THE MOLECULAR BASIS OF *WOLBACHIA*-INDUCED CYTOPLASMIC INCOMPATIBILITY

By

Daniel P. LePage

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Approved:
Seth R. Bordenstein, Ph.D.
Katherine L. Friedman, Ph.D.
Brandt F. Eichman, Ph.D.
Julian F. Hillyer, Ph.D.
Andrea Page-McCaw, Ph.D.

To my family, whose support has been unwavering, and to the legions before on whose shoulders I stand.

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The data shown here represent the best of five years of hard work and dedication. These results come from 20,227 individual *Drosophila* matings and (conservatively) over 750,000 counted embryos. This required over 42,000 grape juice-agar plates and was accompanied by at least 7,500 qPCR reactions. All of this, though, would not have been possible without the support of many key people in my life. I am still, and always will be, deeply grateful to my undergraduate research adviser at the University of Tulsa- Dr. Robert Sheaff. I learned more in two and a half years in his lab than I ever did in four years of classes. I've been fortunate to have just as insightful mentors at Vanderbilt as well. My thesis committee- Dr. Katherine Friedman (chair), Dr. Brandt Eichman, Dr. Julian Hillyer, and Dr. Andrea Page-McCaw- have pushed, prodded, and aided me with their intellectual input every step of the way. This dissertation, and my scientific acumen, is much better because of it.

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LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
cDNA	Complementary DNA
CI	Cytoplasmic incompatibility
DCV	Drosophila C virus
DNA	Deoxyribonucleic acid
DUB	Deubiquitinase
EDP	Eliminate Dengue Project
FDR	False discovery rate
GFP	Green fluorescent protein
IIT	Incompatible Insect Technique
MudPIT	Multidimensional Protein Identification Technology
NCBI	National Center for Biotechnology Information
ns	Not significant
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with Triton X-100
PCR	Polymerase chain reaction
PRS	Population Replacement Strategy
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
RT-qPCR	Reverse transcription quantitative PCR
SUMO	Small ubiquitin-like modifier
TA	Toxin-antitoxin
qPCR	Quantitative PCR

CHAPTER I. INTRODUCTION*

Wolbachia pipientis- the greatest pandemic on earth

Marshall Hertig and S. Burt Wolbach first described the α-proteobacterium *Wolbachia pipientis* in the 1920's while studying *Culex pipiens* mosquitoes (Hertig and Wolbach, 1924). Their account of the infection refers to "enormous numbers of small rods filling the cytoplasm of certain cells...so densely packed that they can be distinguished at low magnification." This enigmatic intracellular bacterium would remain a biological quirk, however, until five decades later when it became implicated in complex manipulations of insect reproduction (Yen and Barr, 1971). Over the coming years, a growing body of research would find that, far from a scientific oddity, *Wolbachia pipientis* can actually be classified as one of the most successful infections of the animal kingdom. Estimated to infiltrate 40% of all arthropod species (Zug and Hammerstein, 2012), and a stunning number of filarial nematodes (Ferri et al., 2011), the impact of *Wolbachia* on entire ecosystems is only now beginning to be understood.

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The current body of knowledge shows that *Wolbachia* play a role in diverse areas of biology including general nutrition (Hosokawa et al., 2010) and pathogen defense (Hedges et al., 2008; Teixeira et al., 2008). They have also been implicated in developmental pathways through stem cell proliferation and embryogenesis (Fast et al., 2011) as well as deep host genetic changes through horizontal gene transfer (Dunning Hotopp et al., 2007; Duron, 2013; Metcalf et al., 2014b; Woolfit et al., 2009) and, in some cases, speciation (Bordenstein et al., 2001; Jaenike et al., 2006). This broad range of phenotypes underlines the importance of studying *Wolbachia* and the various interactions they have with multitudes of hosts.

Further complicating *Wolbachia* biology is its infection by a temperate virus known as phage WO, first observed within *Culex pipiens* in 1978 (Wright et al., 1978). WO has lytic capabilities and stably inserts its genes into the *Wolbachia* genome with some strains of *Wolbachia* harboring multiple regions of phage genes (Masui et al., 2000). Importantly, phage WO faces a unique challenge in that it must contend with both *Wolbachia*'s defenses as well as the host in which *Wolbachia* reside. Specifically, WO must first lyse its host *Wolbachia*, travel through the eukaryotic cytoplasm, penetrate the multiple eukaryotic membranes surrounding a new *Wolbachia* target, and then infect that cell. This requires WO to carry eukaryotic-interacting factors, many of which are actively being identified (Bordenstein and Bordenstein, 2016).

After several decades of obscurity, phage WO particles were re-discovered in 2001 within *Teleogrylius taiwanemma* crickets (Masui et al., 2001). Sequenced genomes are now available for WO that infect several different strains of *Wolbachia* including *w*Mel (Wu et al., 2004), *w*Ri (Klasson et al., 2009), *w*Kue (Masui et al., 2000), *w*CauB (Fujii et al., 2004; Tanaka et al., 2009), *w*Rec (Metcalf et al., 2014a), and *w*Pip (Klasson et al., 2008), and there's a growing

appreciation for the role WO plays in *Wolbachia* genome architecture through mobile genetic elements and horizontal gene transfer (Bordenstein et al., 2006; Duron et al., 2006; Kent and Bordenstein, 2011; Sinkins et al., 2005). CHAPTER III builds on this knowledge by identifying two phage WO genes that *Wolbachia* use to manipulate host reproduction.

***Wolbachia*-induced reproductive parasitisms**

Wolbachia's stunning penetrance of the animal kingdom can largely be attributed to its efficient maternal transmission coupled with robust and varied techniques to alter host reproduction. These reproductive parasitisms all act to increase the ratio of infected females in the population and thus guarantee *Wolbachia*'s transmission to the next generation. So far, four such manipulations of host reproduction have been ascribed to *Wolbachia* and are outlined below.

Feminization

Perhaps the most conceptually straightforward way for *Wolbachia* to increase its transmission is to simply turn infected males into functional females. Feminization has been observed in several insect hosts of *Wolbachia* including butterflies {*Eurema hecabe*, (Narita et al., 2007)}, leafhoppers {*Zyginidia pullula*, (Negri et al., 2006)}, and woodlouse {*Armadillidium vulgare* and *Cylisticus convexus*, (Badawi et al., 2015)}. The biological complexity of performing this trick cannot be understated, though, and is still very poorly understood. Recent results suggest that a prophage WO gene within *Wolbachia*'s genome may be involved (Pichon

et al., 2012) and that, in at least one model system, improper segregation of sex chromosomes is key (Kern et al., 2015).

Parthenogenesis

In some populations, *Wolbachia* infection allows females to produce daughters without sexual intercourse. This is most commonly observed in haploid/diploid parasitic wasps species where haploid progeny become males and diploid animals develop into females. Parthenogenesis has been recorded in several genera of these wasps including *Trichogramma* and *Asobara* (Furihata et al., 2015; Schilthuizen et al., 1992; Stouthamer et al., 1993; Watanabe et al., 2013) and has been implicated in at least ten separate species of *Megastigmus* (Boivin et al., 2014). While it's quite likely that *Wolbachia*-induced parthenogenesis occurs through either failed chromosome separation or incomplete cytokinesis during the first developmental cycles, the exact mechanism remains elusive (Serbus et al., 2008).

Male-killing

A common mechanism for *Wolbachia* to increase the fitness of infected females is to kill their male siblings. This strategy liberates resources for the females and aids their, and thus *Wolbachia*'s, survival. Male-killing has been observed in numerous hosts including fruit flies {*Drosophila innubila, D. bifasciata,* and *D. subquinaria* (Dyer and Jaenike, 2004; Hurst et al., 2000; Jaenike, 2007)}, ladybirds {*Coccinella undecimpunctata*, (Elnagdy et al., 2013)}, butterflies {*Hypolimnas bolina*, (Charlat et al., 2005; Duplouy et al., 2010)}, and moths {*Ostrinia furnacalis*, *Ostrinia scapulalis*, and *Ephestia kuehniella* (Fujii et al., 2001; Kageyama et al., 2002)}. The mechanism of *Wolbachia*-induced male-killing is slowly being elucidated

with strong evidence showing that the host sex-determination system is hijacked to induce mortality. So far, male killing appears to be accomplished through targeting of masculinizing genes as well as the dosage compensation complex (Fukui et al., 2015; Sugimoto et al., 2015) though the exact *Wolbachia* factors required still remain unknown.

Cytoplasmic incompatibility

The most common reproductive alteration induced by *Wolbachia* infection is known as cytoplasmic incompatibility (CI) (Serbus et al., 2008). CI is observed as selective embryonic lethality following fertilization of eggs from uninfected females by modified sperm from infected males (Figure I-1). However, progeny of the reciprocal cross as well as crosses between males and females infected with the same *Wolbachia* strain are viable (Figure I-1). This phenomenon gives a large fitness advantage to the infected females relative to uninfected females within a population and rapidly spreads the *Wolbachia* infection. In documented cases, CI has allowed *Wolbachia* to spread from Southern to Northern California in less than a decade (Turelli and Hoffmann, 1995) and throughout Eastern Australia in less than two decades (Kriesner et al., 2013). This has led to several proposals to utilize CI as a potential gene-drive mechanism to control insect populations and especially disease vectors.

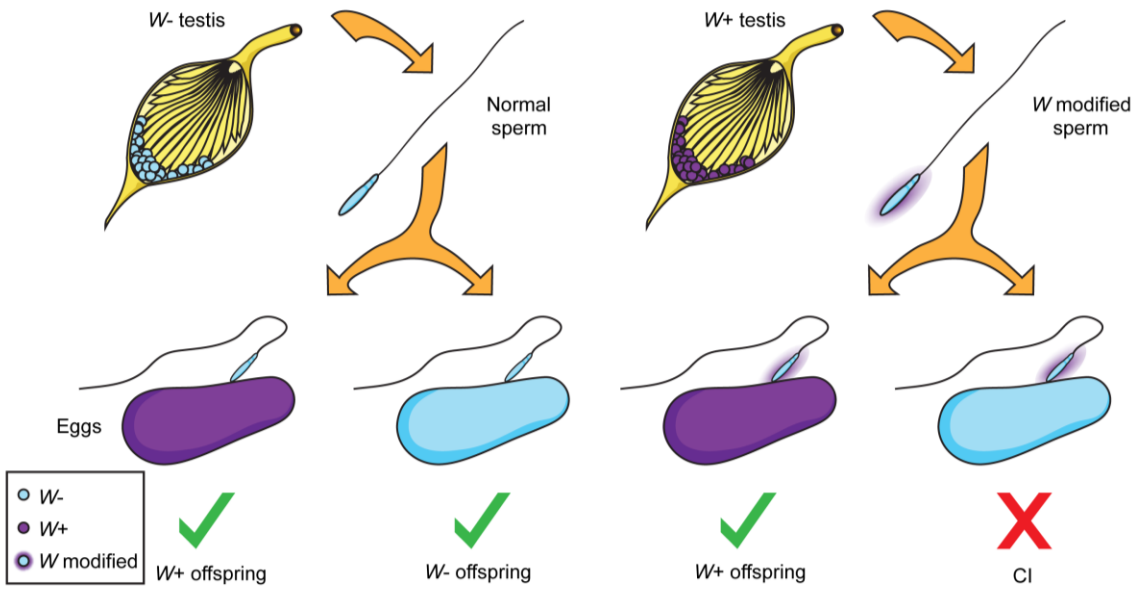

Figure I-1 *Wolbachia*-induced cytoplasmic incompatibility
Wolbachia (*W*, purple) infection causes a modification in the sperm that can be rescued by eggs of infected females but leads to embryonic death in uninfected embryos. Abbreviations: *W*-, *Wolbachia*-uninfected; *W*+, *Wolbachia*-infected; *W* modified, *Wolbachia*-modified sperm. Illustration by Robert M. Brucker.

Commandeering the *Wolbachia* pandemic for vector control

Once studied as an obscure reproductive modification, CI is now at the center stage of efforts to control the transmission of human pathogens through mosquito vectors. This is thanks to a strange quirk in *Wolbachia*-host biology where infection by the bacteria seems to protect against other pathogens. In particular, it has been found that mosquitoes infected with *Wolbachia* have increased resistance against dengue, Chikungunya, yellow fever, Zika, and West Nile viruses, as well as plasmodia and bacteria (Dutra et al., 2016; Glaser and Meola, 2010; van den Hurk et al., 2012; Moreira et al., 2009; Wong et al., 2011). While the biology behind this

Wolbachia-induced defense is still very poorly understood, it does appear to be effective across multiple species (Aliota et al., 2016; Bian et al., 2013; Blagrove et al., 2013; Cattel et al., 2016; Teixeira et al., 2008) and some possible mechanisms, discussed later in this chapter, have been identified.

The twofold advantage of *Wolbachia* to both depress pathogen titers and deterministically spread via CI in insect vectors has direct implications for quelling the transmission of infections to humans. Two strategies take advantage of this system to reduce vector numbers and transmission competency. First, a large release of *Wolbachia*-infected, and therefore pathogen-depleted mosquitoes, could replace the local population of *Wolbachia*-uninfected animals through CI. As discussed below, this Population Replacement Strategy (PRS) (Figure I-2a) has made impressive progress in the past few years via the International Eliminate Dengue Project (EDP). A second strategy, known as the Incompatible Insect Technique (IIT, Figure I-2b), is to release only CI-inducing males into uninfected vector populations, which can then sterilize a large fraction of the females and drastically reduce overall vector numbers (O'Connor et al., 2012). This population suppression has been successfully employed to control farm pests (Apostolaki et al., 2011), and there is an ongoing field study on islands in the Indian Ocean which aims to reduce *Culex pipiens quinquefasciatus* numbers to control filarial parasites and arboviruses (Atyame et al., 2011).

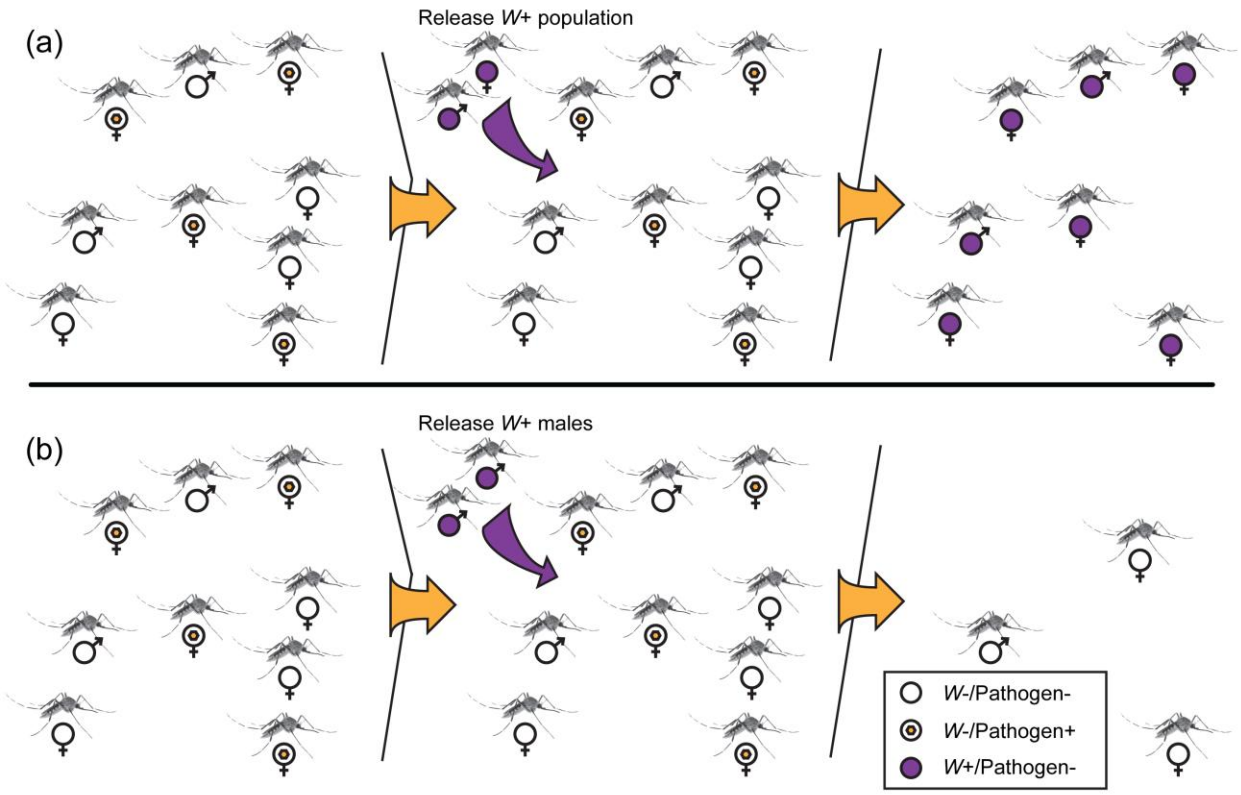

Figure I-2 Vector control strategies
(a) Population Replacement Strategy switches a wild population of mosquitoes (pathogen carrying, *Wolbachia* uninfected) with a pathogen-free one through *Wolbachia*-induced CI. **(b)** In the Incompatible Insect Technique, a release of just *Wolbachia* infected males leads to high levels of CI and a reduction in the total vector population. Abbreviations: *W*-, *Wolbachia*-uninfected; *W*+, *Wolbachia*-infected; Pathogen+, pathogen-infected; Pathogen-, pathogen-uninfected. Illustration by Robert M. Brucker.

Eliminate Dengue Program

The Eliminate Dengue Program was originally established in Australia with the aim of using *Wolbachia*-based strategies to curb the spread of dengue, a mosquito borne disease. Early efforts focused on using the *w*MelPop strain of *Wolbachia* (McMeniman et al., 2009), but in

2011, the EDP stably infected the mosquito vector of dengue, *Aedes aegypti*, with the *w*Mel *Wolbachia* strain from *D. melanogaster* (Walker et al., 2011). The feat was accomplished by passaging the bacteria for several years in an *Aedes albopictus* cell line before microinjection into the mosquitoes. The long term *in vitro* cultivation in mosquito cells led to attenuated virulence in the mosquito species *in vivo* and a normal host lifespan; yet, remarkably, the *w*Mel strain retained high rates of maternal transmission, the capacity to spread through experimental populations by CI, and the crucial ability to repress dengue virus. Controlled release of these mosquitoes into a small number of Australian neighborhoods effectively replaced the native population with a vector that no longer transmits dengue (Hoffmann et al., 2011). While data on whether the population replacement has reduced the incidence of human dengue cases will take many years to assess, the EDP is quickly scaling their approach throughout the world. Recent estimates suggest that dengue infects 390 million people per year with 96 million showing some level of disease severity (Bhatt et al., 2013). The vast majority of these cases are in Southeast Asia and South America where the EDP has research centers in China, Indonesia, Vietnam, and Brazil. These locations will give the EDP a growing influence in the spread of dengue among the most heavily affected areas in the world. This work comes at a critical time as, even though several dengue vaccines are in clinical trials (Schwartz et al., 2015), vaccination has proven to be less effective than hoped and dengue is predicted to spread even further with future climate change (Campbell et al., 2015). The efforts of the EDP are helped by recent work that has analyzed the spread and stability of *Wolbachia* infection in the wild (Dutra et al., 2015; Hoffmann et al., 2014; Ye et al., 2016) as well as updated computer simulations for modeling release programs (Ferguson et al., 2015; Guevara-Souza and Vallejo, 2015; Ndii et al., 2016;

Zhang et al., 2015). Future efforts will also be aided by new research into techniques for increasing the effectiveness of *Wolbachia*-infected mosquito releases (Atyame et al., 2016; Bourtzis et al., 2016; Zhang et al., 2016) as well as contingency plans for any possible developed resistances (Joubert et al., 2016).

The success of the EDP has inspired a broad push to identify applications for *Wolbachia* in other disease vectors. Of particular interest are the anopheline mosquitoes, the main carriers of malaria. Every sampled species of *Anopheles* lacks *Wolbachia*, and while *Anopheles gambiae* can be somatically infected by *Wolbachia* strains from *D. melanogaster* and *Aedes albopictus*, stable germ line infection with high maternal transmission has historically been difficult (Jin et al., 2009; Kambris et al., 2010a). Recently, however, that hurdle was overcome by stably infecting *Anopheline* mosquitoes with microinjections of *Wolbachia* into eggs. The resultant mosquitoes show few defects, induce cytoplasmic incompatibility, and are refractory to *Plasmodium* infection (Bian et al., 2013). This exciting new work now places *Wolbachia*-based control of mosquitoes that transmit malaria within sight.

Caveats to Wolbachia-based vector control

While the discovery of *Wolbachia*-based anti-pathogen resistance (Hedges et al., 2008; Teixeira et al., 2008) has attracted widespread attention for its role in vector control, the mechanism behind this super-charged host protection remains an area of active investigation. It was first hypothesized that *Wolbachia*, by virtue of its transgenerational persistence, simply primes the host immune system and thereby encourages the clearance of viral particles. Indeed, *Wolbachia* heat shock and surface proteins stimulate the expression of innate immunity genes

including cytokines, defensins, proteases, and peptidoglycan recognizing proteins (Kamalakannan et al., 2012; Kambris et al., 2010a; Pinto et al., 2012). These results are countered, however, by data showing that infected cell lines gain protection from viruses (Frentiu et al., 2010) even though they lack several components of the innate immune system, including fat bodies and phagocytosing blood cells. Interestingly, the *Wolbachia* strain *w*MelPop-CLA confers viral protection to both *A. aegypti* and *D. melanogaster* individuals, but it only upregulates immunity genes in the mosquitoes (Rancès et al., 2012). While ′priming′ of the immune system might be insufficient to confer viral resistance, components of the innate immune system such as ROS and the Toll pathway are known to play large roles in *Wolbachia*-induced protection in *A. aegypti* (Pan et al., 2012). Additionally, the innate antiviral siRNA pathway is not required for viral protection in infected *D. melanogaster* (Hedges et al., 2012), and increased immunity is dependent on where *Wolbachia* localizes within *D. simulans* (Osborne et al., 2012). These conflicting results will hopefully be resolved in coming years by comparative studies of species that do not receive pathogen protection from *Wolbachia* infection (Hughes et al., 2012; Longdon et al., 2012) or are actually weakened by the bacteria (Graham et al., 2012). Finally, recent evidence suggests that *Wolbachia*-associated expression of host miRNAs (Hussain et al., 2011; Osei-Amo et al., 2012) assists regulation of dengue virus titers within mosquitoes (Zhang et al., 2013). Finally, release programs such as the EDP have come under renewed scrutiny, particularly with respect to the potential for spread of *Wolbachia* to other species (Loreto and Wallau, 2016), but these issues have largely been addressed by the field (Dobson et al., 2016; Hoffmann et al., 2015; O'Neill, 2016).

How is cytoplasmic incompatibility induced?

Due to its role in insect ecology and disease control, large efforts have focused on the underlying mechanism of CI. Extensive cytological studies of embryonic defects in model insects provide hints as to how CI causes lethality after fertilization. First, the majority of embryonic arrest is associated with shortcomings in the first mitotic division (Tram et al., 2006). They include a failure of the paternal nuclear envelope to break down (Tram, 2002), delayed Cdk1 activation (Tram, 2002), an inability to correctly deposit maternal histones in the paternal genome, and slowed replication of the sperm DNA (Landmann et al., 2009). These delays in cell cycle progression are accompanied by severe chromosomal defects, specifically in the paternal DNA, which include incomplete condensation and failure to segregate correctly. In addition, CI embryos contain an excess of centrosomes that are unassociated with the pronuclei (Callaini et al., 1997; Lassy and Karr, 1996). This latter defect can be explained by a combination of mitotic delays and incomplete chromosome condensation, which are known to dissociate centrosomes from nuclei (Takada et al., 2003).

In the absence of data on the exact sperm modification used to cause embryonic lethality, investigators have turned to conceptual models that may explain CI-associated defects. They are based on the positions that (i) *Wolbachia* modify the sperm to cause severe defects in the timing and progression of mitosis, and (ii) this modification can be ′rescued′ by a female infected with the same strain (Werren, 1997). Males and females infected with genetically-distinct strains are bidirectionally or reciprocally incompatible. For many years, the two predominant models for CI have been (i) the lock and key model and (ii) the mistiming model. The lock and key model posits that *Wolbachia* place certain ′locks′ on the paternal genome. A female infected with the

same strain of *Wolbachia* has the appropriate ′keys′ to remove these locks after fertilization and rescue the mitotic defects that may occur (Poinsot et al., 2003). The mistiming model, however, suggests that CI results from mitotic mistiming between the maternal and paternal pronuclei (Poinsot et al., 2003). An infected female is able to rescue this disparity by making compensatory changes in either pronucleus. The mistiming model can be expanded to suggest that the asynchrony actually occurs between the paternal pronucleus and maternal cytoplasm (Ferree and Sullivan, 2006; Serbus et al., 2008).

While each of the above models has its merits, neither fully explains current observations for CI. For instance, the lock and key model suggests that each strain has its own encrypted locks and keys. The known strain incompatibilities, however, quickly demand a questionably large number of different locks and keys, especially considering the speed with which new incompatibilities arise (Duron et al., 2012). The mistiming model also fails to account for strain specificity. For example, data show that strain *w*San of *Drosophila santomea* is capable of rescuing CI caused by strain *w*Ri of *Drosophila simulans* and that *w*Ri can rescue strain *w*Mel of *D. melanogaster*. When tested, however, *w*San is unable to rescue *w*Mel (Poinsot et al., 1998; Zabalou et al., 2008), an incongruity not explained by the mistiming model. It is obvious that our understanding is incomplete and new simulations, such as the goalkeeper model proposed by Bossan *et al*. (Bossan et al., 2011), are attempting to fill this gap. Briefly, this model posits that a rescuing strain of *Wolbachia* must act as a ′goalkeeper′ to block CI. This action requires the bacteria to utilize two separate ′factors′, which can range from mistiming of the parental genomes to various bacterial proteins or even phage components. These two factors are equivalent to a keeper jumping both far enough and high enough to block a soccer goal. They

could also be altered by host conditions (equivalent to placing the goalkeeper on a stool or in a trench) and thereby explain the dependence on host genotype. This model is supported by growing evidence that multiple factors are involved in rescue (Zabalou et al., 2008).

Finally, it has been known for many years that titers of *Wolbachia* are, in general, positively associated with CI (Bourtzis et al., 1996; Sinkins et al., 1995). Each of the proposed models, whether it's through failure to place enough locks on the host genome, a struggle to induce mistiming, or a lack of sufficient modifying factors, can easily explain how low *Wolbachia* density fails to induce complete cytoplasmic incompatibility. Similar to mistiming, however, infection load cannot fully clarify the mechanism of CI.

Causal factors for CI

Current efforts to identify the causal factor for CI are varied and have largely been unsuccessful. Initial work looking at host gene expression in infected versus uninfected hosts showed that the host histone chaperone *Hira* (Zheng et al., 2011a) and the *D. melanogaster* gene *JhI-26* (Liu et al., 2014) are each partially involved in CI. Unfortunately, data (including results shown in APPENDIX A) show that *Hira* and *JhI-26* each only account for ~30% of the CI phenotype (Zheng et al., 2011a, APPENDIX A). Research has also focused on the unusually large number of *Wolbachia* proteins that contain ankyrin repeat domains. This domain is usually implicated in protein-protein interactions, and was thus a tempting candidate for host modifications. Multiple studies have looked at the link between these proteins and cytoplasmic incompatibility and, while some are regulated in a sex-specific manner, none were shown to be involved in CI (Duron et al., 2007; Papafotiou et al., 2011; Yamada et al., 2010). Finally, there

might be a link between reactive oxygen species (ROS) and the induction of CI. Specifically, *Wolbachia* infection leads to increased levels of ROS in testes and ovaries and these reactive oxygen species lead to damaged spermatid DNA (Brennan et al., 2012). This damage may then be rescued by some yet unknown mechanism in the females. This discovery is interesting as DNA damage induced by ROS can account for several hallmarks of CI including defective paternal chromatin, delayed Cdk1 activation, and failed mitosis. Future research should determine how large a role DNA damage induced by reactive species actually plays in the induction of CI.

