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Genomic and Evolutionary Consequences of *Wolbachia*-Mediated Parthenogenesis in *Trichogramma* Wasps

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Amelia Ryan Isis Lindsey

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Dissertation Committee:
Dr. Richard Stouthamer, Chairperson
Dr. Matthew Daugherty
Dr. Jason Stajich

The Dissertation of Amelia Ryan Isis Lindsey is approved:

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Committee Chairperson

University of California, Riverside

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ABSTRACT OF THE DISSERTATION

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by

Amelia Ryan Isis Lindsey

Doctor of Philosophy, Graduate Program in Entomology
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Dr. Richard Stouthamer, Chairperson

Wolbachia is perhaps the most ubiquitous symbiotic bacterium in the animal world. It is known to infect the majority of insect species, in which it induces a wide array of reproductive phenotypes. In *Trichogramma*, tiny parasitoid wasps often used for biological control of insect pests, *Wolbachia* converts the wasps to an asexual mode of reproduction through the induction of parthenogenesis. We do not have a thorough understanding of 1) the genetic and environmental factors that mediate the penetrance of the parthenogenesis phenotype, thus allowing *Wolbachia* to fix in a population, and 2) how the fixation of *Wolbachia* in a population affects genome evolution of the partners. In Chapters 1 and 2, I develop genomic resources for both the *Wolbachia* strain *w*Tpre (Chapter 1), and its native host *Trichogramma pretiosum* (Chapter 2), and use comparative genomics to identify unique features of these genomes that could be implicated in the co-evolution of host and symbiont. In Chapters 3 and 4, I explore some of the genetic and environmental factors that affect the expression of *w*Tpre's parthenogenesis-inducing phenotype. In Chapter 3, I show that the rate of *Trichogramma*

reproduction mediates the penetrance of the parthenogenesis phenotype. In Chapter 4, I look at the effects of outbreeding, and how the resulting combinations of new host-symbiont genotypes affect *w*Tpre's ability to induce parthenogenesis. By generating genomic resources for the system, and teasing apart the factors that determine the final host-symbiont phenotype, we can improve our understanding of how *Wolbachia* spreads and fixes in a population, and what the long-term consequences of fixation are for both symbiotic partners.

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INTRODUCTION

Wolbachia: A reproductive parasite of arthropods and nematodes

Prokaryotes are intricately woven into the lives of most eukaryotic organisms. Well-known examples include the organelles of eukaryotic cells, the zooxanthellae of corals, and the gut bacteria of herbivores. As symbiotic partners, microbes provide essential metabolic functions, protect against parasites, drive speciation, introduce novel genes, and allow for niche expansion (Moran 2006). Despite the ubiquity of microbes as symbiotic partners, we know comparatively little about the forces that drive the evolution of these associations.

Insects and bacteria are two of the most prevalent life forms, and are often closely associated with each other. *Wolbachia*, a bacterium well known for altering host reproduction to select for females, is widely associated with arthropods and nematodes. *Wolbachia* infects an estimated 40-60 percent of insect species (Hilgenboecker et al. 2008; Zug and Hammerstein 2012). It is advantageous for *Wolbachia* to select for females, as the bacterium is maternally transmitted through the germline, but absent from sperm (Werren 1997). Reproductive modifications include cytoplasmic incompatibility (CI), where *Wolbachia* modifies sperm such that offspring do not develop unless "rescued" by the presence of *Wolbachia* in the egg of an infected female; parthenogenesis-induction (PI), where females are produced asexually; male-killing, where male offspring do not survive; and feminization, where genetic males develop as functional phenotypic females (Werren et al. 2008). These reproductive modifications

resulting from infection with *Wolbachia* have drastic effects on host population dynamics, speciation, and gene flow (Stouthamer et al. 1999). Beyond reproductive manipulations, some *Wolbachia* strains protect the host against pathogens and reduce the ability to transmit pathogens (Teixeira et al. 2008; Bian et al. 2010; Hughes et al. 2011; Kambris et al. 2009; Chrostek et al. 2013; Zele et al. 2012; Walker et al. 2011), are essential for oogenesis (Kremer et al. 2009; Dedeine et al. 2001), provision nutrients to the host (Nikoh et al. 2014; Hosokawa et al. 2010; Moriyama et al. 2015), or are virulent, shortening the lifespan of the host (Min and Benzer 1997). In filarial nematodes, *Wolbachia* is an obligate symbiont that is required for reproduction and likely plays a role in evading the vertebrate host's immune system (Darby et al. 2012; Hoerauf et al. 1999). In the fields of vector- and biological control, there is great interest in transferring *Wolbachia* with particularly favorable phenotypes into a variety of target hosts. This is a promising field that is already being employed for the control of mosquitoes and the diseases they vector (Walker et al. 2011; Hoffmann et al. 2011; Hoffmann et al. 2015; Turelli and Hoffmann 1991). A wide variety of *Wolbachia* strains and host-phenotypes have been identified, but we do not have a thorough understanding of what factors mediate the persistence of these associations. The strength of the manipulation, the frequency of infection and transmission, and the selective pressures to which the relationship is subjected all affect the evolutionary trajectory of the host and symbiont.

This dissertation seeks to identify: 1) variables that mediate the strength of a recently fixed *Wolbachia*-host association, and 2) the genomic consequences of the association for both partners.

Context dependent nature of *Wolbachia*-host interactions

In order to achieve the ultimate goal of predicting the consequences of host-symbiont relationships, we must first work towards identifying the factors that mediate the relationship, and the "mode of action" of the symbiont. So far, a number of genetic and environmental factors that affect *Wolbachia*-host phenotypes have been identified. Many of these factors can act antagonistically or synergistically (Rohrscheib et al. 2016; Chrostek et al. 2013; Bordenstein and Bordenstein 2011; McGraw et al. 2002), with complicated genotype-by-genotype-environment (GxGxE) interactions governing the strength of manipulations, fitness costs for the host, and level of maternal transmission: critical variables in determining persistence and spread of *Wolbachia* in a population (Turelli and Hoffmann 1995; Hoffmann et al. 2011; Hoffmann et al. 1990).

There is a growing body of evidence that suggests *Wolbachia* and host genotypes mediate the success of *Wolbachia*-induced reproductive manipulations. This is likely due, in part, to the fact that *Wolbachia* is primarily maternally transmitted (Werren et al. 2008), affording the opportunity to co-evolve with a particular host genetic background (Huigens et al. 2004; Pintureau et al. 2000; Grenier et al. 1998; Rigaud et al. 2001). Introduction of *Wolbachia* into novel host species or new host genotypes has documented

effects on *Wolbachia* titers (Watanabe et al. 2013; Kondo et al. 2005; Clancy and Hoffmann 1998), host fitness (Mouton et al. 2007; Dean 2006; Rigaud et al. 2001), penetrance of reproductive manipulations (Bordenstein and Werren 1998; Grenier et al. 1998; McGraw et al. 2001; Bordenstein et al. 2003; Huigens et al. 2004; Watanabe et al. 2013), and levels of maternal transmission (Grenier et al. 1998; Huigens et al. 2004). Likewise, different *Wolbachia* variants show differential protection against pathogens (Osborne et al. 2009; Bian et al. 2013; Chrostek et al. 2013), and varying levels of reproductive manipulation (Hoffmann et al. 2015; Cooper et al. 2017). *Wolbachia*-host relationships can be subsequently sculpted by co-infection with other *Wolbachia* strains (Kondo et al. 2005; Hiroki et al. 2004), and the presence of other symbionts (Hughes et al. 2014; Ye et al. 2016; Shokal et al. 2016).

Environmental and ecological conditions add an additional layer to the aforementioned genotype-by-genotype interactions. High temperatures have curing effects that lower *Wolbachia* densities and can result in decreased penetrance of the reproductive manipulation (Hurst et al. 2000; Bordenstein and Bordenstein 2011; Pascal et al. 2004; Hohmann and Luck 2000). This effect can be both direct and indirect, as increased temperatures can activate *Wolbachia*'s bacteriophage, WO, further reducing titers (Bordenstein and Bordenstein 2011). *Wolbachia*'s pathogen protection abilities are likewise affected by temperatures (Rohrscheib et al. 2016; Reynolds et al. 2003), as is the male-killing trait (Hurst et al. 2000). In populations of *Trichogramma* wasps there is potential for a seasonal thermal cure in particularly hot summer climates (Pintureau and

Bolland 2001; Pintureau et al. 2002). Larval crowding, population density, and nutrient availability can interact to have effects on *Wolbachia* titers (Dutton and Sinkins 2004; Wiwatanaratanabutr and Kittayapong 2009; Serbus et al. 2015). Male development time has an effect on the strength of CI induction (Yamada et al. 2007).

Most of the research that has been done on the genetic and environmental determinants of *Wolbachia*-host symbioses has focused on CI-*Wolbachia* and those that protect their host against pathogens. Less is known about the other reproductive phenotypes, which likely have different mechanisms for manipulation of their hosts (LePage et al. 2017; Beckmann et al. 2017), and thus different consequences for host populations. PI-*Wolbachia* are found across a range of haplodiploid arthropods, and exploit the sex determination system to ensure female offspring are produced (Stouthamer and Kazmer 1994; Gottlieb et al. 2002). In parasitoid wasps that are used as biological control agents, *Wolbachia* might improve pest control, as a more female-biased population would be beneficial, because females are the sex capable of attacking pests (Stouthamer 1993; Silva et al. 2000; Pannebakker et al. 2004b). Like CI-*Wolbachia*, PI-*Wolbachia* are able to invade a population. However, PI-*Wolbachia* are unique in that there are several examples of strains that have gone to fixation in their host populations such that the host is dependent upon *Wolbachia* for reproduction (Russell and Stouthamer 2011; Stouthamer et al. 2010; Kremer et al. 2009; Dedeine et al. 2001; Jeong and Stouthamer 2005). It is not clear why only some populations become fixed for infection, allowing for the evolution of irreversible asexuality. It is likely that a combination of genetic and

environmental factors determine the potential for PI-*Wolbachia* to fix in a population. The parasitoid *Trichogramma* wasps are one such example, where there is extensive inter- and intra-specific variation for the frequency of infection, with populations found to never have *Wolbachia* (Pinto 1998), populations with low stable infection levels (Stouthamer and Kazmer 1994; Stouthamer et al. 2001), and those that are fixed for infection and irreversibly asexual (Russell and Stouthamer 2011; Jeong and Stouthamer 2005).

