male-killer strain NSRO than of its non-male-killing counterpart (NSRO-A) at female adult ages 1–3 weeks in the OR host background (lacking *Wolbachia*). Similarly, Kageyama et al. (2006) reported that infection densities of a non-male-killer strain native to *D. hydei*—derived from fly strain TKB163 and identical to our Hyd1 for the three genes examined to date—, in both, its native and non-native host (i.e., *D. melanogaster* OR), were much lower than the densities of NSRO, and similar to the densities of NSRO-A. The observation of lower titers of the non-male-killing strains compared to the male-killing strains is consistent with the threshold density hypothesis, whereby a minimum density of male-killing bacteria must be reached in a female host, for her male progeny to be killed. Accordingly, non-male-killer strains do not reach this threshold. In the present study, infection densities did not follow the pattern of higher densities of the male-killing strains (NSRO and MSRO), compared to the non-male-killing strain Hyd1 (derived from fly strain TEN104-106) over the first three weeks after adult emergence. The only time point at which the densities of the male-killers were consistently higher than those of the non-male-killer was on adult Day 0 (maximum difference between mean densities was ~5 relative copies). In addition, whereas Kageyama et al. (2006) were unable to examine Hyd1 (strain TKB163) titers in *D. melanogaster* OR adults beyond two weeks of age due to high levels of mortality, little to no mortality was observed in the present study, suggesting that differences in the host and/or CS background contribute to longevity.

In addition to not finding consistently higher densities of male-killers compared to non-male-killers throughout weeks 1–3 post-adult emergence, the results differed from Anbutsu and Fukatsu (2003, 2006) in the levels of *Spiroplasma* densities observed.

Relative copy numbers (based on *Spiroplasma DnaA* to host *Rps15A* estimated gene copies) were much lower (< 1 to < 33) than those reported for the OR background (i.e., > 1 to > 100 relative copies, based on *Spiroplasma DnaA* to host *Ef1α* estimated gene copies) by Anbutsu and Fukatsu (2003, 2006) in the absence of *Wolbachia*, and by the same research group in the presence and absence of *Wolbachia* (Goto et al. 2006). Differences between this study and the aforementioned studies may be explained by one or more differences in experimental conditions: host genetic background (CS vs. OR); quantitative PCR method and equipment (SYBR green in BioRad machine vs. TaqMan in ABI machine); and origin and time of original transfection (recently derived from *D. nebulosa* vs. derived ~40 years ago and maintained in *D. melanogaster* since). Nevertheless, a more recent study by the same research group (Kageyama et al. 2009), reported lower NSRO relative copies in several *D. melanogaster* backgrounds, including OR, than those reported in previous studies (Anbutsu and Fukatsu 2003, Anbutsu and Fukatsu 2006, Goto et al. 2006). Mean densities at all time points examined by Kageyama et al. (2009) (i.e., 2, 10, and 20 days after emergence), were generally below ~10 relative copies, and thus, more similar to those observed in our study. As far as we know, the NSRO densities reported in Kageyama et al. (2009) should be equivalent to the NSRO densities reported in Anbutsu and Fukatsu (2003, 2006) and Goto et al. (2006), at least for the OR background, as the methodology and experiments appear to be essentially the equivalent. The comparison between NSRO and NSRO-A has not been repeated in the recent studies; thus, it is unclear whether the pattern of lower titers of the non-male-killer NSRO-A compared to the male-killer NSRO reported in the previous studies (Anbutsu and Fukatsu 2003, Anbutsu and Fukatsu 2006), would be repeatable under the experimental conditions of Kageyama et al. (2009).

Densities of the male-killing *Spiroplasma* strain native to *D. melanogaster* (MSRO) have recently been reported in a study by Herren and Lemaitre (2011), who examined the interaction between *Spiroplasma* infection and host immunity. They evaluated MSRO titers (i.e., *Spiroplasma DnaA* to host *Rps17* estimated gene copies) in the OR background (lacking *Wolbachia*) across several larval stages and adult ages. At

day 1 after adult emergence, mean relative copies in Herren and Lemaitre (2011) were ~4, and by day 21, mean relative copies were ~33. These densities are slightly higher than, but similar to, the densities in our comparable treatment (i.e., MSRO^{W-} mean maximum titers = 19.35–27.16; Table 1).

The results suggest that co-infection by *Wolbachia* does not affect densities of the *Spiroplasma* strains MSRO and Hyd1 in the CS host background. The effect of *Wolbachia* on NSRO titers in the OR background was examined in a previous study (Goto et al. 2006). These authors reported no effect of *Wolbachia* on NSRO titers throughout development and adult ages 0–5 weeks. Thus, it appears that *Wolbachia* does not influence densities of *Spiroplasma* in *D. melanogaster*.

Although Goto et al. (2006) reported no influence of *Wolbachia* on NSRO's male killing ability, our results suggest that *Wolbachia* affects MSRO's ability to kill males. Once vertical transmission was established (i.e., by G2), MSRO^{W-} achieved complete male killing (i.e., 0% males over the period examined), whereas MSRO^{W+} continued to produce 8–9% males. This proportion of males contrasts with the 100% male killing reported by Hutchence et al. (2011) for 2–3 day-old MSRO^{W+} females. Since flies in Hutchence et al. (2011) and my study were derived from the same fly strains (i.e., same donor Red42 and recipient CS), the only apparent differences are female age (2–3-days-old vs. 7-days-old) and the generation post-transfection at which male-killing was measured (at least 18 generations in Hutchence et al. (2011) vs. 1–3 generations post-transfection in our study). Although maternal age is reported to affect the degree of male killing in NSRO (Kageyama et al. 2007), it is the younger females that exhibit lower male killing. Thus, differences in female age do not appear to explain the different degree of male killing in the two studies. One possible explanation for the different degree of male killing achieved by MSRO^{W+} females, is that the fly maintenance regime over the course of many generations inadvertently selected for *w*Mel, MSRO, and/or host background combinations that result in a higher degree of male killing. This scenario assumes that the initial generations post-transfection exhibited a lower degree male killing such as that observed in my study. Artificial selection of the host

background itself appears less likely than selection of the endosymbionts, given that every generation, MSRO^{W+} females were mated to CS males that were subjected to a different maintenance regime. The observations reflect a natural interaction between *Spiroplasma* and *Wolbachia*, and could be attributed to the opposing reproductive manipulation strategies of the two symbionts. *w*Mel causes CI, in which crosses between *Wolbachia*-infected males and *Wolbachia*-uninfected females are incompatible. Co-infection by a male-killer is potentially detrimental to *w*Mel, unless it relies on means other than CI for persistence (e.g., host protection against natural enemies; Hedges et al. 2008, Teixeira et al. 2008a). MSRO and *w*Mel co-infect *D. melanogaster* in nature (Montenegro et al. 2005b), thus, it is possible that *w*Mel evolved to reduce the male-killing ability of MSRO.

Furthermore, the study examined the effect of generation post-infection on both, male-killing ability and titers. Generation post-infection only affected titers of MSRO in the presence of *Wolbachia*, where they were highest in G3 and lowest in G1. As expected, generation post-infection affected the ability to kill males; a higher proportion of males was observed in G1 than in subsequent generations for all male-killing treatments (NSRO^{W+}, MSRO^{W+} and MSRO^{W-}; Table 4a). Nevertheless, despite being previously uninfected, G0 females were able to produce strictly female offspring 5–7 days after transfection. Interestingly, NSRO was able to establish all-female broods in the transfected G0 hosts (*Wolbachia*-infected) earlier than the native strain (Table 4a).

In conclusion, the results are not consistent with the density threshold hypothesis because the male-killers NSRO and MSRO did not exhibit higher densities than non-male-killer Hyd1 throughout adult weeks 1–3. The results do not refute the hypothesis either, as it is possible that lower densities of the male-killers than those observed in this study could lead to reduced male-killing. Observation of higher titers of the male-killers in newly emerged adults (Day 0) compared to the non-male-killer Hyd1 was the only consistent pattern we found, but whether or not it is relevant to male-killing is unclear, because male-killing occurs early in development. Thus, this study suggests that assuming they are repeatable, the results obtained by the pre-2009 studies of NSRO and

NSRO-A in the OR background, cannot be generalized to other *D. melanogaster* backgrounds and to the *D. melanogaster*-native male-killing strain MSRO. Finally, the finding that MSRO exhibits lower male-killing ability in the presence of *Wolbachia* is intriguing and suggests an area that needs further study.

CHAPTER III

A SCREEN FOR MATERNAL TRANSCRIPTS AFFECTED BY *SPIROPLASMA* AND *WOLBACHIA* INFECTIONS

3.1 Introduction

Our knowledge about maternally inherited bacteria in arthropods and nematodes has improved vastly during the recent years. The discovery of previously unknown symbiotic associations between host species and inherited bacteria has become increasingly common. To date 33% of all surveyed arthropod species harbor one or more inherited symbiont (Duron et al. 2008). Bacteria of the genus *Wolbachia* are recognized as the most successful heritable symbionts (Werren et al. 2008). Predictions based on models estimate 66% of all insects to be infected with *Wolbachia* (Hilgenboecker et al. 2008). Evidence indicates however, that other groups such as *Spiroplasma*, *Arsenophonus* and *Cardinium,* have independently evolved heritability, have diverse host species, and replace *Wolbachia* as the dominant symbionts in some arthropod taxa (Duron et al. 2008, Russell et al. 2012).

The influence of inherited bacteria on host species can broadly be based on whether they are obligate or facultative symbionts. Obligate symbionts are essential for host survival or reproduction (Hoerauf et al. 1999, Dedeine et al. 2001, Hosokawa et al. 2010). Facultative symbionts are not essential, but can provide condition dependent benefits to their hosts (reviewed in Moran et al. 2008). Unlike obligate symbionts, facultative bacteria have imperfect transmission and can consequently be lost from a host population (Hurst et al. 2001, Darby and Douglas 2003). Many of them therefore have evolved to manipulate host reproduction (male-killing, cytoplasmic incompatibility (CI), parthenogenesis, and feminization) to enhance their transmission within a host population (Werren et al. 2008). Many studies indicate however, that facultative symbionts interact with their hosts through means beyond the classic effects on

reproduction. Symbiont-mediated resistance, to viruses, bacterial pathogens, fungi, nematodes and parasitoids, has been documented in a variety of hosts (Teixeira et al. 2008, Jaenike et al. 2010, Xie et al. 2010, Wong et al. 2011, Teixeira 2012, Parker et al. 2013). Symbiont infection is thought to prime the immune response of the host, resulting in the defense against commonly encountered pathogens. Recent findings demonstrate *Wolbachia*'s ability to use host microRNAs, and induce epigenetic modification by controlling methylation, to manipulate host gene expression (Osei-Amo et al. 2012, Ye et al. 2013).

Most maternally inherited bacteria transmit transovarially. Therefore, the egg represents an important junction between the old and new host. The process of oogenesis is complex and naturally protected by host defenses from invasion by pathogens (reveiwed in Bastock and St Johnston 2008, Short and Lazzaro 2013). Yet, inherited symbionts get through the spatial and temporal boundaries to be 'in the right place at the right time'. The egg is a highly structured chamber that carries the maternal genome. It interacts with two types of cells; follicle cells of somatic origin that synthesize the chorion and vitelline membranes that surround the egg, and nurse cells of germline origin that produce and supply the RNA, proteins, organelles (mitochondria, Golgi apparatus, etc.), and yolk (reviewed in Bastock and St Johnston 2008). Maternal gene products are exclusively used for early embryonic activity (axis formation, cell fate and patterning, sex determination) when the zygotic genome is transcriptionally silent (Tadros and Lipshitz 2009). Shortly after fertilization, the abundance of maternal RNAs is high, but these degrade rapidly during the maternal-zygotic-transition (MZT). During this period, the embryo begins taking over transcription, which, in Drosophila takes place during cycle 8–10, ~1.5hr after fertilization.

The genus Drosophila is naturally infected by a number of strains of *Wolbachia* and *Spiroplasma* (Mateos et al. 2006). *D. melanogaster* in particular can naturally harbor a male-killing strain of *Spiroplasma* known as the Melanogaster Sex Ratio Organism (MSRO) (Montenegro et al. 2005), which causes embryonic death of sons of infected females, and a *Wolbachia* (*w*Mel) strain that induces a weak form of the CI

phenotype (Hoffmann 1988), which usually results in unviable offspring if the infection type in the males is not matched by his mate. Recent studies that used cytological and mutant analysis have shed light on the mechanisms that are employed by *Wolbachia* and *Spiroplasma* to transmit to a new host. In Drosophila, *Wolbachia* aggregate in the germline stem cell niche (GSCN) and somatic stem cell niche (SSCN) during early oogenesis and travel to the posterior end of the oocyte, which eventually localizes to the adult ovary/germline of the new host (Fast et al. 2011, Toomey et al. 2013). The host microtubule network is exploited by *Wolbachia* to eventually localize within the oocyte (Ferree et al. 2005). *Spiroplasma*, which thrives mainly in host hemolymph, enter the oocyte during late stages of oogenesis through the process of yolk uptake (i.e. vitellogenesis; Herren et al. 2013). Little is known, however, about the global gene expression changes that take place in reproductive females as a result of being infected. Particularly, whether the changes manifest in the egg and thereby influence the new host. In an attempt to answer this question, I examined maternal mRNAs of *D. melanogaster* infected by the native strains of *Spiroplasma* (MSRO) and *Wolbachia* (*w*Mel). In addition, we compared a second *Spiroplasma* strain that was artificially transferred to and maintained in *D melanogaster*, from its native host *D. hydei* (hereafter, strain Hyd1), which lacks a reproductive phenotype.