Tackling the unknown: finding a mechanism for CI

Through the control of vector (and especially mosquito) populations, *Wolbachia*-induced CI has the possibility to protect hundreds of millions from the debilitating consequences of everything from dengue and Yellow fever to West Nile and Zika (Dutra et al., 2016; Slatko et al., 2014). This promise has led to intense research into the mechanism of CI and how it can be modulated to better control insect ecology. As outlined above, however, the underlying mechanism of CI is still poorly understood. This paucity of data surrounding CI can largely be attributed to the difficulty of working with *Wolbachia.* From a staunch refusal to succumb to genetic manipulations, to defying any growth outside host cells, *Wolbachia* have stymied research into their biology at every step along the way (Serbus et al., 2008). This means that many of the largest questions in the field, questions that were first posited decades ago, remain unanswered. These include:

(i) What *Wolbachia* genes are required for CI?

(ii) What is the full complement of host pathways required for CI induction?

(iii) How are infected females able to rescue CI in a strain-specific manner?

The work presented here attempts to at least partially answer all three of these questions. First, CHAPTER II investigates the role of host DNA methylation and whether this one pathway can account for the missing links still known to exist on the host side of CI biology. Secondly, CHAPTER III demonstrates the first confirmed *Wolbachia* genes to induce CI. Finally, CHAPTER III also tentatively suggests possible mechanisms into the strain-specificity of CI and why there is bidirectional incompatibility between parents infected with different strains of *Wolbachia*. While the following results do not fully answer the largest questions surrounding CI, they do provide an important and long-sought steppingstone that will prove critical in understanding *Wolbachia* and the multifaceted role it plays in evolution, ecology, and human disease.

CHAPTER II. THE RELATIVE IMPORTANCE OF DNA METHYLATION AND DNMT2-MEDIATED EPIGENETIC REGULATION ON WOLBACHIA DENSITIES AND CYTOPLASMIC INCOMPATIBILITY†

Abstract

Wolbachia pipientis is a worldwide bacterial parasite of arthropods that infects host germline cells and manipulates host reproduction to increase the ratio of infected females, the transmitting sex of the bacteria. The most common reproductive manipulation, cytoplasmic incompatibility (CI), is expressed as embryonic death in crosses between infected males and uninfected females. Specifically, *Wolbachia* modify developing sperm in the testes by unknown means to cause a post-fertilization disruption of the sperm chromatin that incapacitates the first mitosis of the embryo. As these *Wolbachia*-induced changes are stable, reversible, and affect the host cell cycle machinery including DNA replication and chromosome segregation, we hypothesized that the host methylation pathway is targeted for modulation during cytoplasmic incompatibility because it accounts for all of these traits. Here we show that infection of the testes is associated with a 55% increase of host DNA methylation in *Drosophila melanogaster*,

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but methylation of the paternal genome does not correlate with penetrance of CI. Overexpression and knock out of the *Drosophila* DNA methyltransferase Dnmt2 neither induces nor increases cytoplasmic incompatibility. Instead, overexpression decreases *Wolbachia* titers in host testes by approximately 17%, leading to a similar reduction in CI levels. Finally, strength of CI induced by several different strains of *Wolbachia* does not correlate with levels of DNA methylation in the host testes. We conclude that DNA methylation mediated by *Drosophila*'s only known methyltransferase is not required for the transgenerational sperm modification that causes CI.

Introduction

Wolbachia pipientis, an obligate intracellular bacteria, is estimated to infect approximately 40% of all arthropod species (Zug and Hammerstein, 2012). This widespread prevalence can be attributed to efficient maternal transmission of the infection, intermediate rates of horizontal transmission to new hosts, and strong manipulations of the host reproductive system to enhance its maternal transmission (Serbus et al., 2008; Stouthamer et al., 1999). These sexual alterations all act to increase the number of infected females within a population and include male-killing, feminization, parthenogenesis, and cytoplasmic incompatibility (CI). CI is the most common defect observed in *Wolbachia*-infected hosts and has been documented in numerous species (Serbus et al., 2008).

CI acts as a post-fertilization mating barrier by preventing the development of embryos from uninfected females that are mated with *Wolbachia*-infected males. This zygotic defect can be rescued, however, by females infected with the same strain of *Wolbachia* present in the male. This rescue capability gives a strong fitness advantage to *Wolbachia*-infected females and can

lead to rapid sweeps of the infection through host populations. For instance, CI-inducing *Wolbachia* have been able to spread across most of the *Drosophila simulans* population in eastern Australia in less than a decade (Kriesner et al., 2013). Cytoplasmic incompatibility is also a major isolation barrier between young sibling species (Bordenstein et al., 2001; Jaenike et al., 2006; Miller et al., 2010) and is currently being used as a genetic drive mechanism to eliminate dengue virus in *Aedes aegypti* populations (Bian et al., 2010; Moreira et al., 2009; Walker et al., 2011) and to generally reduce mosquito population sizes (Laven, 1967; O'Connor et al., 2012).

The evolutionary, ecological, and medical importance of cytoplasmic incompatibility has fueled decades of research seeking to understand its underlying mechanisms. However, apart from studies that suggest the host genes JhI-26 and HIRA are involved (Liu et al., 2014; Zheng et al., 2011a), it remains unknown how *Wolbachia* in the testes encode a sperm modification that renders embryos inviable. Previous work elucidated a few post-fertilization hallmarks of CI, most of which are associated with defects in the paternal genome during embryogenesis. These changes include a failure of maternal histones to deposit correctly, prolonged or incomplete replication of the paternal DNA, and failed condensation of the paternal chromosomes (Breeuwer and Werren, 1990; Callaini et al., 1997; Landmann et al., 2009). The alterations of the paternal chromatin and host cell cycle lead to a failure of the first mitosis followed by embryonic death. Interestingly, *Wolbachia* are not actually present within the sperm of their hosts, indicating a semi-permanent modification of the paternal genome that is transgenerationally transmitted to the egg (Clark et al., 2008).

Several assumptions can be made about the paternal genome modification underlying cytoplasmic incompatibility including:

(i) It targets host pathways that are highly conserved across numerous host species

(ii) It involves a semi-permanent but reversible alteration to the paternal genome

(iii) It must be able to affect histone recruitment, DNA replication, and chromosome condensation

Working under these assumptions, we selected the host DNA methylation pathway as a probable target for *Wolbachia*. Methylation is a stable, yet reversible, modification to DNA that could be sex-specific and easily rescued by infected females. It also has the capability to modulate many cell cycle functions including chromosome condensation and histone recruitment (Bird, 2001; Harris and Braig, 2003; Weber and Schübeler, 2007) and has previously been hypothesized to play a role in CI (Liu et al., 2014; Negri, 2011; Saridaki et al., 2011; Ye et al., 2013a). While the role of DNA methylation in insects is not fully understood it is a highly conserved pathway that shows strong upregulation during embryogenesis (Field et al., 2004). Finally, the ability of bacteria to alter host methylation and chromatin structure is increasingly being recognized (Bierne et al., 2012; Gómez-Díaz et al., 2012) and previous work shows that *Wolbachia* infection in particular alters the host methylation profile in both leafhoppers and mosquitoes (Negri et al., 2009; Ye et al., 2013b).

Here we use the model organism *Drosophila melanogaster* infected with the *w*Mel strain of *Wolbachia* to determine the role of host DNA methylation in cytoplasmic incompatibility. *D. melanogaster* flies utilize just one canonical DNA methyltransferase, *Dnmt2* (Lyko et al., 2000), which enables easy genetic manipulation of the host methylation pathway without the confounding influence of other DNA methyltransferases (*Dnmt1* and *Dnmt3*) present in most other insect species (Group et al., 2010). While the role of *Dnmt2*-dependent methylation is

debated and multifaceted (Raddatz et al., 2013; Schaefer and Lyko, 2010; Takayama et al., 2014), evidence demonstrates that the methylation machinery in *D. melanogaster* is not only present but also functional (Gou et al., 2010; Kunert et al., 2003; Lyko et al., 2000; Schaefer et al., 2008). Moreover, overexpression of the mouse *Dnmt3a* in *D. melanogaster* induces CI-like defects such as reduced rates of cell cycle progression and altered chromosome condensation (Weissmann et al., 2003).

Results

Wolbachia wMel increases levels of testes DNA methylation

MethylFlash analysis of host DNA from testes revealed that infection with the *Wolbachia* strain *w*Mel in *Drosophila melanogaster* increases levels of genome-wide cytosine methylation (Figure II-1a). More importantly, this methylation is specific to the host testes (55% increase, P = 0.0015, Mann Whitney U test) and is not observed in the ovaries, consistent with the prediction that only the paternal genome is modified during cytoplasmic incompatibility. The overall levels of methylation are extremely low, which is consistent with previously reported levels of methylation in *Drosophila melanogaster* (Kunert et al., 2003; Lyko et al., 2000). Conflicting reports over the strength and prevalence of DNA methylation in *D. melanogaster* (Lyko et al., 2000; Raddatz et al., 2013; Schaefer and Lyko, 2010) led us to test the validity of our initial results with genome-wide bisulfite sequencing. Results indicate that, contrary to most other species, DNA methylation in *Drosophila melanogaster* is not CpG specific and is evenly distributed over cytosine residues (Figure II-1b, Table II-1). Sequencing results also mirror those of MethylFlash and show that infection with *w*Mel increases testes DNA methylation 46% across

all cytosine residues with a range of 43-54% depending upon the type of cytosine residue (CpG, CHG, or CHH) (Figure II-1b). The minor discrepancies between MethylFlash and bisulfite sequencing (55% and 46% increase in methylation, respectively) are likely due to the sensitivity of the MethylFlash system on such low quantities of methylation. A more thorough investigation of the bisulfite sequencing, including changes in promoter and gene body methylation has failed to find large discrepancies between *Wolbachia* infected and uninfected individuals.

Table II-1 Bisulfite sequencing of *D. melanogaster* testes DNA

	Uninfected	**Infected**
Sequences analyzed	108555753	105859973
Mapping efficiency:	70.80%	57.90%
Total number of cytosines analysed	859563011	762501109
Total methylated cytosines in CpG context	2373418	3030855
Total methylated cytosines in CHG context	2206386	2823679
Total methylated cytosines in CHH context	7459059	9767927
C methylated in CpG context	1.40%	2.00%
C methylated in CHG context	1.30%	2.00%
C methylated in CHH context	1.40%	2.10%

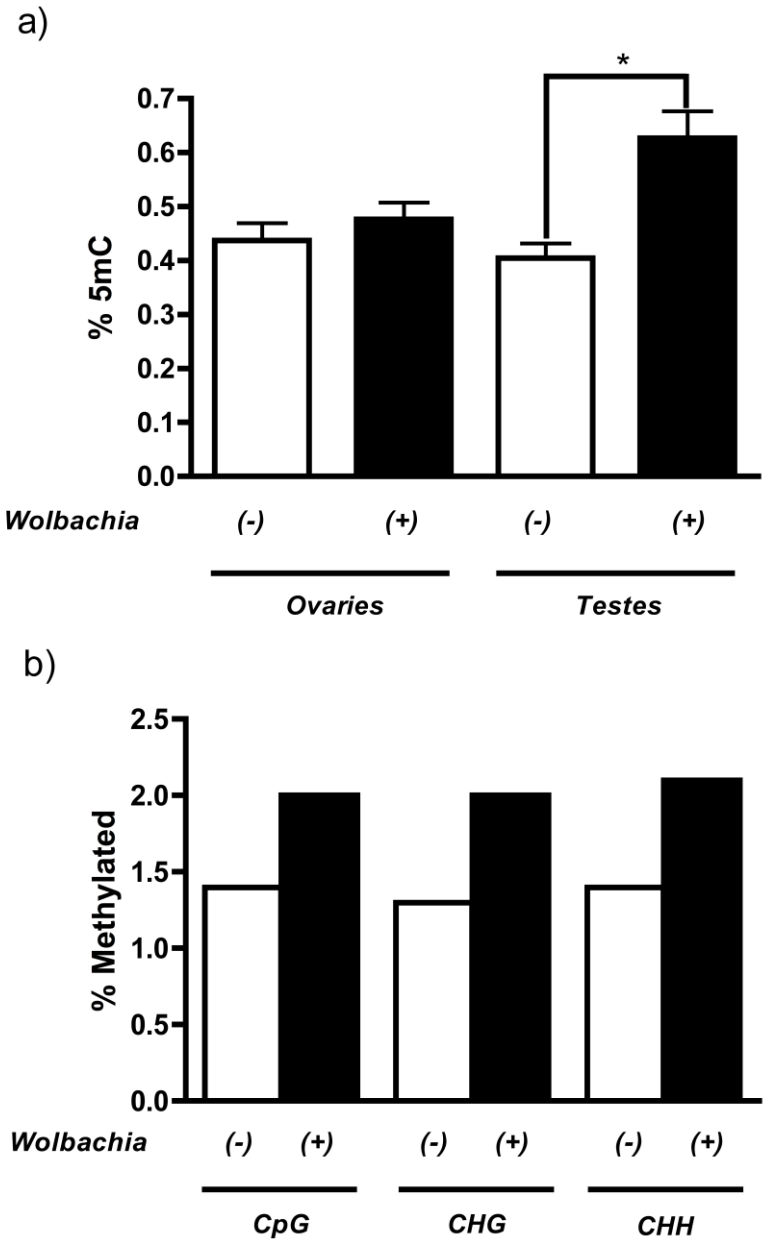

Figure II-1 *Wolbachia* increase host levels of DNA methylation

(a) *Wolbachia* infection (*w*Mel) of *Drosophila melanogaster* increases DNA methylation in host testes by 55% (P = 0.0015, Mann-Whitney U (MWU) test, two-tailed), as measured by the ELISA-based MethylFlash kit. This increase is not observed in host ovaries (P = 0.25). Bars denote standard error of the mean (SEM) (b) Bisulfite sequencing of *Drosophila melanogaster* testes DNA shows that infection by *w*Mel increases methylation of all cytosine residues including CpG (43%), CHG (54%), and CHH (50%).

Overexpression of Dnmt2 neither induces nor strengthens CI

Drosophila melanogaster possess just one canonical DNA methyltransferase, *Dnmt2*, and overexpression of this enzyme in fruit flies has previously been shown to increase levels of DNA methylation (Kunert et al., 2003; Schaefer et al., 2008). Utilizing the Gal4-UAS expression system, we overexpressed *Dnmt2* in uninfected males to test if an increase in host methylation alone could induce the CI defect of reduced embryo hatching rates. Figure II-2 shows that there was no discernable difference in hatching rates with uninfected males expressing increased or wild type levels of *Dnmt2* (P = 0.91, MWU).

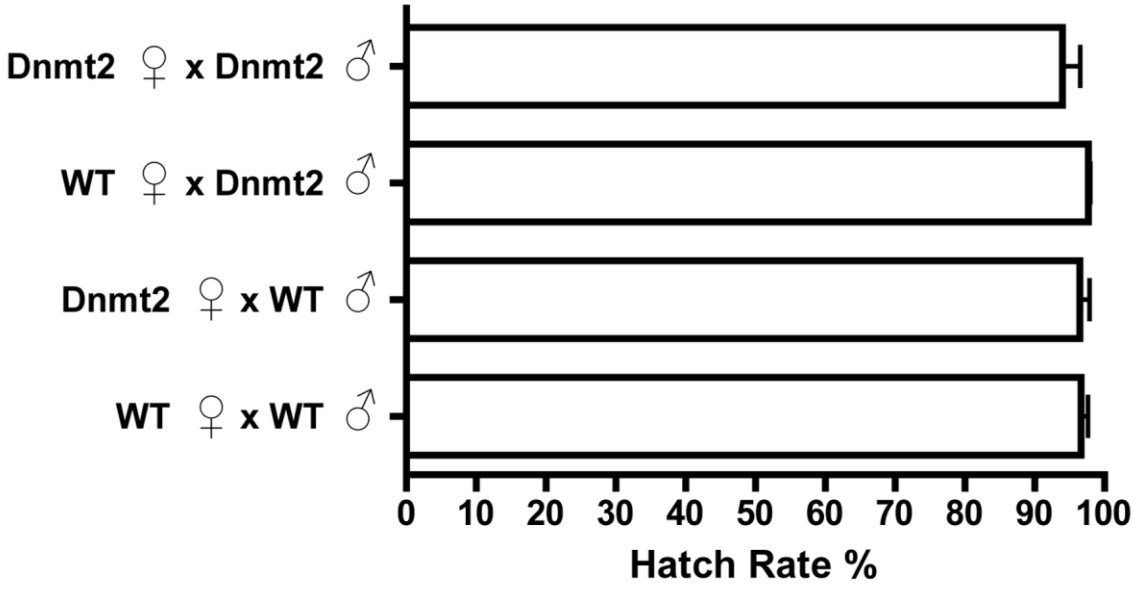

Figure II-2 Expression of DNA methyltransferase 2 does not induce CI
Overexpression of the DNA methyltransferase *Dnmt2* in uninfected males, utilizing the Gal4-UAS system with a *nos* driver, does not reduce hatching rates. Dnmt2 = overexpressing flies; WT = wild type flies. Bars denote standard error of the mean (SEM).

The result was confirmed using an Actin-based driver that again yielded no discernable differences in hatch rates compared to wild type flies (Figure II-3, P = 0.83, MWU). These

findings specify that amplified levels of *Dnmt2*-mediated epigenetic regulation are not sufficient to recapitulate cytoplasmic incompatibility.

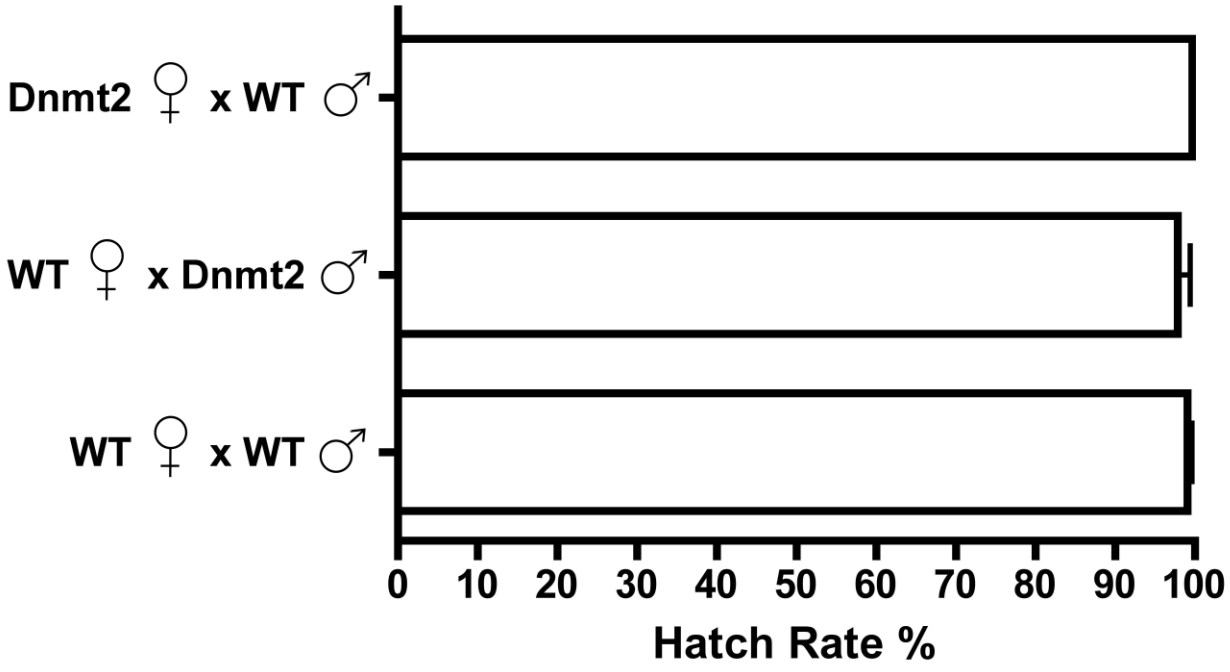

Figure II-3 Dnmt2 overexpression by an Actin driver does not induce CI
Overexpression of *Dnmt2* by an Actin-Gal4 driver does not induce CI in uninfected males. Bars denote SEM. Dnmt2 = overexpression flies; WT = wild type flies.

If multiple factors are responsible for CI, it is possible that overexpression of *Dnmt2*, while unable to induce CI in uninfected flies, may be able to strengthen the modification in the presence of *Wolbachia*. To test this hypothesis, we overexpressed *Dnmt2* in *Wolbachia*-infected males that were then mated to uninfected virgin females. Surprisingly, *Dnmt*2 overexpression in males decreased the level of cytoplasmic incompatibility by an average of 17.4% (Figure II-4). This effect is not dependent on the *Dnmt*2 expression status of the female and suggests that increased methylation of host DNA can diminish the penetrance of cytoplasmic incompatibility.

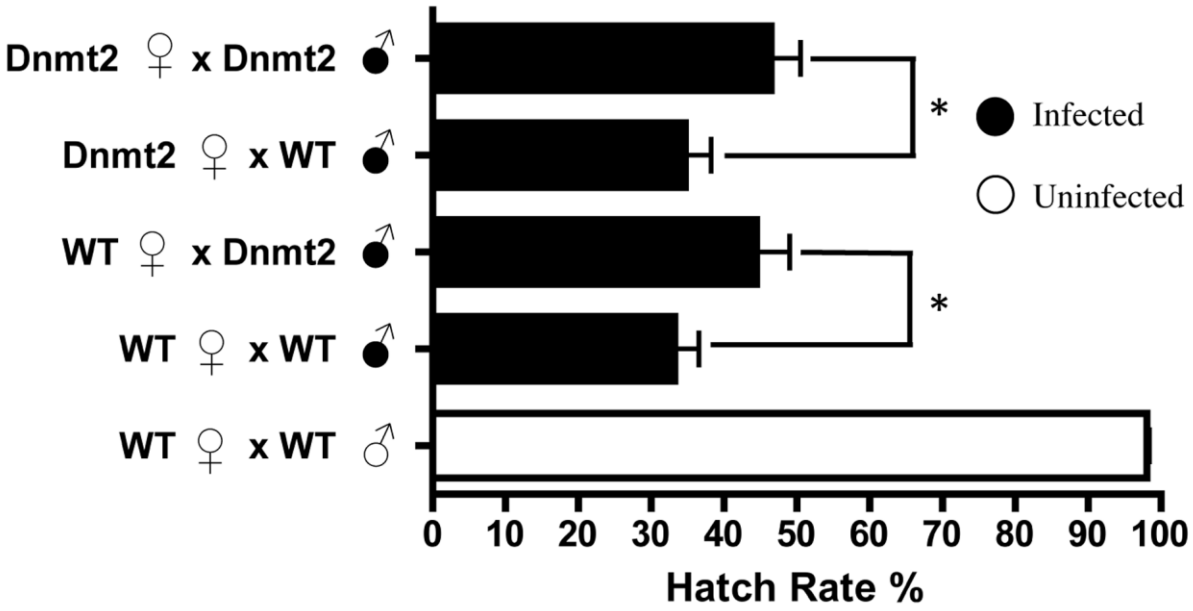

Figure II-4 Overexpression of *Dnmt2* reduces levels of CI
The overexpression of *Dnmt2* in *Wolbachia*-infected males decreases rates of CI ($P < 0.05$, Mann-Whitney U test). *Dnmt2* expression in the mother has no affect. Bars denote standard error of the mean (SEM). Dnmt2 = overexpressing flies; WT = wild type flies.

Overexpression of Dnmt2 reduces Wolbachia titers in host testes

Previous work suggested that *Dnmt*2 is detrimental to *Wolbachia* proliferation in mosquitoes. In fact, *Wolbachia* strain *w*MelPop-CLA utilizes a host miRNA to downregulate *Dnmt2* expression when infecting *Aedes aegypti* (Zhang et al., 2013). We observed no differences in Dnmt2 expression between *w*Mel infected and uninfected *D. melanogaster* testes (data not shown) but hypothesized that overexpression of *Dnmt2* in the host may adversely affect *Wolbachia* titers. In support of this prediction, we found that *Wolbachia* density (as measured by the ratio of *Wolbachia groEL* gene copy number / *Drosophila Actin* gene copy number)

decreased by 17.3% in adult testes overexpressing *Dnmt2* transcripts by 9.6% (Figure II-5 and Figure II-6, respectively).

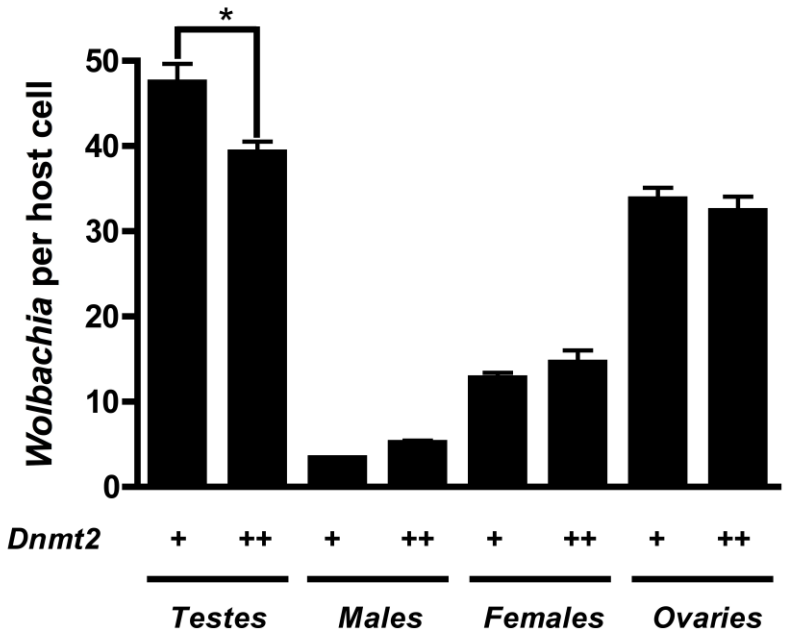

Figure II-5 *Dnmt2* overexpression alters *Wolbachia* titers
Overexpression of *Dnmt2* reduces *Wolbachia* titers within the testes ($P < 0.01$, MWU test) but has no affect on titers within ovaries or whole flies. *Wolbachia* infection is derived from the y^1w^* *Drosophila* background. Bars denote standard error of the mean (SEM). Dnmt2 ++ = overexpressing flies; Dnmt2 + = wild type flies. ($P = 0.007$, Mann-Whitney U test, two-tailed).

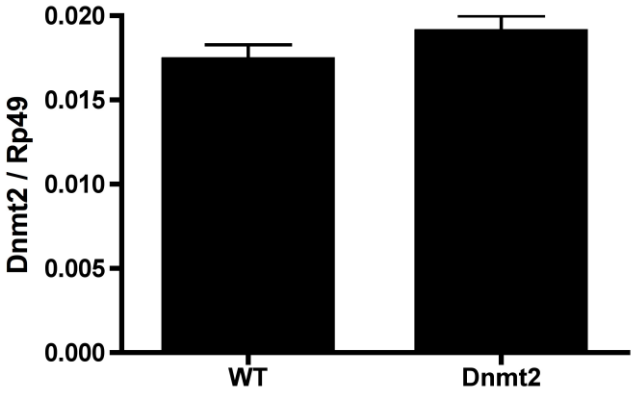

Figure II-6 Overexpression of *Dnmt2* in host testes
Dnmt2 is overexpressed 9.6% compared to wild type in testes using a *nanos*-Gal4 driver. Bars denote SEM. WT = wild type. Dnmt2 = *Dnmt2* overexpressing. Rp49 is used as a control for gene expression.

The low level of transcript overexpression could be specific to the developmental stage of the experimental sample or due to usage of a pUAST vector for germline expression instead of the more efficient pUASP (Kunert et al., 2003). While the upregulation of *Dnmt2* in infected males is not statistically significant, it remains possible that actual protein levels are much higher than those represented by RNA transcripts. Strong protein expression, as measured by Western blot, was seen in uninfected ovaries (data not shown). Nevertheless, the 17.3% decrease in *Wolbachia* titers compares well with the 17.4% reduction in CI penetrance reported above. Expression of the negative control green fluorescent protein (GFP) did not reduce *Wolbachia* titers, as expected (Figure II-7). As the bacterial and phage density models of CI specify that *Wolbachia* titers in the testes are linked to the strength of CI (Bordenstein et al., 2006; Breeuwer and Werren, 1993), we conclude that the reduction of CI observed in *Dnmt2*-overexpressing males is likely due to reduced *Wolbachia* density.