Consequences of asexuality

Irreversible asexuality has been documented in *Trichogramma pretiosum* populations that have become fixed for their *Wolbachia* infection (Russell and Stouthamer 2011). *Wolbachia* duplicates chromosomes in unfertilized *Trichogramma* eggs, turning what would have been a haploid male offspring into a diploid female offspring through a failed anaphase during the first mitotic division of the egg (Stouthamer and Kazmer 1994). Despite this, parthenogenetic females will occasionally mate with conspecific sexual males and fertilize their eggs (Stouthamer and Kazmer 1994). If the egg is fertilized, gamete duplication becomes unnecessary, and *Wolbachia* will not affect chromosome segregation (Stouthamer and Kazmer 1994). When *Trichogramma* populations are a mix of infected and uninfected individuals, there is a selective advantage for uninfected females to produce more males, accomplished through halting egg fertilization: a so-called "virginity mutation" (Stouthamer et al. 2010; Jeong and Stouthamer 2005). The males produced in this way will carry the virginity mutation, and can then mate with the

infected females in the population who will fertilize their eggs, simultaneously transmitting *Wolbachia* and the virginity mutation, both of which become fixed in the population over time (Stouthamer et al. 2010). If populations with virginity mutations are cured of their *Wolbachia* infections with heat or antibiotics, they will not fertilize their eggs at high enough frequencies to maintain a sexual line: they are dependent upon *Wolbachia*-mediated gamete duplication to produce females (Russell and Stouthamer 2011; Jeong and Stouthamer 2005).

After the transition to asexuality, theory predicts that sexual traits will decay, further impeding any possibility for a reversal to sexuality (van der Kooi and Schwander 2014; Neiman et al. 2014; Carson et al. 1982). Traits can decay through drift, which is more likely for male traits as they are not exposed to selection, or by positive selection for mutations that impede costly female function, such as the production of pheromones (van der Kooi and Schwander 2014; Dorken et al. 2004). Decayed male traits such as dysfunctional spermatogenesis and reduced fertilization efficiency have been reported in a variety of lineages (van der Kooi and Schwander 2014; Russell and Stouthamer 2011; Gottlieb and Zchori−Fein 2001; Pannebakker et al. 2004a). Loss of female function has been reported more frequently and can be attributed to reduced or no fertilization of eggs (Ma et al. 2014; Gottlieb and Zchori−Fein 2001; Kraaijeveld et al. 2009), loss of sperm storage abilities (Gottlieb and Zchori−Fein 2001; Kraaijeveld et al. 2009), as well as altered behavior and pheromone production (Carson et al. 1982; Schwander et al. 2013).

Beyond genes that directly relate to sexual function, there are genome-wide consequences of asexuality. Asexual lineages will rapidly accumulate deleterious mutations (Neiman et al. 2010; Henry et al. 2012; Hollister et al. 2015; Paland and Lynch 2006), undergo changes in transposon frequencies (Kraaijeveld et al. 2012; Hurst and Werren 2001; Wright and Finnegan 2001; Arkhipova and Meselson 2000; Schaack et al. 2010), and experience changes in effective recombination and population size (Charlesworth and Wright 2001).

In the case of the irreversibly asexual *Trichogramma* wasps, we do not yet know the genetic architecture of virginity mutations, and how this reproductive shift affects genome evolution of wasp and symbiont. As one of the most commonly used biological control agents against lepidopteran eggs (Knutson 1998), it is especially relevant to biological control programs to understand the effects of asexuality in *Trichogramma*.

Consequences of symbiont domestication

Finally, there are consequences for symbionts that have become domesticated. Genome evolution is well understood for primary symbionts in mutualistic relationships with the host (Moran et al. 2003). Insects that live off of nutrient imbalanced diets such as xylem, phloem, or blood require essential amino acids and vitamins that are frequently provided by maternally transmitted bacteria (Douglas 1998; Snyder et al. 2010; Hansen and Moran 2014; Moran and Telang 1998). In these associations, symbionts are strictly maternally transmitted, and host and symbiont become completely co-dependent, often times

incurring fitness costs as a result of their association (Bennett and Moran 2015). The primary symbiont genome becomes streamlined, undergoing huge reductions in size, coding ability, and functional capacity (McCutcheon and Moran 2012; Moran et al. 2008; Moran 2002; Wernegreen 2002). Maternally transmitted endosymbionts are subject to Muller's ratchet due to small effective population size and little effective recombination. This leads to an accumulation of deleterious mutations, and an A+T rich genome due to mutational bias (Moran 1996; Haigh 1978; Muller 1964).

Wolbachia is, however, not subject to the same fate. While *Wolbachia* is endosymbiotic and the primary mode of transmission is vertical, *Wolbachia* does undergo horizontal transfer between host species (as is evident by discordant host-symbiont phylogenetic histories) (Vavre et al. 1999; Werren et al. 1995; Boyle et al. 1993; O'Neill et al. 1992; Raychoudhury et al. 2009), and shows signatures of recombination (Baldo et al. 2006; Jiggins 2002; Ellegaard et al. 2013). *Wolbachia* genomes are smaller than free-living bacteria, with a size around 1 - 1.5 million basepairs, but are nowhere near as streamlined as strictly maternally transmitted endosymbionts (McCutcheon and Moran 2012; Fenn and Blaxter 2006). *Wolbachia* genomes have an abundance of mobile genetic elements (Wu et al. 2004; Kent et al. 2011; Fenn and Blaxter 2006), and lack synteny, even between closely related strains (Ellegaard et al. 2013; Klasson et al. 2009; Ishmael et al. 2009). There is some evidence that the filarial nematode-infecting *Wolbachia*, obligate for the host and more strictly vertically transmitted, have more genome reduction than the parasitic arthropod infecting strains (Godel et al. 2012; Darby et al. 2012; Foster et al.

2005); mobile genetic element loads are lower, and they have no association with the bacteriophages present in the arthropod-infecting strains (Kent and Bordenstein 2010; Gavotte et al. 2007; Desjardins et al. 2013; Godel et al. 2012; Darby et al. 2012; Foster et al. 2005). Besides the filarial worms, the only other places WO phages are missing are in the *Trichogramma*-infecting *Wolbachia* (Gavotte et al. 2007).

Little attention has been given to genomics of the arthropod-infecting *Wolbachia* currently undergoing domestication. As *Wolbachia* becomes obligate for the host, we might expect to see signs of genome evolution similar to that of the obligate primary symbionts of plant feeding insects.

Summary

The dynamics of *Wolbachia* infection are poorly understood. Genetic and environmental factors can have drastic effects on the titers of *Wolbachia* in the host, and the strength of the manipulations or fitness effects on the host, which are critical for *Wolbachia*'s ability to spread in a population. *Trichogramma* wasps are a great system to look at this due to the facts that 1) PI-*Wolbachia* have received relatively little attention despite their presence in agriculturally important arthropods, 2) the wide range of infection scenarios present, and 3) the possibility for irreversible asexuality due to the infection. We can use this system to ask questions about the factors responsible for *Wolbachia* fixation, and then look at the genomic and evolutionary consequences of host-symbiont associations that have become fixed.

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CHAPTER 1

Comparative genomics of a parthenogenesis-inducing *Wolbachia* symbiont

Amelia R. I. Lindsey[1], John H. Werren[2], Stephen Richards[3], and Richard Stouthamer[1]

[1]Department of Entomology, University of California Riverside, Riverside, California, 92521; [2]Biology Department, University of Rochester, Rochester, New York, 14627; [3]Department of Human and Molecular Genetics, Baylor College of Medicine, Houston, Texas, 77030

ABSTRACT

Wolbachia is an intracellular symbiont of invertebrates responsible for inducing a wide variety of phenotypes in its host. These host-*Wolbachia* relationships span the continuum from reproductive parasitism to obligate mutualism, and provide a unique system to study genomic changes associated with the evolution of symbiosis. We present the genome sequence from a parthenogenesis-inducing *Wolbachia* strain (*w*Tpre) infecting the minute parasitoid wasp *Trichogramma pretiosum.* The *w*Tpre genome is the most complete parthenogenesis-inducing *Wolbachia* genome available to date. We use comparative genomics across 16 *Wolbachia* strains, representing five supergroups, to identify a core *Wolbachia* genome of 496 sets of orthologous genes. Only 14 of these sets are unique to *Wolbachia* when compared to other bacteria from the Rickettsiales. We show that the B-supergroup of *Wolbachia*, of which *w*Tpre is a member, contains a significantly higher number of ankyrin repeat-containing genes than other supergroups. In the *w*Tpre genome, there is evidence for truncation of the protein coding sequences in 20% of open reading frames, mostly as a result of frameshift mutations. The *w*Tpre strain represents a conversion from cytoplasmic incompatibility to a parthenogenesis-inducing lifestyle, and is required for reproduction in the *Trichogramma* host it infects. We hypothesize that the large number of coding frame truncations has accompanied the change in reproductive mode of the *w*Tpre strain.

INTRODUCTION

Wolbachia is a maternally transmitted, intracellular symbiont of arthropods and nematodes that exhibits a range of complex interactions with its hosts (Werren 1997; Werren et al. 2008; Stouthamer et al. 1999a). It is estimated to infect 40-60 percent of arthropod species (Zug and Hammerstein 2012; Hilgenboecker et al. 2008). Across the arthropods, *Wolbachia* is well known for modifying host reproduction, by utilizing various mechanisms that enhance fitness or numbers of infected females. By promoting infected females, *Wolbachia* ensures its own maternal transmission, and has the ability to spread rapidly through a population (Walker et al. 2011; Weeks et al. 2007; Turelli and Hoffmann 1991). These reproductive modifications include: cytoplasmic incompatibility (**CI**), male killing, feminization, and parthenogenesis-induction (**PI**) (Werren 1997). In addition to these reproductive phenotypes, some *Wolbachia* strains protect against pathogens (Chrostek et al. 2013; Moreira et al. 2009; Kambris et al. 2010), supply essential nutrients to its host (Nikoh et al. 2014; Hosokawa et al. 2010), are required for successful egg development (Kremer et al. 2009; Timmermans and Ellers 2009; Dedeine et al. 2001), or are essential for the production of female offspring (Russell and Stouthamer 2011; Stouthamer et al. 2010). In filarial nematodes, *Wolbachia* is an obligate mutualist providing a diversity of benefits to its host, including evasion of the vertebrate immune system (Darby et al. 2012). For these reasons, *Wolbachia* has captured considerable interest in applied fields as a potential "agent" to modify pest populations, reduce pathogen loads in vectors, and specifically target filarial nematodes by way of their obligate symbionts (Zabalou et al. 2004; Bourtzis et al. 2014; Taylor et al. 2000).