3.2 Materials and Methods

3.2.1 Generation of symbiont treatments

I used three infection treatments, one *Wolbachia* and two *Spiroplasma* strains, and a symbiont-free control. Laboratory stocks of *D. melanogaster* (Canton S strain; CS) that naturally harbor the *Wolbachia* strain *w*Mel were used to generate the *Wolbachia* treatment (W^+S^-). Positive infection for *w*Mel was confirmed based on PCR with *Wolbachia*-specific primers targeting the *wsp* gene (Jeyaprakash and Hoy 2000). The same stock was reared in tetracycline food (final concentration 0.02g/ml) for two generations, followed by three generations of antibiotic-free food to generate a *Wolbachia*-free (W^-) stock. The W^- flies served as the symbiont-free control. The *Spiroplasma* infection treatments (W^-S^+) were generated by artificially infecting *Wolbachia*-free (W^-) flies with the strain MSRO (Red 42) native to *D. melanogaster* (Montenegro et al. 2005) or Hyd1 (TEN-104-106) native to *D. hydei* (Mateos et al. 2006). Fifteen *Wolbachia*-free (W^-) females (15 lines) were infected per *Spiroplasma* strain. These artificially infected lines were maintained for 3–5 generations before being used for the experiment. *Spiroplasma*-infected (W^-S^+) lines were selected every generation to ensure positive infection status, based on PCR with *Spiroplasma*-specific 16S ribosomal DNA primers (Montenegro et al. 2005). MSRO treatment lines were backcrossed to *Wolbachia*-free (W^-) CS males every generation, as male-killing by this strain is nearly perfect. A minimum of four infected lines were combined, per replicate, at the start of the experiment to create a total of three biological replicates for each *Spiroplasma* treatment. The biological replicates for the *Wolbachia* treatment and control were maintained as three different populations for four generations prior to the start of the experiment.

3.2.2 Embryo collection

Approximately 40–50 three-day-old virgin females, from each replicate of each treatment, were allowed to mate in cages with *Wolbachia*-free (W^-) CS males during the collection period, and allowed to lay eggs on cornmeal food plates. The initial batch of eggs was discarded to improve the chances of collecting fertilized eggs from the same stage (Dobson 2007). Thereafter, egg laying was monitored and cornmeal plates were changed approximately every 45 minutes in order to collect embryos that were on average ~60–75 min old. Eggs were collected from each replicate with a small brush 6–8 times over a 2-day period for each treatment and the control. The eggs were placed in sterile RNase-free Eppendorf tubes, and immediately put on dry ice during the collection period, after which they were transferred to –80°C for storage.

3.2.3 RNA extraction and library preparation

Three biological replicates per condition (MSRO, Hyd1, *w*Mel, & CS) were used for the extractions. RNA was extracted per collection tube of eggs (mentioned above) with Trizol® Plus RNA Purification System (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. The tubes that belonged to the same biological replicate within a treatment were pooled. All RNA samples were DNase-treated with Ambion® DNA-free (Invitrogen) to remove any DNA contamination. Total RNA was quantified with a NanoDrop® ND-1000 UV spectrophotometer (NanoDrop Technologies, Wilmington, DE), and sample quality and integrity were further tested with the Agilent 2100 BioAnalyzer (Agilent Inc., Santa Clara, CA).

Twelve Illumina mRNA-Seq libraries were constructed with 1µg of total RNA as recommended by the Truseq kit (3 biological replicates x 4 conditions). Six libraries were randomly selected and pooled per sequencing lane (two sequencing lanes) and sequenced on an Illumina HiSeq 2000 platform (San Diego, CA), according to manufacturer's recommendations. Each library produced ~30 million 100bp single-end reads.

3.2.4 Bioinformatics

Reads from each library were filtered for quality and trimmed using the Fastx-toolkit v.0.0.13 (Assaf Gordon). Reads were independently mapped to the Drosophila genome (dm3, BDGP Release 5) with Tophat2 (Trapnell et al. 2009, Kim et al. 2013) implementing the following options : " -i 40 -I 200000 -p 8". Cufflinks version 2.1.1 was used to estimate transcript abundance with the default library normalization method of classic-FPKM (Fragments per Kilobase of exon per Million fragments mapped) and options: "-G -I 200000 -u -M -b" (Trapnell et al. 2013). Cufflink assemblies were merged using Cuffmerge to produce a GTF file as input for Cuffdiff. Cuffdiff v.2.1 was used to test for differentially expressed genes between conditions with the options: "-N -c 10 -u -b -p 8 ". The library normalization method option was changed to 'geometric' for the 'per-condition' option to apply the cross-replicate dispersion estimation method implemented in Cuffdiff v.2. CummeRbund v.2.0 (Trapnell et al. 2012) was used to visualize and explore the output of Cufflinks. The functional annotation tools of DAVID (Huang da et al. 2009b, a) were used to identify enriched Gene Ontology (GO) terms for the significant (FDR = 0.05) maternal genes in each infection treatment.

3.3 Results

As shown by the volcano plots (Figure 5), *Spiroplasma* treatments (MSRO and Hyd1) produced greater changes in gene expression than the *Wolbachia* treatment (*w*Mel). In general, Hyd1 infection caused gene down regulation, MSRO infection led to both up regulated and down regulated gene expression changes, and *w*Mel infection caused mostly up regulated gene expression. The results of the differential expression (DE) analysis reported ~2X more significant genes for the non-native infection Hyd1 than for the native infection MSRO. Of the reported significant genes however, 50, 20, and 10%, respectively, for Hyd1,

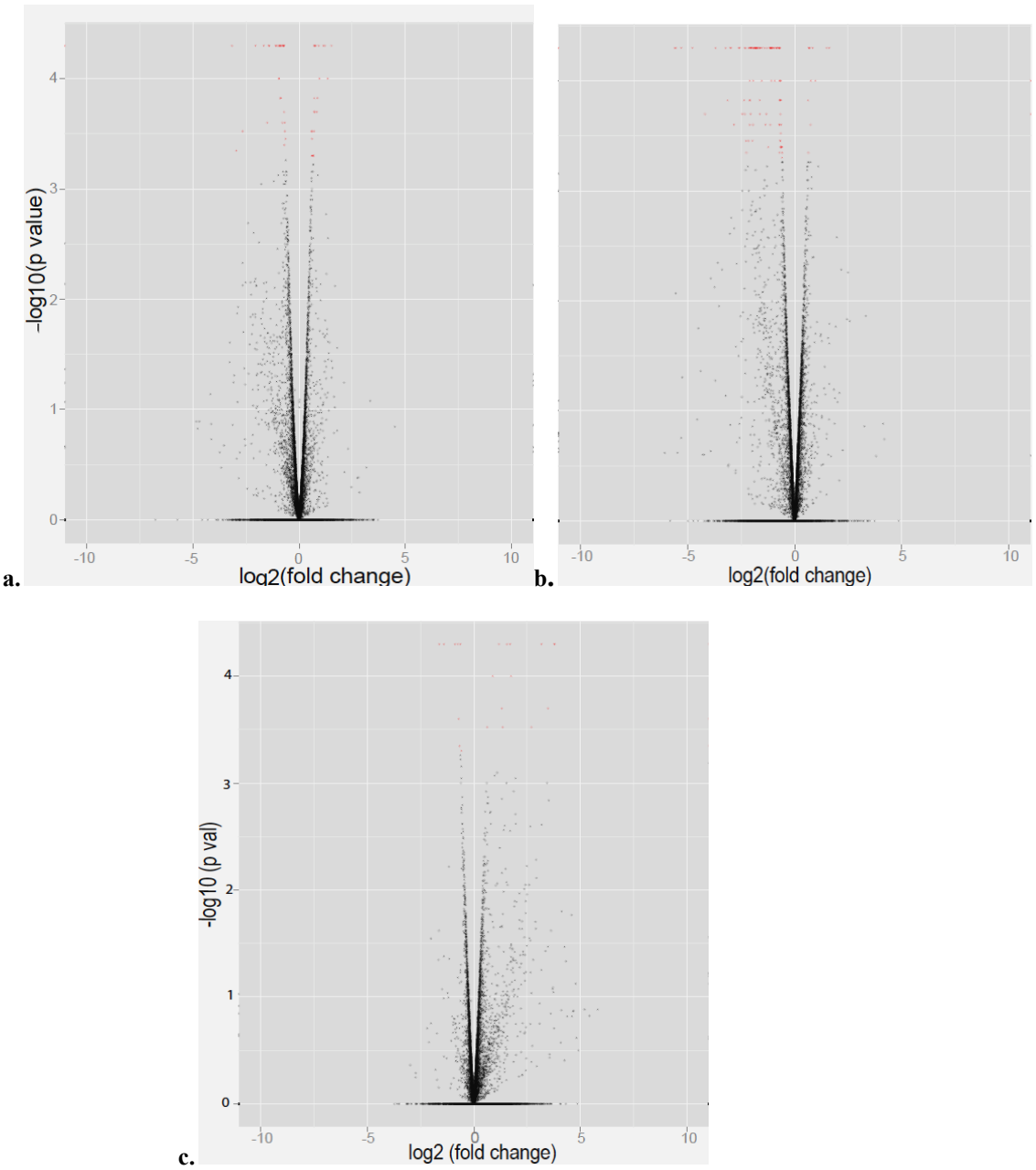

Figure 5. Volcano plots of fold change vs. significance for pairwise comparisons between treatments and control. Values to the right of the center indicate up regulated genes and values to the left indicate down regulated genes in comparison to the control. **a**. MSRO vs. Control; **b**. Hyd1 vs. Control; **c**. *w*Mel vs. Control. Red data points indicate significant genes.

*w*Mel, and MSRO were omitted from further analysis, as their FPKM values were less than five. Because the embryo collection method was likely to contain some older embryos (i.e., those that had reached MZT), we used the gene classification of (Lott et al. 2011) and the Berkeley Drosophila Genome Project expression database, to classify the remaining DE genes as maternal, maternal/zygotic, or zygotic. Compared to the control, MSRO, Hyd1, and *w*Mel treatments had 40, 41, and 9 significantly expressed maternal genes, respectively (Table 5).

Table 5. Summary of filtered genes by class. Number of genes that had FPKM values of five or greater and their classification according to their known expression timing, based on (Lott et al. 2011) and the Berkeley Drosophila Genome Project expression database.

Treatment	Total Genes	Maternal		Maternal/Zygotic		Zygotic		Unclassified[a]	
MSRO	52	40	(76.9)	3	(5.7)	5	(9.61)	4	(7.69)
Hyd1	61	41	(67)	3	(4.91)	13	(21.3)	4	(6.55)
*w*Mel	20	9	(45)	NA		5	(25)	6	(30)

[a] Unclassified - genes with ambiguous classification

The functional annotation terms associated with MSRO and Hyd1 infections are summarized in Table 6, and include genes that were classified as maternal, maternal/zygotic, zygotic, and those that have not been unclassified. The number of genes that were DE in the *w*Mel treatment was insufficient to provide enrichment of any annotation terms. Significant genes in the MSRO treatment were associated with the Gene Ontology (GO) terms of translation, cytoskeletal organization, cell–cycle, and phosphorylation, whereas the Hyd1 treatment was enriched for the terms of cellularization, growth, and gliogenesis (Table 6). The enriched GO terms of the MSRO treatment included more genes than those of the Hyd1 treatment.

Table 6 Gene ontology terms associated with significant genes. **a.** MSRO vs. Control. **b.** Hyd1 vs. Control.

a.

Biological process (GO Term)	Number of genes[a]	Percentage[b]	P-Value
Translation	7	16.7	3.70E-03
Cytoskeletal organization	6	14.3	1.10E-02
Cell–cycle	6	14.3	3.40E-02
Phosphorylation	5	11.9	3.70E-02

b.

Biological process (GO Term)	Number of genes[a]	Percentage[b]	P-Value
Cellularization	4	9.1	4.20E-03
Growth	4	9.1	5.90E-03
Gliogenesis	3	6.8	1.10E-02

[a] Number of involved genes from the significant list
[b] Percentage = involved genes/total significant genes

Table 7 Results of transcript abundance for treatments and control. The FPKM (± SD) values indicate normalized transcript abundance for each gene. Genes were selected based on FPKM values, fold change, and significance. These genes were shortlisted from the maternal and maternal/zygotic genes represented in Table 5.