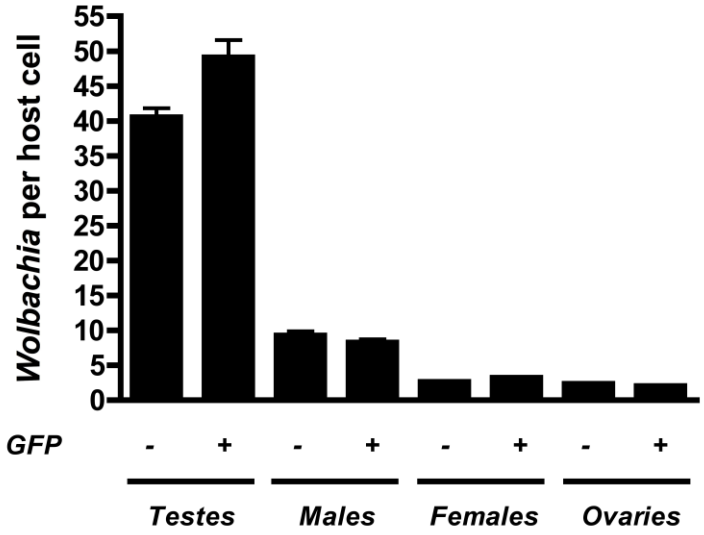

Figure II-7 *Wolbachia* titers are not decreased by GFP expression
Expression of green fluorescent protein (GFP) does not reduce *Wolbachia* titers, as measured in whole males, females, testes, and ovaries. *Wolbachia* infection arises from the y^1w^* background. Bars denote SEM. +/- indicates whether sample express GFP.

Even though we do not observe any change in *Dnmt2* mRNA levels after *Wolbachia* infection, we cannot rule out that *Wolbachia* may be affecting intracellular *Dnmt2* localization rather than levels of gene expression. An increase in localization of *Dnmt2* to the nucleus would not only protect the cytosolic *Wolbachia* but also explain the additional genomic methylation associated with infection. In this scenario, the testes-specific increase in host methylation initially observed would simply be a by-product of high *Wolbachia* activity. Additionally, an immunomodulatory role for *Dnmt2* in *Drosophila* has already been documented in protection against RNA viruses (Durdevic et al., 2013) though we believe the findings in this report are the first evidence for a putative antibacterial role for *Dnmt2* in fruit flies.

Hosts defective in DNA methylation still exhibit CI

As *Dnmt2* overexpression did not induce nor increase cytoplasmic incompatibility, we next tested the strength of CI in hosts defective in the methyltransferase pathway. Knockout mutants for *Dnmt2* characterized by Goll et al (Goll et al., 2006) were acquired and found by PCR and amplicon sequencing to be infected by the *w*Mel strain of *Wolbachia*. The strain is hereafter referred to as Mut and was tetracycline treated for 3 generations to create the uninfected line MutT. We show by MethylFlash that the increase in host DNA methylation induced by *Wolbachia* infection is abolished in the knockout Mut background (Figure II-8) and is thus *Dnmt2*-dependent.

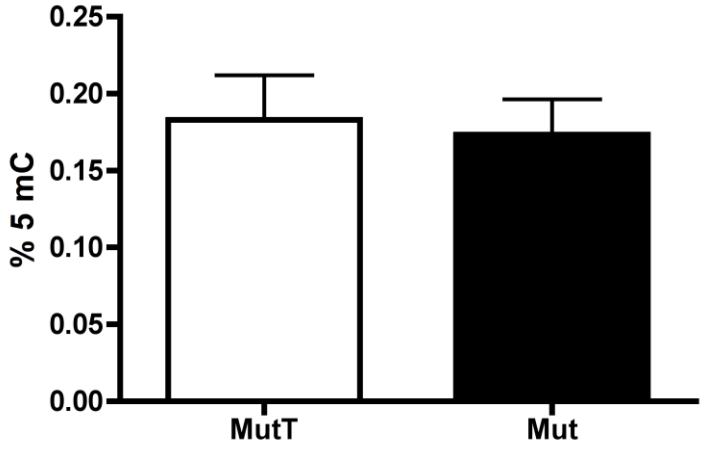

Figure II-8 *Wolbachia*-induced change in host methylation is *Dnmt2* dependent
Testes from Drosophila melanogaster Dnmt2 mutants do not exhibit Wolbachia-induced increase in DNA methylation as measured by MethylFlash. Wolbachia infection arises from the w^{1118} background. Bars denote SEM. MutT = uninfected, Dnmt2 mutant. Mut =Wolbachiainfected, Dnmt2 mutant.

However, loss of this crucial enzyme in the DNA methylation pathway has no effect on the penetrance of CI (Figure II-9), as shown in comparisons between mutant and wild type males mated to uninfected females (P = 0.13, MWU). The low level of DNA methylation still present

in mutants has recently been observed by others (Boffelli et al., 2014) and suggests a possible mechanism of DNA methylation in *Drosophila* that is independent of canonical DNA methyltransferases. Thus, it is possible that CI could be induced by alterations in genomic methylation but in a *Dnmt2*-independent manner.

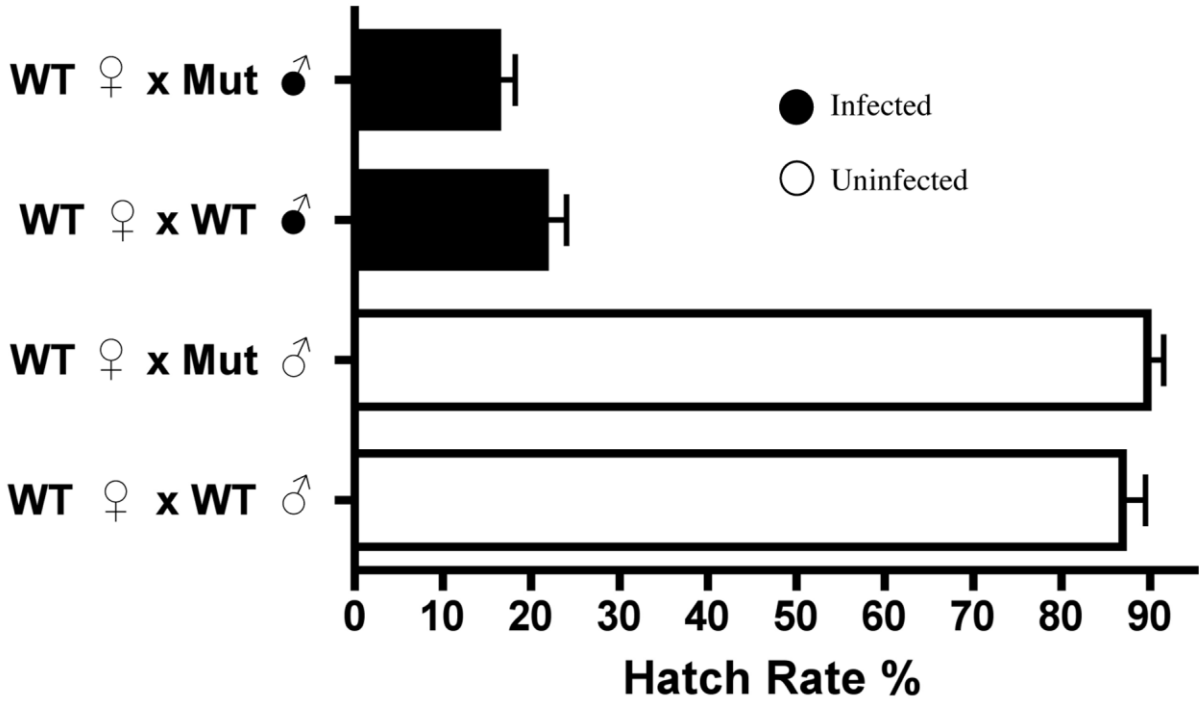

Figure II-9 *Dnmt2* mutants express wild-type levels of CI
Crosses with *Dnmt2*-mutant males ("Mut") show that *Dnmt2* expression within the father is not necessary for expression of CI. Mut = Dnmt2 mutant flies; WT = wild type flies. Bars denote standard error of the mean (SEM).

Curiously, despite the previously observed role for *Dnmt2* in host immunity (Durdevic et al., 2013; Zhang et al., 2013), the mutants observed here exhibit no increase in *Wolbachia* titers within any of the tissues tested (Figure II-10). It is interesting to note that *Dnmt2* mutant *Drosophila*, derived from the w^{1118} background line, have titers that are, on average, half of those

seen in y^1w^* background lines (see Figure II-5 and Figure II-10). This difference has been observed several times in our experiments and suggests either a differing ability of the host lines to control *Wolbachia* titers or an as yet unclassified difference in the *w*Mel strains infecting these flies.

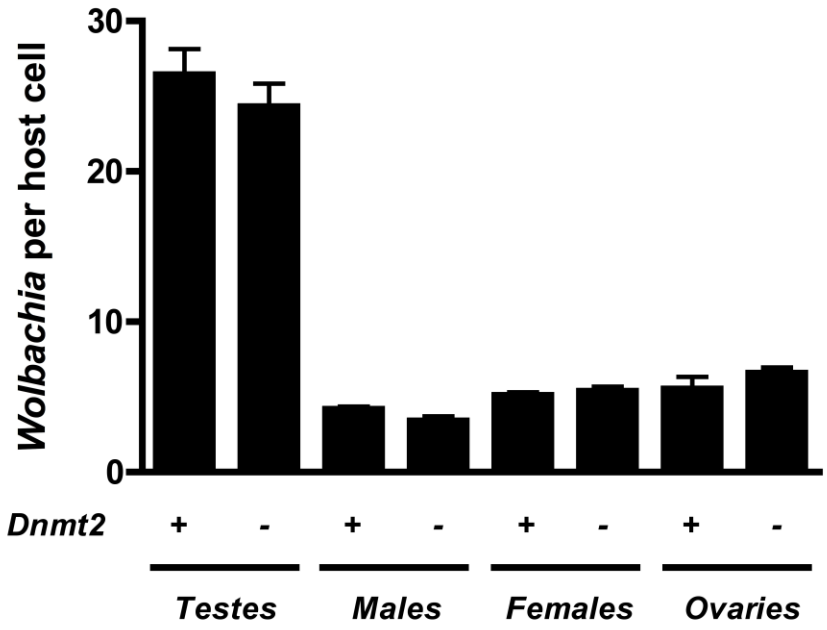

Figure II-10 *Dnmt2* mutants harbor normal *Wolbachia* titers
Loss of *Dnmt2* does not affect *Wolbachia* titers in *Drosophila melanogaster*. Bars denote SEM. Dnmt2 + = wild type flies. Dnmt2 - = *Dnmt2* mutant flies

Host levels of DNA methylation do not correlate with strength of CI

To substantiate the claim that DNA methylation is not involved in the induction of CI for other *Wolbachia* strains and/or host species, we tested the DNA methylation status of testes DNA from *Drosophila* species infected with various strains of *Wolbachia*. These taxa include *D. simulans* infected with strains *w*Ri, *w*No, and *w*Au, which express strong, moderate, and no CI, respectively. We also tested a different *D. melanogaster*-infecting strain *w*Mel derived from the

w^{1118} background strain instead of y^1w^*. As previously mentioned, while the w^{1118} line induces strong CI, *Wolbachia* titers are much lower in these animals compared to the infection found in y^1w^*.

Results show that methylation status of the infected host testes is random with regards to the strength of CI (Figure II-11). While infection with the high CI-inducer *w*Ri exhibits higher methylation in infected testes as compared to uninfecteds, this effect is marginally insignificant (P = 0.072, MWU) and is countered by data from the *w*Au strain, which causes no CI but still significantly increases host DNA methylation in testes (P = 0.0047, MWU). Furthermore, infection with the *w*No strain of *Wolbachia*, which causes moderate CI, actually has less methylation in host testes. Finally, a low-titer infection of *w*Mel (w^{1118}), while still inducing CI, does not induce the same level of DNA methylation associated with a high-density infection (y^1w^*).

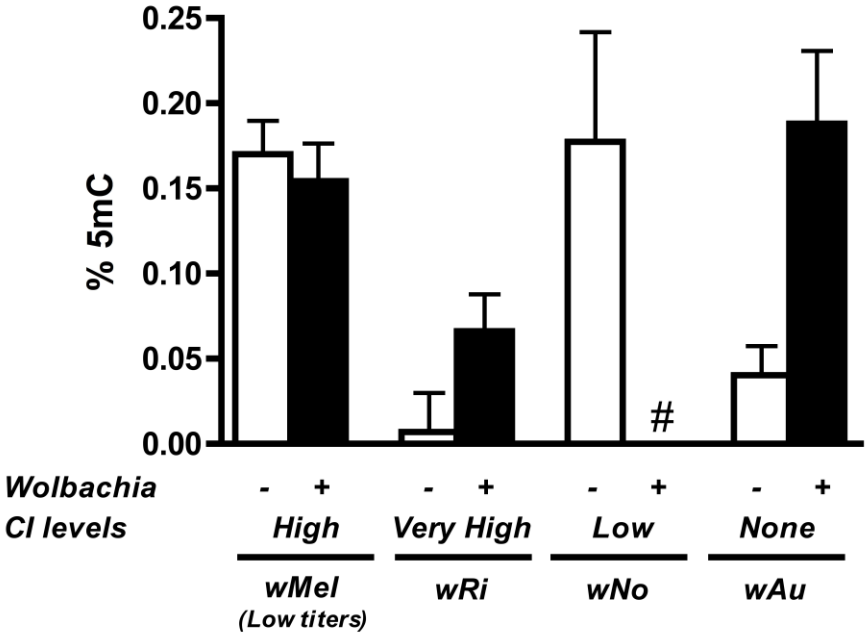

Figure II-11 Levels of host DNA methylation do not correlate with strength of CI
Testing of several different *Wolbachia* infections, capable of inducing various levels of CI in their respective hosts, shows that levels of host DNA methylation and strength of CI are not correlated. Bars denote standard error of the mean (SEM) of testes DNA methylation, as measured by MethylFlash. White bars (-) denote uninfected flies and black bars (+) denote infected flies. # indicates levels of methylation too low for detection.

Discussion

The underlying mechanism of *Wolbachia*-induced CI largely remains elusive after several decades of research. Here we show that host DNA methylation, a promising candidate pathway hypothesized to play a role (Liu et al., 2014; Negri, 2011; Saridaki et al., 2011; Ye et al., 2013a), does not seem to be involved in the induction of cytoplasmic incompatibility. While *Wolbachia* infection preferentially increases host DNA methylation in *Drosophila melanogaster* testes (Figure II-1), this modification is not conserved across other CI-causing strains of *Wolbachia* (Figure II-11) and overexpression of a host methyltransferase neither induces nor

increases rates of CI. We have also found that *Wolbachia*-induced changes in host methylation are dependent on the DNA methyltransferase *Dnmt2* (Figure II-8) but that *Drosophila melanogaster* lacking *Dnmt2* still suffer from CI (Figure II-9). Finally, we found *Dnmt2* has anti-*Wolbachia* properties, as previously reported in *Aedes aegypti* (Zhang et al., 2013), and overexpression of *Dnmt2* reduces the strength of CI.

Taken together, we show that one of the canonical chromatin modification pathways, *Dnmt2*-dependent DNA methylation, likely has no role in *Wolbachia*-induced cytoplasmic incompatibility. *Wolbachia* infection can be associated with changes in host methylation levels, but it is most likely a consequence of the bacteria modulating host immune response or the host defending itself against the infection. The possibility also remains that infection alters gene-specific, and *Dnmt2*-independent, levels of methylation that our current study of genomic methylation levels has not detected. While further investigation of the *Dnmt2* epigenetic pathway will not elucidate a CI mechanism, it may be useful in studying the complex nature of pathogen-host interactions between *Wolbachia* and the many species it infects. It remains possible that a novel methyltransferase, recently suggested to exist in *Drosophila* (Boffelli et al., 2014; Takayama et al., 2014), could affect CI.

Conclusions

This study, published in 2014, marks the first attempt to investigate the link between host epigenetic changes and *Wolbachia*-induced CI. Host DNA methylation was an intriguing possibility for modification by *Wolbachia* as it accounts for many of the hallmarks of CI previously seen. Specifically, DNA methylation plays roles in transcription, histone deposition,

and chromosome condensation, all of which are altered within embryos suffering from CI (Landmann et al., 2009). DNA methylation is also reversible, providing a ready mechanism for infected females to rescue chromatin defects, and is found throughout the animal kingdom, which could account for the prevalence of CI among diverse species.

The work outlined here shows that, despite the interesting prospects and apparent relevance to CI, host methylation pathways do not appear to be required for *Wolbachia*-induced cytoplasmic incompatibility. Important results do suggest, though, that *Wolbachia* alter DNA methylation within *D. melanogaster* either as a direct mechanism for, or perhaps a side effect of, increasing *Wolbachia* titers. This expands on previous knowledge showing that *Wolbachia* alter methylation within other species (Negri et al., 2009; Ye et al., 2013b). Interestingly, it is well established that dengue virus requires the host DNA methylation pathway for efficient replication. *Wolbachia*, on the other hand, seeks to downregulate or shuttle DNA methylation factors out of the host cytoplasm (Durdevic et al., 2013), thus placing them out of the virus' reach. This could at least partially explain the mechanism through which *Wolbachia* provides resistance to dengue within mosquitoes- one of the largest outstanding questions for the field. Future work could determine if this mechanism is conserved throughout other hosts and whether it could also explain *Wolbachia*-induced refractoriness to other diseases such as West Nile, Chikingunya, and yellow fever.

Materials and Methods

Fly rearing and dissections

All flies were reared on a cornmeal and molasses-based media at 25°C. The *Dnmt2* loss-of-function mutant has been previously described (Goll et al., 2006). Briefly, the mutant contains a 28bp insertion with multiple stop codons as well as a frameshift within the coding region of *Dnmt2*. Overexpressing flies were created through the Gal4-UAS system. Crosses were performed between virgin *nos*-Gal4 driver females (y^1w^*; *P{w[+mC]=GAL4-nos.NGT}40* (either *Wolbachia*-infected or uninfected) and 5-6 uninfected UAS-*Dnmt2* (Kunert et al., 2003), UAS-GFP or w^{1118} males. Crosses for Figure II-3 were conducted between virgin *Act5c-Gal4* driver females (y^1w^*;*P{w[+mC]=Act5C-GAL4}25FO1/CyO,*y^+ , *Wolbachia* infected or uninfected depending on desired progeny) and *UAS-Dnmt2* males. For *Act5c-Gal4* crosses, straight-winged progeny were assumed to be overexpressing *Dnmt2* while *CyO* expressing lines were used as the wild-type expressing lines. *Wolbachia*-uninfected lines were created through tetracycline treatment (20ug/mL for 3 generations) and infection status was confirmed through PCR using primers which target the 16S rRNA gene of *Wolbachia* (see APPENDIX B). These lines were further reared for at least three generations on undrugged media before experimentation to avoid detrimental paternal effects seen in other systems (Zeh et al., 2012).

*w*Au, *w*No, and *w*Ri (also known as *w*Ri Agadir) strains of *Drosophila simulans* were kindly provided by Charlat Sylvain (University Lyon, France). All testes and ovary dissections were performed in cold phosphate buffered saline (PBS). Males were dissected within 24 hours of emergence while females were aged 3-4 days before dissections. Testes

samples consisted of tissue obtained from a minimum of 20 males while ovary samples were pooled from 10 females each. Tissues were frozen and stored at -80^{o}C before analysis.

Hatch rate assays

Assays were performed using a grape juice/agar media in 30mm plates for egg laying. For each cross 32-48 individual crosses of one male and one female were set up in separate mating chambers with individual grape juice plates. A minimal amount of a 1:2 dry yeast and water mix was added to each plate and the parents were allowed to mate for 16 hours before the grape juice plates were discarded. Fresh plates were then used for 24 hours, removed, and the number of eggs laid was counted for each cross. The number of unhatched eggs was counted again at 36 hours after the plates had been removed to determine hatch rates.

MethylFlash quantification of DNA methylation

Genomic levels of cytosine methylation (5-mC) were measured using the MethylFlash kit (Epigentek; Farmingdale, NY, USA). 8-10 replicate sets of testes (20-40 testes pairs each replicate) were dissected and DNA was isolated using the Puregene Tissue kit (Qiagen, Venlo, Netherlands). 100ng of genomic DNA from each sample was used and each sample was analyzed in duplicate on a BMG LabTech FLOUstar OPTIMA plate reader (Ortenberg, Germany) according to manufacturer instructions.

Wolbachia density

Eight replicates each of whole animals (pools of 3), testes (pools of 20 pairs), and ovaries (pools of 10 pairs) were collected and DNA was isolated. All males were less than 24 hours old

while females had been aged 3-4 days. Quantitative PCR was performed on a Bio-Rad CFX96 Real-Time System using iTaq Universal SYBR Green Supermix (Bio-Rad; Hercules, CA, USA). *groEL* copy number, determined against a standard curve, was compared to counts for the host gene *Actin*, also determined against a standard curve. It was assumed that one copy of *groEL* was present in each *Wolbachia* genome and 1 or 2 copies of Act5c (for males and females, respectively, as the gene is on the X chromosome) in each *Drosophila* genome. Primers: Act5c and groEL (see APPENDIX B). qPCR conditions: 50^{o} 10 min, 95^{o} 5 min, 40x (95^{o} 10 sec, 55^{o} 30 sec), 95^{o} 30 sec. Followed by melt curve analysis (0.5^{o} steps from 65-95^{o} for 5 sec each).

Gene expression

Quantitative PCR was performed on a Bio-Rad CFX96 Real-Time System using iTaq Universal SYBR Green Supermix (Bio-Rad; Hercules, CA, USA). RNA was isolated from 8 sets of testes (20 pairs each) using the RNeasy Mini kit (Qiagen; Venlo, Netherlands) and DNA was removed with the TURBO DNA-free DNase kit (Ambion; Grand Island, NY, USA). cDNA was synthesized using a SuperScript III First-Strand kit (Invitrogen; Grand Island, NY, USA) and diluted 1:20. All calculations were done using delta delta Ct with Rp49 expression used for normalization of results. Primers: Dnmt2 and Rp49 (see APPENDIX B). qRT-PCR conditions are the same as used in qPCR for *Wolbachia* densities.

Bisulfite sequencing

100 testes were dissected in PBS from *Wolbachia* infected ($y^{1}w^{*}$) and uninfected males and flash frozen. gDNA was then isolated using the Puregene kit (Qiagen; Venlo, Netherlands) and fragmented by Covaris shearing. gDNA was submitted to Vanderbilt Technologies for

Advanced genomics (VANTAGE) where the PE-75 bp library was generated using the TruSeq sample preparation kit (with methylated adapters), bisulfite treated, PCR amplified (EpiMark and ZymoTaq) and sequenced (Illumina HiSeq 2000, 86bp PE read). Sequences with $\geq$10x coverage were analyzed using Bismark (Krueger and Andrews, 2011) and cytosines which were methylated in at least one read were counted.

CHAPTER III. WOLBACHIA-INDUCED CYTOPLASMIC INCOMPATIBILITY IS CAUSED BY PROPHAGE WO GENES[‡]

Introduction

The genus *Wolbachia* is an archetype of maternally inherited intracellular bacteria that infect the germline of millions of invertebrate species worldwide and parasitically alter arthropod sex ratios and reproductive strategies to increase the proportion of infected females (the transmitting sex) in the population. The most common of these reproductive manipulations is cytoplasmic incompatibility (CI), typically expressed as embryonic lethality in crosses between infected males and uninfected females. This lethality is completely rescued by females infected with the same or a similar *Wolbachia* strain. Despite more than 40 years of research (Yen and Barr, 1971), the genes by which *Wolbachia* cause CI remain unknown. Here, we use comparative genomic, transcriptomic, proteomic and transgenic approaches to elucidate two genes that are CI effectors. In the *Wolbachia* strain *w*Mel, the phage WO-encoded operon (Kent and Bordenstein, 2011) consisting of WD0631 and WD0632 recapitulates significant degrees of CI in transgenic

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male *Drosophila melanogaster* that express both genes. The transgene-induced CI causes cytological defects similar to wild type CI, and it is fully rescued by *w*Mel-infected females. The discovery of these two cytoplasmic incompatibility factor genes (*cifA* and *cifB*) represents an important step forward in understanding the genetics of reproductive parasitism and has implications for symbiont-induced speciation (Brucker and Bordenstein, 2012; Shropshire and Bordenstein, 2016) and control of agricultural pests (Zabalou et al., 2004) and disease vectors that spread dengue virus (O'Connor et al., 2012; Walker et al., 2011), Zika virus (Dutra et al., 2016), and other human pathogens.

Results and Discussion

CI candidate gene discovery and evolution

We hypothesized that the genes responsible for CI would be present in all CI-inducing *Wolbachia* strains but absent or divergent in strains that are mutualists or that do not induce CI; we also predicted that these genes would be relatively highly expressed in the gonads of infected insects. To elucidate CI effector candidates, we determined the core genome shared by the CI-inducing *Wolbachia* strains *w*Mel, *w*Ri, *w*Pip (Pel), and the recently sequenced *w*Rec, which helped narrow the list of candidate prophage WO genes associated with reproductive parasitism (Metcalf et al., 2014a), while excluding the pan-genome of the mutualistic strain *w*Bm. This analysis yielded 113 gene families representing 161 unique *w*Mel genes (Figure III-1a, Table C-1).

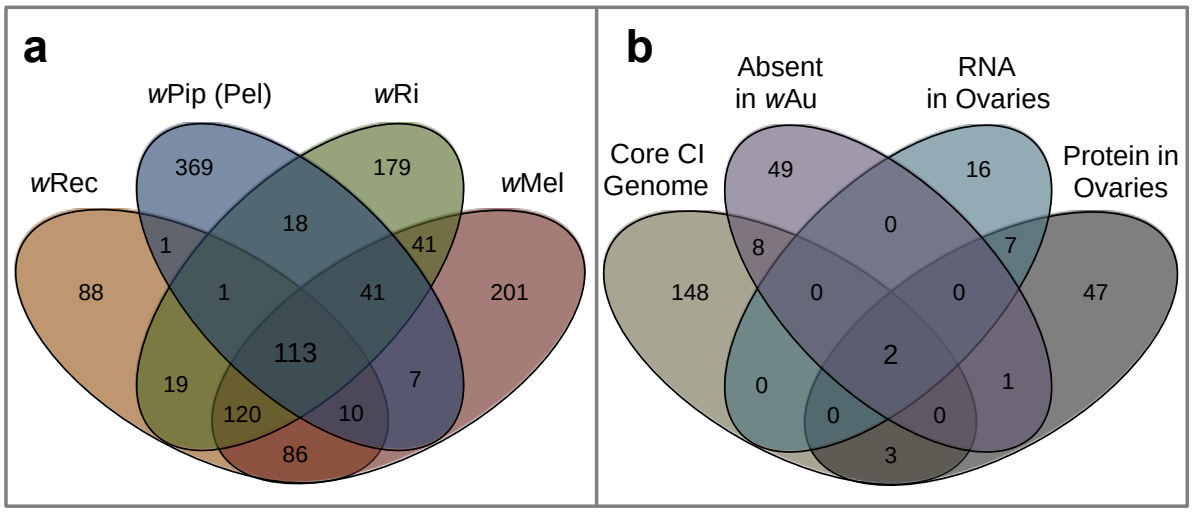

Figure III-1 CI candidate gene selection
(a) Venn diagram illustrating unique and shared gene sets from four CI-inducing *Wolbachia* strains. The number of gene families in common between strains is indicated for each combination. (b) Venn diagram illustrating the number of unique *w*Mel genes matching each criteria combination.