In addition to the practical applications of studying *Wolbachia*, the complexity of interactions with diverse hosts provides an opportunity to explore genomic changes accompanying the evolution of such unique life histories. Nested within a clade of other symbiotic and pathogenic bacteria, *Wolbachia* are members of the Rickettsiales, an order of α-proteobacteria (O'Neill et al. 1992; Dumler et al. 2001). The *Wolbachia* clade is composed of 16 reported supergroups, denoted A – F and H – Q (Ros et al. 2009; Augustinos et al. 2011; Bing et al. 2014; Haegeman et al. 2009; Lo et al. 2002; Glowska et al. 2015), with supergroups A – D being the most well studied. Supergroup G is no longer considered a distinct *Wolbachia* lineage, as it represents a recombinant between supergroups A and B (Baldo and Werren 2007). Supergroups A and B are a monophyletic assemblage infecting arthropods (Gerth et al. 2014), whereas supergroups C and D are the major nematode-infecting lineages (Bandi et al. 1998). Supergroup F is unique as it contains both nematode and arthropod infecting strains (Casiraghi et al. 2005), including the bed bug infecting *Wolbachia* strain *w*Cle that supplements B vitamins to its obligate blood-feeding hosts (Nikoh et al. 2014; Hosokawa et al. 2010). The less studied supergroups H – Q infect a variety of hosts, including termites, aphids, whiteflies, mites, fleas and a plant-parasitic nematode (Ros et al. 2009; Augustinos et al. 2011; Bing et al. 2014; Haegeman et al. 2009; Lo et al. 2002; Glowska et al. 2015).

While co-cladogenesis of *Wolbachia* and their hosts does occur (Raychoudhury et al. 2009), it is relatively uncommon, and host-switching is a prominent feature of

Wolbachia's evolutionary history (Vavre et al. 1999; van Meer et al. 1999; Zhou et al. 1998; Baldo et al. 2006). In addition to the incongruence of host and symbiont phylogenies, there is little conservation of the induced phenotypes. For example, independently derived parthenogenesis-inducing (**PI**) *Wolbachia* are found in the A and B supergroups (Stouthamer et al. 1993), and likely the F supergroup (Baldo et al. 2007). These PI-*Wolbachia* strains induce parthenogenesis through different mechanisms including the merging of nuclei (Gottlieb et al. 2002), a failed anaphase during the first embryonic cell division (Stouthamer and Kazmer 1994; Pannebakker et al. 2004), and functional apomixis (Weeks and Breeuwer 2001). Uninfected parasitoid wasps of the genus *Trichogramma* are arrhenotokous, but infection with PI-*Wolbachia* strains causes gamete duplication in unfertilized eggs by preventing chromosome segregation during anaphase of the first mitotic division of the egg, resulting in a diploid female (Stouthamer and Kazmer 1994). The PI-*Wolbachia* strains infecting *Trichogramma* spp. are unique for at least three reasons: there is a single origin of *Wolbachia* infection for the genus (Werren et al. 1995; van Meer et al. 1999); the *Trichogramma* hosts can evolve dependencies upon their *Wolbachia* infection for the production of females (Russell and Stouthamer 2011; Stouthamer et al. 2010); and, unlike other arthropod-infecting strains, the PI-*Wolbachia* infecting *Trichogramma* do not have relationships with phages (Gavotte et al. 2007).

Wolbachia genomes are small in size, ranging from 0.9 – 1.5 Mbp, and contain a number of unique features. The arthropod infecting genomes have a large number of repetitive

and mobile elements, including ankyrin repeat domain containing (ANK) genes (Iturbe-Ormaetxe et al. 2005; Siozios et al. 2013b; Papafotiou et al. 2011), bacteriophage sequences (Gavotte et al. 2007), transposons, and many copies of short open reading frames (ORFs) of unknown function (Wu et al. 2004). Little is known about the role these short, unannotated ORFs play in the biology of *Wolbachia.*

Here, we explore the changes in genome content across *Wolbachia*, and present a draft genome for the PI-*Wolbachia* strain, *w*Tpre, infecting the parasitoid wasp *Trichogramma pretiosum*. The *w*Tpre genome represents the most complete PI-*Wolbachia* genome assembly to date, and the first B-supergroup PI-*Wolbachia* genome. We show evidence for protein sequence truncation in 20% of the *w*Tpre gene set, and hypothesize that these truncations are a feature of the change in reproductive phenotype.

MATERIALS AND METHODS

Biological materials

A unisexual colony of naturally *Wolbachia*-infected *Trichogramma pretiosum* was chosen for genome sequencing. Originally collected in the Puira Valley of Peru, this colony has been maintained in a commercial insectary since 1966 (Beneficial Insectary, Guelph, Ontario, Canada), and herein is referred to as the "Insectary Line". Species identifications were confirmed by molecular protocols from Stouthamer et al. (1999b), and *Wolbachia* infection status was confirmed using the protocols from Stouthamer et al. (1990) and Werren and Windsor (2000). Attempts to initiate *Wolbachia*-free replicates of

this colony following antibiotic treatment protocols from Stouthamer et al. (1990) have not been successful due to severe fertility reduction, as seen in Russell and Stouthamer (2011), indicating that this infection is obligate for this particular *Trichogramma* line.

Identification of a *w*Tpre genome

The genome of the *Trichogramma pretiosum* Insectary Line (GenBank Accession Number: JARR00000000) (Lindsey et al., in prep) was sequenced in collaboration with the i5k initiative to sequence 5,000 arthropod genomes (www.arthropodgenomes.org/wiki/i5K) and made publicly available prior to publication under the Fort Lauderdale agreement. The *T. pretiosum* assembly was scanned for evidence of *Wolbachia* DNA using two methods. First, total DNA was extracted from 10 wasps using a Chelex method (Walsh et al. 1991) as implemented by Stouthamer et al. (Stouthamer et al. 1999b). The *Wolbachia* 16S rRNA gene was amplified and sequenced with W-Specf and W-Specr primers (Werren and Windsor 2000). Sequences were aligned and primer sequencees excised in Sequencher® 4.9. The 16S rRNA gene was then queried against the *T. pretiosum* genome assembly using nucleotide BLASTN at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The remaining scaffolds were checked for bacterial DNA sequence by querying them against Bacteria (taxid: 2) in NCBI GenBank with blastn. Second, the *T. pretiosum* assembly was scanned with the bioinformatics pipeline developed by Wheeler et al. (2013), in order to identify bacterial sequence from a eukaryotic background.

Genome annotation, clusters of orthologous genes, and completeness estimates

The IGS Annotation Engine was used for structural and functional annotation of the *w*Tpre genome. (http://ae.igs.umaryland.edu/cgi/index.cgi, (Galens et al. 2011)). Manatee was used to view annotations (http://manatee.sourceforge.net/). The *w*Tpre genome and 17 other previously published genomes (see Table 1.1) were used in comparative analyses. Previously published genomes were re-annotated with the IGS Annotation Engine, and Clusters of Orthologous Genes (COGs) across all 18 genomes were defined using Sybil (http://sybil.sourceforge.net/index.html) (Riley et al. 2012; Crabtree et al. 2007). Genome completeness was assessed with the BUSCO pipeline (Simão et al. 2015) using the 40 core bacterial genes from Mende et al. (2013) compared to the gene set from each *Wolbachia* genome (-m = OGS).

Phylogenetic analyses

A phylogenetic reconstruction of *Wolbachia* strains was inferred using the five Multi Locus Sequence Typing (MLST) genes (Baldo et al. 2006), with *Anaplasma marginale* str. *Florida* (GenBank Accession Number: PRJNA58577) "Ama" as an outgroup. In addition to the strains in Table 1.1 (minus *w*Wb, see results), we included *Wolbachia* strains from the MLST database (*w*Ajap infecting *Asobara japonica*, *w*Uni infecting *Muscidifurax uniraptor, w*Dali infecting *Diaphorencyrtus aligarhensis*, *w*Tdei infecting *Trichogramma deion*, *w*Efor infecting *Encarsia formosa, w*PsiaB infecting *Protocalliphora sialia*, and *w*Lcla infecting *Leptopilina clavipes*) and the *w*Tbras strain infecting *Trichogramma brassicae* (downloaded from GenBank, Accession Numbers:

JF920468.1, JF920470.1, JF920472.1, JF920464.1, and JF920466.1). Multiple alignments were created for each gene using the L-INS-i algorithm in MAFFT version 7 (Katoh and Standley 2013), and were concatenated prior to maximum likelihood analyses in RAxML version 8.2.4 (Stamatakis 2014) using the GTRGAMMA substitution model and 1000 bootstrap replicates. A second phylogenetic reconstruction was made using the same methods, but with only the strains used in our comparative analyses. Trees were visualized in FigTree version 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/), and annotated in Inkscape (https://inkscape.org/en/).

Identification of core and unique genomes

Unique and core genome assessments were performed using Sybil results loaded on a Chado relational database (Galens et al. 2011; Mungall et al. 2007). The core genome was determined by identifying all COGs that had at least one gene member from each *Wolbachia* strain being considered. COGs were considered unique to a monophyletic assemblage when all members of the COG belonged exclusively to the clade, and were found in all members of the clade. To determine the uniqueness of the *Wolbachia* core, a representative *w*Tpre gene for each of the core COGs was queried against a database of the protein coding sequences of *Rickettsia rickettsii*, *Ehrlichia chaffeensi*s, and *Anaplasma marginale*, (respective GenBank Accession Numbers CP003318, CP000236, and CP001079) using BLASTP. A cutoff e-value of 1e-10 was used to determine significance. The comparison of the core was done with both the 496-COG core

(excluding *w*Wb and *w*Gmm), and the 436-COG core (excluding only *w*Wb, and *w*Gmm included).

Analysis of genome content and ankyrin genes

Role category annotations from the IGS annotation pipeline were used to compare genome content across 17 *Wolbachia* strains, excluding unannotated genes. The number of genes in each role category for each genome was plotted according to standard deviation, then subjected to a Principle Components Analysis (PCA) based on the standardized proportion of genes in each role category, using `prcomp` in R version 3.1.2 (R Core Team 2014). Due to the high variance of the hyper-variable "mobile and extrachromosomal element functions" category, a second PCA analysis was performed after removing the category and re-calculating proportions.

The term "ankyrin" was queried against all gene annotations, and the number of positive matches was tabulated for each genome. Ankyrin genes are most commonly found in eukaryotes, but are common in *Wolbachia* and may mediate protein-protein interactions (Iturbe-Ormaetxe et al. 2005). The number of ankyrin repeat-containing genes was plotted in R, and a Mann-Whitney U test was used to test for a significant difference in abundance between supergroups A and B. Supergroups C, D, and F were not included in the statistical analyses due to the small number of representative genomes available.