Gene ID	Treatment	Treatment FPKM		Control FPKM		Fold Change	P-value
CR32028	MSRO	44.99	(11.4)	17.00	(7.57)	2.65	0.0082
	Hyd1	48.18	(6.19)			2.83	0.0082
	*w*Mel	31.57	(7.18)			1.86	0.0149
RpL38	MSRO	4613.32	(331.0)	2166.91	(149.29)	2.13	0.0082
	Hyd1	3872.91	(674.5)			1.79	0.0082
	*w*Mel	3360.25	(495.23)			1.55	0.0330
RpL36	MSRO	3483.13	(656.46)	1874.87	(293.82)	1.86	0.0082
RpL23	MSRO	4327.57	(712.14)	2394.61	(44.76)	1.81	0.0206
RpL24	MSRO	1742.37	(297.61)	934.63	(75.84)	1.86	0.0206
RpLP2	MSRO	2864.60	(590.0)	1501.18	(218.36)	1.91	0.0149
mRpS21	MSRO	233.70	(31.58)	124.01	(23.90)	1.88	0.0082
mRpL10	MSRO	159.64	(17.0)	96.63	(17.45)	1.65	0.0082
CG12400	MSRO	349.90	(13.76)	182.79	(0.01)	1.91	0.0082
CG3621	MSRO	181.09	(1.14)	85.97	(7.06)	2.11	0.0082
SmB	MSRO	295.40	(38.80)	177.72	(27.07)	1.66	0.0082
LSm7	MSRO	234.65	(30.67)	133.68	(21.80)	1.76	0.0082
hoip	Hyd1	711.38	(54.97)	449.22	(31.04)	1.58	0.0082
pncr009:3L	MSRO	73.75	(6.40)	38.02	(5.34)	1.94	0.0149
CG40228	MSRO	79.11	(1.97)	43.83	(3.81)	1.81	0.0082
fs(1)N	MSRO	29.38	(3.81)	47.76	(7.05)	1.63	0.0082
	Hyd1	29.79	(2.31)			1.60	0.0206
CG5355	Hyd1	68.56	(6.24)	106.79	(15.78)	1.56	0.0149
CG7510	Hyd1	21.24	(1.91)	33.06	(5.09)	1.56	0.0206
MESR4	Hyd1	34.06	(2.63)	55.15	(8.86)	1.62	0.0293
CG2789	MSRO	191.66	(25.30)	114.30	(17.54)	1.68	0.0082
CG14036	Hyd1	564.98	(87.72)	349.83	(7.85)	1.61	0.0082

From the maternal and maternal/zygotic genes recovered in Table 1, a subset of 21 genes was selected for further analysis based on transcript abundance, fold change, p-value, and functional interest (Table 7). These 21 selected genes had relative fold changes ranging from a 1.5 to 2.8. Accordingly, the MSRO treatment had thirteen differentially expressed (DE) genes, the Hyd1 treatment had only five; and the *w*Mel

treatment had none. Two genes, *CR32028* and *RpL38,* were up regulated in all three symbiont treatments with respect to the control. *fs(1)N* was the only gene uniquely DE in both *Spiroplasma* treatments, exhibiting lower expression levels than the control. In total, four genes (*fs(1)N*, *CG5355*, *CG7510*, and *MESR4*) were down regulated. Three of them were only significantly DE in the Hyd1 treatment. Functional information for the 21 genes listed in Table 7, based on direct assays, mutant analysis, and predicted functions (flybase.org), is summarized in Table 8.

The greatest change in expression, ~2.8 fold change, was seen in *CR32028*/*Unr*, a gene required for the dose-sensitive expression of the X chromosome (Abaza et al. 2006, Patalano et al. 2009). *CR32028*/*Unr* had higher expression in all three symbiont treatments than in the control (Figure 6a). Both, symbiont-infected and control embryos had relatively low expression levels of this gene at the stage examined.

Several ribosomal protein (r-protein) genes exhibited fold-changes of at least 1.5, in the comparison of symbiont treatments versus the control. The mRNA levels of ribosomal protein (r-protein) genes are very high during oocyte maturation and early embryogenesis, during which they remain translationally quiescent, so as to coordinate protein synthesis in early embryos (Al-Atia et al. 1985, Hongo and Jacobs-Lorena 1991). *RpL38* was the only r-protein gene DE by all three symbiont treatments. Several other cytoplasmic and mitochondrial r-protein genes were significantly up regulated only in the MSRO treatment. Similarly, *CG12400* and *CG3621,* which are predicted to be involved in mitochondrial electron transport, were also significantly up regulated only in the MSRO treatment.

The small nuclear RNA associated protein (snRNP) genes, *SmB*, *LSm7* (Figure 6b), and *hoip* were DE in the *Spiroplasma* treatments. These genes code for proteins that form a complex with small nuclear RNA (snRNA), which controls RNA processing and modification (reviewed in Matera et al. 2007). *SmB* and *LSm7* were significantly up regulated in the MSRO treatment, whereas *hoip* was significantly up regulated in the Hyd1 treatment.

Table 8. Twenty-one DE genes and their hypothesized functions.

Gene ID	Summary Information
CR32028	dosage compensation by hyperactivation of X chromosome; lateral inhibition
RpL38	translation
RpL36	translation
RpL23	translation; mitotic spindle elongation & organization
RpL24	translation; mitotic spindle elongation; centrosome duplication
RpLP2	translation
mRpS21	translation ,mitochondrial
mRpL10	translation; mitochondrial
CG12400	predicted, mitochondrial electron transport, NADH to ubiquinone
CG3621	predicted, mitochondrial electron transport, NADH to ubiquinone
SmB	gonad development; mitotic spindle organization; germ cell development
LSm7	neurogenesis
hoip	nervous system development
pncr009:3L	putative non-coding RNA
CG40228	predicted, transcription elongation factor implicated in the maintenance of chromatin structure in actively transcribed genes
fs(1)N	vitelline membrane formation; involved in chorion-containing eggshell formation
CG5355	proteolysis; repressed by miRNA during oocyte maturation
CG7510	predicted, transmembrane transport; intracellular signal transduction
MESR4	negative regulation of Ras protein signal transduction; cellular response to hypoxia; regulation of response to DNA damage stimulus.
CG2789	unknown
CG14036	unknown; peak expression in 00-06hr and adult females; moderate in adult testes

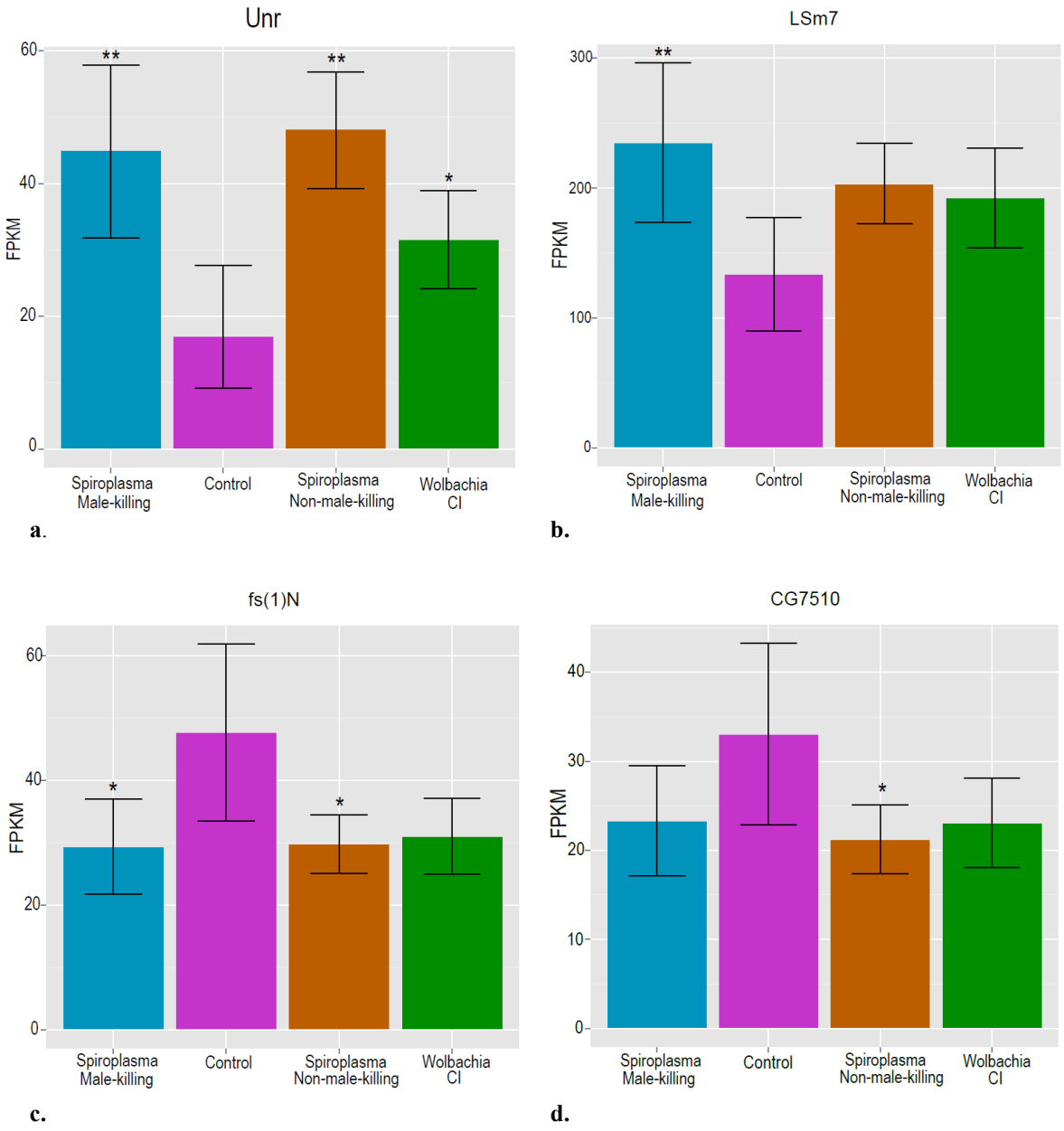

Figure 6 Mean transcript abundance of selected genes for each treatment and the control. Error bars are standard deviation.

Two of the DE genes were involved in oogenesis. *fs(1)N* was down regulated in both *Spiroplasma* treatments (Figure 6c), whereas *CG5355* was significantly down regulated only in the Hyd1 treatment. *fs(1)N* is required for the formation of the egg shell, which consists of a vitelline membrane and a chorion layer (reviewed in Cavaliere et al. 2008). *CG5355* encodes a serine protease, which is transcriptionally repressed during oocyte maturation (Nakahara et al. 2005).

Additional genes involved with different or unknown functions were differentially expressed. *CG7510* (Figure 6d) and *MESR4*, which are associated with intracellular signal transduction and Ras/MAPK signaling pathway (Huang and Rubin 2000, FlyBase et al. 2004) were significantly down regulated in the Hyd1 treatment. *CG40228,* a predicted transcription elongation factor, and *pncr009:3L*, a putative non-coding RNA (Tupy et al. 2005), were up regulated in the MSRO treatment. Finally, transcript levels of *CG2789* and *CG14036*, which have no known function, were moderately high in control embryos, and ~1.6 fold higher in the MSRO and Hyd1 treatments, respectively.

3.4 Discussion

Endosymbionts transmitted within the egg cytoplasm may alter aspects of oogenesis as a cause or consequence of their presence in, or entry into, the egg cytoplasm. In this study, we compared the maternal mRNA composition of early *D. melanogaster* embryos in the presence and absence of three different heritable symbionts that differ in: (a) the phenotype exerted during early host development (male-killing, CI rescue, or none); (b) their native host species (*D. melanogaster* or *D. hydei*), and/or (c) the mode and time of entry into the egg cytoplasm (*Wolbachia* is present in the germline, whereas *Spiroplasma* appears to colonize the egg cytoplasm during vitellogenesis).

3.4.1 Effect of Wolbachia *on maternal mRNA composition of embryos*

The results indicate that *w*Mel infection causes little to no change in the maternally-deposited mRNAs of eggs (Tables S1). The r-protein gene RPL38 and dosage compensation gene *CR32028* /*Unr* were the only DE genes retained following our selection criteria (see Table 7). These results imply that the reproductive phenotype of *w*Mel does not rely on manipulation of maternally deposited rRNAs. The ability of *w*Mel to cause CI is considered weak, but it is capable of rescuing sperm modified by *Wolbachia* strains that cause strong CI; suggesting that alterations to the egg or zygote by *Wolbachia* can be independent of the strength of the CI (Zabalou et al. 2008). Although my results suggest that the CI rescue mechanism of *w*Mel does not rely on alteration of the maternal mRNAs deposited in the egg, alteration of small RNAs, which play an important role in gene regulation, cannot be ruled out, because the methods used were not suited for such molecules. Indeed, recent findings exemplify *Wolbachia*'s ability to control host micro RNA (miRNA) in the mosquito *A. aegypti* (Hussain et al. 2011, Osei-Amo et al. 2012, Zhang et al. 2013). *Wolbachia* was shown to regulate a host miRNA to express a metalloprotease gene, which is crucial for symbiont maintenance and replication (Hussain et al. 2011). The CI rescue mechanism by *w*Mel might thus involve alteration of maternally deposited regulatory RNAs, as well as of proteins, or epigenetic modifications. Furthermore, our findings do not rule out the possibility that other reproductive manipulation strategies of *Wolbachia* (e.g. male killing) alter the composition of maternally-deposited mRNAs.