Next we streamlined this candidate list by comparing it to (i) homologs of genes previously determined by comparative genomic hybridization to be absent or divergent in the strain *w*Au (Ishmael et al., 2009), which does not induce CI, (ii) homologs to genes that are highly expressed at the RNA level in *w*VitA-infected *Nasonia vitripennis* ovaries, and (iii) homologs detected at the protein level in *w*Pip (Buckeye)-infected ovaries of *Culex pipiens* mosquitoes. Remarkably, only two genes, those whose *w*Mel locus tags are WD0631 and WD0632, were shared among all four gene subsets (Figure III-1b, Table C-2). Notably, the homolog of WD0631 in the *Wolbachia* strain *w*Pip, *w*Pa_0282, was found at the protein level in the fertilized spermathecae of infected mosquitoes, lending support to the gene's role in reproductive manipulation (Beckmann and Fallon, 2013).

We found that homologs of both genes are always associated with prophage WO in the *Wolbachia* chromosome (Bordenstein and Bordenstein, 2016). This is interesting as the WO phage that infects *w*Mel is incomplete and unlikely to make viable phage particles (Figure III-4). Therefore, these candidate genes have been maintained within the *Wolbachia* genome independent of an active phage infection and are likely providing some benefit to *Wolbachia.* Further, we analyzed the evolution and predicted protein domains of these two genes and found that they codiverge into three distinct phylogenetic groups that we designate type I, II, and III (Figure III-2a,c). These relationships are not recapitulated in the phylogeny of the *Wolbachia* cell division gene *ftsZ*, which exhibits the typical bifurcation of A and B *Wolbachia* (Figure III-3a), or in the phylogeny of phage WO baseplate assembly gene *gpW* (Figure III-3b). This suggests that WD0631 and WD0632 are evolving under different evolutionary pressures than the core *Wolbachia* genome and active phage WO haplotypes.

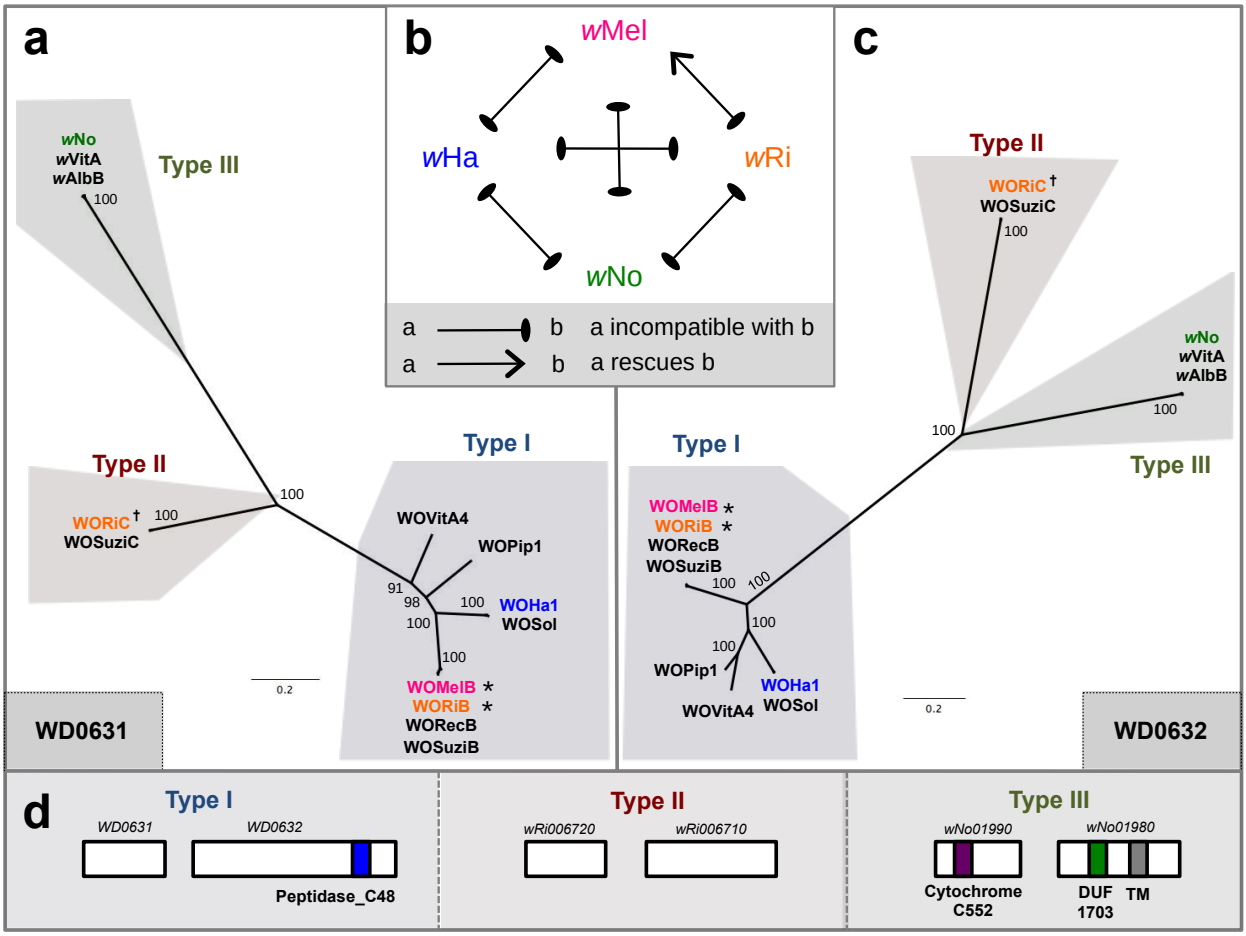

Figure III-2 CI candidate gene evolution

Bayesian phylogenies of WD0631 (a) and WD0632 (c) and their homologs are shown based on a 256-aa alignment of WD0631 reciprocal BLASTp hits and a 462-aa alignment of WD0632 reciprocal BLASTp hits. When multiple similar copies of the same operon exist in the same strain, only one copy is shown. Consensus support values are shown at the nodes. Both trees are based on the JTT+G model of evolution and are unrooted. (b) CI patterns correlate with WD0631/WD0632 operon homology. *w*Ri rescues *w*Mel and both share a similar operon (*). The inability of *w*Mel to rescue *w*Ri correlates with an operon type (†) that is present in *w*Ri but absent in *w*Mel. Likewise, bidirectional incompatibility of all other crosses correlates to divergent operons. This diagram was adapted from Bossan et. al. 2011. (d) Protein architecture of WD0631/WD0632 homologs is conserved for each clade and is classified according to the WD0632-like domain: Type I features Peptidase_C48; Type II lacks an annotated functional domain; and Type III features DUF1703. TM stands for transmembrane domain. For (a) and (c), the WO-prefix indicates a specific phage WO haplotype and the w-prefix refers to a "WO-like island," a small subset of conserved phage genes, within that specific *Wolbachia* strain.

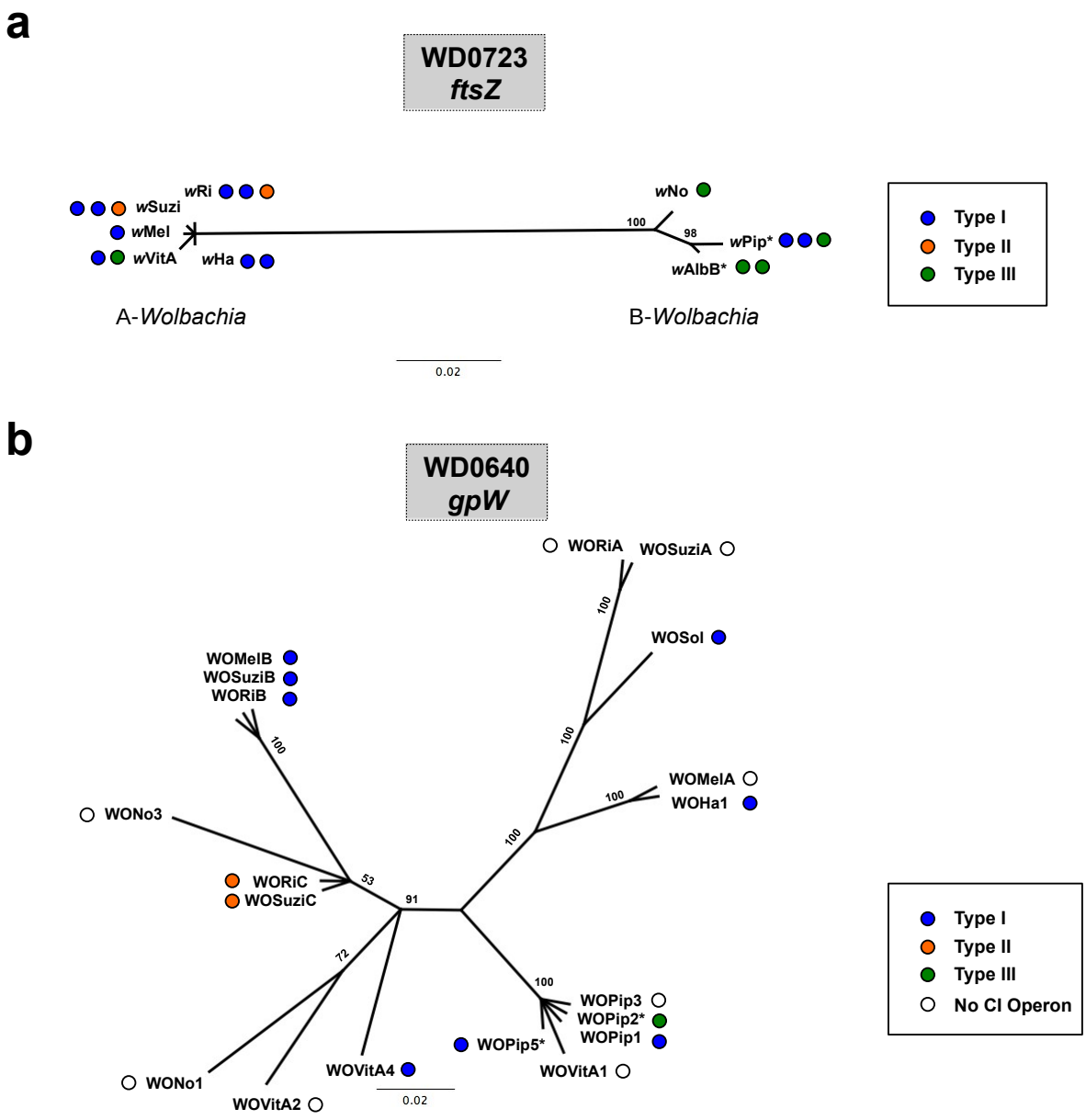

Figure III-3 Evolution of *Wolbachia* or phage WO

(a) Bayesian phylogenies based on a 393-aa alignment of WD0723, the *w*Mel *ftsZ* gene, and its homologs and (b) a 70-aa alignment of WD0640, the phage WO *gpW* gene, and its homologs. Trees are based on JTT+G and CpRev+I models of evolution, respectively, and are unrooted. Consensus support values are shown at the nodes. (*) indicates that the CI operon is not included in Figure III-2. The WOPip5 operon is truncated while the WOPip2 and second *w*AlbB operons are highly divergent from WD0632.

Type I genes are the most prevalent amongst sequenced *Wolbachia* strains, and are always associated with large but incomplete phage WO regions that are missing important tail genes likely needed for active phage (Figure III-4). Although the function of type I WD0631 homologs are unknown, type I WD0632 homologs contain a peptidase_C48 domain (Figure III-2d), a key feature of Ulp1 (ubiquitin-like-specific protease) proteases (Beckmann and Fallon, 2013), which catalyze the maturation of small ubiquitin-like modifier (SUMO) propeptides and can play a role in regulating cell cycle progression in eukaryotes (Li and Hochstrasser, 1999). A number of bacteria and viruses are known to usurp SUMOylation pathways in the manipulation of their hosts (Wimmer and Schreiner, 2015; Wimmer et al., 2012). Type II WD0631 and WD0632 homologs are located within more complete phage haplotypes (Figure III-4), but the WD0632 homologs are truncated and lack recognized protein domains (Figure III-2d). Notably, all *Wolbachia* strains that contain type II homologs invariably contain at least one other copy of the operon that is type I and intact. Type III WD0631 homologs possess a cytochrome C552 domain involved in nitrate reduction, while type III WD0632 homologs contain a domain of unknown function (DUF1703) and a transmembrane domain (Figure III-2d). The functions of these domains are less well understood, but DUF1703 likely possesses nuclease activity (Knizewski et al., 2007) and was previously found in a selfish genetic element that mediates embryonic lethality in *Tribolium* beetles (Lorenzen et al., 2008).

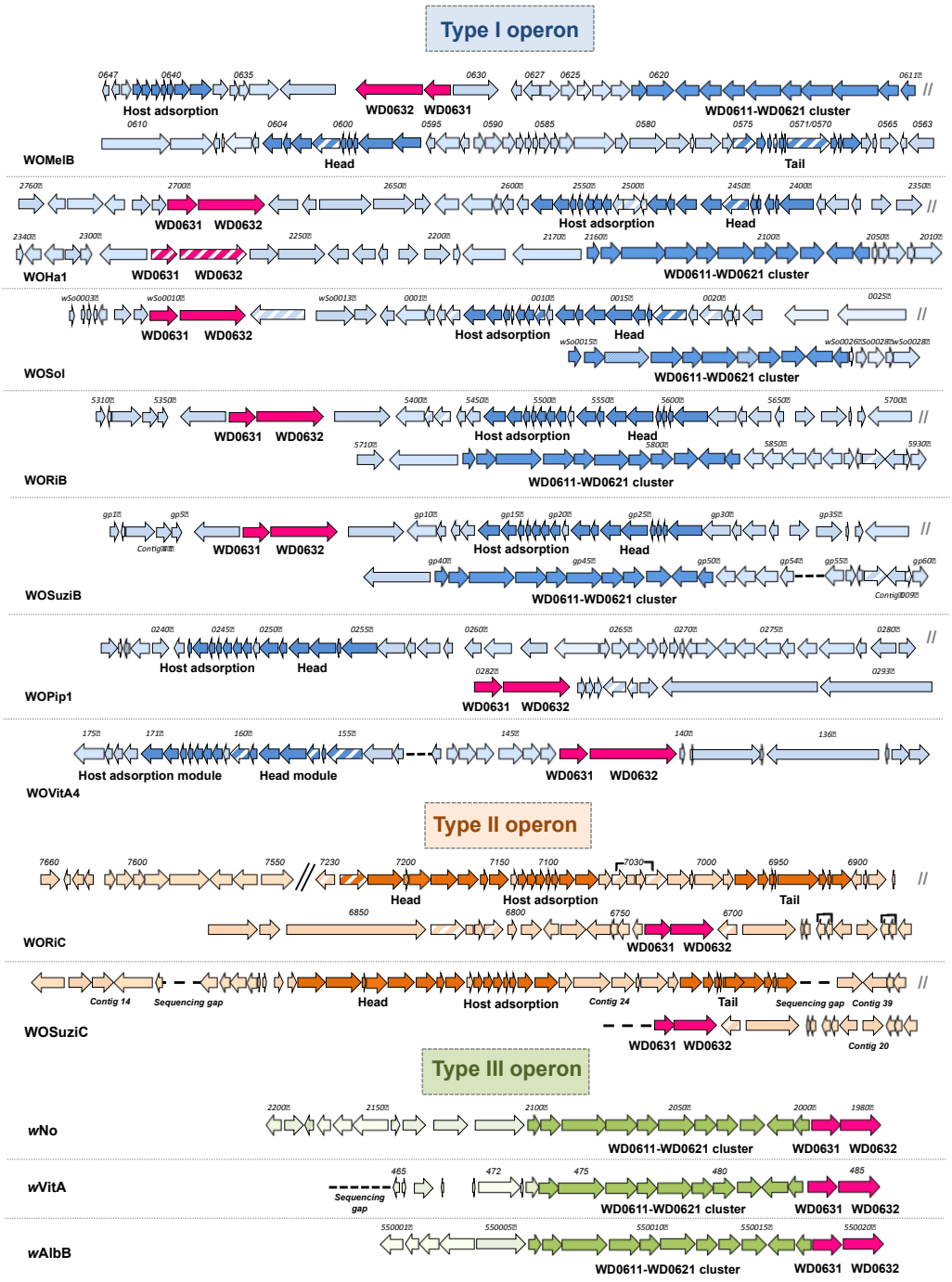

Figure III-4 WD0631/WD0632 operon is always associated with prophage WO regions

CI operons are labeled and colored pink. Structural modules are labeled as host adsorption, head or tail. The WD0611-WD0621 label highlights a conserved gene cluster that is often associated with the CI operon. Only one phage haplotype is shown per *Wolbachia* strain when multiple copies of the same operon type are present.

CI patterns correspond to candidate gene homology and copy number

Consistent with these genes' role in CI, the degree of relatedness and presence or absence of shared operons of WD0631 and WD0632 between *Wolbachia* strains correlates with known patterns of bidirectional incompatibility (Figure III-2b). Among the strains *w*Ri, *w*Ha, and *w*No, only *w*Ri is able to rescue *w*Mel-induced CI (Poinsot et al., 1998; Zabalou et al., 2008). We postulate that this is due to the fact that *w*Ri and *w*Mel share a highly related type I operon (99% amino acid identity), and thus likely also have a shared rescue factor, while *w*Ri has an additional type II operon that may explain its ability to induce CI against *w*Mel. Meanwhile, *w*Ha has at most a 67% identity in the amino acid sequence of these proteins when compared to *w*Mel, while *w*No contains a type II operon that is only 31% identical (Figure III-5a). Additionally, the strength of CI varies considerably between different *Wolbachia* strains, and the relative degree of offspring lethality correlates with the number of copies of the WD0631/WD0632 operon that are present in each strain (Figure III-5b). Those strains with only one copy, such as *w*Mel, have a comparatively weak CI phenotype, while those with two or three copies of the operon, such as *w*Ri and *w*Ha, cause strong CI (Poinsot et al., 1998).

a

WD0631-like % aa identity

	WORiB	WORiC	WOHa1	*w*No
WOMelB	99	46	67	31
WORiB		46	68	31
WORiC			44	33
WOHa1				31

WD0632-like % aa identity

	WORiB	WORiC	WOHa1	*w*No
WOMelB	99	30	62	29
WORiB		30	62	29
WORiC			31	36
WOHa1				30

b

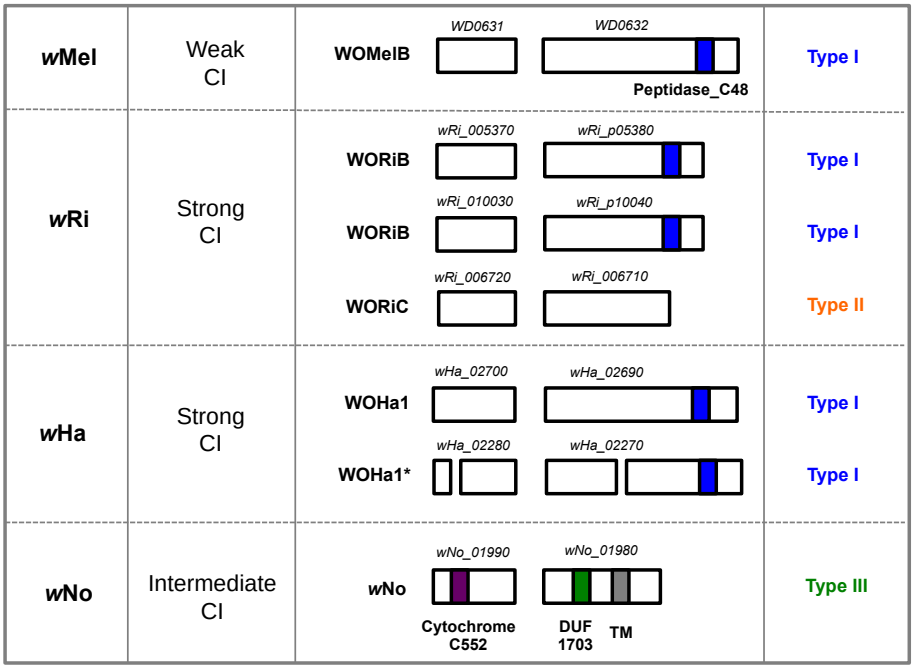

Figure III-5 *Wolbachia* CI patterns correlate with WD0631/WD0632 operon similarity and copy number

(a) The % amino acid (aa) identity between homologs for each cif protein correlates with *Wolbachia* compatibility patterns. The only compatible cross, *w*Mel males x *w*Ri females, features a shared operon between WOMelB and WORiB. All other crosses are greater than 30% divergent and are bidirectionally incompatible. Each "% aa identity" value is based on the region of query coverage in a 1:1 BLASTp analysis. (b) CI strength, protein architecture and operon type are listed for each of the *Wolbachia* strains shown in Figure III-2b. (*) indicates the proteins are disrupted and not included in comparison analyses.

WO phage gene expression correlates with CI penetrance

Given the many lines of evidence in support of these two genes, we next examined whether expression of WD0631 and WD0632, as well as control *w*Mel genes, correlate with

expression patterns expected of putative CI factors. These control genes are WD0034, which encodes a PAZ (Piwi, Argonaut, and Zwille) domain containing protein, and two prophage WO genes - WD0508, which encodes a putative transcriptional regulator, and WD0625, which encodes a DUF2466 domain likely acting as a nuclease or regulatory protein. We first examined the expression of CI effector candidates in the testes of *w*Mel-infected, one-day-old and seven-day-old *D. melanogaster* males through qRT-PCR. Since the magnitude of CI is known to decrease dramatically between newly emerged and one-week-old males (Yamada et al., 2007), we predicted that a CI effector would be expressed at a lower level in older male testes. Indeed, while WD0631 and WD0632 are expressed at different levels, both show a significantly lower transcription level in older versus younger males (Figure III-6a,b), as measured relative to the *Wolbachia* housekeeping gene *groEL*. Both phage-encoded control genes, WD0508 and WD0625, also exhibited this pattern, but the non-phage gene WD0034, did not (Figure III-6c-e). WD0640, which encodes phage WO structural protein gpW, was also reduced in older males, suggesting that phage genes in general are relatively downregulated in seven-day-old testes (Figure III-6f). The phenomenon of decreased CI in older males is not due to decreases in *Wolbachia* titer over time, as the copy number of *Wolbachia groEL* relative to *D. melanogaster Rp49* increases as males age, and there is no significant difference in the absolute *Wolbachia* gene copies between one-day-old and seven-day-old males (Figure III-7a,b).

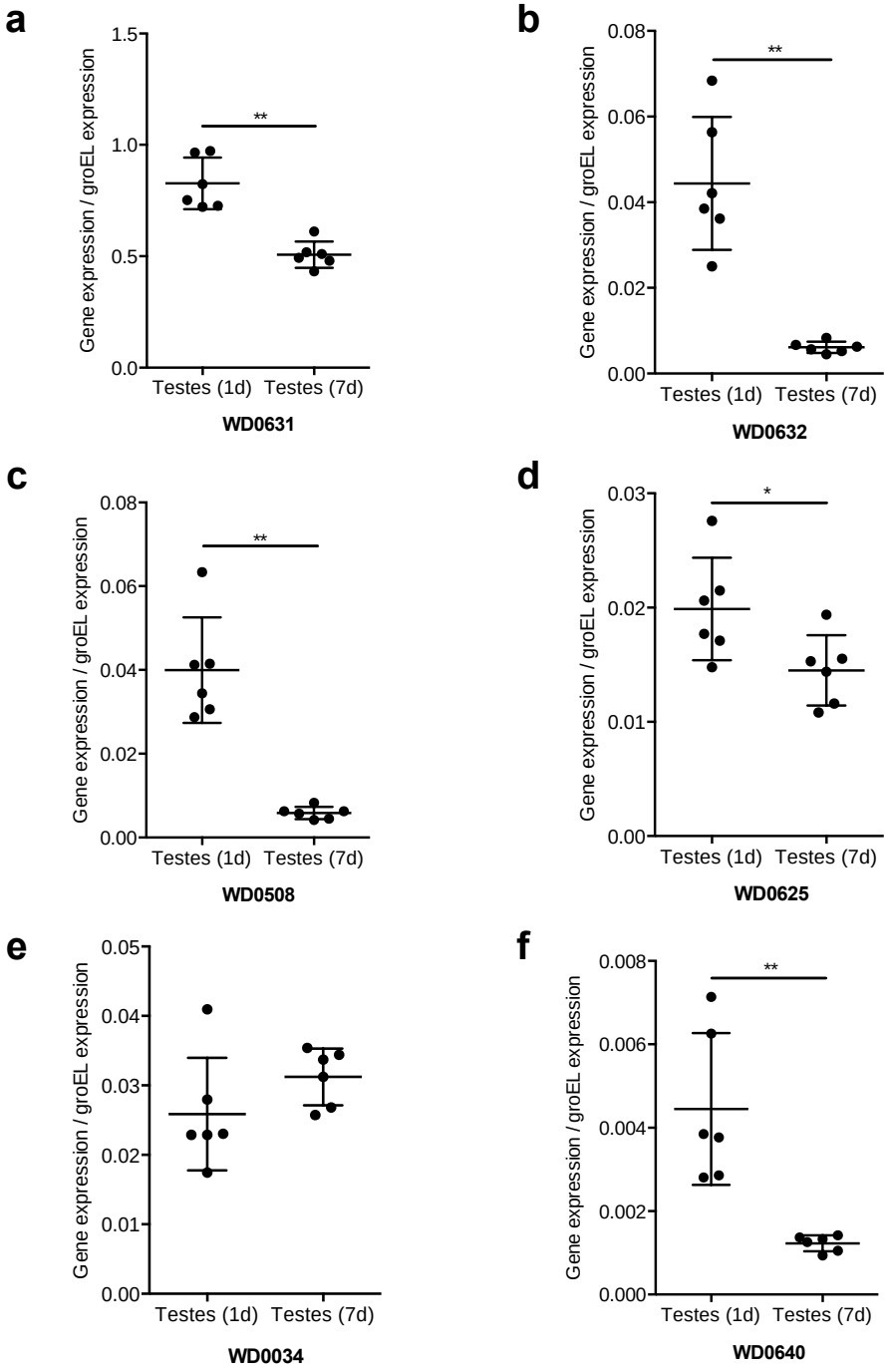

Figure III-6 Expression of CI effector candidates decreases as males age

Expression of each gene in one-day-old and seven-day-old *w*Mel-infected *D. melanogaster* testes, as determined by quantitative RT-PCR, is shown relative to *groEL*. Error bars indicate standard deviation. * = P<0.05, ** = P<0.01 by Mann-Whitney U test.

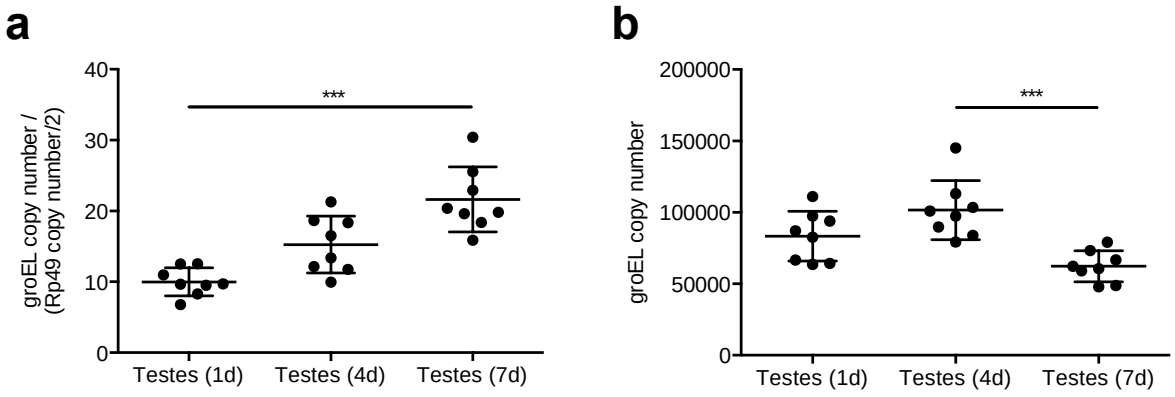

Figure III-7 *Wolbachia* titers in wild type lines

(a) Relative *Wolbachia* titers do not decrease with age. DNA copy number of *w*Mel *groEL* gene is shown normalized to *D. melanogaster Rp49* gene copy number in testes at the indicated ages. (b) Absolute *Wolbachia* titers do not decrease with male age. Titers determined by real-time PCR detecting absolute copy number of *w*Mel *groEL* gene compared to absolute copy number of the *D. melanogaster Rp49* gene. Error bars show standard deviation. *** = P<0.001 by ANOVA with Kruskal-Wallis test and Dunn's multiple test correction.