Identification of truncated ORFs in *w*Tpre

The nucleotide sequence of all *w*Tpre genes determined not to be a member of any orthologous clusters (see Results), were queried against a database of all *Wolbachia* genes from the remaining 16 genomes (*w*Wb not included due to low BUSCO score), using BLASTN. The full nucleotide sequence of the best match was then queried back against the *w*Tpre genome sequence to look for regions of homology beyond the *w*Tpre gene ORF. To be further considered as evidence of protein sequence truncation, the BLASTN best match to the genome was required to meet an 85% identity cutoff, and the best match had to align to *w*Tpre across at least 70% of its length, or at least three times the length of the *w*Tpre gene in question. Alignments that passed these quality measures were scanned for the presence of mutations that would result in premature stop codons, and categorized by mutation type. ORF length comparisons were performed in R and a Mann-Whitney U test was used to determine significance.

Comparison to inactive genes in *Wolbachia* strain *w*Au

The set of *w*Mel genes that were found to be potentially inactive in *Wolbachia* strain *w*Au (Sutton et al. 2014) was compared to the *w*Tpre gene set. *w*Au was not included in previous analyses because it was published after COG assessment was completed. The *w*Mel genes were classified as either 1) having an ortholog in *w*Tpre (as determined by Sybil COG assessment), 2) being truncated in *w*Tpre (as determined by the homolog of a truncated *w*Tpre gene sharing COG membership with the respective *w*Mel gene), or 3) absent in *w*Tpre.

Data availability

The *w*Tpre Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession LKEQ00000000. The version described in this paper is version LKEQ01000000.

RESULTS

The *w*Tpre genome: A parthenogenesis-inducing *Wolbachia* strain

The genome sequence of *w*Tpre was extracted from a whole genome assembly of its host, *Trichogramma pretiosum*, performed as a part of the i5k genome project (Lindsey et al., in prep). The *w*Tpre genome was recovered in a single scaffold, composed of nine contigs. The scaffold was 1,133,709 bp in length and BLASTN searches against the NCBI GenBank database revealed 97% nucleotide similarity to the *Wolbachia* symbiont *w*Pip_Pel infecting *Culex quinquefasciatus* (GenBank accession number: AM999887). No other bacterial sequence was identified in the *T. pretiosum* assembly. Average scaffold coverage for the *Wolbachia* scaffold was the lowest of all scaffolds in the i5k genome project assembly, indicating that the recovered genome is not the result of a lateral transfer into the *Trichogramma pretiosum* genome (*Wolbachia* scaffold =35.6x coverage, *T. pretiosum* assembly = 232.7x coverage). The *w*Tpre genome was structurally and functionally annotated with the Institute for Genome Sciences (IGS) pipeline at the University of Maryland (http://ae.igs.umaryland.edu/cgi/index.cgi, (Galens et al. 2011)), revealing 1405 open reading frames, 35 tRNA coding genes, and a single

set of rRNA genes (one each of 5S, 23S and 16S), giving a coding density of 81.8%. The size and number of coding sequences fell within the range of previously sequenced *Wolbachia* genomes (Table 1.1). While the arthropod-infecting *Wolbachia* genomes are known to carry a large number of mobile elements, the *w*Tpre genome was depauperate in these features. Only nine genes related to prophage function, and 14 transposon function genes were identified in the genome.

Completeness and phylogenetic relationships of sequenced *Wolbachia* genomes

Seventeen previously published *Wolbachia* genomes, representing supergroups A – D, and F, were examined alongside the *w*Tpre genome in phylogenetic and comparative analyses (Table 1.1). All genomes were re-annotated with the same IGS pipeline used to annotate *w*Tpre. BUSCO (Simão et al. 2015) was used to scan for the 40 core bacterial genes defined by Mende et al. (2013) to estimate completeness for each sequenced genome based on the proportion of missing BUSCO genes. Scores from these analyses are reported in Table 1.1. Notably, none of the *Wolbachia* strains, including completely sequenced genomes, contained all 40 BUSCO genes. All 18 strains are missing the BUSCO orthologs that encode for ribosomal proteins S7, L11, L4, and L14 (COG0049, COG0080, COG0088, COG0093, respectively). The *w*Wb strain (from the nematode *Wuchereria bancrofti*) appeared to be an outlier, as 22 of the 40 orthologs were missing or fragmented. Additionally, *w*Wb was missing a duplication of COG0552 (Signal recognition particle GTPase) that is present in all 17 other strains. The draft *Wolbachia* genomes have BUSCO scores that fall within the range of scores from the complete

genomes, with the exception of *w*Wb. The *w*Wb assembly is the expected size for a *Wolbachia* genome, but has an abnormally large number of ORFs (n = 2144), almost 600 more than the other *Wolbachia* genomes (Table 1.1). For these reasons, the *w*Wb strain was excluded from additional analyses.

Phylogenetic reconstruction based on maximum likelihood analysis was conducted using Multilocus Sequence Typing (MLST) genes (Baldo et al. 2006) to determine relationships amongst the PI-*Wolbachia.* This analysis confirms multiple independent origins of PI-*Wolbachia*, placement of the *w*Tpre strain in the B supergroup, and the monophyly of the *Trichogramma*-infecting *Wolbachia* (Figure 1.1A). All supergroups with multiple members were recovered as monophyletic. The major arthropod infecting lineages, supergroups A and B, formed a monophyletic clade, and supergroups C and F also formed a monophyletic clade. The nematode-infecting supergroup D was sister to the rest of the *Wolbachia* lineage. The *w*Pip strains have identical MLST sequences, and are represented as a polytomy.

The core *Wolbachia* genome

The core genome of the 17 *Wolbachia* strains was made up of 436 Clusters of Orthologous Genes (COGs) (Figure 1.1B). The core genomes of the A (655 COGs) and B (659 COGs) supergroups were similar in size despite the B supergroup being represented by four more strains than the A supergroup. Together, these two supergroups had a core genome of 541 COGs. As expected, including additional supergroups lead to a reduction

in the size of the core genome. Sampling more heavily among more distantly related groups yields a decrease in shared similarities. It is important to note that the positions of *w*Gmm and *w*Ha have changed: in the phylogenetic reconstruction including more strains (Figure 1.1A) *w*Ha is sister to the rest of the A supergroup, and *w*Gmm is sister to the rest of the A supergroup when the phylogeny is reconstructed with only the strains for which genomes are available (Figure 1.1B). That node in both trees is supported by a bootstrap value of 100, so we kept the topologies and calculated core and unique genome sizes with *w*Gmm as sister to the rest of the A supergroup.

The size of the core genome for the 8 *Wolbachia* strains with completely sequenced genomes (*w*Bm, *w*Cle, *w*Mel, *w*No, *w*Oo, *w*Pip_Pel, *w*Pip_Mol, and *w*Ri) was 511 COGs. Inclusion of *w*Ha, which has a genome assembly of a single scaffold with 2 gaps, did not reduce the core size. Addition of *w*Tpre, the remaining single-scaffold assembly, only reduced the core genome by one COG, to 510 COGs, indicating that the *w*Tpre assembly is relatively complete. These 10 complete and single-scaffold genomes were used to determine which genome(s) were having the largest effect on the final core genome size of all 17 strains. One at a time, the core genome was determined for the aforementioned 10 genomes, plus one of the seven remaining assemblies. *w*Di and *w*Suzi had a small effect on the core size, each resulting in one less COG in the core. *w*Pip_JBH reduced the core genome by two COGs. *w*AlbB and *w*Bol1 were each responsible for a loss of three COGs from the core, and *w*VitB for five COGs. The *w*Gmm strain had the most drastic effect on the size of the *Wolbachia* core, as the *w*Gmm assembly (infecting

the tsetse fly *Glossina morsitans morsitans*) is missing 63 of the 510 COGs found in the 10 complete and single-scaffold genomes. Its low BUSCO score (Table 1.1), in combination with the effect on the core genome, indicate that a significant portion of sequence data may be missing or miss-assembled for *w*Gmm. Elimination of *w*Gmm from the analysis resulted in a core *Wolbachia* genome of 496 COGs for the remaining 16 strains, which is likely closer to the true size of the *Wolbachia* core. This 496 COG core was searched against *Rickettsia rickettsii*, *Ehrlichia chaffeensi*s, and *Anaplasma marginale*. Fourteen *Wolbachia* core COGs did not have hits to the other Rickettsiales: 11 hypothetical or predicted proteins, a cutA1 divalent ion tolerance family protein, a surface antigen family protein, and a nitroreductase family protein. Four of these 14 *Wolbachia*-unique COGs, all conserved hypothetical proteins, are missing from the 436-COG core than includes *w*Gmm.

Ordination of *Wolbachia* strains based on genome content

The number of genes in each role category, for each genome, as determined by the IGS annotation pipeline, was used in comparative analyses of genome content. The role categories with the most variation in gene number per genome were: mobile and extrachromosomal element functions, transport and binding proteins, and cell envelope (Figure 1.2A). *Wolbachia* genomes showed little variance in the number of genes devoted to central intermediary metabolism, signal transduction, and amino acid biosynthesis. All *Wolbachia* genomes had a high (median = 106), but relatively conserved number of genes devoted to protein synthesis. Principal Components Analysis (PCA) was used to visualize

the similarity of genomes based on the proportion of genes in each of these role categories (Figure 1.3A). While the A supergroup genomes ordinate to the upper left quadrant, the B supergroup strains showed greater diversity in genome content across strains. Bed bug-infecting *w*Cle clustered with the distantly related, yet also arthropod-infecting A supergroup strains, although phylogenetically *w*Cle belongs to the F supergroup (Rasgon and Scott 2004). *w*Tpre's closest neighbor in the genome content based ordination was the obligate, nematode-infecting *w*Oo strain. We suspect that the highly variable number of genes in the mobile and extrachromosomal element functions role category could strongly influence these patterns. Therefore, proportions were re-calculated without this category and again subjected to PCA (Figure 1.3B). Without the mobile and extrachromosomal element functions role category, the *w*Cle genome neighbored B supergroup strains, and the *w*Tpre genome neighbored the group of A supergroup strains. This category had a dominant effect on the ordination of *w*Tpre and *w*Cle. However, the overall pattern of a loose A supergroup cluster and B supergroup diversity was maintained in the absence of the mobile and extrachromosomal element functions category, indicating support from other role categories for this patterning.