3.4.2 Effect of Spiroplasma *on maternal mRNA composition of embryos*

The results suggest that *Spiroplasma* infection alters the transcription of several maternal genes in this host (Table S1, Table 7). Sixteen maternal genes were DE in the MSRO treatment and eight in the Hyd1 treatment. Hyd1 is a strain of *Spiroplasma* that naturally infects *D. hydei*. Unlike MSRO, it does not exert an obvious reproductive phenotype on its host. Nevertheless, Hyd1 is closely related to MSRO and is

phylogenetically grouped with other male-killing strains of *Spiroplasma* that infect Drosophila (Haselkorn 2010).

Inherited symbionts are fastidious and often do not survive or transmit well in surrogate hosts (Veneti et al. 2012). In a previous study, however, we demonstrated that upon transfer into *D. melanogaster*, Hyd1 (TEN104-106) can establish vertical transmission and achieve infection levels equivalent to those of the native MSRO strain (Silva et al. 2012). In contrast to the native strain MSRO, presence of Hyd1 lowered the expression of two genes involved in response to bacterial infections : *CG7510* (Figure 6d), which codes for a predicted intracellular signal transduction protein (FlyBase et al. 2004); and *MESR4*, which regulates the Ras/MAPK signaling pathway. *CG7510* was previously shown to be DE in Drosophila S2 cells infected with *Buchnera*; the obligate heritable bacterial symbiont of the pea aphid (Douglas et al. 2011). In contrast to Hyd1, however, infection by *Buchnera* increases the expression of this gene in the non-native host cells. Changes in *MESR4* have been associated with Gram- and + bacterial infections in Drosophila and mammals (Foley and O'Farrell 2004, Fukuyama et al. 2013). Specifically, in the case of the intracellular Gram+ pathogen *Listeria monocytogenes*, Cheng et al. (2005) reported that *MESR4* limits intracellular bacterial replication in Drosophila cells. Down regulation of these genes may enhance the proliferation of *Spiroplasma* Hyd1 in the early embryo.

A gene involved in the formation of the vitelline and chorion membranes, *fs(1)N*, was down regulated in both *Spiroplasma* treatments (Figure 6c). This gene encodes for the Nasrat protein, which is incorporated into the vitelline membrane that forms the protective outer layers of the egg (Cernilogar et al. 2001, Jiménez et al. 2002, Ventura et al. 2010). These membranes are secreted by the follicle cells during the vitellogenic stages of oogenesis. Recently, Herren et al. (2013) showed that *Spiroplasma* MSRO is endocytosed into the oocyte during vitellogenesis, and thus, uses the yolk uptake receptors of the host to transmit to the new host. Down regulation of *fs(1)N* might be part of the mechanism that allows *Spiroplasma* to traverse the outer membranes of the oocyte during yolk uptake. Significant down regulation of *fs(1)N* in both, the MSRO

and Hyd1 treatments, is consistent with the hypothesis that *Spiroplasma* might exploits this mechanism to achieve maternal transmission.

The gene *CG5355* was down regulated in the Hyd1 treatment. A previous study showed that this protein is enriched in *dicer-1* mutant oocytes, suggesting that *CG5355* is negatively regulated by microRNAs (miRNAs) during oocyte maturation (Nakahara et al. 2005). miRNAs are processed by the Dicer class of endoribonucleases, and typically repress expression in animals via translation inhibition. The repression of *CG5355*, however, appears to be mediated via transcript production or stability (transcript levels were also enhanced in *dicer-1* mutant oocytes), rather than translation inhibition (Nakahara et al. 2005). The reduced *CG5355* transcript levels observed in the Hyd1 treatment may reflect an interaction of Hyd1 with a miRNA that results in further repression of *CG5355* during this stage.

Translational control is the primary means by which gene expression is regulated during oocyte maturation and early embryogenesis (reviewed in Johnstone and Lasko 2001). This process controls the biogenesis and accumulation of proteins required for the early stages of embryonic development. Large quantities of maternal r-protein mRNAs are generated during Drosophila oogenesis, to be used by the developing embryo. R-protein mRNAs remain selectively untranslated until later stages of embryogenesis, when embryonic ribosomes are synthesized (Al-Atia et al. 1985, Kay and Jacobs-Lorena 1985, Hongo and Jacobs-Lorena 1991). Very high amounts of r-protein transcripts therefore are expected in early embryos. This was evident in all of our treatments and control. In general, however, infected embryos had increased levels of r-protein transcripts relative to the control, especially the MSRO treatment. It is possible that indirect effects of infection by a symbiont causes more transcription of these genes or reduced degradation of such transcripts.

Spiroplasma-infected embryos had significant increases in expression of three genes that code for proteins that interact with small nuclear RNAs (snRNAs) (Herold et al. 2009). *SmB* and *LSm7* (Figure 6b) were significantly up regulated in the MSRO treatment, whereas *hoip* was up regulated in the Hyd1 treatment (Table 7). The protein

products of these genes are RNA binding components of spliceosomes required for mRNA processing (reviewed in Matera et al. 2007). In addition, these genes have been implicated in other developmental roles (Table 8), such as germline development and neurogenesis (Prokopenko et al. 2000, Anne 2010, Gonsalvez et al. 2010). *SmB* in particular, is expressed in the oocyte and pre-blastoderm embryo and is involved in splicing *oskar* pre-mRNA. The SmB-mRNA complex of *oskar* also facilitates the localization of these transcripts to the posterior end of the oocyte, which is required for proper germline establishment and embryonic patterning (Anne 2010, Gonsalvez et al. 2010). The use of an endogenous localization mechanism of the host could allow *Spiroplasma* to localize to future tissues that enhance its proliferation or phenotype (e.g. by moving to "targeted" tissues).

This study identified a maternally and zygotically expressed gene that functions in a sex specific manner. *CR32028/Unr* in *Spiroplasma* (Figure 6a) treatments had one of the highest changes in expression compared to the control (~2.6–2.8 fold). This gene was also significantly up regulated in the *w*Mel treatment, albeit to a lesser extent (1.86 fold; Table 7). Several lines of evidence suggest that this is gene is a likely candidate for the *Spiroplasma* male-killing mechanism.

Sex in Drosophila is determined by the interplay between the number of X chromosomes in the diploid state and the sex specific gene *Sex-lethal* (*Sxl*) (reviewed in Salz and Erickson 2010). X-linked signaling proteins relay information about the number of X chromosomes, which leads to the expression of *Sxl* in XX females, but not in XY males. The presence or absence of the protein product of this gene determines female versus male somatic differentiation and dosage compensation. When *Sxl* is switched off, the single X chromosome is hypertranscribed in males, by the dosage compensation complex (DCC), to balance the dose of X-linked genes between the sexes. The DCC consists of five proteins including MSL-2. Translation inhibition of *msl-2* mRNA, by the female-specific protein SXL, prevents the assembly of DCC in females. *Unr* was recently found to play an integral role in this process (Abaza et al. 2006, Duncan et al. 2006). In females, UNR and SXL bind to the 3' UTR of *msl-2* transcripts

to inhibit translation, and thus inhibit the formation of the DCC. This was verified with hypomorphic mutant females of *Unr*, which exhibited ectopic DCC formation on the X chromosome (Patalano et al. 2009). Counter intuitively, lowered expression of *Unr* in males, caused reduced DCC binding to the male X chromosome, suggesting that males require this gene to assemble the DCC or to target the X chromosome by the DCC. This was corroborated by the finding that the non-coding RNAs *rox1* and *rox2*, which recruit the DCC onto the high affinity sites of the X chromosome, bind to UNR.

A related male-killing strain of *Spiroplasma* (strain NSRO) was previously shown to require a functional DCC to achieve male-killing. Veneti et al. (2005) examined the efficiency of male-killing by *Spiroplasma* in mutants of the genes that code for the DCC. This study revealed that male offspring of the mutant females survived beyond the stages at which *Spiroplasma*-induced killing typically occurs in Drosophila (i.e., early embryonic stages).

In this study, the maternal expression of *CR32028*/*Unr* was ~2.5 fold greater in *Spiroplasma* infected embryos (Table 7). Interestingly, Patalano et al. (2009) found that zygotic overexpression of *Unr* in blastoderm embryos caused preferential male lethality, and that a ~2 fold increase in expression resulted in complete lethality of both sexes. Our results, together with the evidence that *Spiroplasma* requires a functional DCC to kill males, as well as the sex-specific and dose-specific effects of *Unr*, especially related to male mortality, suggest that this gene might be involved in the mechanism of male-killing. The observation that the non-male-killing strain Hyd1 caused an equivalent increase in *Unr* expression, however, suggests that manipulation of this gene alone is not sufficient to cause male death.

In conclusion, the goal of this study was to determine whether facultative symbionts alter the maternally-derived mRNA composition of its host *D. melanogaster*. Infection by *Spiroplasma* (two strains) altered expression of several genes, whereas infection by *Wolbachia* had a minimal effect. This study provides evidence for the repression of a protein-coding gene that forms the outer membranes of developing oocytes, and thus contributes to the existing evidence for the mechanism by which

Spiroplasma colonizes the egg to transmit maternally. Down regulation of genes known to be altered by intracellular pathogens, and as a result of infection by a non-native obligate symbiont, could imply *Spiroplasma*'s (Hyd1) need and ability to alter host immunity during reproductive stages/oogenesis to enhance its transmission/proliferation in the non-native host. I highlight the possibility that *Spiroplasma* uses host regulatory RNAs during oocyte maturation and early embryogenesis, due to the enhanced down regulation of genes that are normally repressed by miRNAs during this time. I also point out that *Spiroplasma* infection affected the expression of protein coding genes that bind snRNA, which together are involved in pre-mRNA processing. Finally, I identify a promising candidate gene (*Unr*), that might be targeted by *Spiroplasma* to achieve male-killing in Drosophila. To test the role of the DE genes identified by our screening, future studies should examine the phenotype and transmission efficiency of *Spiroplasma* in hosts with altered expression of such genes.

CHAPTER IV

CONCLUSION

The purpose of my dissertation work was to expand the current knowledge on the association of maternally inherited endosymbionts and their hosts, with a special interest in host reproductive manipulation. I examined three strains of symbionts from two distinct groups that have independently evolved the ability to be inherited through the egg cytoplasm of a host. I took advantage of the genomic tools afforded by the model organism *D. melanogaster*, which is a natural host to *Spiroplasma* and *Wolbachia* strains that exert the reproductive phenotypes of male-killing and cytoplasmic incompatibility, respectively. Furthermore, I successfully transfected and used two other *Spiroplasma* strains, another male-killing strain and one lacking a phenotype, but phylogenetically similar to that of the native strain of *D. melanogaster*, to compare effects on the host and of male-killing.

Chapter one described the dynamics of infection densities of male-killing and non-male-killing *Spiroplasma* in female hosts. Quantitative PCR was used to trace bacterial titers of females, from emergence through the reproductive stages. In contrast to several previous studies, I found no evidence for the correlation between bacterial densities and the male-killing phenotype of *Spiroplasma*. This was also the first instance, a male-killing strain of *Spiroplasma* was being tested for the proliferation of *Spiroplasma* in its natural host. This study also noted the decrease in male-killing efficiency of *Spiroplasma* in hosts that are simultaneously infected with weak CI-causing *Wolbachia.* This study quantified bacterial densities in adults, which could have a direct impact on efficient transmission of the bacteria to the new host via the egg, but not on male-killing. A future study could benefit from doing a similar experiment, but with single or pooled eggs from females infected with male-killing and non-male-killing *Spiroplasma*, to determine a correlation between the phenotype and the bacteria loaded into the egg.