Transgenic expression of single candidate genes does not induce CI

To directly test the function of these genes in CI, we generated transgenic *D. melanogaster* that express the candidate genes alone under the direction of an upstream activating sequence (UAS), since *Wolbachia* itself cannot be genetically transformed. We utilized a *nanos*-Gal4 driver line for tissue-specific expression predominantly in the germline (Rørth, 1998; White-Cooper, 2012). CI was determined by measuring the percentage of embryos that hatched into larvae. While wild type (WT) CI between infected males (less than one day old) and uninfected females led to significantly reduced hatch rates, transgene-expressing, uninfected males with each of the four candidate genes did not affect hatch rates when crossed to uninfected females (Figure III-8).

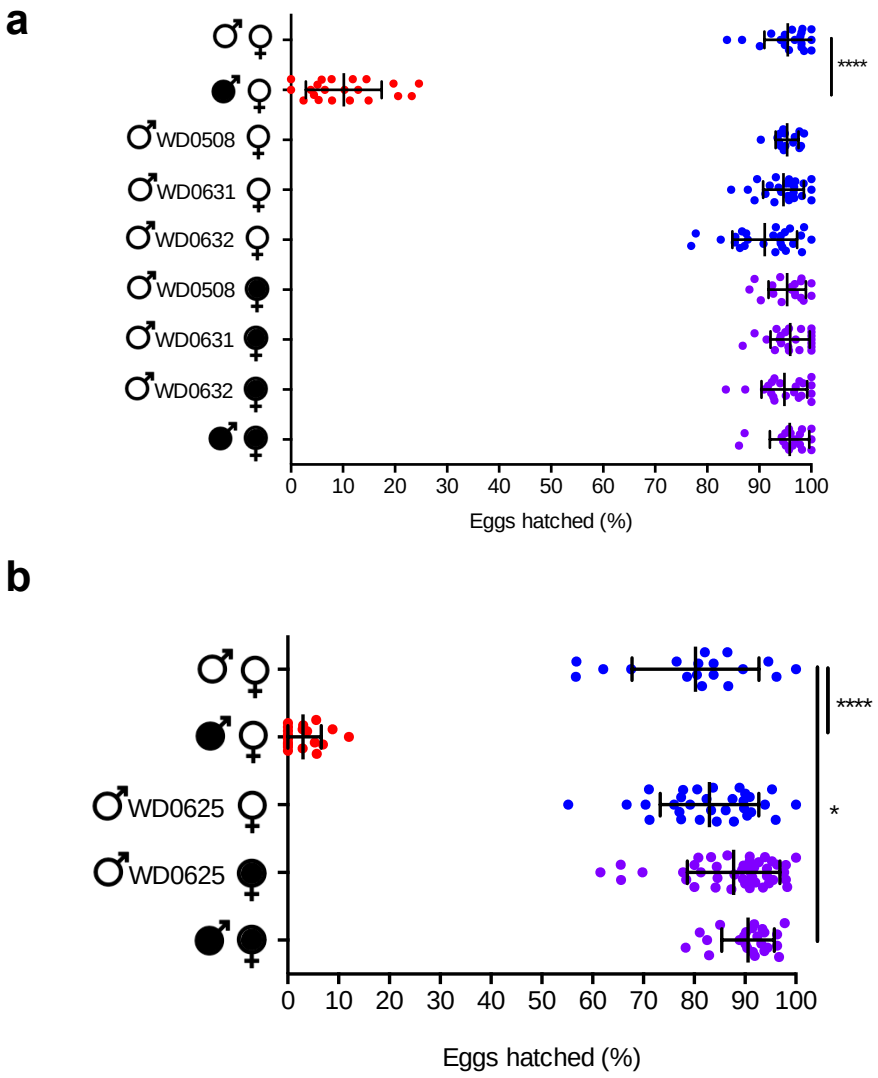

Figure III-8 Single expression of candidates does not induce CI

Expression of WD0631, WD0632, WD0508 (a) or WD0625 (b) alone in uninfected males does not induce CI. Infection status is designated with filled-in symbols for a *w*Mel-infected parent or open symbols for an uninfected parent. Transgenic flies are labeled with their transgene to the right of their gender symbol. Unlabeled gender symbols represent wild type flies. Data points are colored according to the type of cross, with blue indicating no *Wolbachia* infection, red indicating a CI cross with male-only *w*Mel infections, and purple indicating a rescue cross with *w*Mel-infected females. Error bars indicate standard deviation. * = P<0.05, **** = P<0.0001 by ANOVA with Kruskal-Wallis test and Dunn's multiple test correction.

In addition, none of the four genes had an effect on sex ratios (Figure III-9). Confirmed expression of each transgene in the testes (Figure III-10a-d) shows that this lack of effect is not due to nonexistent transgene expression.

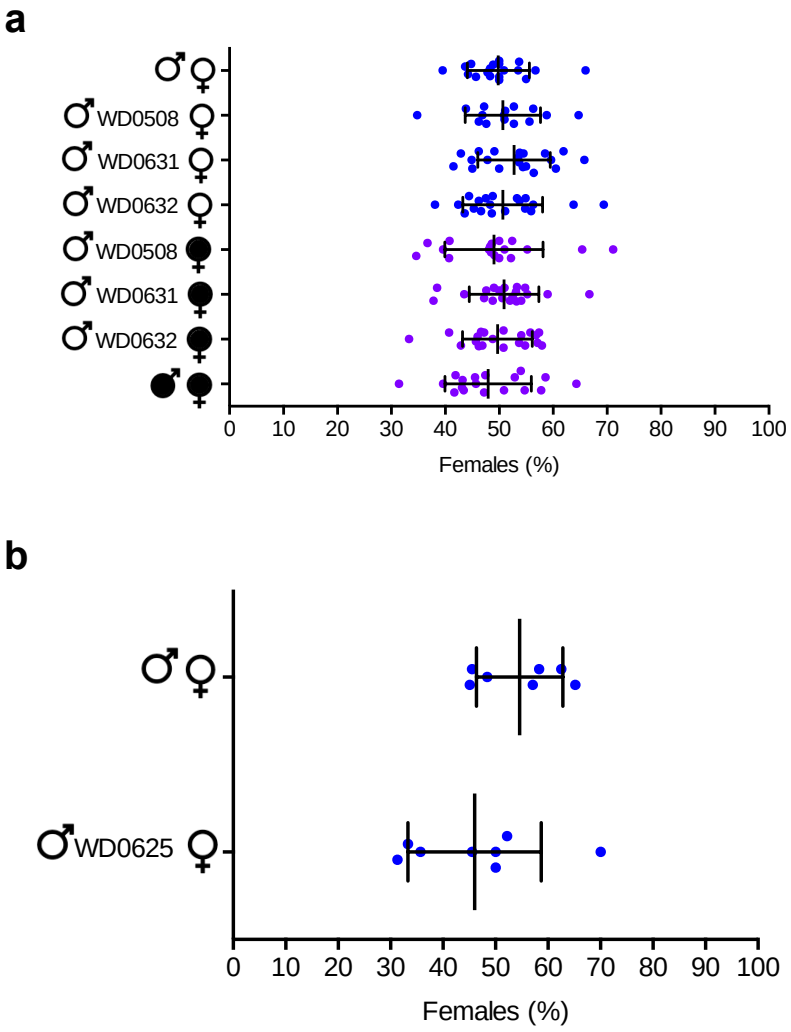

Figure III-9 Expression of single candidate genes does not alter sex ratios
Graphs correspond to the same crosses as Figure III-9. Infection status is designated with filled in symbols for a *w*Mel-infected parent or open symbols for an uninfected parent. Transgenic flies are labeled with their transgene to the right of their gender symbol. Unlabeled gender symbols represent WT flies. Data points are colored according to the type of cross, with blue indicating no *Wolbachia* infection, red indicating a CI cross with male-only *w*Mel infections, and purple indicating a rescue cross with *w*Mel-infected females. Error bars indicate standard deviation.

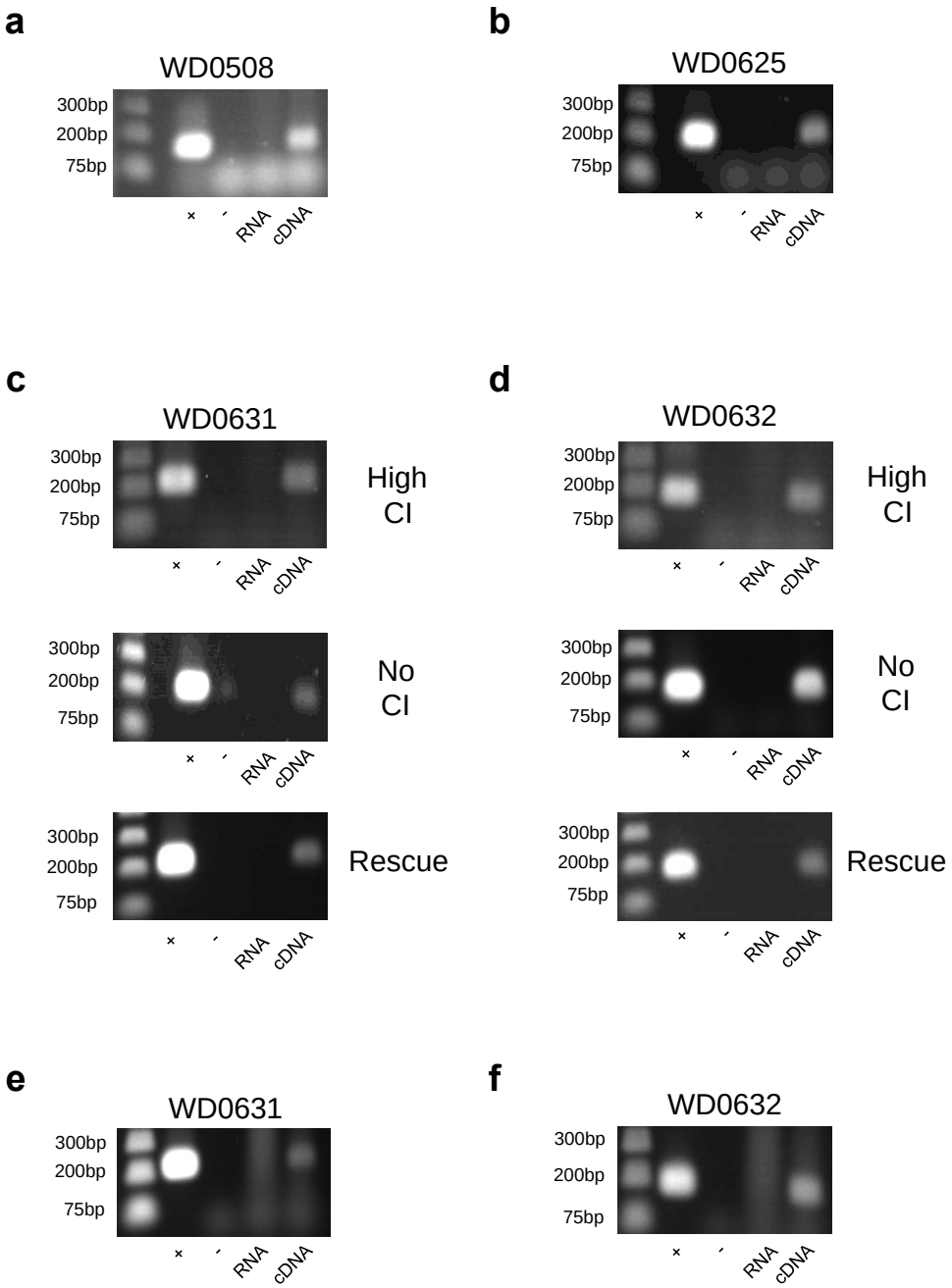

Figure III-10 CI effector candidates are expressed in transgenic fly reproductive organs
WD0508 **(a)** and WD0625 **(b)** are expressed in testes as evident by PCR performed against cDNA generated from dissected males utilized in Figure III-9. **(c,d)** WD0631 and WD0632 are expressed in the testes from transgenic males inducing high CI, no CI, or rescued CI. Testes were removed from males used in Figure III-11a. **(e,f)** WD0631 and WD0632 are expressed in ovaries from transgenic females. Ovaries were dissected from females utilized in Figure III-18a.

Dual transgene expression of WD0631 and WD0632 induces partial CI

As WD0631 and WD0632 are adjacent genes natively expressed as an operon (Beckmann and Fallon, 2013), we reasoned that dual transgene expression of WD0631 and WD0632 in males may be required to induce CI. Indeed, dual expression significantly reduced hatch rates (74.2 ± 18.5%) in comparison to that of uninfected males (96.2 ± 2.5%) when mated to uninfected females (Figure III-11a). While this level of CI is incomplete, several individual crosses with transgenic males yielded hatch rates at levels comparable to the median hatch rate of WT CI (39.8 ± 24.2%). It is possible that full induction of CI requires other factors or that our transgenic system does not express the genes at the ideal time, place, or amount to induce complete CI, though the genes do have confirmed expression in adult testes (Figure III-10c,d). Importantly, the observed defects are fully rescued by *w*Mel-infected females (Figure III-11a), indicating that these genes are bona fide *Wolbachia*-induced CI genes rather than genes that artificially reduce hatch rates through off target effects. We provisionally name them here cytoplasmic incompatibility factors, *cifA* and *cifB*, for WD0631 and WD0632, respectively.

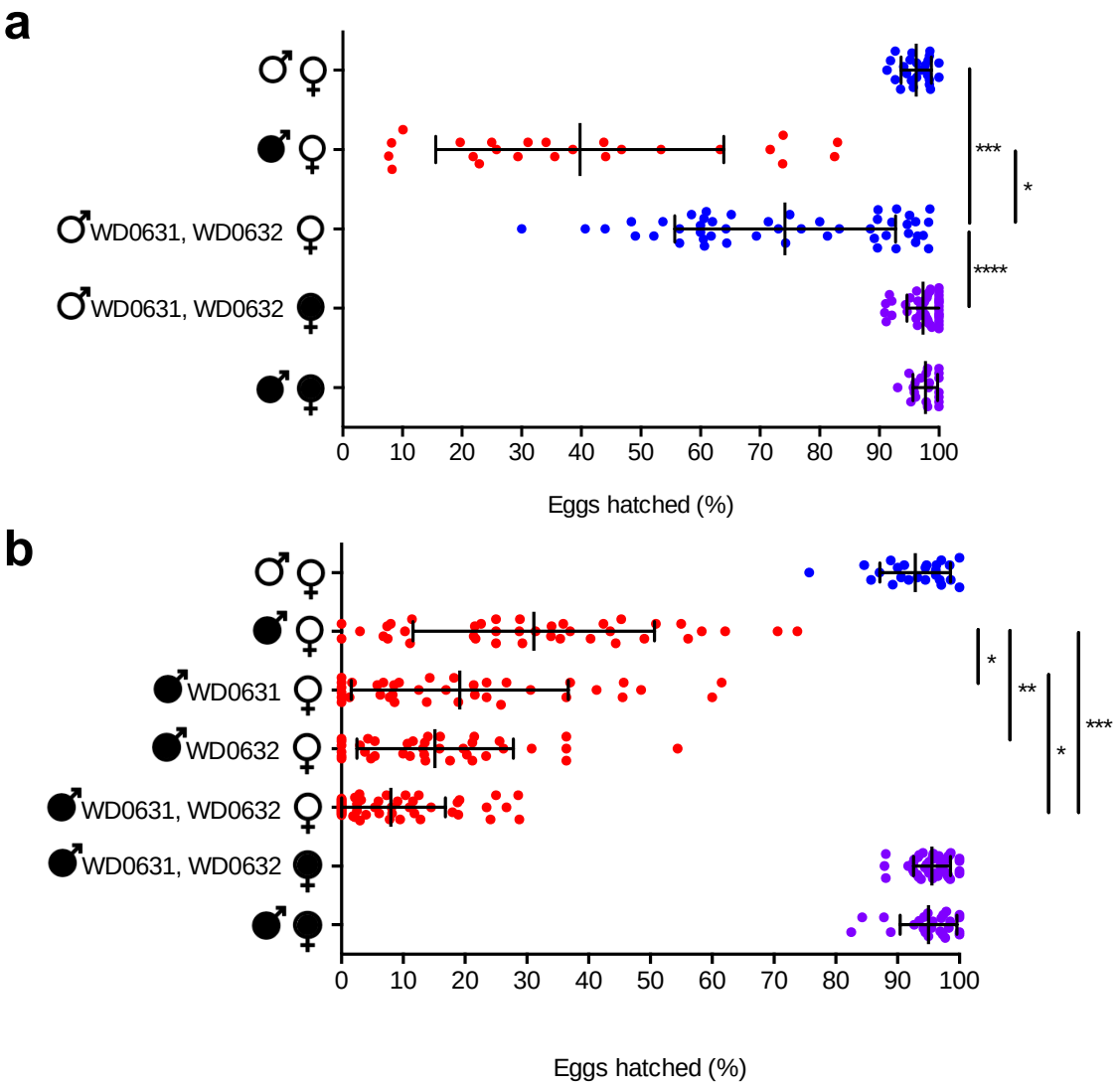

Figure III-11 Dual expression of WD0631 and WD0632 induces CI

(a) Dual expression of WD0631 and WD0632 in uninfected males induces partial CI. (b) Expression of either gene in infected males increases wild-type CI. Dual expression in infected males has an additive effect. Infection status is designated with filled-in symbols for a *w*Mel-infected parent or open symbols for an uninfected parent. Transgenic flies are labeled with their transgene to the right of their gender symbol. Unlabeled gender symbols represent wild type flies. Data points are colored according to the type of cross, with blue indicating no *Wolbachia* infection, red indicating a CI cross with male-only *w*Mel infections, and purple indicating a rescue cross with *w*Mel-infected females. Error bars indicate standard deviation. * = P<0.05, ** = P<0.01, *** = P<0.001, **** = P<0.0001 by ANOVA with Kruskal-Wallis test and Dunn's multiple test correction. Statistical comparisons are between all groups for panel a; comparisons for panel b are between CI crosses (red) only.

To test if the genes enhance WT CI levels that are naturally incomplete in *D. melanogaster*, we expressed WD0631 or WD0632 separately in *w*Mel-infected male flies and found that hatch rates decreased significantly compared to WT CI crosses (Figure III-11b). In this context, we reason that both genes are adding to the quantity of CI effector molecules in *w*Mel-infected tissues. This effect is not seen when control genes are expressed in *w*Mel-infected males (Figure III-12a,b). Moreover, dual expression of the genes in *w*Mel-infected flies reduces hatch rates still further than either gene alone, yet remains fully rescuable by *w*Mel-infected females (Figure III-11b). Adding WD0625 to WD0632 in *w*Mel-infected males does not increase CI beyond WD0632 alone (Figure III-12b), and the combination of WD0625 and WD0632 in uninfected males has no effect on hatching (Figure III-12c), indicating that the combination of WD0631 and WD0632 is uniquely required for induction of CI and that these findings are not an artifact of the transgenic system.

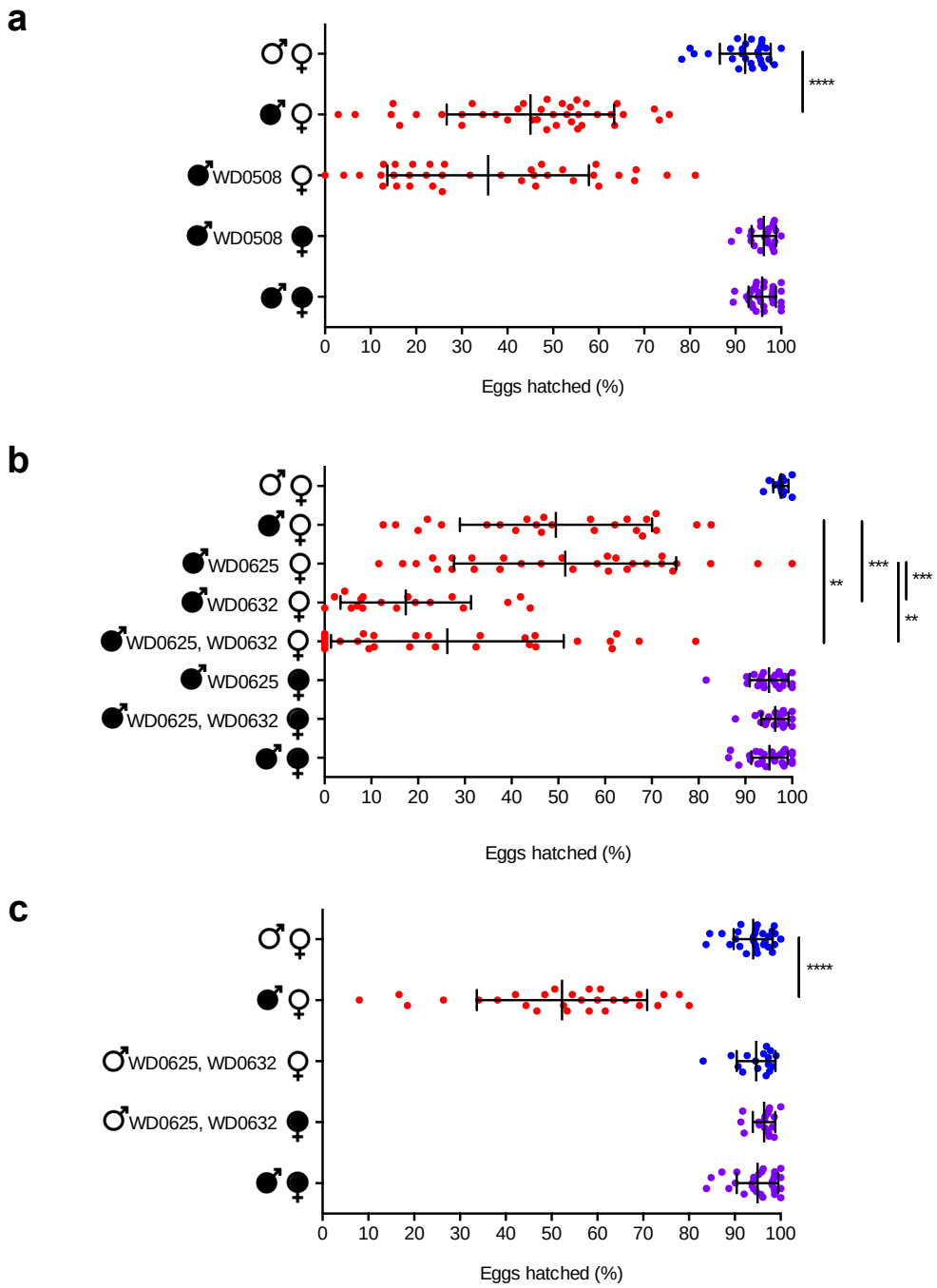

Figure III-12 Expression of genes other than WD0631/WD0632 has no effect on hatch rates
(a) The WD0508 transgene does not increase CI in infected males. (b) Addition of WD0625 to WD0632 in *w*Mel-infected males does not lower hatch rates further than WD0632 alone. (c) WD0625/WD0632 dual expression cannot induce CI. Error bars indicate standard deviation. ** = P<0.01, *** = P<0.001, **** = P<0.0001 by ANOVA with Kruskal-Wallis test and Dunn's multiple test correction.

To rule out the possibility that enhancement of CI in the infected transgenic lines is due to an increase in *Wolbachia* titers, we monitored symbiont densities by measuring amplicons of single copy genes from *Wolbachia* and *D. melanogaster*. Although there were some differences in *Wolbachia* titers between the infected transgenic lines (Figure III-13), these differences did not correlate with changes in the magnitude of CI, suggesting that decreased offspring viability was due to the direct effect of the transgenes rather than increased *Wolbachia* proliferation. Most notably, densities are significantly increased in control transgene WD0508 lines (Figure III-13a), but there is no effect on CI (Figure III-8a). Finally, none of these gene combinations had any effect on the sex ratios of offspring (Figure III-14, Figure III-15).

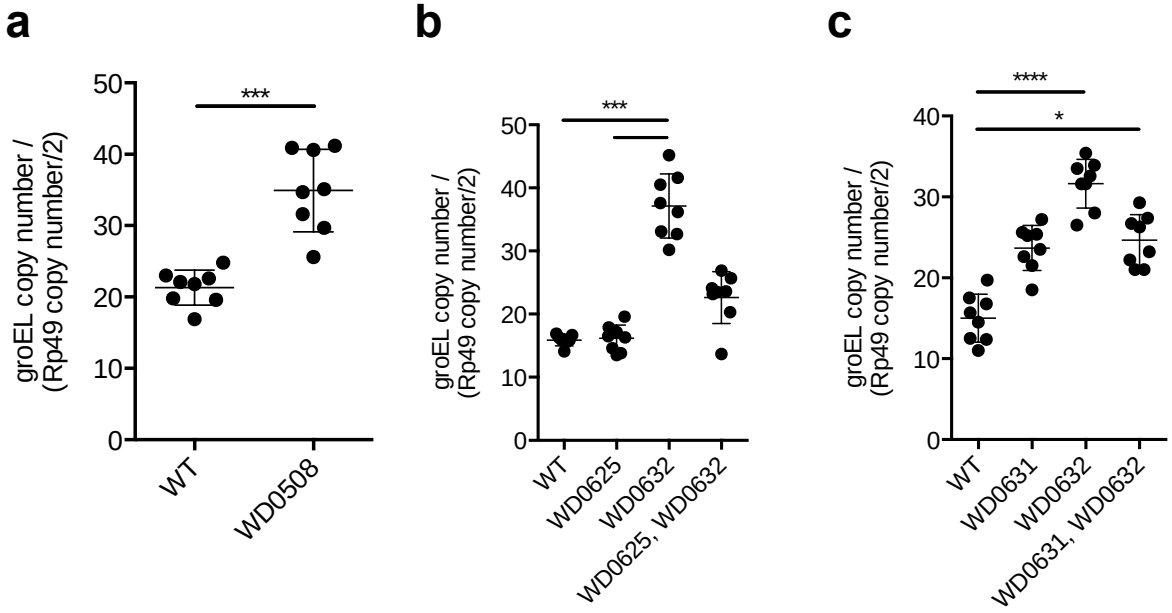

Figure III-13 *Wolbachia* titers in transgenic lines

(a-c) Relative *Wolbachia* titers are increased in WD0508, WD0631, or WD0632 transgenic lines. This does not occur in the WD0625 transgenic line nor does there appear to be an additive effect. Titers determined by real-time PCR detecting absolute copy number of *w*Mel *groEL* gene compared to absolute copy number of the *D. melanogaster Rp49* gene. Error bars show standard deviation. * = P<0.05, *** = P<0.001, **** = P<0.0001 by ANOVA with Kruskal-Wallis test and Dunn's multiple test correction. Two-tailed Mann-Whitney U test used for (a).