Supergroup B has significantly more ankyrin repeat containing genes

We specifically looked at the number of ankyrin repeat-containing (ANK) genes in each of the *Wolbachia* genomes. ANK genes are involved in protein-protein interactions and are rare in bacteria, but are found in *Wolbachia,* where they may modulate host phenotypes (Iturbe-Ormaetxe et al. 2005; Papafotiou et al. 2011). The *w*Tpre strain has

54 ANK genes. With 48 ANK genes, the *w*AlbB strain has the fewest number of ANK genes in the B-supergroup. We demonstrate a significant difference in the number of ANK genes between supergroups A and B (Mann Whitney-U, $p = 0.003$) (Figure 1.2B). The B supergroup has, on average, more than double the number of ANK genes than any other supergroup. The median number of ANK genes in supergroup A is 30, and in supergroup B, 64. While supergroups C, D, and F were not subjected to statistical analysis due to the low number of representative genomes available, the numbers of ANK genes present in those genomes was low when compared to supergroup B. The *w*Oo (C), *w*Bm (D), and *w*Cle (F) genomes have 3, 20, and 39 ANK genes, respectively.

"Unique" *w*Tpre genes are derived from truncated versions of *Wolbachia* genes

The newly sequenced *w*Tpre strain has one of the largest sets of "unique genes", and the largest of all the arthropod-infecting *Wolbachia* strains, with 482 genes not assigned any orthologs (Figure 1.1B). This represents 34% of the total genes in the *w*Tpre genome. Nucleotide BLAST searches of the *w*Tpre "unique genes" against a database of all the other coding sequences from the other *Wolbachia* genomes in Table 1.1 reveal that 367 of *w*Tpre "unique genes" show similarity with other *Wolbachia* genes (Table 1.2). However, the predicted coding regions of *w*Tpre "unique genes" were on average 77.5% shorter than their corresponding homologs in other *Wolbachia* genomes (Mann-Whitney U test, $p < 0.0001$) (Figure 1.4A). The significant difference in size could indicate that these genes are truncated versions of the coding sequence, either due to deletions, or premature stop codons. To explore this, the nucleotide sequences of the best matches

were aligned to the *w*Tpre genome sequence to look for homology of the *w*Tpre "unique gene" up- and down-stream of the ORF. Of the 367 *w*Tpre "unique genes" with sequence similarity to other *Wolbachia* genes, 86 genes were excluded from analyses based on low identity values and/or lack of evidence for up/down-stream homology, and 281 genes showed evidence of truncation of the predicted protein sequence and potential pseudogenization due to nonsense and frameshift mutations (Table 1.2). Many of the *w*Tpre "unique genes" occur in tandem, where an early frameshift or nonsense mutation resulted in a premature stop codon, and subsequent annotation of additional short, downstream, ORFs with sequence homology to the downstream portions of the same ORF in the other *Wolbachia* genome. Figure 1.4B shows a schematic representation of this phenomenon, where the *w*Tpre "unique genes" *w*Tpre_380, *w*Tpre_381, and *w*Tpre_382 all align to sequential portions of the *w*Pip_Pel gene, WD0152. A single base-pair deletion at position 421 in *w*Tpre_380, relative to *w*Pip_167, resulted in a premature stop codon. The intergenic spaces between these *w*Tpre "unique genes" also showed sequence similarity to corresponding locations in the *w*Pip_Pel gene. The short ORFs downstream of the nonsense or frameshift mutation are hereafter referred to as "post-nonsense" or "post-frameshift" ORFs, respectively. In the *w*Tpre genome, 52% (n=146) of these "unique genes" with evidence of truncation were post-frameshift ORFs (Table 1.2). The coding frame truncated *w*Tpre genes were more likely to have a hypothetical annotation than their counterparts from other *Wolbachia* genomes (Chi-Square, $p < 0.0001$). Of the 281 truncated *w*Tpre genes, 149 (53%) had a hypothetical annotation. This contrasts to the 188 genes that the truncated *w*Tpre genes match to,

where only 62 (33%) had a hypothetical annotation. Fifty-seven of the truncated *w*Tpre genes are of phage or transposon origin, and 45 are homologs of ANK genes. We therefore conclude that the majority of these "unique genes" are artifacts of ORF prediction, and are actually degenerated protein coding sequences of genes found in other *Wolbachia*.

Comparison to inactive genes in *Wolbachia* strain *w*Au

The genome for the *w*Au strain infecting *Drosophila simulans* was recently sequenced, and also found to be missing or have potentially inactive versions of homologous genes present in the closely related *w*Mel strain (Sutton et al. 2014). While *w*Mel induces strong CI, *w*Au has lost this function (Hoffmann et al. 1996). The *w*Au genome was not included in the aforementioned comparative analyses because the genome was published after our completion of COG assessment and genome content analyses. All of the 46 *w*Mel genes found to be inactive in *w*Au were members of COGs, and were not unique to *w*Mel. Of these 46 *w*Mel genes, 36 were either absent (n=24), truncated (n=9), or "unique genes" that did not meet criteria to be considered truncations (n=3) in the *w*Tpre genome. Ten of the *w*Mel genes shared the same fate in both the *w*Tpre and *w*Au genomes. Five hypothetical proteins, an ANK protein, and DNA repair protein RadC are absent in both *w*Tpre and *w*Au. Multidrug resistance protein D and a hypothetical protein both have frameshift mutations in *w*Tpre and *w*Au. Lastly, a prophage gene has a nonsense mutation in both strains.

DISCUSSION

The *w*Tpre assembly represents the most complete genome sequence of a parthenogenesis-inducing *Wolbachia* to date. This particular PI-*Wolbachia* strain is required for reproduction in its host; attempts to initiate *Wolbachia*-free replicates of this *Trichogramma* colony, following protocols from Stouthamer et al. (1990) have not been successful (e.g. Russell and Stouthamer (2011)). The only other available PI-*Wolbachia* genome is strain *w*Uni from the parasitic wasp *Muscidifurax uniraptor*, an A-supergroup *Wolbachia* (Klasson et al. 2009). *w*Uni was not included in analyses as the record contains only partial genome data that was generated by amplification with primers based on the *w*Mel genome.

In some ways, the *w*Tpre genome is similar to the other arthropod infecting strains. *w*Tpre contains a large number of ANK genes, as is common in the *Wolbachia* clade. In regards to the number of phage genes, the *w*Tpre genome is more similar to the obligate, nematode-infecting *Wolbachia*: *w*Tpre contains 9 annotated phage genes and 14 transposon function genes. As a comparison, the same annotation pipeline identified 55 prophage function genes, and 132 transposon function genes in the *w*Pip_Pel genome, and 30 prophage and 81 transposon genes in the *w*Mel strain (infecting *Drosophila melanogaster*). This corroborates previous analyses which discovered a diversity of phages in many other arthropod-infecting *Wolbachia*, but no evidence of functional bacteriophages in the *Trichogramma*-infecting *Wolbachia* (Gavotte et al. 2007). Phylogenetic analyses confirmed the multiple origins of PI-*Wolbachia*, and monophyly

of the *Trichogramma*-infecting strains (van Meer et al. 1999). The relationship of the supergroups using the five MLST genes (Baldo et al. 2006) replicated results from phylogenomic analyses using 90 informative loci (Gerth et al. 2014).

We attempted to assess completeness of the *Wolbachia* genomes using the BUSCO pipeline, and 40 core bacterial genes. Completely sequenced genomes varied widely in the number of genes recovered, indicating that this gene set may not be ideal for assessing completeness in *Wolbachia*. Four ribosomal proteins were absent from all *Wolbachia* genomes. Genome sequencing of the primary-symbionts of insects has revealed that not all ribosomal proteins are retained in these highly reduced genomes (McCutcheon 2010). While *Wolbachia* is not considered a primary-symbiont, and is not strictly maternally transmitted (Raychoudhury et al. 2009), some degree of genome-reduction has taken place. There was a trend towards lower BUSCO scores in the obligate *Wolbachia* strains, indicating more extensive reductions in genomic content.

Due to the draft status of some of the *Wolbachia* genomes, we relied on the proportions of genes in role categories to assess similarity of genome content. The *w*Tpre strain clusters with the nematode infecting strains when mobile and extrachromosomal elements are included, likely driven by the similarity in the number of phage genes. Without this category of genes, *w*Tpre neighbors A-supergroup *Wolbachia*. The ordination of *w*Cle also changes drastically when the mobile and extrachromosomal element genes are removed from the analysis, going from neighboring A-supergroup strains to neighboring

B-supergroup strains. While the mobile and extrachromosomal elements role category appears to have a dominant effect on ordination for certain strains, the overall pattern of the A and B supergroups was more strongly supported.

The size of the core genome here (496 COGs) was lower than estimates from previous studies. Duplouy et al. (2013) estimated a core of 654 genes based on five strains (from 3 supergroups); *w*Bol1, *w*Pip_Pel, *w*Mel, *w*Ri, and *w*Bm. Similarly, Ishmael et al. (2009) used exponential regression to estimate a core genome size of 621 genes, but their study examined only *Drosophila*-infecting *Wolbachia* strains. It is likely that our inclusion of additional *Wolbachia* strains, from more diverse hosts and supergroups, is responsible for the smaller core genome size. Comparison of the core *Wolbachia* genome to other members of the Rickettsiales revealed that only 2.8% of the core is unique to *Wolbachia*. This finding parallels the discovery of high conservation of two-component systems across 12 *Wolbachia* strains, *Anaplasma phagocytophilum*, and *Ehrlichia chaffeensis* (Christensen and Serbus 2015). These similarities with other closely related rickettsial pathogens may indicate the core genome comprises genes required for life within an arthropod host, and the accessory genomes are responsible for the phenotypes that various strains induce.

In *w*Tpre, 482 (34%) of the ORFs were apparently unique: the largest number of any of the arthropod-infecting strains. Only the two nematode-infecting strains, *w*Bm and *w*Oo had more "unique genes" than *w*Tpre. This may be a feature of the obligate nature of the

symbiotic relationships that these strains share with their hosts. However, *w*Bm and *w*Oo are the only representatives from their respective supergroups, and it is likely that inclusion of additional C and D supergroup members would result in a reduction in the number of "unique genes" found in these strains. The *w*Gmm strain also contained a high number of "unique genes". This may be a result of a problematic assembly, as *w*Gmm had one of the lower BUSCO scores and was responsible for a drastic effect on the size of the core *Wolbachia* genome.