Chapter two provides the results of a screen for host maternal genes whose expression is altered as a result of infection by three maternally inherited endosymbionts. I used RNA-sequencing and associated bioinformatics tools to select genes that were significantly differentially expressed due to infection. The results of this experiment suggest that *Spiroplasma* causes changes to the maternal mRNA expressed in the egg, whereas *Wolbachia* has little to no effect on maternal mRNA composition. Specifically, I found both strains of *Spiroplasma* to down regulate a gene that could facilitate its entrance into the egg. This hypothesis fits well with a new study that provides cytological evidence for the invasion of the egg by *Spiroplasma* during late oogenesis. This study also suggests and interaction of the non-native *Spiroplasma* strain with host immune genes, indicating the possibility that inherited symbionts do not go unnoticed, and have perhaps evolved, as not to alarm host defenses. I also, highlight the intriguing possibility that *Spiroplasma* can regulate host gene expression through silencing (miRNA) and mRNA splicing. Finally, I describe a gene involved in the sex determination pathway of the host, as that a potential candidate gene necessary for the mechanism of male-killing by *Spiroplasma*. The results of this screen provide a basis for future studies. Many of the genes described can be manipulated with mutant analyses. The candidate male-killing gene (*Unr*) for example, has a ~2.8 fold increase in expression due to infection. The male killing phenotype could be affected in females that do not produce this transcript, and hence the protein (through heterozygote mutants or RNA-interference). If *Unr* is indeed involved in male killing, I would expect mutant or RNA-interference *Spiroplasma*-infected females to produce male offspring in what is generally ~100% female biased brood.

REFERENCES

Abaza, I., O. Coll, S. Patalano, and F. Gebauer. 2006. Drosophila UNR is required for translational repression of male-specific lethal 2 mRNA during regulation of X-chromosome dosage compensation. Genes & Development **20**:380-389.

Al-Atia, G. R., P. Fruscoloni, and M. Jacobs-Lorena. 1985. Translational regulation of mRNAs for ribosomal proteins during early Drosophila development. Biochemistry **24**:5798-5803.

Anbutsu, H. and T. Fukatsu. 2003. Population dynamics of male-killing and non-male-killing Spiroplasmas in *Drosophila melanogaster*. Applied and Environmental Microbiology **69**:1428–1434.

Anbutsu, H. and T. Fukatsu. 2006. Tissue-specific infection dynamics of male-killing and nonmale-killing spiroplasmas in *Drosophila melanogaster*. FEMS Microbiology Ecology **57**:40-46.

Anbutsu, H. and T. Fukatsu. 2011. *Spiroplasma* as a model insect endosymbiont. Environmental Microbiology Reports **3**:144-153.

Anne, J. 2010. Arginine methylation of SmB is required for Drosophila germ cell development. Development **137**:2819-2828.

Baldo, L., J. C. Dunning Hotopp, K. A. Jolley, S. R. Bordenstein, S. A. Biber, R. R. Choudhury, C. Hayashi, M. C. J. Maiden, H. Tettelin, and J. H. Werren. 2006. Multilocus sequence typing system for the endosymbiont *Wolbachia pipientis*. Applied and Environmental Microbiology **72**:7098-7110.

Bandi, C., A. M. Dunn, G. D. Hurst, and T. Rigaud. 2001. Inherited microorganisms, sex-specific virulence and reproductive parasitism. Trends Parasitology **17**:88-94.

Bastock, R. and D. St Johnston. 2008. Drosophila oogenesis. Current Biology **18**:R1082-R1087.

Baumann, P. 2005. Biology bacteriocyte-associated endosymbionts of plant sap-sucking insects. Annual Review of Microbiology **59**:155-189.

Baumann, P., C. Y. Lai, L. Baumann, D. Rouhbakhsh, N. A. Moran, and M. A. Clark. 1995. Mutualistic associations of aphids and prokaryotes: biology of the genus *Buchnera*. Appl. Environmental. Microbiology. **61**:1–7.

Bentley, J. K., Z. Veneti, J. Heraty, and G. D. D. Hurst. 2007. The pathology of embryo death caused by the male-killing *Spiroplasma* bacterium in *Drosophila nebulosa*. BMC Biology **5**.

Bourtzis, K., A. Nirgianaki, G. Markakis, and C. Savakis. 1996. *Wolbachia* infection and cytoplasmic incompatibility in Drosophila species. Genetics **144**:1063-1073.

Breeuwer, J. A. and J. H. Werren. 1993. Cytoplasmic incompatibility and bacterial density in *Nasonia vitripennis*. Genetics **135**:565-574.

Brownlie, J. C. and K. N. Johnson. 2009. Symbiont-mediated protection in insect hosts. Trends in Microbiology **17**:348-354.

Buchner, P. 1965. Endosymbiosis of animals with plant microorganisms. Interscience, New York.

Callaini, G., R. Dallai, and M. G. Riparbelli. 1997. Wolbachia-induced delay of paternal chromatin condensation does not prevent maternal chromosomes from entering anaphase in incompatible crosses of *Drosophila simulans*. Journal of Cell Science **110**:271-280.

Cavaliere, V., F. Bernardi, P. Romani, S. Duchi, and G. Gargiulo. 2008. Building up the Drosophila eggshell: First of all the eggshell genes must be transcribed. Developmental Dynamics **237**:2061-2072.

Cernilogar, F. M., F. Fabbri, D. Andrenacci, C. Taddei, and G. Gargiulo. 2001. Drosophila vitelline membrane cross-linking requires the *fs(1)Nasrat*, *fs(1)polehole* and chorion genes activities. Development genes and evolution **211**:573-580.

Chandler, J. A., J. M. Lang, S. Bhatnagar, J. A. Eisen, and A. Kopp. 2011. Bacterial communities of diverse Drosophila species: ecological context of a host-microbe model system. PLoS Genetics **7**:e1002272.

Charlat, S., J. W. O. Ballard, and H. Merçot. 2004. What maintains noncytoplasmic incompatibility inducing *Wolbachia* in their hosts: a case study from a natural *Drosophila yakuba* population. Journal of Evolutionary Biology **17**:322-330.

Cheng, L. W., J. P. M. Viala, N. Stuurman, U. Wiedemann, R. D. Vale, and D. A. Portnoy. 2005. Use of RNA interference in Drosophila S2 cells to identify host pathways controlling compartmentalization of an intracellular pathogen. Proceedings of the National Academy of Sciences of the United States of America **102**:13646-13651.

Cline, T. W. 1986. A female-specific lethal lesion in an X-linked positive regulator of the Drosophila sex determination gene, Sex-lethal. Genetics **113**:641-663.

Counce, S. J. and D. F. Poulson. 1962. Developmental effects of the sex-ratio agent in embryos of *Drosophila willistoni*. Journal of Experimental Zoology **151**:17–31.

Dale, C. and N. A. Moran. 2006. Molecular interactions between bacterial symbionts and their hosts. Cell **126**:453-465.

Darby, A. C. and A. E. Douglas. 2003. Elucidation of the transmission patterns of an insect-borne bacterium. Applied Environmental Microbiology **69**:4403-4407.

Dedeine, F., F. Vavre, F. Fleury, B. Loppin, M. E. Hochberg, and M. Bouletreau. 2001. Removing symbiotic *Wolbachia* bacteria specifically inhibits oogenesis in a

parasitic wasp. Proceedings of the National Academy of Sciences of the United States of America **98**:6247-6252.

Dobson, S. L., K. Bourtzis, H. R. Braig, B. F. Jones, W. Zhou, F. Rousset, and S. L. O'Neill. 1999. *Wolbachia* infections are distributed throughout insect somatic and germ line tissues. Insect Biochemistry and Molecular Biology **29**:153-160.

Dobson, S. L. 2007. Transfection of *Wolbachia pipientis* into Drosophila Embryos. Current Protocols in Microbiology. **5**:3A.4.1–3A.4.11.

Douglas, A. E., S. Bouvaine, and R. R. Russell. 2011. How the insect immune system interacts with an obligate symbiotic bacterium. Proceedings of the Royal Society B: Biological Sciences **278**:333-338.

Duncan, K., M. Grskovic, C. Strein, K. Beckmann, R. Niggeweg, I. Abaza, F. Gebauer, M. Wilm, and M. W. Hentze. 2006. Sex-lethal imparts a sex-specific function to UNR by recruiting it to the *msl-2* mRNA 3′ UTR: translational repression for dosage compensation. Genes & Development **20**:368-379.

Duron, O., D. Bouchon, S. Boutin, L. Bellamy, L. Zhou, J. Engelstadter, and G. Hurst. 2008. The diversity of reproductive parasites among arthropods: *Wolbachia* do not walk alone. BMC Biology **6**:27.

Duron, O. and G. Hurst. 2013. Arthropods and inherited bacteria: from counting the symbionts to understanding how symbionts count. BMC Biology **11**:45.

Dyer, K. A., J. Jaenike, and M. Noor. 2005. Evolutionary dynamics of a spatially structured host-parasite association: *Drosophila innubila* and male-killing *Wolbachia*. Evolution **59**:1518-1528.

Engelstädter, J. and G. D. D. Hurst. 2007. The Impact of male-killing bacteria on host evolutionary processes. Genetics **175**:245-254.

Fast, E. M., M. E. Toomey, K. Panaram, D. Desjardins, E. D. Kolaczyk, and H. M. Frydman. 2011. *Wolbachia* enhance Drosophila stem cell proliferation and target the germline stem cell niche. Science **334**:990-992.

Ferree, P. M., H. M. Frydman, J. M. Li, J. Cao, E. Wieschaus, and W. Sullivan. 2005. *Wolbachia* utilizes host microtubules and dynein for anterior localization in the Drosophila oocyte. PLoS Pathogens **1**:e14.

FlyBase, C., M. Swiss-Prot Project, and M. InterPro Project. 2004. Gene Ontology annotation in FlyBase through association of InterPro records with GO terms.

Foley, E. and P. H. O'Farrell. 2004. Functional dissection of an innate immune response by a genome-wide RNAi screen. PLoS Biology **2**:e203.

Fukatsu, T., T. Tsuchida, N. Nikoh, and R. Koga. 2001. *Spiroplasma* symbiont of the Pea aphid, *Acyrthosiphon pisum* (Insecta: Homoptera). Applied and Environmental Microbiology **67**:1284-1291.

Fukuyama, H., Y. Verdier, Y. Guan, C. Makino-Okamura, V. Shilova, X. Liu, E. Maksoud, J. Matsubayashi, I. Haddad, K. Spirohn, K. Ono, C. Hetru, J. Rossier, T. Ideker, M. Boutros, J. Vinh, and J. A. Hoffmann. 2013. Landscape of protein-protein interactions in Drosophila immune deficiency signaling during bacterial challenge. Proceedings of the National Academy of Sciences of the United States of America **110**:10717-10722.

Gasparich, G. E. 2002. *Spiroplasmas*: evolution, adaptation and diversity. Frontiers in Bioscience **7**:D619-D640.

Giordano, R., S. L. O'Neill, and H. M. Robertson. 1995. *Wolbachia* infections and the expression of cytoplasmic incompatibility in *Drosophila sechellia* and *D. mauritiana*. Genetics **140**:1307-1317.

Giulietti, A., L. Overbergh, D. Valckx, B. Decallonne, R. Bouillon, and C. Mathieu. 2001. An overview of Real-Time Quantitative PCR: applications to quantify cytokine gene expression. Methods **25**:386-401.

Gonsalvez, G. B., T. K. Rajendra, Y. Wen, K. Praveen, and A. G. Matera. 2010. Sm proteins specify germ cell fate by facilitating *oskar* mRNA localization. Development **137**:2341-2351.

Goto, S., H. Anbutsu, and T. Fukatsu. 2006. Asymmetrical interactions between *Wolbachia* and *Spiroplasma* endosymbionts coexisting in the same insect host. Applied and Environmental Microbiology **72**:4805-4810.

Hansen, A.K. and Moran, N.A. 2011. Aphid genome expression reveals host-symbiont cooperation in the production of amino acids. Proceedings of the National Academy of Sciences of the United States of America **108**:2849-2854.

Haselkorn, T. S. 2010. The *Spiroplasma* heritable bacterial endosymbiont of Drosophila. Fly **4**:80-87.

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.

Hedges, L. M., J. C. Brownlie, S. L. O'Neill, and K. N. Johnson. 2008. *Wolbachia* and virus protection in insects. Science **322**:702.

Herold, N., C. L. Will, E. Wolf, B. Kastner, H. Urlaub, and R. Lührmann. 2009. Conservation of the protein composition and electron microscopy structure of *Drosophila melanogaster* and human spliceosomal complexes. Molecular and Cellular Biology **29**:281-301.

Herren, J. K. and B. Lemaitre. 2011. *Spiroplasma* and host immunity: activation of humoral immune responses increases endosymbiont load and susceptibility to

certain Gram-negative bacterial pathogens in *Drosophila melanogaster*. Cellular Microbiology **13**:1385-1396.

Herren, J. K., J. C. Paredes, F. Schüpfer, and B. Lemaitre. 2013. Vertical transmission of a Drosophila endosymbiont via cooption of the yolk transport and internalization machinery. mBio **4**: 10.1128/mBio.00532-12

Hertig, M. and S. B. Wolbach. 1924. Studies on *Rickettsia*-like micro-organisms in insects. Journal of Medical Research **44**:329-374.

Hilgenboecker, K., P. Hammerstein, P. Schlattmann, A. Telschow, and J. H. Werren. 2008. How many species are infected with *Wolbachia*? – a statistical analysis of current data. FEMS Microbiology Letters **281**:215-220.