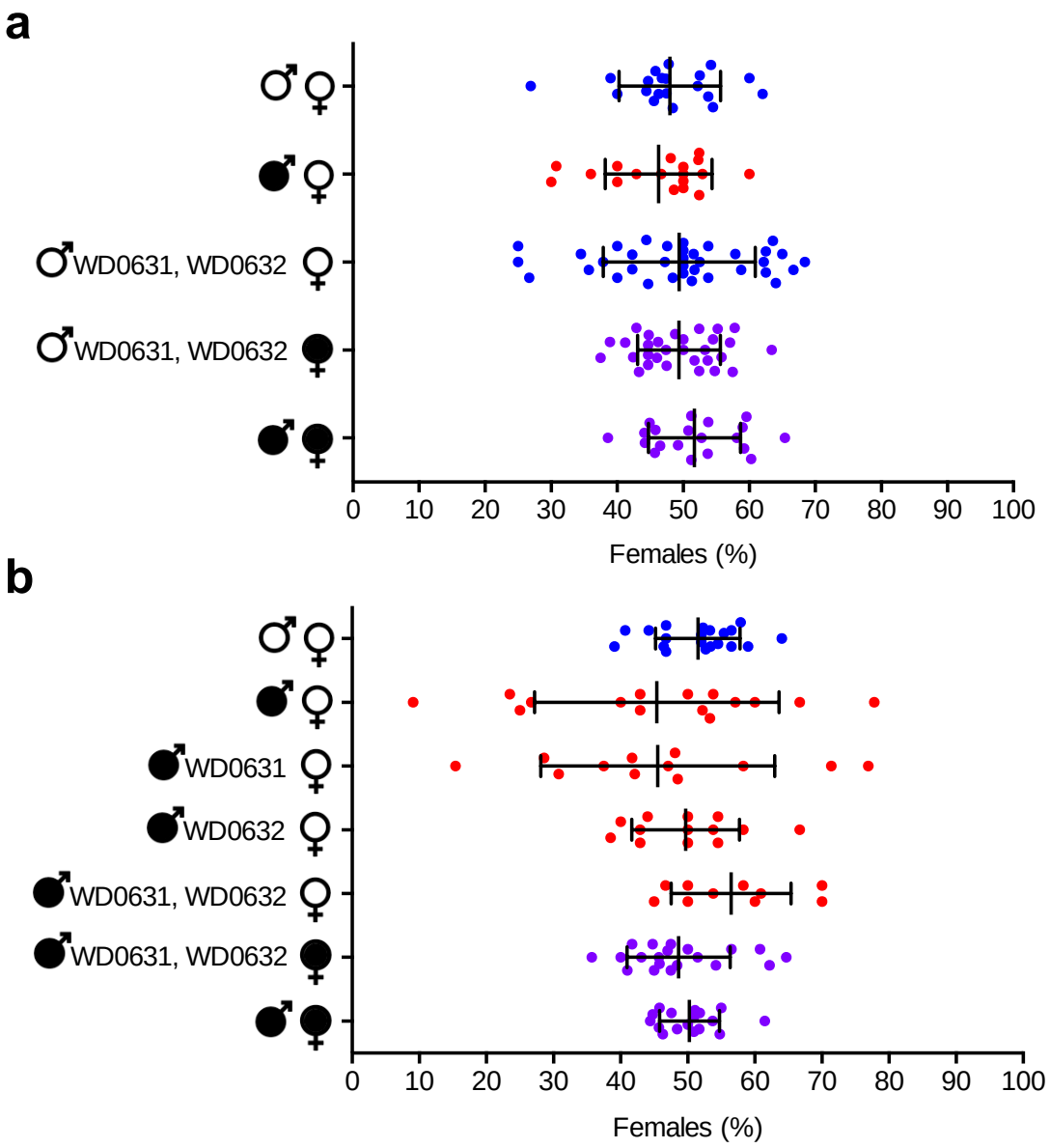

Figure III-14 Expression of CI effector candidates does not alter sex ratios

Graphs correspond to the same crosses as Figure III-11. Infection status is designated with filled in symbols for a *w*Mel-infected parent or open symbols for an uninfected parent. Transgenic flies are labeled with their transgene to the right of their gender symbol. Unlabeled gender symbols represent WT flies. Data points are colored according to the type of cross, with blue indicating no *Wolbachia* infection, red indicating a CI cross with male-only *w*Mel infections, and purple indicating a rescue cross with *w*Mel-infected females. Error bars indicate standard deviation.

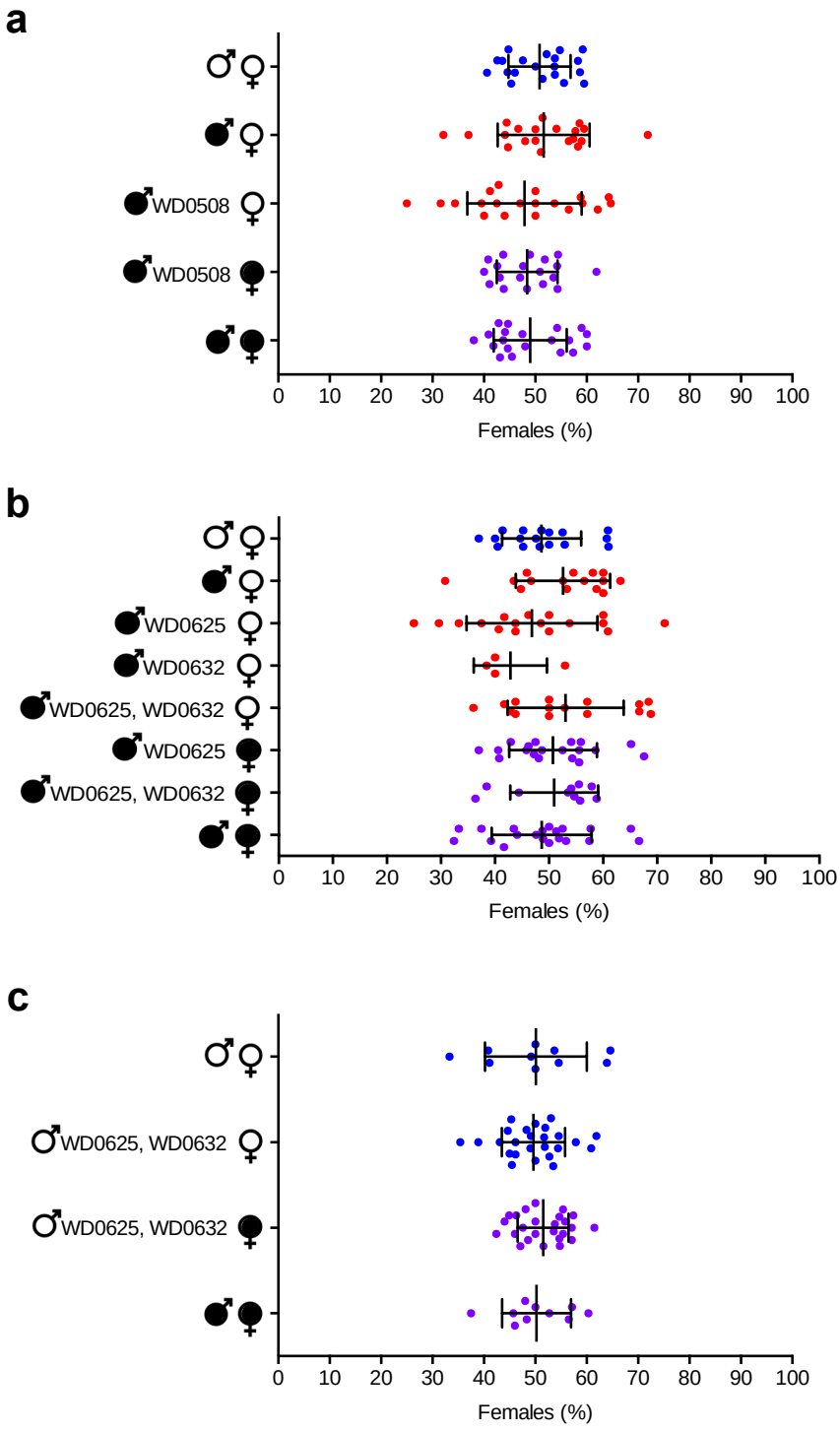

Figure III-15 Expression of genes other than WD0631/WD0632 has no effect on sex ratios

Graphs correspond to crosses in Figure III-12 Error bars indicate standard deviation. Statistics performed by ANOVA with Kruskal-Wallis test and Dunn's multiple test correction.

WD0631/WD0632 induce cytological defects similar to wild-type CI

Next we determined the similarity between the cytological defects observed during embryonic development in *Wolbachia*-induced CI versus CI from dual WD0631/WD0632 expressing transgenic flies. Although CI is classically recognized to cause failure of the first mitotic division (Landmann et al., 2009; Serbus et al., 2008), nearly half of the embryonic arrest in incompatible crosses occurs during advanced developmental stages in *Drosophila simulans* (Callaini et al., 1996), a result that was first reported in *Aedes polinesiensis* mosquitoes. We examined embryos resulting from uninfected, *w*Mel-induced CI, and transgenic crosses after one to two hours of development and, scoring blindly, binned their cytology into one of six phenotypes. While a few eggs in each cross were unfertilized (Figure III-16a), most embryos in WT crosses were either in normal late-stage preblastoderm (Figure III-16b), or in the syncytial blastoderm stage (Figure III-16c) (Bate and Arias, 1993). In the CI induced by *w*Mel, embryos had one of three defects: arrest of cellular division after two to three mitotic divisions (Figure III-16d), arrest throughout development associated with moderate to extensive chromatin bridging as is classically associated with strong CI in *D. simulans* (Figure III-16e) (Lassy and Karr, 1996), or arrest associated with regional failure of division in one segment of the embryo (Figure III-16f). After blindly scoring the number of embryos demonstrating each phenotype, we determined that arrest phenotypes d, e, and f were significantly more common in the offspring of dual WD0631/WD0632 transgenic males mated to uninfected females, but that these abnormalities were rescued in embryos from *w*Mel-infected females (Figure III-17a). These effects were not seen with control gene WD0508 or with singular expression of WD0631 or

WD0632 (Figure III-17b). These data again validate that *Wolbachia*-induced CI is recapitulated in dual WD0631/WD0632 transgenic flies.

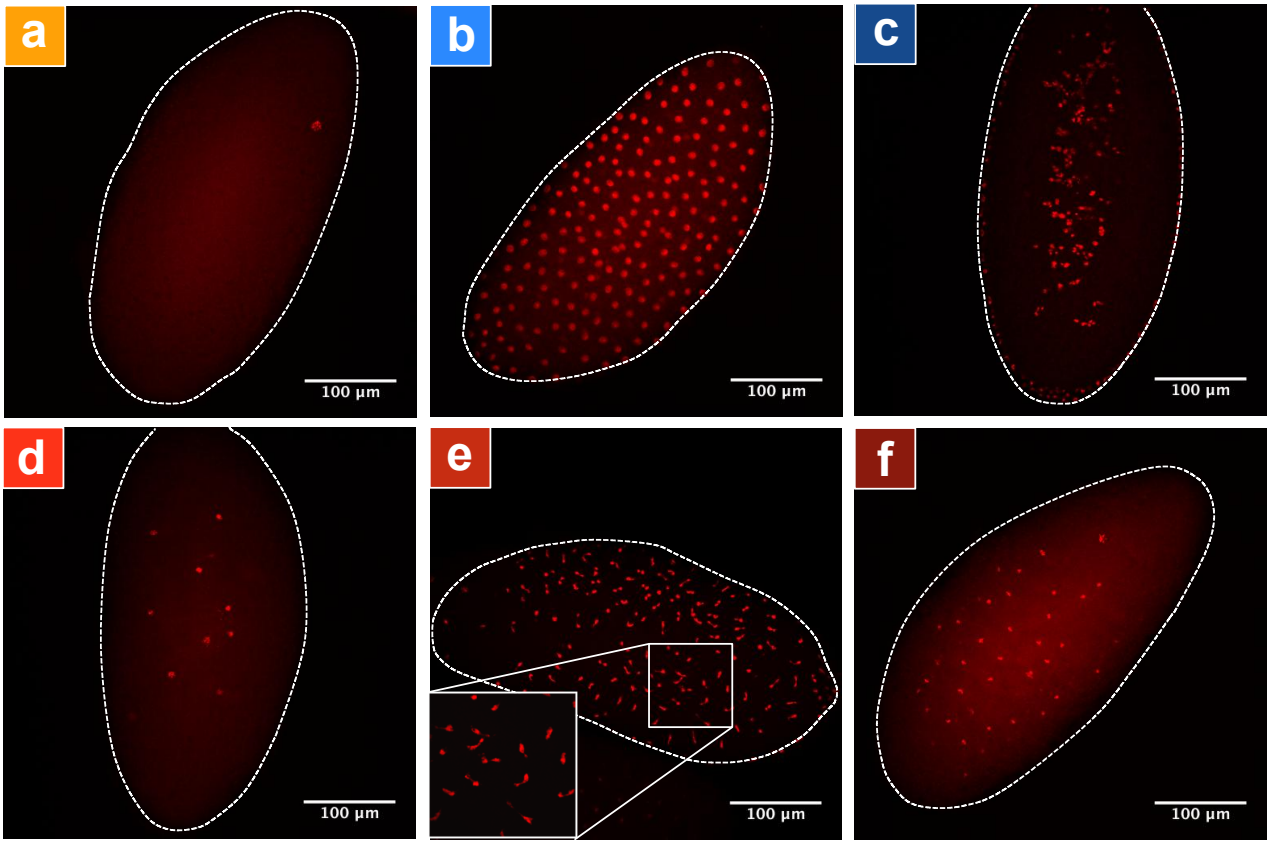

Figure III-16 Cytological defects associated with CI

Representative embryo cytology is shown for (**a**) unfertilized eggs, (**b**) normal embryos at one hour of development, (**c**) normal embryos at two hours of development, and three different mitotic abnormalities: (**d**) failure of cell division after two to three mitoses, (**e**) chromatin bridging, and (**f**) regional mitotic failure (here in the upper right portion of the embryo).

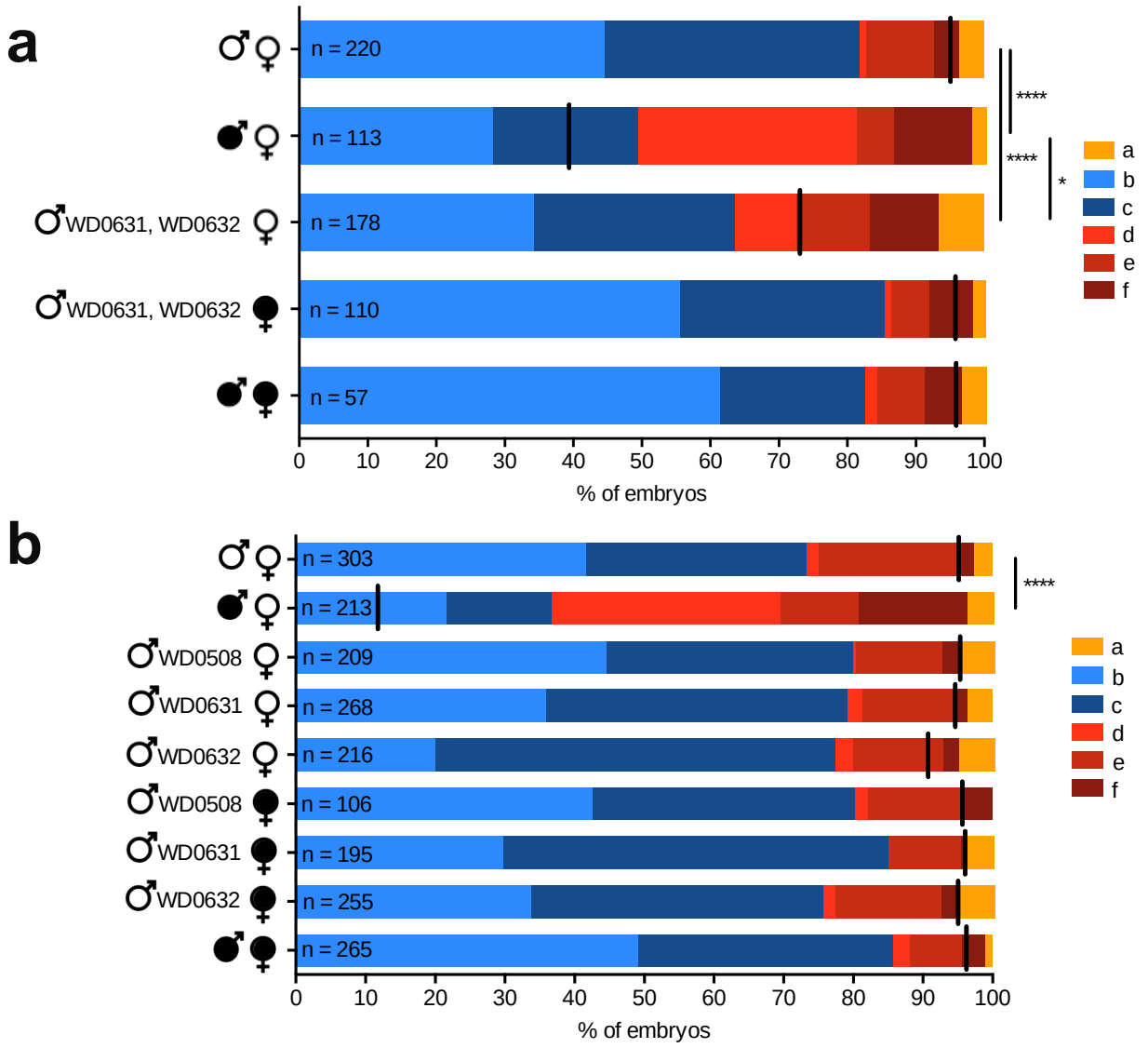

Figure III-17 Dual expression of WD0631 and WD0632 recapitulates CI cytological defects

Scoring corresponds to CI defects outlined in Figure III-16. (a) The number of embryos with each cytological phenotype resulting from crosses of dual-expressing WD0631/WD0632 males and uninfected females along with control crosses were counted. Infection status is designated with filled in symbols for a *w*Mel-infected parent or open symbols for an uninfected parent. Transgenic flies are labeled with their transgene to the right of their gender symbol. Unlabeled gender symbols represent wild type flies. Black lines on each graph indicates mean hatch rate for the cross and corresponds to the sibling crosses in Figure III-8a and Figure III-11a. * = P<0.05, **** = P<0.0001 by two-tailed Fisher's exact test comparing normal (phenotypes b and c) to abnormal (phenotypes a, d, e, and f) for each cross. (b) Quantitation of cytological defects in crosses utilizing WD0508, WD0631, or WD0632 uninfected males.

Finally, we evaluated whether WD0631 and WD0632 can rescue CI. Neither WD0631 nor WD0632, whether alone or combined, had an effect on hatch rates when expressed in uninfected females (Figure III-18). WD0631- or WD0632-expressing females could not rescue *w*Mel-induced CI, nor could WD0631/WD0632 dual-expressing females rescue CI induced by dual transgenic males (Figure III-18), despite confirmed expression in ovaries (Figure III-10e,f). Transgene expression also had no effect on sex ratios (Figure III-19). These data suggest that the genes underlying incompatibility and rescue are different.

This study identifies, for the first time, genes that are responsible for inducing CI. While protein domain predictions suggest that the mechanism may involve nuclease or ubiquitin-modifying activity, the molecular basis of CI is further elucidated in a companion publication by Beckmann, *et al* (Submitted, 2016). The discovery of CI effector genes is the first inroad to solving the genetic basis of reproductive parasitism, a phenomenon induced worldwide in an estimated hundreds of thousands to millions of arthropod species (Zug and Hammerstein, 2012). The genes also have major implications for studying microbe-assisted speciation, because these genes likely underlie the CI-induced hybrid lethality observed between closely related species of *Nasonia* and *Drosophila* (Bordenstein et al., 2001; Jaenike et al., 2006). Finally, these genes are important for arthropod pest or vector control strategies, as they could potentially be used as an alternative or adjunctive strategy to current *Wolbachia*-based paradigms aimed at controlling agricultural pests or curbing arthropod-borne transmission of infectious diseases (Dutra et al., 2016; O'Connor et al., 2012; Walker et al., 2011; Zabalou et al., 2004).

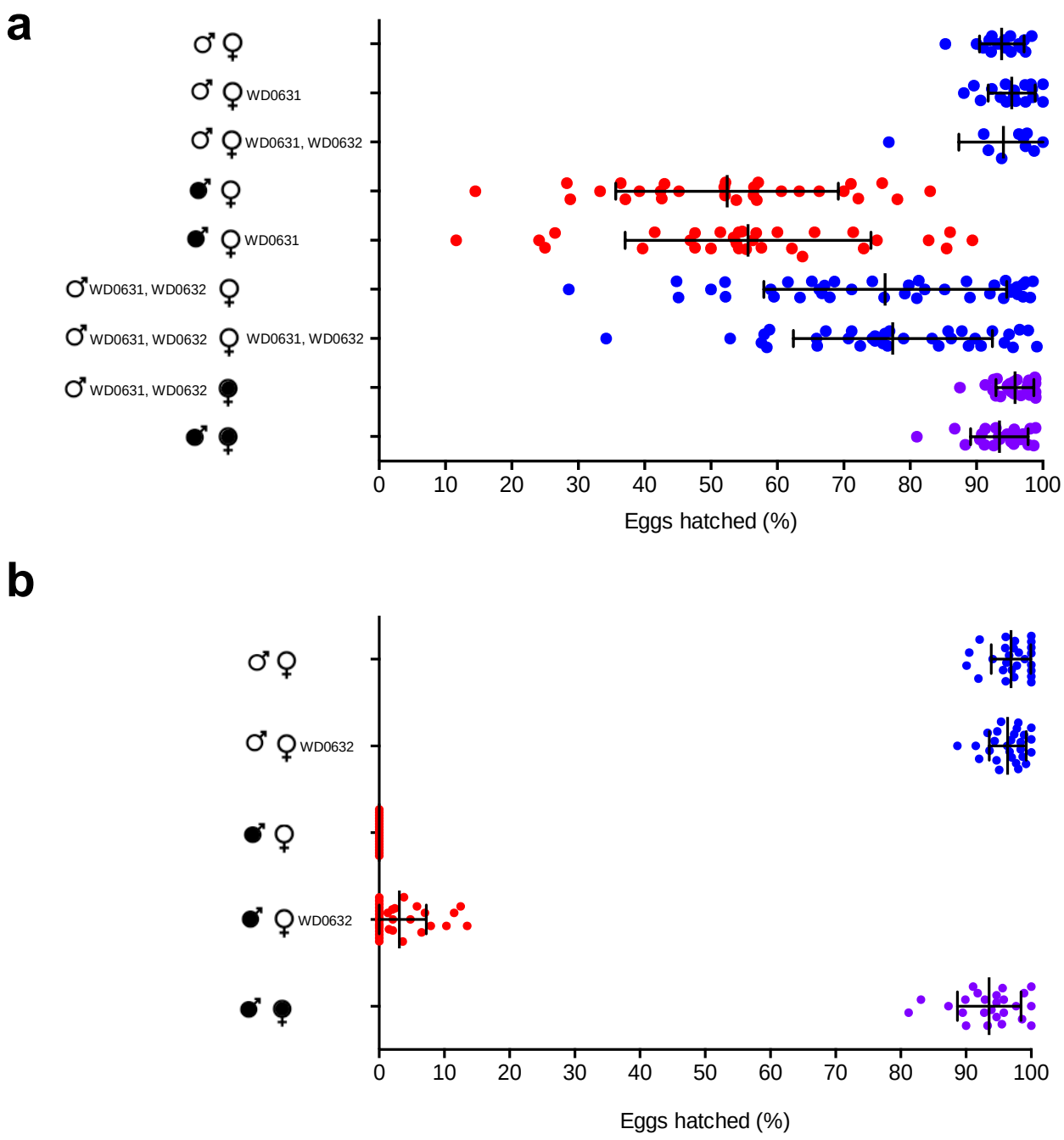

Figure III-18 WD0631/WD0632 expression in females cannot rescue CI

(**a, b**) Hatch rates for the indicated crosses are shown. Single expression or dual expression of WD0631 and WD0632 in uninfected females does not reduce embryo hatching or rescue wild-type or induced CI defects. Error bars indicate standard deviation. Statistics performed by ANOVA with Kruskal-Wallis test and Dunn's multiple test correction.

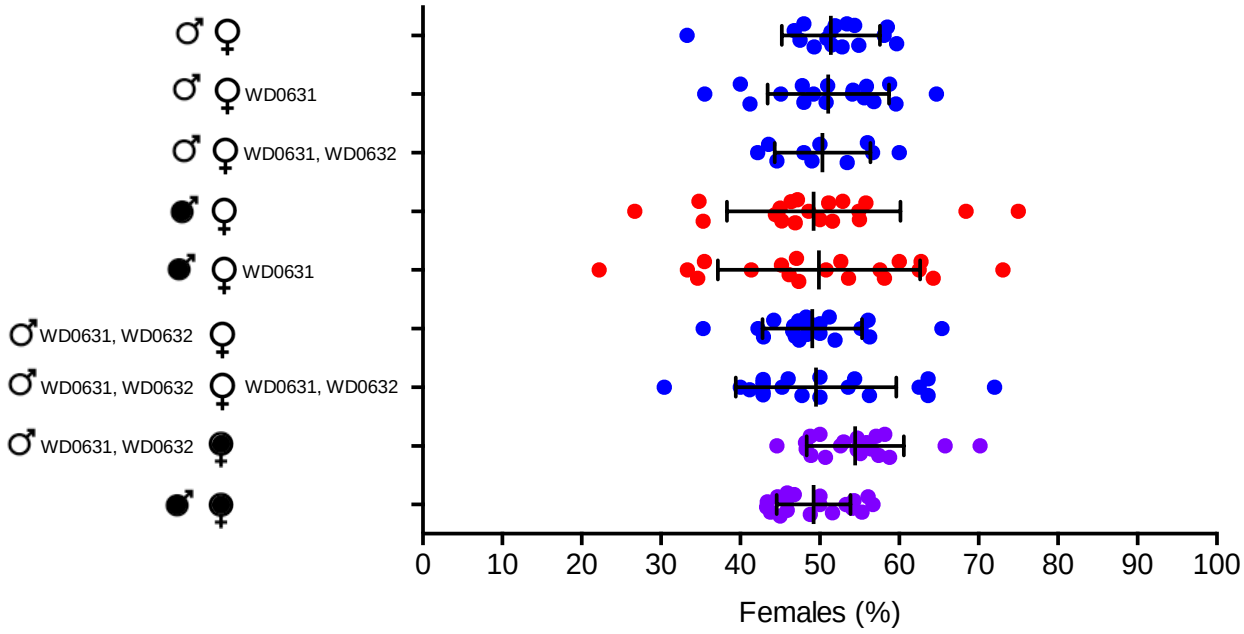

Figure III-19 Dual expression of WD0631/WD0632 in females does not alter sex ratios
Crosses correspond to those used in Figure III-18a. Single expression or dual expression of WD0631 and WD0632 in uninfected females does not reduce embryo hatching or rescue wild-type or induced CI defects. Infection status is designated with shading for a *w*Mel-infected parent or no shading for an uninfected parent. Transgenic flies are labeled with their transgene to the right of their gender symbol. Unlabeled gender symbols represent WT flies. Data points are colored according to the type of cross, with blue indicating no *Wolbachia* infection, red indicating a CI cross with male-only *w*Mel infections, and purple indicating a rescue cross with *w*Mel-infected females. Error bars indicate standard deviation. Statistics performed by ANOVA with Kruskal-Wallis test and Dunn's multiple test correction.

Conclusions

The results shown here are the first to identify causal factors for *Wolbachia*-induced CI in over forty years of research (Yen and Barr, 1971). Crosses in *D. melanogaster* suggest that two *Wolbachia* genes from *w*Mel, WD0631 and WD0632, act together to induce embryonic lethality and can do so without any other *Wolbachia* factors. While the protein domains of WD0632

suggests a role in SUMOylation, not all homologs from CI-causing strains of *Wolbachia* share that annotation (Figure III-2). This would suggest that either (i) other *Wolbachia* factors are involved in CI or (ii) the SUMOylation domain within WD0632 is not absolutely required.

Transgenic expression of WD0631 and WD0632 together induces cytological defects within developing embryos that are similar to wild-type CI (Figure III-17). Importantly, these defects are fully rescued by *w*Mel-infected females. This shows that the WD0631/WD0632 combination does not arbitrarily stall host development but rather induces the selective sterility found in wild type CI. This rescue does not seem to be mediated by WD0631 and WD0632 themselves, though, making it likely that other factors are required (Figure III-18). Interesting, the sequence of each candidate gene seems to correlate with known bidirectional incompatibilities (Figure III-2), thus providing an intellectual framework to understand this vexing hallmark of CI.