Examination of the *w*Tpre "unique genes" showed evidence for coding frame truncation in 281 genes, representing 20% of the ORFs in the genome. This is likely an under-estimate of the amount of truncation in *w*Tpre. Stringent filtering of sequence similarity, and of up- and downstream homology did not allow for identifying truncation in rapidly evolving genes, or genes that may have been truncated or fragmented through genomic rearrangements or deletions. Mutations resulting in downstream post-nonsense and post-frameshift ORFs were not exclusively located in genes identified as unique to *w*Tpre. If the mutation occurred too early in the coding sequence, the ORF was too short to be considered a gene by the IGS pipeline. Conversely, mutations that occurred more 3' in the coding sequence left an ORF long enough to be considered orthologous with other *Wolbachia* genes, but could still result in the annotation of short downstream *w*Tpre "unique" ORFs. In *w*Tpre, truncated genes were more likely to carry a hypothetical annotation, despite the fact that homologs from other *Wolbachia* genomes were often assigned a function. One explanation for this may be the frameshift mutations that result

in a change of amino acid sequence, and the loss of recognized functional domains or motifs that would assist in assigning function to the gene. Additionally, the fragmentation of a gene into several ORFs would lead to a functional domain or motif only being associated with one of the resulting ORFs, thus making functional assignments difficult for the other ORFs. Therefore, we conclude that the majority of "unique genes" in *w*Tpre are actually truncated orthologs of known *Wolbachia* genes from other strains, and likely are not active protein coding genes, but artifacts or ORF prediction machinery.

A relatively small number of inactive or truncated genes were identified in *w*Au, a *Wolbachia* strain infecting *Drosophila simulans* that does not induce strong CI, but does provide viral protection to its host. While the *w*Tpre genome contains a larger number of truncated genes, 78% of the inactive *w*Au genes were also missing or truncated in *w*Tpre, providing an overlapping set of 36 genes. Both *w*Au and presumably *w*Tpre have lost the capacity for CI induction. This overlap may indicate an important feature of the transition away from a strong CI phenotype. However, many of these genes have hypothetical gene annotations, and therefore we cannot comment on their potential functions.

We identified a significantly higher number of ANK genes in the B supergroup *Wolbachia* strains. ANK genes are unusual in bacteria, and it has been hypothesized that phages, transposons, and recombination may have played a role in proliferation of the ANK gene repertoire in *Wolbachia* (Siozios et al. 2013b; Iturbe-Ormaetxe et al. 2005). The *w*Tpre strain has 54 ANK genes, despite not having associated bacteriophages and

having a reduced number of mobile elements. *w*Tpre may have lost its mobile elements and bacteriophages more recently. Indeed, 57 of the 281 truncated *w*Tpre genes (20.2%) are versions of *Wolbachia* genes with phage or transposon function.

We hypothesize that the extensive protein coding frame truncations present in *w*Tpre reflect the change in reproductive phenotype from CI to PI. In *Trichogramma,* fixation of asexual reproduction can occur through changes in the host genome, which makes *Wolbachia* essential to the production of female offspring; so called virginity mutations (Russell and Stouthamer 2011; Stouthamer et al. 2010). While this *w*Tpre strain does infect a host that is dependent upon *w*Tpre's parthenogenesis-induction, not all *Trichogramma*, or even all *Trichogramma pretiosum*, have this dependent relationship with their resident *Wolbachia* strains. Sequencing of additional *Trichogramma*-infecting *Wolbachia* strains is necessary to determine whether or not these coding frame truncations are pervasive across all PI-*Wolbachia*, just the *Trichogramma*-infecting *Wolbachia*, or are unique to strains such as *w*Tpre that infect irreversibly asexual hosts.

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TABLES AND FIGURES

Table 1.1 *Wolbachia* strains used in comparative and phylogenetic analyses.

Strain	Host	Group	Size (bp)	ORFs	Reference	Accession	BUSCO Score[c]
*w*Gmm	*Glossina morsitans morsitans*	A	1,019,687	1378	(Brelsfoard et al. 2014)	AWUH00000000	C:77.5%[D:6.4%],F:5%,M:17.5%,n:40
*w*Ha	*Drosophila simulans*	A	1,295,804[b]	1342	(Ellegaard et al. 2013)	CP003884	C:85%[D:2.9%],F:5%,M:10%,n:40
*w*Mel	*Drosophila melanogaster*	A	1,267,782[a]	1401	(Wu et al. 2004)	AE017196	C:87.5%[D:2.9%],F:2.5%,M:10%,n:40
*w*Ri	*Drosophila simulans*	A	1,445,873[a]	1493	(Klasson et al. 2009)	CP001391	C:82.5%[D:3%],F:5%,M:12.5%,n:40
*w*Suzi	*Drosophila suzukii*	A	1,415,350	1528	(Siozios et al. 2013a)	CAOU00000000	C:87.5%[D:2.9%],F:2.5%,M:10%,n:40
*w*AlbB	*Aedes albopictus*	B	1,162,431	1187	(Mavingui et al. 2012)	CAGB00000000	C:82.5%[D:3%],F:2.5%,M:15%,n:40
*w*Bol1	*Hypolimnas bolina*	B	1,377,933	1369	(Duplouy et al. 2013)	CAOH00000000	C:80%[D:3.1%],F:5%,M:15%,n:40
*w*Di	*Diaphorina citri*	B	1,240,904	1250	(Saha et al. 2012)	AMZJ00000000	C:80%[D:3.1%],F:2.5%,M:17.5%,n:40
*w*No	*Drosophila simulans*	B	1,301,823[a]	1317	(Ellegaard et al. 2013)	CP003883	C:82.5%[D:3%],F:2.5%,M:15%,n:40
*w*Pip_Pel	*Culex quinquefasciatus* Pel	B	1,482,355[a]	1461	(Klasson et al. 2008)	AM999887	C:80%[D:3.1%],F:5%,M:15%,n:40
*w*Pip_JBH	*Culex quinquefasciatus* JBH	B	1,542,137	1556	(Salzberg et al. 2009)	ABZA00000000	C:75%[D:3.3%],F:2.5%,M:22.5%,n:40
*w*Pip_Mol	*Culex pipiens molestus*	B	1,340,443[a]	1340	(Pinto et al. 2013)	HG428761	C:80%[D:3.1%],F:2.5%,M:17.5%,n:40
*w*Tpre	*Trichogramma pretiosum*	B	1,133,709[b]	1405	This study	LKEQ00000000	C:77.5%[D:3.2%],F:5%,M:17.5%,n:40
*w*VitB	*Nasonia vitripennis*	B	1,107,643	1245	(Kent et al. 2011)	AERW00000000	C:77.5%[D:3.2%],F:2.5%,M:20%,n:40
*w*Oo	*Onchocerca ochengi*	C	957,990[a]	1272	(Darby et al. 2012)	HE660029	C:75%[D:3.3%],F:2.5%,M:22.5%,n:40
*w*Bm	*Brugia malayi*	D	1,080,084[a]	1339	(Foster et al. 2005)	AE017321	C:82.5%[D:3%],F:5%,M:12.5%,n: 40
*w*Wb	*Wuchereria bancrofti*	D	1,052,327	2144	(Desjardins et al. 2013)	ADHD00000000	C:45%[D:0%],F:20%,M:35%,n:40
*w*Cle	*Cimex lectularius*	F	1,250,060[a]	1357	(Nikoh et al. 2014)	AP013028	C:72.5%[D:3.4%],F:2.5%M:25%,n:40

[a]Complete assembly; [b]Single-scaffold assembly; [c]BUSCO scores in standard BUSCO notation (C:complete [D:duplicated], F:fragmented, M:missing, n:number of genes used)

Table 1.2 Classification of *w*Tpre "unique genes".

"Unique Genes" with Evidence of Truncation		**"Unique Genes" without Evidence of Truncation**	
Nonsense mutation	26	No match to other *Wolbachia* genes	115
Post-nonsense	76	Low identity score of alignment	7
Frameshift mutation	30	Homolog is shorter than *w*Tpre gene	11
Post-frameshift	139	No up/down-stream homology	68
Post-start codon mutation	10		
Total truncations	281	Total excluded	201

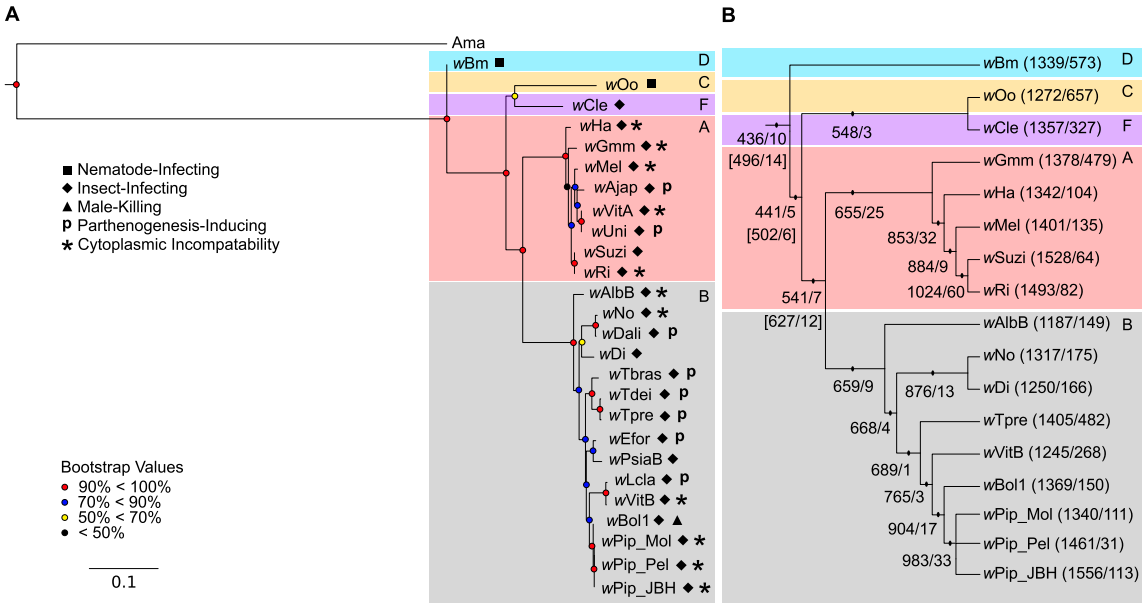

Figure 1.1 Phylogenetic relationships of *Wolbachia*. (A) Phylogeny inferred with RAxML from a nucleotide supermatrix of the five *Wolbachia* MLST genes using 1000 bootstrap replicates. Supergroups are shown in colored boxes, and labeled in the top right corner of each box. Symbols next to taxa denote *Wolbachia* host and phenotypes. Colors at nodes indicate bootstrap values. *Anaplasma marginale* str. *Florida* "Ama" is the outgroup. (B) Cladogram of *Wolbachia* inferred with RAxML using the same methods as in Figure 1A, but analyzing only the strains with sequenced genomes. Numbers in parentheses next to taxon names represent, on the left, the number of genes in the genome, and on the right, the number of genes unique to that genome. Numbers corresponding to points on internodes represent, on the left, the number of core Cluster of Orthologous Genes (COGs) for that clade, and on the right, the number of COGs unique to that clade. Numbers in square brackets represent alternative core and unique genome sizes for the respective clade, calculated without *w*Gmm. Colored boxes denote supergroups, with labels in the top right corner.