Hoerauf, A., K. Nissen-Pahle, C. Schmetz, K. Henkle-Duhrsen, M. L. Blaxter, D. W. Buttner, M. Y. Gallin, K. M. Al-Qaoud, R. Lucius, and B. Fleischer. 1999. Tetracycline therapy targets intracellular bacteria in the filarial nematode Litomosoides sigmodontis and results in filarial infertility. Journal of Clinical Investigastion **103**:11-18.

Hoffmann, A. A. 1988. Partial cytoplasmic incompatibility between two Australian populations of *Drosophila melanogaster*. Entomologia Experimentalis et Applicata **48**:61-67.

Hongo, S. and M. Jacobs-Lorena. 1991. Translational discrimination of ribosomal protein mRNAs in the early Drosophila embryo. Developmental Biolology **145**:338-343.

Hosokawa, T., R. Koga, Y. Kikuchi, X. Y. Meng, and T. Fukatsu. 2010. *Wolbachia* as a bacteriocyte-associated nutritional mutualist. Proceedings of the National Academy of Sciences of the United States of America **107**:769-774.

Huang, A. M. and G. M. Rubin. 2000. A misexpression screen identifies genes that can modulate RAS1pathway signaling in *Drosophila melanogaster*. Genetics **156**:1219-1230.

Huang da, W., B. T. Sherman, and R. A. Lempicki. 2009a. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Research **37**:1-13.

Huang da, W., B. T. Sherman, and R. A. Lempicki. 2009b. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nature Protocols **4**:44-57.

Hurst, G. D., J. H. Graf von der Schulenburg, T. M. Majerus, D. Bertrand, I. A. Zakharov, J. Baungaard, W. Volkl, R. Stouthamer, and M. E. Majerus. 1999. Invasion of one insect species, *Adalia bipunctata*, by two different male-killing bacteria. Insect Molecular Biololgy **8**:133-139.

Hurst, G. D. and F. M. Jiggins. 2000. Male-killing bacteria in insects: mechanisms, incidence, and implications. Emerging Infectectious Diseases **6**:329-336.

Hurst, G. D., F. M. Jiggins, and S. J. Robinson. 2001. What causes inefficient transmission of male-killing *Wolbachia* in Drosophila? Heredity **87**:220-226.

Hurst, G. D. D. and M. E. N. Majerus. 1993. Why do maternally inherited microorganisms kill males? Heredity **71**:81–95.

Hussain, M., F. D. Frentiu, L. A. Moreira, S. L. O'Neill, and S. Asgari. 2011. *Wolbachia* uses host microRNAs to manipulate host gene expression and facilitate colonization of the dengue vector *Aedes aegypti*. Proceedings of the National Academy of Sciences of the United States of America **108**: 9250-9255.

Hutchence, K.J., Fischer, B., Paterson, S., and Hurst, G.D.D. 2011. How do insects react to novel inherited symbionts? A microarray analysis of Drosophila

melanogaster response to the presence of natural and introduced *Spiroplasma*. Molecular Ecology **20**: 950-958.

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

Jeyaprakash, A. and M. A. Hoy. 2000. Long PCR improves Wolbachia DNA amplification: wsp sequences found in 76% of sixty-three arthropod species. Insect Molecular Biology **9**:393-405.

Jiggins, F., G. Hurst, C. v. d. S. Jiggins, JH, and M. Majerus. 2000a. The butterfly *Danaus chrysippus* is infected by a male-killing *Spiroplasma* bacterium. Parasitology **120**:439-446.

Jiménez, G., A. González-Reyes, and J. Casanova. 2002. Cell surface proteins Nasrat and Polehole stabilize the Torso-like extracellular determinant in Drosophila oogenesis. Genes & Development **16**:913-918.

Johnstone, O. and P. Lasko. 2001. Translational regulation and RNA localization in Drosophila oocytes and embryos. Annual Review of Genetics **35**:365-406.

Kageyama, D., H. Anbutsu, M. Shimada, and T. Fukatsu. 2007. *Spiroplasma* infection causes either early or late male killing in Drosophila, depending on maternal host age. Naturwissenschaften **94**:333-337.

Kageyama, D., H. Anbutsu, M. Shimada, and T. Fukatsu. 2009. Effects of host genotype against the expression of spiroplasma-induced male-killing in *Drosophila melanogaster*. Heredity **102**:475–482.

Kageyama, D., H. Anbutsu, M. Watada, T. Hosokawa, M. Shimada, and T. Fukatsu. 2006a. Prevalence of a non-male-killing *Spiroplasma* in natural populations of *Drosophila hydei*. Applied and Environmental Microbiology **72**:6667-6673.

Kay, M. A. and M. Jacobs-Lorena. 1985. Selective translational regulation of ribosomal protein gene expression during early development of *Drosophila melanogaster*. Molecular and Cellular Biology **5**:3583-3592.

Kim, D., G. Pertea, C. Trapnell, H. Pimentel, R. Kelley, and S. Salzberg. 2013. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biology **14**:R36.

Lassy, C. W. and T. L. Karr. 1996. Cytological analysis of fertilization and early embryonic development in incompatible crosses of *Drosophila simulans*. Mechanisms of Development **57**:47-58.

Lott, S. E., J. E. Villalta, G. P. Schroth, S. Luo, L. A. Tonkin, and M. B. Eisen. 2011. Noncanonical compensation of zygotic X transcription in early *Drosophila melanogaster* development revealed through single-embryo RNA-Seq. PLoS Biology **9**:e1000590.

Lucchesi, J. C. and J. E. Manning. 1987. Gene dosage compensation in *Drosophila melanogaster*. Advances in Genetics **24**:371-429.

Mateos, M., S. Castrezana, B. Nankivell, A. Estes, T. A. Markow, and N. A. Moran. 2006. Heritable endosymbionts of Drosophila. Genetics **174**:363–376.

Matera, A. G., R. M. Terns, and M. P. Terns. 2007. Non-coding RNAs: lessons from the small nuclear and small nucleolar RNAs. Nature Reviews Molecular Cell Biology **8**:209-220.

Miller, W. J. and M. Riegler. 2006. Evolutionary dynamics of wAu-like *Wolbachia* variants in neotropical Drosophila spp. Applied and Environmental Microbiology **72**:826-835.

Montenegro, H., L. Hatadani, H. Medeiros, and L. Klaczko. 2006a. Male killing in three species of the tripunctata radiation of Drosophila (Diptera: Drosophilidae). Journal of Zoological Systematics and Evolutionary Research **44**:130-135.

Montenegro, H., A. S. Petherwick, G. D. Hurst, and L. B. Klaczko. 2006b. Fitness effects of *Wolbachia* and *Spiroplasma* in *Drosophila melanogaster*. Genetica **127**:207-215.

Montenegro, H., V. N. Solferini, L. B. Klaczko, and G. D. Hurst. 2005a. Male-killing *Spiroplasma* naturally infecting *Drosophila melanogaster*. Insect Molecular Biology **14**:281-287.

Moran, N. A., J. P. McCutcheon, and A. Nakabachi. 2008. Genomics and evolution of heritable bacterial symbionts. Annual Review of Genetics **42**:165-190.

Nakahara, K., K. Kim, C. Sciulli, S. R. Dowd, J. S. Minden, and R. W. Carthew. 2005. Targets of microRNA regulation in the Drosophila oocyte proteome. Proceedings of the National Academy of Sciences of the United States of America **102**:12023-12028.

O'Neill SL, H. A., Werren JH. 1998. Influential Passengers: inherited microorganisms and arthropod reproduction. Oxford University Press, Oxford, UK.

Osei-Amo, S., M. Hussain, S. L. O'Neill, and S. Asgari. 2012. *Wolbachia*-induced aae-miR-12 miRNA negatively regulates the expression of *MCT1* and *MCM6* genes in *Wolbachia*-infected mosquito cell line. PLoS ONE **7**:e50049.

Parker, B. J., C. J. Spragg, B. Altincicek, and N. M. Gerardo. 2013. Symbiont-mediated protection against fungal pathogens in pea aphids: a role for pathogen specificity? Applied and Environmental Microbiology **79**:2455-2458.

Patalano, S., M. Mihailovich, Y. Belacortu, N. Paricio, and F. Gebauer. 2009. Dual sex-specific functions of Drosophila Upstream of N-ras in the control of X chromosome dosage compensation. Development **136**:689-698.

Poulson, D. F. and B. Sakaguchi. 1961. Hereditary infections in Drosophila. Genetics:890-891.

Prokopenko, S. N., Y. He, Y. Lu, and H. J. Bellen. 2000. Mutations affecting the development of the peripheral nervous system in Drosophila: a molecular screen for novel proteins. Genetics **156**:1691-1715.

Reed, K. M. and J. H. Werren. 1995. Induction of paternal genome loss by the paternal-sex-ratio chromosome and cytoplasmic incompatibility bacteria (*Wolbachia*): a comparative study of early embryonic events. Molecular Reproduction and Development **40**:408-418.

Russell, J. A., C. F. Funaro, Y. M. Giraldo, B. Goldman-Huertas, D. Suh, D. J. Kronauer, C. S. Moreau, and N. E. Pierce. 2012. A veritable menagerie of heritable bacteria from ants, butterflies, and beyond: broad molecular surveys and a systematic review. PLoS ONE **7**:20.

Sakaguchi, B. and D. F. Poulson. 1963. Interspecific transfer of the "sex ratio" condition from *Drosophila willistoni* to *D. melanogaster*. Genetics **48**:841–861.

Salz, H. K. and J. W. Erickson. 2010. Sex determination in Drosophila: The view from the top. Fly **4**:60-70.

Serbus, L. R., C. Casper-Lindley, F. Landmann, and W. Sullivan. 2008. The genetics and cell biology of *Wolbachia*-host interactions. Annual Review of Genetics **42**:683-707.

Short, S. M. and B. P. Lazzaro. 2013. Reproductive status alters transcriptomic response to infection in female *Drosophila melanogaster*. G3 **3**:827-840.

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

Tadros, W. and H. D. Lipshitz. 2009. The maternal-to-zygotic transition: a play in two acts. Development **136**:3033-3042.

Teixeira, L. 2012. Whole-genome expression profile analysis of *Drosophila melanogaster* immune responses. Briefings in Functional Genomics **11**:375-386.

Teixeira, L., A. Ferreira, and M. Ashburner. 2008a. The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. Plos Biology **6**:2753-2763.

Toomey, M. E., K. Panaram, E. M. Fast, C. Beatty, and H. M. Frydman. 2013. Evolutionarily conserved *Wolbachia*-encoded factors control pattern of stem-cell niche tropism in Drosophila ovaries and favor infection. Proceedings of the National Academy of Sciences **110**:10788-10793

Tram, U. and W. Sullivan. 2002. Role of delayed nuclear envelope breakdown and mitosis in Wolbachia-induced cytoplasmic incompatibility. Science **296**:1124-1126.

Trapnell, C., D. G. Hendrickson, M. Sauvageau, L. Goff, J. L. Rinn, and L. Pachter. 2013. Differential analysis of gene regulation at transcript resolution with RNA-seq. Nature Biotechnology **31**:46-53.

Trapnell, C., L. Pachter, and S. L. Salzberg. 2009. TopHat: discovering splice junctions with RNA-Seq. Bioinformatics **25**:1105-1111.

Trapnell, C., A. Roberts, L. Goff, G. Pertea, D. Kim, D. R. Kelley, H. Pimentel, S. L. Salzberg, J. L. Rinn, and L. Pachter. 2012. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nature Protocols **7**:562-578.

Tupy, J. L., A. M. Bailey, G. Dailey, M. Evans-Holm, C. W. Siebel, S. Misra, S. E. Celniker, and G. M. Rubin. 2005. Identification of putative noncoding polyadenylated transcripts in Drosophila melanogaster. Proceedings of the National Academy of Sciences of the United States of America **102**:5495-5500.

Turelli, M. and A. A. Hoffmann. 1995. Cytoplasmic incompatibility in *Drosophila simulans*: dynamics and parameter estimates from natural populations. Genetics **140**:1319-1338.

Veneti, Z., J. K. Bentley, T. Koana, H. R. Braig, and G. D. Hurst. 2005. A functional dosage compensation complex required for male killing in Drosophila. Science **307**:1461-1463.

Veneti, Z., S. Zabalou, G. Papafotiou, C. Paraskevopoulos, S. Pattas, I. Livadaras, G. Markakis, J. K. Herren, J. Jaenike, and K. Bourtzis. 2012. Loss of reproductive parasitism following transfer of male-killing *Wolbachia* to *Drosophila melanogaster* and *Drosophila simulans*. Heredity **109**:306-312.

Ventura, G., M. Furriols, N. Martín, V. Barbosa, and J. Casanova. 2010. closca, a new gene required for both Torso RTK activation and vitelline membrane integrity. Germline proteins contribute to Drosophila eggshell composition. Developmental Biology **344**:224-232.