The identification of verified CI factors is highly informative for many fields and provides a critical breakthrough in understanding the role of *Wolbachia* in ecology, speciation, and insect control. A deeper comprehension of CI biology will greatly aid current efforts utilizing *Wolbachia* to eliminate dengue and opens the possibility for rapidly expanding these programs by modulating wild-type rates of CI.

Materials and Methods

Comparative genomics and transcriptomics

MicroScope (Vallenet et al., 2014) was used to select the set of genes comprising the core genomes of CI-inducing *Wolbachia* strains *w*Mel [NC_002978.6] (Wu et al., 2004), *w*Ri [NC_012416.1] (Klasson et al., 2009), *w*Pip (Pel) [NC_010981.1] (Klasson et al., 2008), and the recently sequenced *w*Rec [RefSeq 1449268] (Metcalf et al., 2014a), while excluding the pan-genome of the mutualistic strain *w*Bm [NC_006833.1] (Foster et al., 2005), using cutoffs of 50% amino acid identity and 80% alignment coverage. *w*Au microarray data were obtained from the original authors (Ishmael et al., 2009) and genes that were present in CI-inducing strains *w*Ri and *w*Sim but absent or divergent in the non-CI strain *w*Au were selected.

For ovarian transcriptomics, one-day old females from *w*VitA infected-*Nasonia vitripennis* 12.1 were hosted as virgins on *Sarcophaga bullata* pupae for 48 hours to stimulate feeding and oogenesis. Females were then dissected in RNase-free 1X PBS buffer, and their ovaries were immediately transferred to RNase-free Eppendorf tubes in liquid nitrogen. Fifty ovaries were pooled for each of three biological replicates. Ovaries were manually homogenized with RNase-free pestles, and their RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol for purification of total RNA from animal tissues. After RNA purification, samples were treated with RQ1 RNase-free DNase (Promega), and ethanol precipitation was performed. PCR of RNA samples with *Nasonia* primers NvS6KQTF4 and NVS6KQTR4 (Bordenstein and Bordenstein, 2011) confirmed that all samples were free of DNA contamination. RNA concentrations were measured with a Qubit 2.0 Fluorometer (Life

Technologies) using the RNA HS Assay kit (Life Technologies), and approximately 5 µg of total RNA from each sample was used as input for the MICROB*Enrich* Kit (Ambion) in order to enrich for *Wolbachia* RNA in the samples. Microbially-enriched RNA was then ethanol-precipitated, and rRNA was depleted from the samples using the Ribo-Zero Magnetic kit (Illumina) according to manufacturer's protocol. Approximately 1.5 µg of microbially-enriched, rRNA-depleted RNA for each replicate was shipped to the University of Rochester Genomics Research Center for sequencing. Library preparation was performed using the Illumina ScriptSeq v2 RNA-Seq Library Preparation kit, and all samples were run multiplexed on a single lane of the Illumina HiSeq2500 (single-end, 100 bp reads). Raw reads were trimmed and mapped to the *w*VitA genome (PRJDB1504) in CLC Genomics Workbench 8.5.1 using a minimum length fraction of 0.9, a minimum similarity fraction of 0.8, and allowing one gene hit per read. With all three replicates combined, a total of 364,765 reads out of 41,894,651 (0.87%) mapped to the *w*VitA genome with the remaining reads mapping to the *N. vitripennis* host genome (GCF_000002325.3). All *Wolbachia* genes with greater than or equal to five RNA-seq reads, with the exception of the 16S and 23S RNA genes, were selected. For non-*w*Mel data sets, the closest homologs in *w*Mel were found using blastp in Geneious Pro v5.5.6 (Kearse et al., 2012).

Protein extraction and mass spectrometry

Protein was extracted from *Culex pipiens* tissues as described previously (Beckmann and Fallon, 2013). Ovaries from 30 *w*Pip (Buckeye)-infected mosquitoes were dissected in 100% ethanol and collected in a 1.5 ml tube filled with 100% ethanol. Pooled tissues were sonicated at 40 mA for 10 seconds in a Kontes GE 70.1 ultrasonic processor, and trichloroacetic acid (TCA)

was added to a final concentration of 10% (v/v). After centrifugation at 13,000 rpm in a microcentrifuge, pellets were washed with acetone:water (9:1), dried, and stored at -20°C. Samples were directly submitted to the University of Minnesota's Center for Mass Spectrometry and Proteomics for iTRAQ (isobaric tagging for relative and absolute quantification) analysis. Proteins were sorted according to their relative abundance as determined by the number of spectra from the single most abundant peptide. Because proteins can often produce varying amounts of detectable tryptic peptides depending upon protein size and lysine/arginine content, we counted only the single most abundant peptide for each protein. This quantification is justified by previous reports (Beckmann and Fallon, 2013) showing that the two most abundant proteins are the *Wolbachia* surface protein (WSP; gi|190571332) and another putative membrane protein (gi|190570988). Only proteins with at least three unique peptides (95% confidence) detected were reported, and using this criterion the false discovery rate was zero.

Gene Expression Assays

Expression of CI candidates was tested with RT-qPCR on pools of 20 pairs of testes from one-day-old and seven-day-old virgin males. RNA was extracted with the Qiagen RNeasy mini kit, DNase treated with TURBO DNase (Life Technologies) and cDNA was generated with Superscript III Reverse Transcriptase (Invitrogen). Delta delta Ct analysis against the housekeeping gene *groEL* was used to determine relative gene expression.

Evolutionary analyses

WD0631 and WD0632 were used as queries to perform a BLASTp search of NCBI's nonredundant (nr) protein sequence database with algorithm parameters based on a word-size of

six and BLOSUM62 scoring matrix (Johnson et al., 2008). Homologs were selected based on the satisfaction of three criteria: (i) E-value $\leq 10^{-20}$, (ii) query coverage greater than 60%, and (iii) presence in fully sequenced *Wolbachia* and/or phage WO genomes. FtsZ and gpW proteins were identified for all representative *Wolbachia* and phage WO genomes, respectively. Protein alignments were performed using the MUSCLE plugin (Edgar, 2004) in Geneious Pro v8.1.7 (Kearse et al., 2012); the best models of selection, according to the corrected Akaike Information Criteria {AICc, (Hurvich and Tsai, 1993)}, were estimated using the ProtTest server (Abascal et al., 2005); and phylogenetic trees were built using the MrBayes plugin in Geneious (Ronquist et al., 2012). Putative functional domains were identified using NCBI's BLASTP, Wellcome Trust Sanger Institute's PFAM database (Finn et al., 2015) and EMBL's Simple Modular Architecture Research Tool {SMART, (Letunic et al., 2012)}.

Fly rearing

D. melanogaster were reared on standard cornmeal and molasses based media. Stocks were maintained at 25C while virgin flies were stored at room temperature. During virgin collections, stocks were kept at 18C overnight and 25C during the day. *Wolbachia* uninfected lines were generated through tetracycline treatment for three generations. Briefly, tetracycline was dissolved in ethanol and then diluted in water to a final concentration of 1mg/mL. 1mL of this solution was added to 50mL of media (final concentration of 20ug/mL). Freshly treated media was used for each generation. Infection status was confirmed with PCR using Wolb_F and Wolb_R3 primers (Bordenstein et al., 2001), and flies were reared on untreated media for at least three additional generations before being utilized.

Transgenic flies

Each CI candidate gene was cloned into the pTIGER plasmid for transformation and expression in *D. melanogaster* (Ferguson et al., 2012). pTIGER was designed for targeted integration into the *D. melanogaster* genome using PhiC31 integrase (Groth et al., 2004) and tissue-specific, inducible expression through the Gal4-UAS system (Elliott and Brand, 2008). Cloning was performed using standard molecular biology techniques and plasmids were purified and sequence-confirmed before injection. At least 200 *D. melanogaster* embryos were injected per gene by Best Gene, Inc (Chino Hills, CA), and transformants were selected based on w+ eye color. Isogenic, homozygous lines were maintained when possible, or isogenic heterozygous flies were maintained when homozygous transgenics were inviable (WD0625/CyO). WD0508 and WD0631 insertion was carried out with the y^1 *M{vas-int.Dm}ZH-2A w*; P{CaryP}attP4*0 line. WD0625 was inserted into BSC9723 with the genotype: y^1 *M{vas-int.Dm}ZH-2A w*; PBac{y+-attP-3B}VK00002*. WD0632 insertion was done using BSC8622 with the genotype: y^1 w^{67c23}; *P{CaryP}attP2*.

Wolbachia titers

For Extended Data Fig. 4c-e, brothers of those used in the corresponding hatch rates were utilized. Testes were dissected from males in cold PBS. Pools of testes from 15 males were used for each sample, and DNA was extracted using the Gentra Puregene Tissue kit (Qiagen). Quantitative PCR was performed on a Bio-Rad CFX-96 Real-Time System using iTaq Universal SYBR Green Supermix (Bio-Rad). Absolute quantification was achieved by comparing all experimental samples to a standard curve generated on the same plate. The Rp49 standard

template was generated using the same primers as those used to determine quantity while the groEL standard template was generated using groELstd_F and groELstd_R primers that we designed. qPCR conditions: 50C 10 min, 95C 5 min, 40x (95C 10 sec, 55C 30 sec), 95C 30 sec. Followed by melt curve analysis (0.5C steps from 65-95C for 5 sec each). To obtain a more accurate *Wolbachia*:host cell ratio, it was assumed that each host cell has two copies of Rp49 and each *Wolbachia* cell has one copy of groEL.

Hatch Rate Assays

Parental females, unless expressing a transgene, were WT y^1w^* flies (*w*Mel-infected or uninfected) and aged for 2-5 days before crossing. Parental males were created by crossing *nanos-Gal4* virgin females (*w*Mel-infected or uninfected) with either WT or UAS-candidate gene-transgenic males. Only the first males emerging from these crosses were used to control for the older-brother effect associated with CI (Yamada et al., 2007). In assays to determine whether CI was increased, virgin males were aged for 3-4 days before crossing to reduce the level of WT CI. In these experiments, care was taken to match the age of males between experimental and control crosses. In all other assays, virgin males were used within 30 hours of emergence. 32-64 individual crosses were used for each crossing condition. To perform the hatch rate assays, a single male and single female were placed in an 8oz, round bottom, polypropylene *Drosophila* stock bottle. A grape juice-agar plate with a small amount of yeast mix (1 part water: 2 parts dry yeast) smeared on top was placed in the bottle opening and affixed with tape. Grape juice-agar plates consist of the lids from 35x10mm culture dishes (CytoOne). 12.5g of agar is mixed in 350mL of ddH2O and autoclaved. In a separate flask, 10mL of ethanol is used to dissolve 0.25g

tegosept (methyl 4-hyrdoxybenzoate). 150mL of Welch's grape juice is added to the tegosept mix, combined with the agar, and poured into plates.

Hatch rate bottles were placed in a 25C incubator overnight (~16 hours). After this initial incubation the grape plates were discarded and replaced with freshly yeasted plates. After an additional 24 hours the adult flies were then removed and frozen for expression analysis and the embryos on each plate were counted. These plates were then incubated at 25C for 36 hours before the number of unhatched embryos was counted. Larvae were moved from these plates and placed in vials of fly media with one vial being used for each individual grape plate to be assayed for sex ratios at adulthood. A total of 10-20 vials were used for each cross type. Any crosses with fewer than 25 embryos laid were discarded from the hatching analysis while vials with fewer than 10 adults emerging were discarded from the sex ratio analysis. Statistical analysis and outlier removal, utilizing the ROUT method, were performed using Graphpad Prism v6 software.

Transgene RT-PCR

Pools of six pairs of testes or ovaries were dissected from parents utilized in hatch rate assays. In samples designated "High CI" and "No CI", the males correspond to crosses that had low or normal hatch rates, respectively. For all other samples the flies utilized were chosen at random. RNA was extracted using the Direct-zol RNA MiniPrep Kit (Zymo), DNase treated with DNA-free (Ambion, Life Technologies) and cDNA was generated with SuperScript VILO (Invitrogen). 30 cycles of PCR were performed against positive controls (extracted DNA), negative controls (water), RNA, and cDNA with the following conditions: 95C 2 min, 30x (95C 15 sec, 56C 30 sec, 72C 30 sec), 72C 5 min.

Embryo imaging

Embryos were collected in a fashion similar to hatch rate assays except bottles contained 60-80 females and 15-20 males. After an initial 16 hours of mating, fresh grape plates were added and embryos were removed after 60 minutes. The embryo-covered plates were then placed in the incubator at 25C for a further 60 minutes to ensure each embryo was at least 1 hour old. Embryos were then moved to a small mesh basket and dechorionated in 50% bleach for 1-3 minutes. These were then washed in embryo wash solution (7% NaCl, 0.5% Triton X-100) and moved to a small vial with ~2mL heptane. An equal amount of methanol was added to the vial and then vigorously shaken for 15 seconds. The upper heptane layer, and most of the methanol, was then removed and the embryos moved to fresh methanol in a 1.5mL microcentrifuge tube. Embryos were stored overnight at 4^{o}C for clearing. The old methanol was then removed and replaced with 250uL of fresh methanol along with 750uL of PBTA (1x PBS, 1% BSA, 0.05% Triton X-100, 0.02% sodium azide). After inverting the tube several times, the solution was removed and replaced with 500uL PBTA. Embryos were then rehydrated for 15 minutes on a rotator at room temperature. After rehydrating, the PBTA was replaced with 100uL of a 10mg/mL RNase solution and incubated at 37^{o}C for 2 hours. The RNase was then removed and embryos were washed several times with PBS followed by a final wash with PBS-Azide (1x PBS, 0.02% sodium azide). After removing the PBS-Azide, embryos were mounted on glass slides with ProLong Diamond Antifade (Life Technologies) spiked with propidium iodide (Sigma-Aldrich) to a final concentration of 1ug/mL. Imaging was performed at the Vanderbilt Cell Imaging Shared Resource using a Zeiss LSM 510 META inverted confocal microscope. All

scores were performed blind and image analysis was done using ImageJ software (Schneider et al., 2012).

CHAPTER IV. CONCLUSIONS AND FUTURE DIRECTIONS

Wolbachia pipientis represent one of the most widespread pandemics in the animal kingdom. Surprisingly little is understood about *Wolbachia* biology, however, and knowledge surrounding the reproductive parasitisms they utilize against hosts is especially sparse. The results shown here make significant inroads to understanding the most common and medically important of *Wolbachia*'s reproductive manipulations- cytoplasmic incompatibility- and establish a strong framework for possible future studies that are outlined below.

The role of host DNA methylation in CI

The role host DNA methylation plays during CI induction was examined in CHAPTER II. This work found that *Wolbachia* infection within *Drosophila melanogaster* increases DNA methylation in host testes, suggesting a link to the male-specific defects that occur during CI (Figure II-1). This phenotype was not consistently found within other *Wolbachia* infections, however, and males deficient in DNA methylation machinery were still able to induce CI (Figure II-11 and Figure II-9, respectively). A negative correlation was observed, though, between expression of the DNA methyltransferase *Dnmt2* and *Wolbachia* titers (Figure II-5). This finding expands on previous knowledge showing that *Dnmt2* is toxic to *Wolbachia* in *A. aegypti* (Zhang et al., 2013) and opens the prospect that *Wolbachia* interaction with DNA methyltransferases is a universal trait across hosts.

DNA methylation and virus suppression

A large outstanding question for the field is how *Wolbachia* protect their host from certain viral and parasitic infections. Understanding this biology is absolutely critical to informing current efforts that utilize *Wolbachia* infection to combat disease, as outlined in CHAPTER I. While research into this area has been intense, the results are conflicting and no mechanism has been found that explains all known host-*Wolbachia*-pathogen interactions. For example, some data indicate that *Wolbachia* activate the host immune system (Kambris et al., 2010a, 2010b) and others suggest this activation requires reactive oxygen species (ROS) (Pan et al., 2012). These hypotheses are countered by results that claim immune activation is not required (Rancès et al., 2012) and, even if it is, does not require ROS (Molloy and Sinkins, 2015).

One possible mechanism for *Wolbachia* to provide pathogen resistance is through DNA methyltransferases. This phenomenon has been established in *Aedes aegypti*, where *Wolbachia* downregulate host *Dnmt2* and thus block dengue replication (Zhang et al., 2013). Data described in CHAPTER II shows that interaction between *Dnmt2* and *Wolbachia* is not specific to mosquitos and creates the possibility that pathogen resistance in other hosts is also mediated through DNA methyltransferases. This hypothesis could easily be tested in the *D. melanogaster* system, where *Wolbachia* infection provides resistance against Drosophila C virus (DCV) (Teixeira et al., 2008) and *Dnmt2* is also known to protect against DCV independent of *Wolbachia* (Durdevic et al., 2013). Experiments testing the extent to which DCV titers are reduced in *Wolbachia*-infected *Dnmt2* mutants would determine whether *Wolbachia* use this

pathway to reduce viral load. This avenue of research could establish a fundamental mechanism for *Wolbachia*-mediated pathogen blocking that spans host species and virus types.

Identifying *Wolbachia* genes required for CI

While CHAPTER II highlights experiments seeking to identify the potential host pathways required for induction of CI, CHAPTER III represents efforts to characterize the *Wolbachia* genes necessary. This work identified the first two *Wolbachia* factors, WD0631 and WD0632 from the *w*Mel strain in *D. melanogaster*, that are able to induce CI. Expression of these two genes in transgenic, uninfected males induces embryonic lethality in crosses with uninfected females (Figure III-11). Infected females fully rescue this defect and the sex ratios of progeny are not affected, similar to wild-type CI (Figure III-14). Critically, imaging of embryos during early development shows that WD0631/WD0632 expression induces cytological defects similar to those seen in CI (Figure III-17). While these findings represent an important milestone for the field, many questions remain and future studies outlined below will further illuminate the full molecular mechanism of CI.

Phage WO genes as modulators of arthropod reproduction

It's interesting to note that both WD0631 and WD0632 are phage WO genes that have been incorporated into the *Wolbachia* genome. WO is unique in that, to spread to a new *Wolbachia* host, it must also contend with the eukaryotic environment in which *Wolbachia* reside. This makes it highly likely that WO phage has tools to modulate and navigate the eukaryotic cell and these genes could be a rich reservoir for *Wolbachia* to utilize in their

manipulations of host processes. WD0631 and WD0632 do not recapitulate the full strength of CI so it is reasonable to assume that other phage genes may be required. In fact, data show that WO genes in general follow an expression pattern that would be expected of CI factors (Figure III-6). It is also possible that WO genes may be involved in other reproductive parasitisms induced by *Wolbachia* infection such as feminization and male-killing. Current work in the Bordenstein lab has found one such phage WO gene that is able to selectively kill males in *D. melanogaster* (Jessamyn I. Perlmutter, unpublished). Finally, other phage genes such as WD0508 are clearly able to influence *Wolbachia* titers when expressed transgenically (Figure III-13), possibly by acting against host pathways that suppress *Wolbachia* proliferation. These preliminary results suggest that more research into phage WO genes is warranted as they may act as a repository for factors utilized by *Wolbachia* to alter host processes.

Identifying the host targets of CI effectors

While it is now shown that WD0631 and WD0632 are capable of inducing CI within *D. melanogaster*, their targets within the host are still unknown. Protein domain analysis of WD0632 suggests that it may function as a deubiquitinase (DUB), which is further supported by evidence showing it is capable of DUB-like activity *in vitro* (John F. Beckmann, personal communication). Whether this DUB activity is required for CI-induction, however, is debatable. In the *w*No strain of *Wolbachia*, which induces CI, only one homologue of WD0632 has been identified and it lacks the DUB domain. Whether this protein still functions as a CI factor, or whether other genes compensate for the lack of DUB activity, remains to be determined.

WD0631, on the other hand, lacks any recognizable conserved domains and its function is entirely speculative.

Efforts are already underway to find what WD0631 and WD0632 target within the host through the creation of transgenic *D. melanogaster* lines expressing HA-tagged versions of the genes. 3' and 5' tagged versions of each gene have been generated and initial experiments will test whether these lines are still able to induce CI at a level consistent with lines expressing the untagged transgenes. Any HA-tagged lines that lack activity will be removed from future analysis. Immunoprecipitation using publicly available monoclonal antibodies against the HA tag will be performed in multiple tissues, including testes and embryos, to determine which host factors are bound to candidate proteins. Given the DUB domain of WD0632, it would be logical to follow immunoprecipitation with mass spectrometry analysis to identify potential protein substrates. If, however, interactions with nucleic acids are suspected, as in the nuclease domain-containing *w*No homologue, then sequencing to find the specific host target could follow crosslinking and immunoprecipitation. Any analysis of WD0631 host targets must be approached with caution as a lack of conserved protein domains makes substrate prediction difficult. The only available information is from homologues that contain a cytochrome C552 domain suggesting a possible role as an electron donor. To aid the identification of WD0631 targets, localization assays utilizing HA-tagged versions of candidate genes, immunofluorescence, and microscopy of host tissue could indicate whether WD0631 is binding to chromatin or cytoplasmic targets. It is likely, however, that WD0631 and WD0632 act in concert as each is required for CI. This leaves the possibility that these two candidate genes work as a single complex and identifying the target of just one will provide critical information about the function

of the other. Finally, experiments utilizing truncated and/or mutated versions of WD0631 and WD0632 will be able to determine what regions and amino acids within each protein are necessary for inducing CI and could provide information about where these two factors may bind each other. Co-immunoprecipitation could also be performed to study WD0631-WD0632 binding, though additional transgenic lines utilizing a different epitope tag for one of the candidates would be required.

Immunoprecipitation efforts are supplemented by independent experiments seeking to find the host pathways required for CI. This work does not rely on candidate *Wolbachia* genes but instead utilizes omics-based screening that includes a large proteome for host sperm as well as previously published RNA sequencing data from *D. melanogaster* testes (Liu et al., 2014). Current data for this project are outlined in APPENDIX A. Briefly, I have confirmed a previous report that at least one host gene from *D. melanogaster*, JhI-26, is partially required for the induction of full wild-type CI. Future experiments will determine whether WD0631 and/or WD0632 rely on this gene to reduce embryonic survival or if they work through an as-yet-unknown mechanism.

Understanding bidirectional incompatibility

As discussed in CHAPTER III, the distribution and relatedness of WD0631/WD0632 homologs throughout various *Wolbachia* genomes suggests a role in the bidirectional incompatibility of CI-inducing strains. At the moment, however, this is only conjecture and further testing is warranted. This testing could be accomplished by experiments to determine whether strains other than *w*Mel are capable of rescuing CI induced by *w*Mel CI-factors. For

example, sequence homology would suggest that *w*No would not be able to rescue CI induced by WD0631/WD0632 transgenes. Crosses between transgene-expressing, uninfected males and *w*No-infected females could resolve this question. While these experiments would require moving novel *Wolbachia* infections into *D. melanogaster* lines, these techniques are well established (Frydman, 2007) and have been very successful in the Bordenstein lab (unpublished). It would also be highly informative to express other homologs of WD0631 and WD0632 and test whether they are able to induce CI. As many of these homologs do not share conserved domains with WD0631 and WD0632 it remains necessary to discover whether these genes actually induce CI and, if so, through what mechanism.

CI effectors as a toxin-antitoxin system

It is interesting to note that WD0631 and WD0632, and their homologs, are always found as a two-gene operon (Figure III-4). This observation opens the possibility that they may be acting as a toxin-antitoxin (TA) system. Classically, TA systems consist of two genes: one that functions as a "toxin" and the other as an "antitoxin." The labile, easily degraded antitoxin either binds directly to the toxin or regulates its expression. Under stress conditions, the antitoxin is quickly degraded and the cognate toxin released. The toxin can then target various cell processes including DNA replication, cell-wall biosynthesis, mRNA stability, and ribosome function. TA systems are widely prevalent in bacteria and archaea and have been linked to higher levels of pathogenicity in human infections (Van Melderen, 2010; Van Melderen and Saavedra De Bast, 2009; Pandey and Gerdes, 2005).

Several groups have hypothesized that CI may be induced by a TA-like system (Clark et al., 2003; Hurst, 1991; Poinsot et al., 2003; Presgraves, 2000). The proposed model is that the putative toxin would be used to induce CI by modifying sperm or being transported within the sperm of infected males. The cognate antitoxin, carried by infected females, would be capable of neutralizing the toxin and its effects within the embryo. This model is attractive for several reasons, including simplicity, the ability to explain bidirectional incompatibility (through different toxin-antitoxin pairings), and the fact that TA systems are a well-established means for bacteria to control cell cycles and development (Yamaguchi et al., 2011). Working from this model, others have found that a homolog of WD0632, wPa_0283 from *Culex*, functions as a toxin by inhibiting growth when expressed in yeast. Interestingly, they also found that the putative antitoxin (wPa_0282) binds to the toxin and, when expressed together in yeast, the toxin's effects are stopped (John F. Beckmann, personal communication). While these results are not corroborated by our work within insects, where both factors are needed to induce lethality (Figure III-11), they do suggest that this CI operon functions as a TA system in at least one model organism: yeast. It remains possible that, within insects, both toxin and antitoxin are needed for correct localization of the toxin. This is not unreasonable as in other systems one would expect the two to be bound together until the toxin needs to act. Future experiments looking at the localization of WD0631 and WD0632 through antibody tagging and fluorescent microscopy within *Drosophila* tissues should be able to clarify whether this is the case. It's also possible that infected females could provide additional, unidentified rescue factors and searches are currently under way to identify these elements (Lisa Klasson, personal communication).

Links between CI and other reproductive parasitisms

A renewed focus on the other reproductive alterations induced by *Wolbachia* should also prove informative. Exciting, recent work demonstrates that male-killing, like CI, is associated with damaged paternal chromatin (Riparbelli et al., 2012). Further links between CI and male-killing are evident as CI-inducing *Wolbachia* from *Drosophila recens* elicit male-killing when transferred to *Drosophila subquinaria,* with similar effects observed in strains transferred between moths (Jaenike, 2007; Sasaki et al., 2005). Finally, strains from *Drosophila bifasciata* that exhibit incomplete male-killing can also induce CI (Hurst et al., 2000). The growing links between the types of *Wolbachia*-induced sexual parasitism suggest a related underlying mechanism that warrants future exploration and modeling. While WD0631 and WD0632 do not seem to induce male-killing in *D. melanogaster* (Figure III-14) it is possible that these could selectively kill males in other host species. It will also be informative to understand whether current male-killing genes identified in the Bordenstein lab act through a mechanism similar to WD0631 and WD0632 (Jessamyn I. Perlmutter, unpublished).

Concluding remarks

The work presented here forms an important steppingstone in understanding *Wolbachia* and cytoplasmic incompatibility that has been forty years in the making. While many large questions remain, the identification of two verified CI factors and continued work seeking host pathways that they target provides key knowledge into how this once little-known bacteria has become a global force in ecology and disease.

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APPENDIX A. IDENTIFICATION OF POSSIBLE HOST PATHWAYS REQUIRED FOR CYTOPLASMIC INCOMPATIBILITY

Introduction

While the host DNA methylation pathway does not seem to be required for induction of cytoplasmic incompatibility (CHAPTER II) it remains likely that *Wolbachia* hijack other host systems to influence embryonic development. Others have sought to identify these candidate factors by screening for changes in host mRNA through RNA sequencing of third instar *D. melanogaster* testes (Liu et al., 2014; Zheng et al., 2011b). I expanded on these results by confirming the role of at least one host gene identified with this method: Juvenile hormone Inducible-26 (*JhI-26*). RNA sequencing of testes, however, is only a substitute for identifying any changes that *Wolbachia* may induce in the actual sperm. Therefore, in an attempt to more accurately elucidate changes occurring within the sperm of *Wolbachia*-infected males, I used protein purification and mass spectrometry analysis to detect any differences in host proteins as well as any *Wolbachia* proteins that may be localized in the sperm. These analyses, outlined below, provide an important stepping-stone for future work by identifying possible modes of action for WD0631 and WD0632 and which pathways they may be targeting within the host.