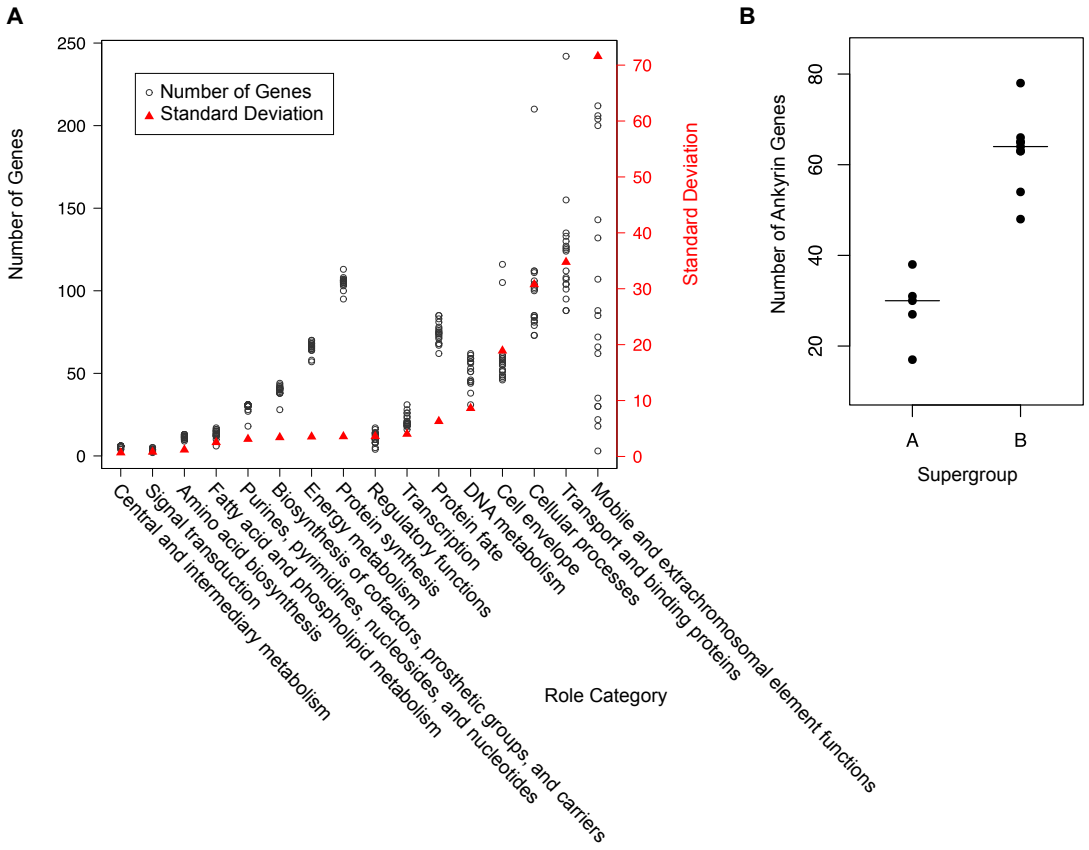

Figure 1.2 Gene content of *Wolbachia*. (A) The numbers of genes in each role category, for each *Wolbachia* genome are plotted with open circles and correspond to the left axis. Role categories are sorted by standard deviation, represented by the red triangles, and the right axis. (B) Number of ankyrin repeat containing genes per genome, by supergroup. The B supergroup has a significantly higher number of ankyrin genes than the A supergroup (Mann-Whitney U test, $p = 0.003$).

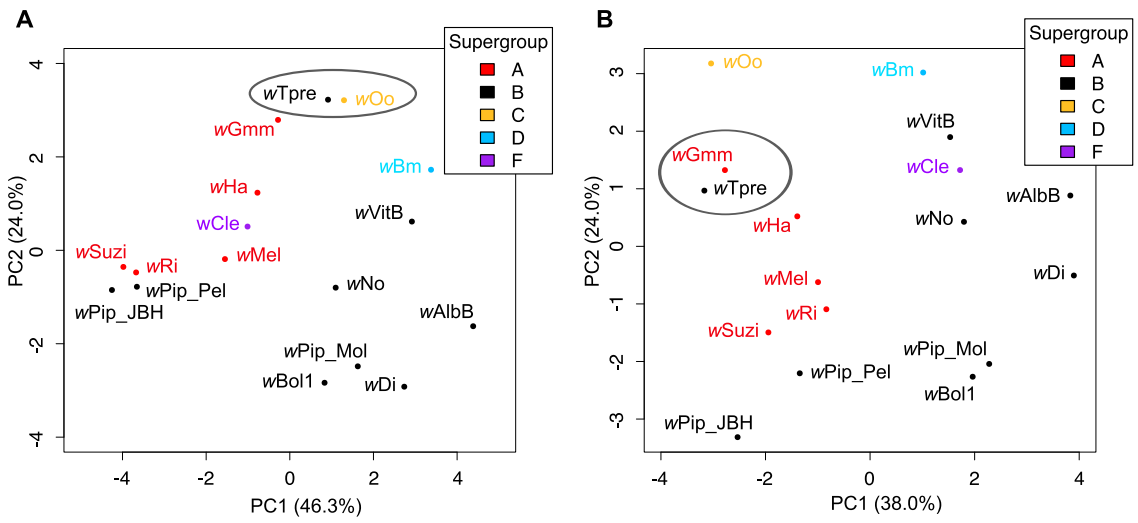

Figure 1.3 Principal Components Analysis of *Wolbachia* genomes based on proportion of annotated genes devoted to each role category, with *w*Tpre and closest neighbor circled. (A) All annotated role categories analyzed. The strongest factor loadings along PC1 (46.3% of total variance) and PC2 (34.0% of total variance), respectively, are energy metabolism and regulatory functions. (B) Mobile and extrachromosomal elements functions category excluded. The strongest factor loadings along PC1 (38.0% of total variance) and PC2 (24.0% of total variance), respectively, are cellular processes and DNA metabolism.

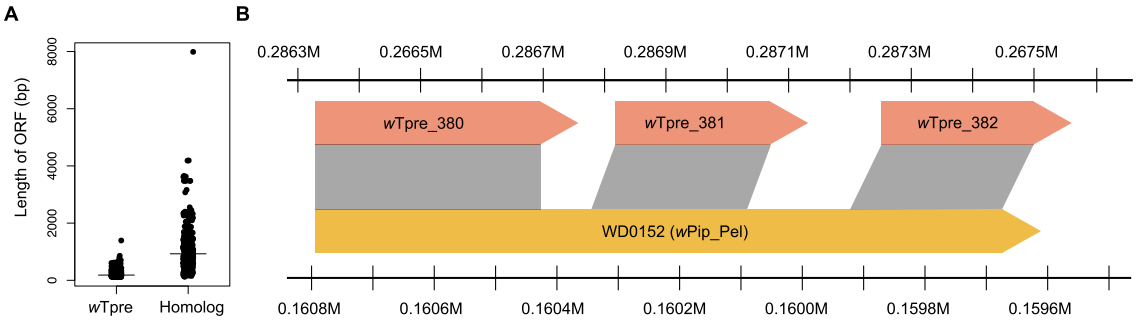

Figure 1.4 Evidence for truncation in *w*Tpre genes. (A) Length of *w*Tpre "unique genes" and their homologous genes from other *Wolbachia* genomes. There is a significant difference in the size of the *w*Tpre unique gene set as compared to their homologous counterparts (Mann-Whitney U test, $p < 0.0001$). (B) Schematic representation of *w*Tpre coding frame truncation and fragmentation. The *w*Tpre "unique genes", *w*Tpre_380, *w*Tpre_381, and *w*Tpre_382 are homologous to sequential locations in the WD0152 gene from *w*Pip_Pel. A frameshift mutation at basepair 421 in *w*Tpre_380 resulted in a premature stop codon and the subsequent annotation of downstream ORFs, or "post-frameshift" ORFS.

CHAPTER 2

The genome of an irreversibly asexual parasitoid wasp: *Trichogramma pretiosum*

Amelia R. I. Lindsey[1], Yogeshwar D. Kelkar[2], Xin Wu[3], Dan Sun[3], Ellen O. Martinson[2], Paul F. Rugman-Jones[1], Daniel S.T. Hughes[4], Shwetha C. Murali[4], Jiaxin Qu[4], Shannon Dugan[4], Sandra L. Lee[4], Hsu Chao[4], Huyen Dinh[4], Yi Han[4], HarshaVardhan Doddapaneli[4], Kim C. Worley[4], Donna M. Muzny[4], Richard A. Gibbs[4], Stephen Richards[4], Soojin V. Yi[3], Richard Stouthamer[1], John H. Werren[2]

[1]Department of Entomology, University of California Riverside, Riverside, California, 92521; [2]Biology Department, University of Rochester, Rochester, New York, 14627; [3]School of Biological Sciences, Institute of Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, Georgia, 30332; [4]Human Genome Sequencing Center, Department of Human and Molecular Genetics, Baylor College of Medicine, Houston, Texas, 77030

ABSTRACT

Trichogramma wasps are parasitoids of other insect eggs, frequently used in agricultural systems against damaging moth and butterfly pests. In addition to their use in agriculture, *Trichogramma* are known for their unique relationships with symbionts and selfish genetic elements, and for their complex patterns of reproductive incompatibilities. As a member of the super-diverse superfamily Chalcidoidea which contains an estimated half million species of wasps, most of them parasitic, the *Trichogramma* genome may help us to understand the evolution of parasitism more broadly. We present the 195 Mb complete genome assembly of a line of *Trichogramma pretiosum* that is asexual due to infection with the bacterial symbiont *Wolbachia*. The genome assembly is contained in 357 scaffolds. Across Hymenoptera, *Trichogramma pretiosum* shows the largest number of missing or contracting gene families, in addition to having a suite of lineage specific genes and expansions relating to nucleic acid metabolism, DNA recombination and integration, transcription, nitrogen metabolism, and epigenetic modifications. We show gene loss is a prominent feature of Chalcid wasp evolution, which may have been an important step in the transition to a parasitic lifestyle. *Trichogramma* has a rapidly evolving gene set: protein rate evolution tests reveal rapid evolution of genes relating to signaling, regulation of metabolic processes, and gene expression. These genomic resources will allow for further studies on the genomic basis of symbiont-mediated parthenogenesis, sex determination, and the evolution of parasitism, as well as a more comprehensive understanding of the consequences of diverse symbiotic interactions.