Watts, T., T. S. Haselkorn, N. A. Moran, and T. A. Markow. 2009. Variable incidence of *Spiroplasma* infections in natural populations of Drosophila species. PLoS ONE **4**:e5703.

Weeks, A. R., M. Turelli, W. R. Harcombe, K. T. Reynolds, and A. A. Hoffmann. 2007. From parasite to mutualist: rapid evolution of *Wolbachia* in natural populations of Drosophila. PLoS Biology **5**:e114.

Werren, J. H., L. Baldo, and M. E. Clark. 2008. *Wolbachia:* master manipulators of invertebrate biology. Nature Review Microbiology **6**:741-751.

Werren, J. H., W. Zhang, and L. R. Guo. 1995. Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. Proceedings of the Royal Society of London. Series B: Biological Sciences **261**:55-63.

Williamson, D. L. and D. F. Poulson. 1979. Sex ratio organisms (*Spiroplasmas*) of Drosophila. Pages 175–208 *in* R. F. Whitcomb and J. G. Tully, editors. The Mycoplasmas. Academic Press, New York.

Wong, Z. S., L. M. Hedges, J. C. Brownlie, and K. N. Johnson. 2011. *Wolbachia*-Mediated antibacterial protection and immune gene regulation in Drosophila. PLoS ONE **6**:e25430.

Xie, J., I. Vilchez, and M. Mateos. 2010. *Spiroplasma* bacteria enhance survival of *Drosophila hydei* attacked by the parasitic wasp *Leptopilina heterotoma*. PLoS ONE **5**:0012149.

Ye, Y. H., M. Woolfit, G. A. Huttley, E. Rances, E. P. Caragata, J. Popovici, S. L. O'Neill, and E. A. McGraw. 2013. Infection with a virulent strain of *Wolbachia* disrupts genome wide-patterns of cytosine methylation in the mosquito. PLoS ONE **8**: e66482. doi:10.1371/journal.pone.0066482

Zabalou, S., A. Apostolaki, S. Pattas, Z. Veneti, C. Paraskevopoulos, I. Livadaras, G. Markakis, T. Brissac, H. Mercot, and K. Bourtzis. 2008. Multiple rescue factors within a *Wolbachia* strain. Genetics **178**:2145-2160.

Zhang, G., M. Hussain, S. L. O'Neill, and S. Asgari. 2013. *Wolbachia* uses a host microRNA to regulate transcripts of a methyltransferase, contributing to dengue virus inhibition in *Aedes aegypti*. Proceedings of the National Academy of Sciences of the United States of America **110**:10276-10281.

APPENDIX

Table S1 Transcript abundance for treatments vs. control.

Gene ID	MSRO	Control	FPKM MSRO	FPKM Control	Fold Change C/M	Test Stat	Un-adj P	Adj P
TwdlM	eggM	eggC	8.52168	0.960323	-3.14955	-4.70794	5.00E-05	0.008238
TwdlL	eggM	eggC	3.15731	0.409043	-2.94837	-2.80941	0.00045	0.04452
Tor	eggM	eggC	15.2572	23.6082	0.629804	2.25316	0.0003	0.033035
tefu	eggM	eggC	5.24909	8.4238	0.682404	2.10442	0.0005	0.048294
SmB	eggM	eggC	295.401	177.719	-0.733082	-2.4636	5.00E-05	0.008238
sec71	eggM	eggC	5.6944	9.25153	0.700148	2.1495	0.0003	0.033035
RpLP2	eggM	eggC	2864.6	1501.18	-0.932238	-2.49666	0.0001	0.014889
RpL38	eggM	eggC	4613.32	2166.91	-1.09016	-6.23679	5.00E-05	0.008238
RpL36	eggM	eggC	3483.13	1874.87	-0.893596	-2.46109	5.00E-05	0.008238
RpL24	eggM	eggC	1742.37	934.631	-0.898584	-3.36398	0.00015	0.020558
RpL23	eggM	eggC	4327.57	2394.61	-0.853766	-3.65341	0.00015	0.020558
robl	eggM	eggC	203.743	110.241	-0.886095	-4.31802	5.00E-05	0.008238
RhoGEF2	eggM	eggC	20.5258	31.2311	0.605543	2.05014	0.00035	0.036995
Prestin	eggM	eggC	33.0813	54.181	0.711772	2.75511	0.0002	0.025108
poe	eggM	eggC	19.5762	30.28	0.629268	1.9558	0.0005	0.048294
pncr009:3L	eggM	eggC	73.7477	38.0235	-0.955708	-2.54472	0.0001	0.014889
Obp99a	eggM	eggC	66.407	16.0904	-2.04514	-6.41695	5.00E-05	0.008238
Nplp2	eggM	eggC	54.7347	17.3626	-1.65647	-5.13647	5.00E-05	0.008238
mRpS21	eggM	eggC	233.704	124.009	-0.914237	-2.62004	5.00E-05	0.008238
mRpL10	eggM	eggC	159.642	96.6269	-0.724343	-3.09946	5.00E-05	0.008238

Table S1 Continued

Gene ID	MSRO	Control	FPKM MSRO	FPKM Control	Fold Change C/M	Test Stat	Un-adj P	Adj-P
LSm7	eggM	eggC	234.65	133.676	-0.811774	-2.63963	5.00E-05	0.008238
l(2)k09022	eggM	eggC	16.11	26.6571	0.726563	2.58377	5.00E-05	0.008238
Hem	eggM	eggC	14.7098	24.4838	0.735047	2.46842	0.0002	0.025108
fs(1)N	eggM	eggC	29.3801	47.7571	0.700879	2.50799	5.00E-05	0.008238
Exn	eggM	eggC	5.50712	9.82868	0.835698	2.2944	0.0002	0.025108
dpr6	eggM	eggC	4.85786	1.71467	-1.50239	-2.78124	0.00025	0.029254
dj-1beta	eggM	eggC	151.199	93.4558	-0.694092	-3.12845	0.0004	0.04065
dgt2	eggM	eggC	141.833	89.727	-0.660585	-2.84397	0.00025	0.029254
CR32028	eggM	eggC	44.9896	16.9985	-1.40419	-3.55122	5.00E-05	0.008238
Cpr65Ea	eggM	eggC	14.9651	2.39193	-2.64536	-4.97737	0.0003	0.033035
CG6752	eggM	eggC	9.68113	14.9968	0.631408	2.03228	0.0005	0.048294
CG6180	eggM	eggC	353.62	216.578	-0.707314	-2.93204	5.00E-05	0.008238
CG4554	eggM	eggC	6.90717	11.3449	0.715879	2.36467	0.00015	0.020558
CG40228	eggM	eggC	79.1131	43.8284	-0.852049	-3.02166	5.00E-05	0.008238
CG3621	eggM	eggC	181.087	85.9737	-1.07472	-5.05056	5.00E-05	0.008238
CG31999	eggM	eggC	1.2594	3.59863	1.51471	2.52035	5.00E-05	0.008238
CG31715	eggM	eggC	220.528	138.162	-0.674596	-3.29527	0.0003	0.033035
CG2789	eggM	eggC	191.659	114.302	-0.745691	-2.49667	5.00E-05	0.008238
CG18808,Dhc64C	eggM	eggC	10.1787	15.2745	0.585565	2.05687	0.0003	0.033035
CG1673	eggM	eggC	2.5581	5.91169	1.2085	2.62473	5.00E-05	0.008238
CG15628	eggM	eggC	1.18791	3.01083	1.34173	2.45734	0.0001	0.014889
CG15523	eggM	eggC	1.50688	2.73582	0.860412	2.2127	0.00015	0.020558

Table S1 Continued

Gene ID	MSRO	Control	FPKM MSRO	FPKM Control	[a]Fold Change C/M	Test Stat	Un-Adj P	Adj P
CG14464	eggM	eggC	635.329	386.808	-0.715885	-3.37535	5.00E-05	0.008238
CG14036	eggM	eggC	545.094	349.833	-0.639838	-3.98964	0.00035	0.036995
CG13679	eggM	eggC	0.473353	0	NA	NA	5.00E-05	0.008238
CG13427	eggM	eggC	54.6335	105.779	0.953191	4.28228	0.0001	0.014889
CG1332	eggM	eggC	12.178	20.5166	0.752519	2.40448	5.00E-05	0.008238
CG13185	eggM	eggC	6.48501	11.128	0.77902	2.4389	5.00E-05	0.008238
CG12400	eggM	eggC	349.9	182.787	-0.936781	-5.28692	5.00E-05	0.008238
CG12384	eggM	eggC	231.755	142.996	-0.696625	-3.414	0.0002	0.025108
CG12011	eggM	eggC	18.6773	6.95141	-1.42591	-3.48133	5.00E-05	0.008238
CG11198	eggM	eggC	5.74315	10.8216	0.914005	2.99828	5.00E-05	0.008238
CG11137	eggM	eggC	408.005	238.031	-0.777437	-2.2291	0.00025	0.029254
CG10631	eggM	eggC	8.29268	12.4502	0.586259	2.01565	0.0005	0.048294
CG10035	eggM	eggC	22.9219	50.3457	1.13515	3.99902	5.00E-05	0.008238
Arc1	eggM	eggC	16.2981	8.18849	-0.993034	-2.89945	5.00E-05	0.008238

Table S1 Continued

Gene ID	Control	Hyd1	FPKM Control	FPKM Hyd1	[a]Fold Change H/C	Test Stat	Un-adj P	Adj P
verm	eggC	eggH	2.58769	0.603755	-2.09963	-2.88786	5.00E-05	0.008238
uif	eggC	eggH	0.578749	0.140184	-2.04562	-3.10863	5.00E-05	0.008238
trn	eggC	eggH	2.09217	0.587188	-1.83311	-2.91877	5.00E-05	0.008238
Tor	eggC	eggH	23.6082	14.0841	-0.745227	-3.19031	5.00E-05	0.008238
Tom70	eggC	eggH	57.4002	36.8132	-0.640832	-2.62976	0.0004	0.04065
Tom	eggC	eggH	33.936	16.4915	-1.04109	-3.8234	5.00E-05	0.008238
Toll-6	eggC	eggH	0.661019	0.17227	-1.94002	-2.33957	0.00025	0.029254
stumps	eggC	eggH	0.947361	0.22886	-2.04945	-2.32369	0.0002	0.025108
Sr-CII	eggC	eggH	2.78006	0.811179	-1.77702	-2.40357	5.00E-05	0.008238
shtd	eggC	eggH	38.3093	20.2819	-0.917504	-2.65644	0.0001	0.014889
serp	eggC	eggH	1.09938	0.281895	-1.96346	-2.40795	0.00035	0.036995
sec71	eggC	eggH	9.25153	4.72525	-0.969303	-3.42994	5.00E-05	0.008238
sca	eggC	eggH	1.561	0.412365	-1.92048	-2.66657	5.00E-05	0.008238
sala	eggC	eggH	35.718	17.0367	-1.06801	-3.83117	5.00E-05	0.008238
RpS4	eggC	eggH	1655.3	1033.67	-0.679318	-2.78354	0.00035	0.036995
RpL38	eggC	eggH	2166.91	3872.91	0.837776	6.27718	5.00E-05	0.008238
RpL23	eggC	eggH	2394.61	3707.66	0.63072	3.90303	0.00015	0.020558
roX1	eggC	eggH	6.13037	2.8793	-1.09026	-2.73302	5.00E-05	0.008238
rib	eggC	eggH	3.08794	1.45985	-1.08083	-2.44016	0.0001	0.014889
Rfabg	eggC	eggH	2.47044	1.21648	-1.02205	-2.45743	5.00E-05	0.008238
Ptr	eggC	eggH	1.60098	0.328525	-2.28488	-3.41125	5.00E-05	0.008238
ptc	eggC	eggH	1.19371	0.338322	-1.81899	-2.87669	5.00E-05	0.008238
Pros26	eggC	eggH	294.362	196.857	-0.580442	-3.11455	0.00045	0.04452
Prestin	eggC	eggH	54.181	33.3265	-0.701116	-3.23836	5.00E-05	0.008238