Results

Previous work by Zheng *et al* found that the *D. melanogaster* gene *JhI-26* is significantly upregulated in the testes of *Wolbachia*-infected third instar larvae (Zheng et al., 2011b).

Additionally, this group was able to show that induced overexpression of this gene in the testes of uninfected males can dramatically decrease embryonic hatch rates in crosses with uninfected females. Crosses between overexpressing males and *w*Mel infected females, however, are perfectly viable, as one would expect from CI (Liu et al., 2014). While this seminal work gives strong evidence that *JhI-26* can play a role in the induction of CI, it failed to prove whether it is necessary in the wild-type context. To answer this question, I crossed a publicly available *JhI-26* mutant line into a *w*Mel-infected background. I then tested the ability of this line to induce CI and found that mutant infected males mated to uninfected females generated progeny with a 39.8% embryonic hatch rate. While this represents significant levels of CI, this is still less lethal than the WT CI hatch rate of 16.1% (Figure A-1). This suggests that *JhI-26* is likely one of many factors involved in the induction of wild-type CI but that it is not absolutely necessary.

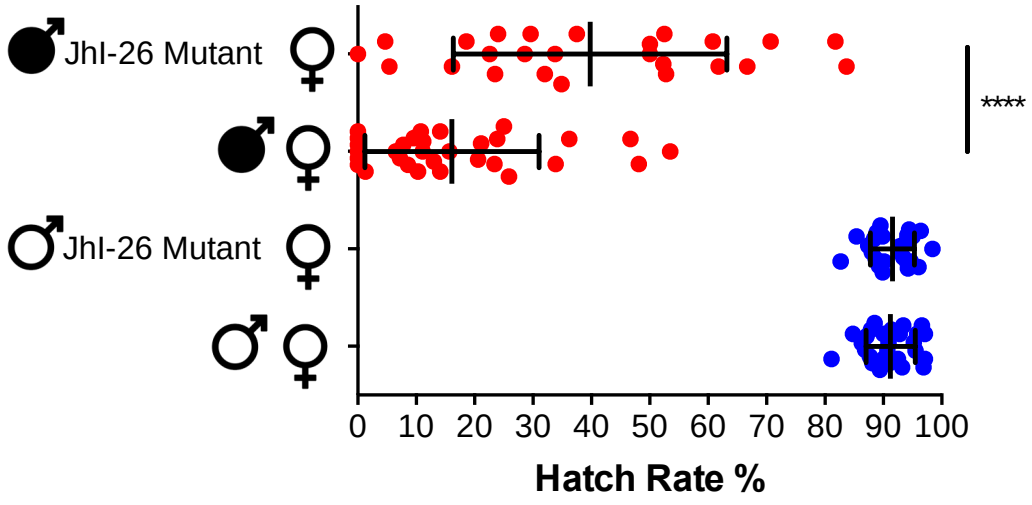

Figure A-1 Induction of CI by *JhI-26* mutant males
D. melanogaster males which lack a functional copy of *JhI-26* still induce CI (39.8% hatching) but this is weaker than wild-type CI (16.1% hatching, **** = $P < 0.0001$ by ANOVA with Kruskal-Wallis test and Dunn's multiple test correction). Red represents CI crosses while blue represents crosses between uninfected parents.

Consistent with my observed results, Liu *et al* suggest that JhI-26 could be functioning as a transcriptional regulator. This leaves the possibility that other transcription factors could target the same downstream pathways as *JhI-26* and thus compensate when it is removed from the system. It therefore becomes important to identify these downstream pathways and their role in CI. To this end, I sought to use a screening process that is more proximal to *Wolbachia*-modified sperm than simple mRNA sequencing of the testes to identify any altered host systems. While there are no published results looking at protein differences between the sperm of *Wolbachia*-infected and uninfected insects the techniques for creating a full sperm proteome in *D. melanogaster* through mass spectrometry are well established (Wasbrough et al., 2010). This is therefore a viable, and possibly novel, approach for identifying changes that *Wolbachia* induce in host sperm. Unfortunately, current techniques for generating a sperm proteome in *D. melanogaster* require that virgin males be aged for seven to fourteen days. Males of this age no longer induce CI (Reynolds and Hoffmann, 2002) and thus *D. melanogaster* is not a good model for this analysis. *Drosophila simulans*, however, poses a practical alternative for several reasons including (1) a fully sequenced genome and predicted proteome, (2) a native *Wolbachia* infection (*w*Ri) which induces very strong CI that can still be observed in older males, and (3) a fully sequenced genome and predicted proteome for *w*Ri, thus allowing for screening of *Wolbachia* proteins within the sperm as well.

Before generating a sperm proteome for *D. simulans,* I sought to confirm that aged virgin males with a *w*Ri infection could still induce strong CI. Figure A-2 shows that, despite being aged for seven days, *w*Ri infected *D. simulans* males are still capable of inducing CI with a mean hatch rate of 12.1%.

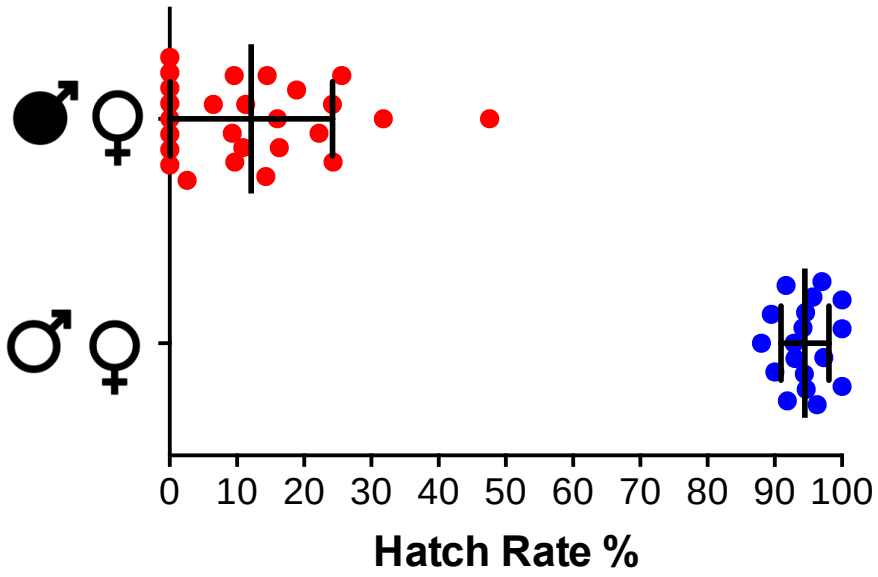

Figure A-2 Aged *D. simulans* induce strong CI
Virgin *D. simulans* males infected with *w*Ri and aged for seven days induce CI with a hatch rate of 12.1%. Red represents a CI cross while blue represents a cross between uninfected parents.

After confirming the strength of CI in aged *D. simulans*, pure sperm was dissected from both infected and uninfected males that had been aged for seven days. Protein lysates were generated and analyzed using MudPIT with protein identification performed using both *Drosophila simulans* and *w*Ri predicted proteomes. This analysis yielded 487 identified proteins with stringent cutoffs of 3 minimum peptides, 99% protein identity confidence, and a 0.8% False Discovery Rate. While this number is lower than the 1,108 proteins predicted in the published *Drosophila melanogaster* sperm proteome (Wasbrough et al., 2010), 19.8% of proteins in that study were identified with just a single confirmed peptide. It is not surprising then that with a lower sample size and more stringent cutoff this work identified fewer proteins. Interestingly, though, several dozen proteins appear to be misregulated in sperm from *Wolbachia* infected males. A sample of the most significant candidates is shown in Table A-1. Many of these

candidates are testes-specific (*Loopin-1*, *Ocnus*, sperm leucylaminopeptidases, and *Male sterile (2) 34Fe*) while others, such as *CG3213* (a chromosome segregation factor), suggest a link to the known mitotic defects that occur during CI. While no *Wolbachia* proteins with more than three confirmed peptides were identified in infected sperm, this approach does suggest a robust method to identify possible host pathways altered in mature sperm from *Wolbachia*-infected hosts.

Table A-1 Host proteins misregulated in sperm from *w*Ri infected *D. simulans*

D. simulans	*D. melanogaster* homologue	Fold Change	P Value (Fisher's Exact Test)
GD19909	Alpha Tubulin 84b	-1.52	<0.0001
GD11203	Loopin-1	-1.46	<0.0001
Ocnus	Ocnus	2.19	<0.0001
GD14112	Sperm leucylaminopeptidase 1	-3.09	<0.0001
GD14113	Sperm leucylaminopeptidase 2	-1.59	<0.0001
GD14243	Sperm leucylaminopeptidase 3	-3.97	<0.0001
GD22749	CG3213 (chromosome segregation)	3.07	<0.0001
GD23958	Male sterile (2) 34Fe	-4.67	0.006

The large similarity between *D. simulans* and *D. melanogaster* allows for testing of candidate gene homologues in a *D. melanogaster* background, thus taking advantage of the vast number of publicly available overexpressing, mutant, and RNAi expressing *D. melanogaster* lines. Specifically, I utilized a line expressing RNAi against *Loopin-1* under UAS control in combination with a *nanos-Gal4* driver line. This allows testes-specific downregulation of *Loopin-1*, similar to what is observed in mature sperm from infected males. Importantly, the *D.*

melanogaster homologue shares 98% protein similarity with *D. simulans*, making the possibility of conserved functions highly likely. Crossing infected or uninfected males expressing RNAi against *Loopin-1* resulted in hatch rates less than 2% in all tested scenarios (Figure A-3). Unfortunately, this embryonic defect is not rescued by infected females leaving the possibility that *Loopin-1* is generally required for embryonic development and not necessarily involved in CI.

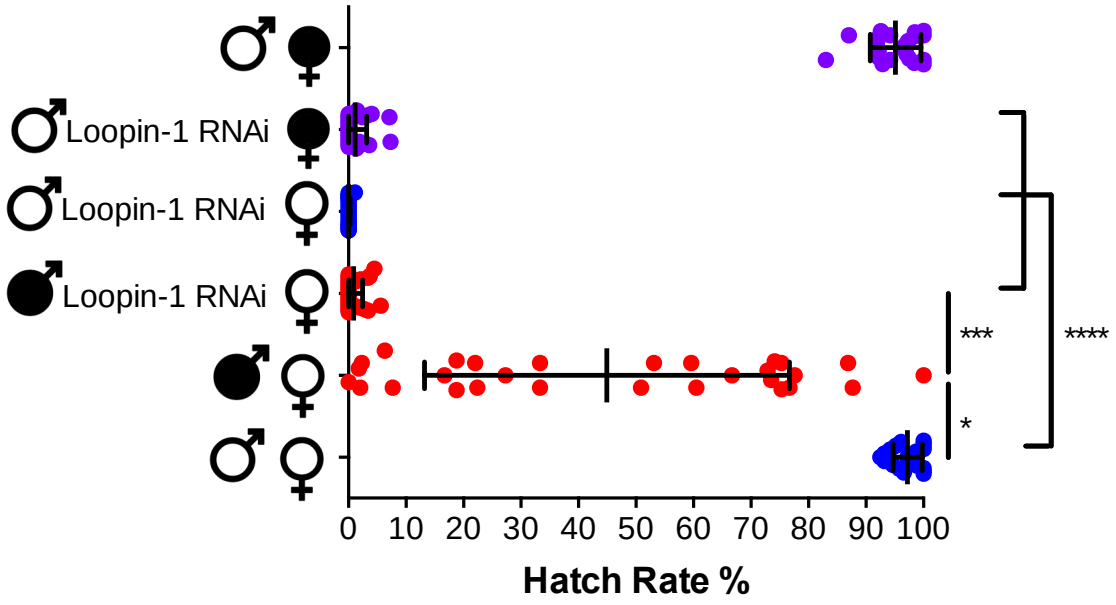

Figure A-3 Knockdown of *Loopin-1* significantly reduces hatch rates
RNAi knockdown of *Loopin-1* reduces hatching to less than 2% in all tested scenarios. Infected females do not rescue this defect. * = P < 0.05, *** = P < 0.001, **** = P < 0.0001 by ANOVA with Kruskal-Wallis test and Dunn's multiple test correction. Red represents CI crosses, blue represents crosses between uninfected parents, while purple represents crosses with and infected female.

Conclusions

This work confirms the identification of at least one host gene, *JhI-26*, that is utilized by *Wolbachia* to induce CI. Experiments are currently under way to determine whether WD0631 and WD0632 also require this host factor by crossing UAS-transgenic and *nanos-Gal4* lines into *JhI-26* mutant backgrounds and measuring whether these candidate genes still induce embryonic defects. The function of *Jhi-26* in *Drosophila* is not completely understood, however. Some evidence suggests that it may act as a transcription factor with at least one downstream target being *CG10433* (Liu et al., 2014). Thus, it remains possible that WD0631 and WD0632 act independently of *JhI-26*, especially as *JhI-26* is not absolutely necessary to induce wild-type CI. In that case, host targets of *Wolbachia* may be identified through proteome analysis of *Drosophila* sperm. This work has already discovered one host gene, *Loopin-1*, that is misregulated in sperm from infected males and required for embryonic development. RNAi knockdown of *Loopin-1* induces embryonic lethality but infected females do not rescue this lethality. Experiments utilizing alternative Gal4 drivers will be able to determine whether weaker knockdown of *Loopin-1* (similar to what is found in the sperm proteome) can more fully recapitulate CI. Finally, future work will hopefully expand on these results to elucidate the full set of host factors required for *Wolbachia*-induced cytoplasmic incompatibility. This may include replication of the sperm proteome experiments to gain better sample size and a smaller candidate list as well as testing of the candidate host factors already identified.

Materials and Methods

Fly lines and hatch rate assays

Hatch rates were performed essentially as previously described (CHAPTER II). The JhI-26 mutant line was obtained from Bloomington Stock Center (BSC28109, y^1 w^*; *P{EP}JhI-26*^{G18921}) and crossed into a *yw* background line (both uninfected and *w*Mel infected). The mutant line contains a large genomic insertion within the *JhI-26* gene, presumably making it inactive. Mutants were followed using a red eye marker. For *Loopin-1* knockdown, *nos-Gal4* virgin females were crossed with y^1v^1; *P{TRiP.HMJ21436}attP40* males.

Sperm proteome generation

Sperm was dissected from 100 hundred *w*Ri infected and uninfected males each. These males were the first to emerge from their respective crosses and maintained as virgins for 7 days before sample preparation. Dissections were performed in PBS with 1x protease inhibitors added (manufacturer's instructions, EDTA free, Pierce) that was filtered through a 0.22 micro syringe filter. Sperm was spun for 1 minute at full speed in a microcentrifuge before and PBS was removed. Samples were washed with PBS and the spin was repeated. Lysis was performed with 20uL of lysis buffer (8M urea, 1% 2-Mercaptoethanol, 0.6M NaCl, 100mM Tris).

Peptides were analyzed via MudPIT (Multidimensional Protein Identification Technology) essentially as previously described ((MacCoss et al., 2002; Martinez et al., 2012)). Briefly, trypsin digested peptides were loaded onto a biphasic pre-column consisting of 4 cm of reversed phase (RP) material followed by 4 cm of strong cation exchange (RP) material. Once loaded this column was placed in line with a 20 cm RP analytical column packed into a

nanospray emitter tip directly coupled to a linear ion trap mass spectrometer (LTQ). A subset of peptides was eluted from the SCX material onto the RP analytical via a pulse of volatile salt, those peptides separated by an RP gradient, and then ionized directly into the mass spectrometry where both the intact masses (MS) and fragmentation patters (MS/MS) of the peptides were collected. These peptide spectral data were searched against a protein database using Sequest (Yates et al., 1995) and the resulting identifications collated and filtered using IDPicker (Ma et al., 2009) and Scaffold (http://www.proteomesoftware.com).

APPENDIX B. PRIMER INFORMATION

Table B-1. Primer targets, sequences, and product sizes

Target	Name of Primer Set	Primer sequences (5' to 3')	Product Size (bp)
16s rRNA (*Wolbachia*)	Wolb	F: GAAGATAATGACGGTACTCAC R3: GTCACTGATCCCACTTTAAATAAC	990
Act5c	Act5c	F: ATGTGTGACGAAGAAGTTGCT R: GTCCCGTTGGTCACGATACC	231
Dnmt2	Dnmt2	F: CCGTGGCGTGAAATAGCG R: ACACCGCTTTCGGAGGACG	150
groEL (qPCR standards)	groELstd	F: GGTGAGCAGTTGCAAGAAGC R: AGATCTTCCATCTTGATTCC	923
groEL (qPCR)	groEL	F: CTAAAGTGCTTAATGCTTCACCTTC R: CAACCTTTACTTCCTATTCTTG	97
Rp49	Rp49	F: CGGTTACGGATCGAACAAGC R: CTTGCGCTTCTTGGAGGAGA	154
WD0034	WD0034	F: GGAAGAAACTTGCACACCACTTAC R: TGCTCTCCGACCATCTGGATATTT	151
WD0508	WD0508	F: TAGAGATCTAGCTTGCGGACAAGA R: TCCTTAACTAAACCCTTTGCCACC	204
WD0625	WD0625	F: GAGCCATCAGAAGAAGATCAAGCA R: TTCTCGAAAGCTGAAATAGCCTCC	120
WD0631	WD0631	F: TGTGGTAGGGAAGGAAAGAGGAAA R: ATTCCAAGGACCATCACCTACAGA	111
WD0632	WD0632	F: TGCGAGAGATTAGAGGGCAAAATC R: CCTAAGAAGGCTAATCTCAGACGC	197
WD0640	Gpw	F: CTACAACCTCATCGAAGCGAATCT R: CTGCAGAAGCTTTGGAAAAATGGG	144
WD0508 (transgene)	WD0508opt	F: GACGTGCTGATCAAGAGCCT R: TGCCCACTGTCTTCAGGATG	136
WD0625 (transgene)	WD0625opt	F: CGCGAGATGGATGACCTGAA R: CTCGCGCTCACTATGTCCAA	180
WD0631 (transgene)	WD0631opt	F: GGTGGATAGTCAGGGCAACC R: AAAAGTACTCCACGCCCTCG	191
WD0632 (transgene)	WD0632opt	F: CCTGCCCTACATTACACGCA R: GGCGACAGATCCAGGTCAAT	159

APPENDIX C. CI CANDIDATE GENES

Table C-1 Core CI genes

*w*Mel Locus	Gene Description
WD0035	ankyrin repeat-containing protein
WD0038	Protein tolB
WD0046	reverse transcriptase, interruption-N
WD0049	hypothetical protein
WD0056	major facilitator family transporter
WD0061	hypothetical protein
WD0064	Pyridoxine 5'-phosphate synthase
WD0069	hypothetical protein
WD0074	hypothetical protein
WD0077	hypothetical protein
WD0078	hypothetical protein
WD0079	hypothetical protein
WD0092	DNA processing chain A
WD0099	multidrug resistance protein
WD0100	sugE protein
WD0131	hypothetical protein
WD0139	TenA family transcription regulator
WD0140	TenA family transcription regulator
WD0168	major facilitator family transporter
WD0200	hypothetical protein
WD0208	hypothetical protein
WD0211	hypothetical protein
WD0214	hypothetical protein
WD0217	phage uncharacterized protein
WD0231	hypothetical protein
WD0234	hypothetical protein
WD0240	transposase, IS5 family, degenerate
WD0255	transcriptional regulator, putative
WD0257	DNA repair protein RadC, truncation
WD0258	Reverse transcriptase, frame shift
WD0274	hypothetical protein
WD0278	Prophage LambdaW1, minor tail protein Z
WD0279	hypothetical protein
WD0281	hypothetical protein

WD0282	prophage LambdaW1, baseplate assembly protein W, putative
WD0283	prophage LambdaW1, baseplate assembly protein J, putative
WD0284	hypothetical protein
WD0285	Prophage LambdaW1, ankyrin repeat protein
WD0286	ankyrin repeat-containing prophage LambdaW1
WD0288	prophage LambdaW1, site-specific recombinase resolvase family protein
WD0315	hypothetical protein
WD0324	hypothetical protein
WD0329	transposase, IS5 family, degenerate
WD0336	transposase, IS5 family, degenerate
WD0338	hypothetical protein
WD0345	RND family efflux transporter MFP subunit
WD0376	potassium uptake protein TrKH, frame shift
WD0382	hypothetical protein
WD0385	ankyrin repeat-containing protein
WD0396	reverse transcriptase, truncation
WD0407	Na+/H+ antiporter, putative
WD0426	hypothetical protein
WD0431	glycosyl transferase, group 2 family protein
WD0447	phage prohead protease
WD0458	HK97 family phage major capsid protein
WD0472	AAA family ATPase
WD0480	hypothetical protein
WD0481	hypothetical protein
WD0482	SPFH domain-containing protein/band 7 family protein
WD0483	M23/M37 peptidase domain-containing protein
WD0498	ankyrin repeat-containing protein
WD0501	surface antigen-related protein
WD0506	Reverse transcriptase, frame shift
WD0507	DNA repair protein RadC, truncation
WD0508	transcriptional regulator, putative
WD0515	reverse transcriptase, interruption-C
WD0518	reverse transcriptase, interruption-N
WD0538	reverse transcriptase, truncation
WD0604	hypothetical protein
WD0606	Reverse transcriptase, frame shift
WD0623	transcriptional regulator, putative
WD0624	conserved domain protein, frame shift

WD0625	DNA repair protein RadC, putative
WD0626	transcriptional regulator, putative
WD0628	hypothetical protein
WD0631	**hypothetical protein**
WD0632	**SUMO protease**
WD0633	Prophage LambdaW5, ankyrin repeat domain protein
WD0634	prophage LambdaW5, site-specific recombinase resolvase family protein
WD0636	ankyrin repeat-containing prophage LambdaW1
WD0638	hypothetical protein
WD0639	prophage LambdaW5, baseplate assembly protein J, putative
WD0640	prophage LambdaW5, baseplate assembly protein W, putative
WD0641	hypothetical protein
WD0642	prophage LambdaW5, baseplate assembly protein V
WD0643	hypothetical protein
WD0644	Prophage LambdaW5, minor tail protein Z
WD0645	reverse transcriptase, truncation
WD0686	hypothetical protein
WD0693	reverse transcriptase, putative
WD0696	hypothetical protein
WD0702	hypothetical protein
WD0713	hypothetical protein
WD0718	conserved hypothetical protein, truncated
WD0721	Mg chelatase-related protein
WD0724	hypothetical protein
WD0730	phosphatidylglycerophosphatase A, putative
WD0733	hypothetical protein
WD0748	hypothetical protein
WD0749	transposase, IS5 family, degenerate
WD0750	PQQ repeat-containing protein
WD0764	hypothetical protein
WD0787	araM protein
WD0790	hypothetical protein
WD0818	hypothetical protein
WD0823	hypothetical protein
WD0826	hypothetical protein
WD0834	conserved hypothetical protein, degenerate
WD0835	hypothetical protein
WD0874	transposase, truncated

WD0875	IS5 family transposase
WD0880	coenzyme PQQ synthesis protein C, putative
WD0882	FolK
WD0883	dihydropteroate synthase, putative
WD0884	dihydrofolate reductase
WD0887	DNA repair protein RadA
WD0901	transposase, IS110 family, degenerate
WD0903	transposase, IS5 family, degenerate
WD0908	transposase, degenerate
WD0911	transposase, IS5 family, degenerate
WD0914	hypothetical protein
WD0932	IS5 family transposase
WD0935	transposase, IS5 family, interruption-C
WD0941	transposase, degenerate
WD0947	IS5 family transposase
WD0958	hypothetical protein
WD0964	hypothetical protein
WD0975	hypothetical protein
WD0995	reverse transcriptase
WD0999	hypothetical protein
WD1002	hypothetical protein
WD1012	HK97 family phage portal protein
WD1015	hypothetical protein
WD1016	phage uncharacterized protein
WD1041	surface protein-related protein
WD1047	sodium/alanine symporter family protein
WD1052	folylpolyglutamate synthase
WD1069	hypothetical protein
WD1073	N-acetylmuramoyl-L-alanine amidase
WD1091	tRNA (guanine-N(7)-)-methyltransferase
WD1114	LipB
WD1118	hypothetical protein
WD1126	hypothetical protein
WD1131	conserved hypothetical protein, degenerate
WD1132	phage uncharacterized protein
WD1138	reverse transcriptase, putative
WD1159	Pyridoxine/pyridoxamine 5'-phosphate oxidase
WD1160	ComEC/Rec2 family protein

WD1161	hypothetical protein
WD1162	ribosomal large subunit pseudouridine synthase D
WD1163	diacylglycerol kinase
WD1175	hypothetical protein
WD1179	hypothetical protein
WD1204	TPR domain-containing protein
WD1212	16S ribosomal RNA methyltransferase RsmE
WD1218	ParB family protein
WD1242	hypothetical protein
WD1272	hypothetical protein
WD1310	hypothetical protein
WD1320	multidrug resistance protein D
WD1321	hypothetical protein

Table C-2 Genes divergent in *w*Au

***w*Mel Locus Tag**	**Gene Description**
WD0019	transcription antitermination protein NusG, putative
WD0022	ribosomal protein L10
WD0034	PAZ Zwille/Arganaut/Piwi/ SiRNA binding domain
WD0072	hypothetical protein
WD0205	hypothetical protein
WD0244	hypothetical protein
WD0255	transcriptional regulator, putative
WD0256	hypothetical protein
WD0257	DNA repair protein RadC, truncation
WD0289	hypothetical protein
WD0297	hypothetical protein
WD0311	hypothetical protein
WD0320	trigger factor, putative
WD0349	hypothetical protein
WD0363	hypothetical protein
WD0366	hypothetical protein
WD0367	hypothetical protein
WD0369	hypothetical protein
WD0389	conserved hypothetical protein
WD0424	hypothetical protein
WD0449	hypothetical protein
WD0508	transcriptional regulator, putative
WD0512	ankyrin repeat domain protein
WD0553	hypothetical protein
WD0576	hypothetical protein
WD0577	hypothetical protein
WD0578	Hypothetical (Eukaryotic DUF812?)
WD0579	hypothetical protein (virulence associated?)
WD0598	hypothetical protein
WD0607	hypothetical protein
WD0623	transcriptional regulator, putative
WD0624	conserved domain protein, authentic frameshift
WD0625	DNA repair protein RadC, putative
WD0626	transcriptional regulator, putative
WD0631	**hypothetical protein**
WD0632	**SUMO protease**

WD0633	prophage LambdaWp5, ankyrin repeat domain protein
WD0704	hypothetical protein
WD0723	cell division protein FtsZ
WD0746	hypothetical protein
WD0747	hypothetical protein
WD0806	hypothetical protein
WD0808	hypothetical protein
WD0809	hypothetical protein
WD0836	hypothetical protein
WD0837	hypothetical protein
WD0840	hypothetical protein
WD0850	rpsU-divergently transcribed protein
WD0854	membrane protein, putative
WD0877	hypothetical protein
WD0940	hypothetical protein
WD0946	hypothetical protein
WD0971	hypothetical protein
WD1038	hypothetical protein
WD1151	citrate synthase
WD1260	hypothetical protein
WD1287	hypothetical protein
WD1291	hypothetical protein
WD1311	Glycoside hydrolase 24
WD1313	conserved domain protein

APPENDIX D. LIST OF PUBLICATIONS

LePage, D.P.,* Metcalf, J.A.,* Bordenstein, S.R., On, J., Funkhouser-Jones, L.J., Beckmann, J.F., Bordenstein, S.R. (2016) *Wolbachia*-induced Cytoplasmic Incompatibility is Caused by Prophage WO genes. *Submitted*.

LePage, D.P., Jernigan, K.K., Bordenstein, S.R. (2014) The relative importance of DNA methylation and Dnmt2-mediated epigenetic regulation on *Wolbachia* densities and cytoplasmic incompatibility. PeerJ. 2:e6778. doi:10.7717/peerj.678

LePage, D.P., Bordenstein, S.R. (2013) *Wolbachia*: Can we save lives with a great pandemic? Trends in Parasitology. **29** (8) p359-416. doi:10.1016/j.pt.2013.06.003.