Table S1 Continued

Gene ID	Control	Hyd1	FPKM Control	FPKM Hyd1	[a]Fold Change H/C	Test-Stat	Un-Adj P	Adj-P
Ppn	eggC	eggH	0.558744	0.181733	-1.62037	-2.22234	0.0002	0.025108
ppa	eggC	eggH	2.89982	1.32943	-1.12516	-2.35746	0.00025	0.029254
poe	eggC	eggH	30.28	19.2834	-0.651005	-2.55558	0.0002	0.025108
Pld	eggC	eggH	32.4526	20.7365	-0.646161	-2.51205	0.0003	0.033035
Osi7	eggC	eggH	7.59211	0.164678	-5.52678	-7.54508	5.00E-05	0.008238
Osi6	eggC	eggH	18.9016	0.391287	-5.59414	-8.26029	5.00E-05	0.008238
Osi20	eggC	eggH	2.25046	0.415172	-2.43844	-3.20384	0.0002	0.025108
Osi15	eggC	eggH	3.75412	0.468695	-3.00175	-4.17539	5.00E-05	0.008238
Osi14	eggC	eggH	2.78793	0.153846	-4.17964	-4.51772	0.0002	0.025108
opa	eggC	eggH	1.06235	0.211942	-2.32552	-2.75547	0.0002	0.025108
nkd	eggC	eggH	1.14018	0.369234	-1.62665	-2.40886	0.00015	0.020558
Nf1	eggC	eggH	10.654	6.83533	-0.640313	-2.46055	0.0004	0.04065
N	eggC	eggH	18.7135	12.074	-0.632177	-2.69174	0.00025	0.029254
Mhc	eggC	eggH	1.64379	0.616725	-1.41432	-2.66365	5.00E-05	0.008238
mfas	eggC	eggH	1.44539	0.289158	-2.32153	-2.74368	5.00E-05	0.008238
MESR4	eggC	eggH	55.1546	34.0596	-0.695423	-2.73582	0.00025	0.029254
mei-41	eggC	eggH	8.77495	5.37987	-0.70582	-2.7649	0.00025	0.029254
LRP1	eggC	eggH	7.00391	3.91295	-0.839903	-3.45693	5.00E-05	0.008238
lea	eggC	eggH	1.40268	0.339615	-2.04622	-3.39706	5.00E-05	0.008238
LanB2	eggC	eggH	1.26967	0.316022	-2.00636	-3.14585	5.00E-05	0.008238
LanA	eggC	eggH	1.42716	0.435358	-1.71287	-3.78588	5.00E-05	0.008238
l(2)k09022	eggC	eggH	26.6571	15.0626	-0.823548	-3.48017	5.00E-05	0.008238

Table S1 Continued

Gene ID	Control	Hyd1	FPKM Control	FPKM Hyd 1	[a]Fold Change H/C	Test-Stat	Un-Adj P	Adj-P
KP78a,KP78b	eggC	eggH	3.92382	1.12111	-1.80734	-3.12992	5.00E-05	0.008238
jbug	eggC	eggH	1.64431	0.494611	-1.73311	-2.85934	5.00E-05	0.008238
ImpL3	eggC	eggH	2.33849	0.525138	-2.15481	-2.92162	0.00035	0.036995
ImpL2	eggC	eggH	6.44024	1.81894	-1.82402	-3.0725	5.00E-05	0.008238
ImpL1	eggC	eggH	2.66359	0.208334	-3.6764	-3.25301	5.00E-05	0.008238
ImpE2	eggC	eggH	2.38557	0.255527	-3.22278	-3.92639	5.00E-05	0.008238
hth	eggC	eggH	2.03502	0.795793	-1.35458	-1.89906	0.00025	0.029254
hoip	eggC	eggH	449.22	711.383	0.663205	4.13191	5.00E-05	0.008238
Gp150	eggC	eggH	5.0034	1.34358	-1.89682	-4.44533	5.00E-05	0.008238
Gasp	eggC	eggH	1.28883	0.252514	-2.35162	-2.13717	0.00015	0.020558
fs(1)N	eggC	eggH	47.7571	29.7941	-0.680688	-2.90863	0.00015	0.020558
Eip75B	eggC	eggH	8.6373	3.87287	-1.15718	-3.96175	5.00E-05	0.008238
ect	eggC	eggH	2.22566	0.0574126	-5.27672	-3.59494	5.00E-05	0.008238
dyl	eggC	eggH	1.06711	0.0391376	-4.76901	-3.21633	5.00E-05	0.008238
Dscam	eggC	eggH	0.481483	0.109024	-2.14284	-1.8747	5.00E-05	0.008238
dpr6	eggC	eggH	1.71467	5.22485	1.60746	3.20602	5.00E-05	0.008238
Dhc64C	eggC	eggH	15.2745	8.60019	-0.828682	-3.50457	5.00E-05	0.008238
dan	eggC	eggH	1.91861	0.577024	-1.73336	-2.54003	5.00E-05	0.008238
CR32028	eggC	eggH	16.9985	48.1835	1.50314	4.09883	5.00E-05	0.008238
ci	eggC	eggH	2.25221	0.733026	-1.6194	-3.25979	5.00E-05	0.008238
CG9926	eggC	eggH	51.5648	87.6305	0.765045	3.36615	0.0001	0.014889
CG9650	eggC	eggH	2.98814	5.87769	0.976002	2.67281	0.0001	0.014889
CG9095	eggC	eggH	0.40653	0.0945776	-2.10379	-2.17292	0.00025	0.029254
CG8486	eggC	eggH	6.02941	3.72159	-0.696096	-2.56967	0.00045	0.04452

Table S1 Continued

Gene ID	Control	Hyd1	FPKM Control	FPKM Hyd1	[a]Fold Change H/C	Test-Stat	Un-Adj P	Adj-p
CG8147	eggC	eggH	1.94896	0.462106	-2.0764	-2.41808	0.00015	0.020558
CG7884	eggC	eggH	0.53807	0.0757137	-2.82917	-2.9803	0.00025	0.029254
CG7510	eggC	eggH	33.0634	21.2435	-0.638216	-2.69136	0.00015	0.020558
CG6770	eggC	eggH	1851.75	3082.61	0.735265	3.48797	0.00025	0.029254
CG6752	eggC	eggH	14.9968	9.22275	-0.701387	-2.61657	5.00E-05	0.008238
CG6734	eggC	eggH	10.4532	6.53303	-0.67812	-2.70426	0.00015	0.020558
CG6234	eggC	eggH	3.47983	0.936206	-1.89412	-3.26903	5.00E-05	0.008238
CG6180	eggC	eggH	216.578	348.36	0.685691	3.42289	5.00E-05	0.008238
CG5604	eggC	eggH	96.9579	61.1297	-0.665486	-2.55197	0.00035	0.036995
CG5355	eggC	eggH	106.788	68.5568	-0.639379	-2.76713	0.0001	0.014889
CG5205	eggC	eggH	17.0118	10.4784	-0.699121	-2.93895	0.0001	0.014889
CG4554	eggC	eggH	11.3449	7.2624	-0.643527	-2.4973	0.0004	0.04065
CG4440	eggC	eggH	133.91	63.0688	-1.08626	-6.42524	5.00E-05	0.008238
CG40228	eggC	eggH	43.8284	68.5055	0.644352	2.58738	0.00045	0.04452
CG3363	eggC	eggH	20.8118	13.8739	-0.585021	-2.39828	0.0005	0.048294
CG32972	eggC	eggH	0.466911	0.0968066	-2.26997	-1.85084	0.00035	0.036995
CG31477	eggC	eggH	0	0.488275	NA	NA	0.0001	0.014889
CG2962	eggC	eggH	4.21648	1.06351	-1.9872	-3.42433	5.00E-05	0.008238
CG18507	eggC	eggH	1.17494	0.248329	-2.24227	-2.25012	0.00045	0.04452
CG17362	eggC	eggH	0.980105	0	NA	NA	5.00E-05	0.008238
CG1673	eggC	eggH	5.91169	2.05051	-1.52759	-3.51663	5.00E-05	0.008238
CG15628	eggC	eggH	3.01083	0.863854	-1.8013	-3.35252	5.00E-05	0.008238
CG15099	eggC	eggH	17.3686	10.6819	-0.701318	-2.4491	0.00015	0.020558
CG14756	eggC	eggH	3.49792	0.402319	-3.12008	-3.85179	0.00015	0.020558

Table S1 Continued

Gene ID	Control	Hyd1	FPKM Control	FPKM Hyd1	[a]Fold Change H/C	Test-Stat	Un-Adj P	Adj-P
CG14309,CG7675	eggC	eggH	4.08053	0.691237	-2.5615	-3.68106	5.00E-05	0.008238
CG14036	eggC	eggH	349.833	564.98	0.691532	5.00427	5.00E-05	0.008238
CG13731	eggC	eggH	1.55749	0.364607	-2.09481	-3.03743	0.00015	0.020558
CG13679	eggC	eggH	0	0.398152	NA	NA	0.0002	0.025108
CG13427	eggC	eggH	105.779	49.3214	-1.10076	-5.69692	5.00E-05	0.008238
CG13333	eggC	eggH	13.9364	4.51581	-1.6258	-4.47849	5.00E-05	0.008238
CG1332	eggC	eggH	20.5166	12.4011	-0.726325	-2.81241	5.00E-05	0.008238
CG13185	eggC	eggH	11.128	5.89236	-0.917282	-3.49696	5.00E-05	0.008238
CG13159	eggC	eggH	33.4095	5.54297	-2.59153	-6.82329	5.00E-05	0.008238
CG12179	eggC	eggH	17.4866	10.8798	-0.684594	-2.48167	0.0001	0.014889
CG12006	eggC	eggH	60.4373	40.0097	-0.595089	-2.50876	0.0004	0.04065
CG11198	eggC	eggH	10.8216	4.94548	-1.12974	-4.37202	5.00E-05	0.008238
CG10631	eggC	eggH	12.4502	7.74602	-0.684643	-2.81934	5.00E-05	0.008238
CG10035	eggC	eggH	50.3457	16.1715	-1.63842	-6.62488	5.00E-05	0.008238
Cad74A	eggC	eggH	0.406957	0.0519379	-2.97002	-2.97374	5.00E-05	0.008238
Bruce	eggC	eggH	17.0187	11.2326	-0.599427	-2.46996	0.0004	0.04065
Brd	eggC	eggH	16.9702	5.83657	-1.53981	-3.56247	0.0001	0.014889
bib	eggC	eggH	2.20883	0.939863	-1.23276	-2.10897	0.0004	0.04065
ASPP	eggC	eggH	0.948725	0.221544	-2.0984	-2.4639	0.0001	0.014889
Ance	eggC	eggH	4.22246	1.69603	-1.31592	-2.38181	0.0002	0.025108
Ama	eggC	eggH	9.47513	4.25154	-1.15616	-2.71341	5.00E-05	0.008238
Act57B	eggC	eggH	26.0863	10.6669	-1.29015	-4.15862	5.00E-05	0.008238
18w	eggC	eggH	0.976906	0.255705	-1.93374	-2.66716	0.0001	0.014889

Table S1 Continued

Gene ID	Control	wMel	FPKM Control	FPKM wMel	[a]Fold Change W/C	Test Stat	Un-adj P	Adj P
sec71	eggC	eggW	9.25153	5.69777	-0.699294	-2.22224	0.00025	0.029254
CG13679	eggC	eggW	0	1.37117	NA	NA	5.00E-05	0.008238
CG13674	eggC	eggW	0	1.08787	NA	NA	0.00045	0.04452
CG13678	eggC	eggW	0	1.14062	NA	NA	0.00025	0.029254
TwdlL	eggC	eggW	0.409043	5.5719	3.76784	3.54801	5.00E-05	0.008238
TwdlB	eggC	eggW	0.657881	7.2431	3.46071	4.42602	0.0002	0.025108
TwdlM	eggC	eggW	0.960323	13.262	3.78764	5.67537	5.00E-05	0.008238
Cpr51A	eggC	eggW	1.48926	9.54802	2.68061	3.68744	0.0003	0.033035
Cpr65Ea	eggC	eggW	2.39193	21.5594	3.17207	5.78778	5.00E-05	0.008238
CG15523	eggC	eggW	2.73582	1.49497	-0.871854	-2.15084	5.00E-05	0.008238
up	eggC	eggW	3.79766	9.4848	1.32051	2.13425	0.0002	0.025108
Mlc2	eggC	eggW	4.57655	10.2941	1.16948	2.27064	5.00E-05	0.008238
Mlc1	eggC	eggW	5.65962	16.613	1.55354	2.4168	5.00E-05	0.008238
Osi7	eggC	eggW	7.59211	2.45315	-1.62987	-2.97701	5.00E-05	0.008238
mei-41	eggC	eggW	8.77495	5.48881	-0.676898	-2.38802	0.00045	0.04452
CG11198	eggC	eggW	10.8216	6.48883	-0.737889	-2.62022	5.00E-05	0.008238
CG18808,Dhc64C	eggC	eggW	15.2745	9.84154	-0.634166	-2.51565	5.00E-05	0.008238
Obp99a	eggC	eggW	16.0904	52.4462	1.70464	5.36626	5.00E-05	0.008238
CR32028	eggC	eggW	16.9985	31.5667	0.892999	2.31129	0.0001	0.014889
Bruce	eggC	eggW	17.0187	11.3016	-0.590596	-2.29476	0.0005	0.048294
Nplp2	eggC	eggW	17.3626	58.172	1.74434	5.3424	0.0001	0.014889
Osi6	eggC	eggW	18.9016	7.22615	-1.38721	-3.37917	5.00E-05	0.008238
RpL38	eggC	eggW	2166.91	3360.25	0.632928	4.41761	0.0003	0.033035

[a]Fold Change=(base 2) log of the fold change