INTRODUCTION

Trichogramma (Hymenoptera: Trichogrammatidae) wasps are minute polyphagous egg-parasitoids used globally for controlling a variety of agricultural insect pests (Knutson 1998). They are some of the smallest known insects, with adults measuring only tenths of a millimeter in length. The family Trichogrammatidae is one of the earliest branching families of the diverse superfamily Chalcidoidea (Heraty et al. 2013; Munro et al. 2011), putting *Trichogramma* in a position to study the evolution of parasitism more broadly. There are estimated to be at least a half million species in the Chalcidoidea (Heraty 2009; Heraty and Gates 2003), with extreme variation in morphological and biological diversity. Most Chalcids are parasitoids of other insects and arthropods, but there are a handful of independent transitions to phytophagy, such as the mutualist fig pollinators of the family Agaonidae (Munro et al. 2011). Chalcid life cycles are equally as diverse, including generalist Trichogrammatids that can oviposit into various orders insect eggs; heteronomous Aphelinids where females develop as primary parasitoids in one host, and males develop as hyperparasitoids in a different host (Hunter and Woolley 2001); and the ant nest parasites in the Eucharitidae that must hitch a ride into and disguise themselves within an ant nest (Vander Meer et al. 1989).

Trichogramma are found worldwide with c. 180 described species in the genus (Pinto and Stouthamer 1994; Pinto 1998; Pinto 2006), many of which are morphologically indistinguishable from each other and exhibit complex patterns of reproductive incompatibility (Li et al. 2004; Stouthamer et al. 2000; Pinto et al. 1997; Pinto et al.

1991). To further confound their identification, many populations of *Trichogramma* are asexual due to infection with parthenogenesis-inducing *Wolbachia* symbionts (Schilthuizen and Stouthamer 1997; Stouthamer and Werren 1993; Stouthamer et al. 1993), rendering keys based on male genitalia useless (Owen et al. 2007; Stouthamer et al. 1990b). Like all other Hymenoptera, *Trichogramma* are haplodiploid, where males develop from haploid eggs, and females develop from diploid eggs. In *Trichogramma* infected with *Wolbachia*, diploidy is obtained through failed chromatid segregation during the first mitotic division of the egg (Stouthamer and Kazmer 1994), resulting in females from unfertilized eggs rather than from fertilization. This creates a genetic conflict between *Wolbachia* and *Trichogramma* that can lead to irreversible asexuality. (Russell and Stouthamer 2011; Stouthamer et al. 2010). In populations with a low incidence of *Wolbachia* infection, there is a selective advantage for females that do not fertilize their eggs, as they will produce males that will mate with *Wolbachia*-infected conspecifics. These so called "virginity mutations" become fixed in a population as *Wolbachia* spreads, resulting in a completely infected population that is dependent upon *Wolbachia* for the production of female offspring (Russell and Stouthamer 2011; Stouthamer et al. 2010; Jeong and Stouthamer 2005). Sexual colonies cannot be maintained after curing these particular wasps of their *Wolbachia* infections, as mothers do not fertilize their eggs at a high enough rate to produce sufficient numbers of females. In *Trichogramma pretiosum*, populations can be either *Wolbachia*-uninfected (sexual), or *Wolbachia*-infected (asexual), with some of the asexual populations being irreversibly so.

We present the genome sequence of one such irreversibly asexual, *Wolbachia*-infected iso-female line of *Trichogramma pretiosum*, and use comparative genomics across Hymenoptera to identify unique features of the *Trichogramma* genome, and the evolution of parasitism in the Chalcidoidea. This genome provides a foundation for further studies in biological control, the evolution of parasitism, and the evolution of asexuality.

MATERIALS AND METHODS

DNA and RNA for sequencing and annotation

An asexual line of *Trichogramma pretiosum* was mass reared for sequencing. This colony has been maintained in a commercial insectary since 1966 (Beneficial Insectary, Guelph, Ontario, Canada), after collection from the Puira Valley of Peru. Laboratory replicates of this colony are maintained in 12 x 75 mm glass culture tubes stopped with cotton and incubated at 24^{o}C, L:D = 16:8, with 50% relative humidity. Every 10 days cultures are offered fresh honey and egg cards made of irradiated *Ephestia kuehniella* host eggs (Beneficial Insectary, Guelph, Ontario, Canada) afixed to card stock with double-sided tape. Species identification was confirmed by molecular protocols from Stouthamer et al. (1999), and *Wolbachia* infection status was confirmed through antibiotic curing (Stouthamer et al. 1990a) and PCR (Werren and Windsor 2000). Establishment of a *Wolbachia*-free replicate of this colony has not been possible, as females do not fertilize their eggs at high enough frequencies, rendering them dependent upon *Wolbachia,* as seen in (Russell and Stouthamer 2011).

DNA was extracted from ~2,000 males obtained by treating a single generation of the Insectary line with antibiotics using procedures from (Stouthamer et al. 1990a). The single dose of antibiotics is strong enough to knock down *Wolbachia* titers to a level that results in the production of predominantly male offspring in the following generation, but does not eliminate *Wolbachia* completely. This was done to prevent sequencing libraries from being saturated with *Wolbachia* reads, as *Wolbachia* titers can be extremely high in *Trichogramma* (Stouthamer and Werren 1993; Jeong and Stouthamer 2009; Pintureau et al. 2000). Approximately 2,000 infected males (10 mg) were collected and euthanized in 100% ethanol in a glass Dounce tissue grinder tube. The ethanol was subsequently decanted and the sample allowed to air dry before being homogenized in 180 µl of PBS using a glass pestle. The homogenate was transferred to a 1.5 ml microcentrifuge tube and DNA was extracted using the QIAGEN® DNeasy Blood and Tissue Kit following the manufacturer's protocol for purification of total DNA from insects (available at: www.qiagen.com/literature/render.aspx?id=528), completed with a final elution of the DNA in 100 µl of Buffer AE. The extraction was replicated 8 times and subsequently combined into a single eluate. Extracted DNA was quantified using a Nanodrop 1000 and diluted to 50 ng/µl. For RNA extractions, additional male wasps were collected, as well as female wasps from a replicate colony of the Insectary line that had not been treated with antibiotics. The male pool and the female pool were separately flash frozen in liquid nitrogen, and RNA was extracted using the QIAGEN® RNeasy Mini Kit, following the manufacturer's protocol. Dr. Paul Rugman-Jones performed DNA and RNA extractions in the summer of 2012.

Genome sequencing

Trichogramma pretiosum is one of 30 arthropod species sequenced as a part of the pilot project for the i5K 5000 arthropod genomes project at the Baylor College of Medicine Human Genome Sequencing Center. This work was performed by the Baylor co-authors on the project (see authors list). For all of these species, an enhanced Illumina-ALLPATHS-LG sequencing and assembly strategy enabled multiple species to be approached in parallel at reduced costs. For *Trichogramma pretiosum* we sequenced four libraries of nominal insert sizes 180bp, 500bp, 1kb, 3kb and 8 kb at genome coverages of 108.2X, 71.8X, 27.0X, 85.1X and 55.8X respectively (assuming a 200 Mb genome size). These raw sequences have been deposited in the NCBI SRA, BioSample ID: SAMN02439301.

To prepare the 180bp and 500bp libraries, we used a gel-cut paired end library protocol. Briefly, 1 μg of the DNA was sheared using a Covaris S-2 system (Covaris, Inc. Woburn, MA) using the 180-bp or 500-bp program. Sheared DNA fragments were purified with Agencourt AMPure XP beads, end-repaired, dA-tailed, and ligated to Illumina universal adapters. After adapter ligation, DNA fragments were further size selected by agarose gel and PCR amplified for 6 to 8 cycles using Illumina P1 and Index primer pair and Phusion® High-Fidelity PCR Master Mix (New England Biolabs). The final library was purified using Agencourt AMPure XP beads and quality assessed by Agilent Bioanalyzer 2100 (DNA 7500 kit) to determine library quantity and fragment size distribution before sequencing.

Long mate-pair libraries with 3kb and 8kb insert sizes were constructed according to the manufacturer's protocol (Mate Pair Library v2 Sample Preparation Guide art # 15001464 Rev. A PILOT RELEASE). Briefly, 5 µg (for 1, 2 and 3-kb gap size library) or 10 µg (8-10 kb gap size library) of genomic DNA was sheared to desired size fragments by Hydroshear (Digilab, Marlborough, MA), then end repaired and biotinylated. Fragment sizes between 1.8 - 2.5 kb (2Kb) 3 - 3.7 kb (3kb) or 8-10 kb (8 kb) were purified from 1% low-melting agarose gel and then circularized by blunt-end ligation. These size selected circular DNA fragments were then sheared to 400-bp (Covaris S-2), purified using Dynabeads M-280 Streptavidin Magnetic Beads, end-repaired, dA-tailed, and ligated to Illumina PE sequencing adapters. DNA fragments with adapter molecules on both ends were amplified for 12 to 15 cycles with Illumina P1 and Index primers. Amplified DNA fragments were purified with Agencourt AMPure XP beads. Quantification and size distribution of the final library was determined before sequencing as described above.

Sequencing was performed on Illumina HiSeq2000s generating 100bp paired-end reads. Reads were assembled using ALLPATHS-LG (v35218) (Gnerre et al. 2011) and further scaffolded and gap-filled using in-house tools Atlas-Link (v.1.0) and Atlas gap-fill (v.2.2) (https://www.hgsc.bcm.edu/software/). This yielded an assembly of size 195Mb with contig N50 of 78.6kb and scaffold N50 of 3.7Mb. The assembly has been deposited in the NCBI: BioProject PRJNA168121.

Gene annotation

Trichogramma pretiosum is one of 30 i5K pilot genome assemblies that were subjected to automatic gene annotation using a Maker 2.0 annotation pipeline tuned specifically for arthropods. This work was performed by the Baylor co-authors on the project (see authors list). The pipeline is designed to be systematic by providing a single, consistent procedure for the species in the pilot study, scalable to handle 100's of genome assemblies, evidence guided using both protein and RNA-seq evidence to guide gene models, and targeted to utilize extant information on arthropod gene sets. The core of the pipeline was a Maker 2 instance, modified slightly to enable efficient running on our computational resources (Holt and Yandell 2011). The genome assembly was first subjected to de-novo repeat prediction and CEGMA (Parra et al. 2007) analysis to generate gene models for initial training of the ab-initio gene predictors. Three rounds of training of the Augustus (Stanke et al. 2008) and SNAP (Korf 2004) gene predictors within Maker were used to bootstrap to a high quality training set. Input protein data included 1 million peptides from a non-redundant reduction (90% identity) of Uniprot Ecdysozoa (1.25 million peptides) supplemented with proteomes from eighteen additional species (*Strigamia maritima, Tetranychus urticae, Caenorhabditis elegans, Loa loa, Trichoplax adhaerens, Amphimedon queenslandica, Strongylocentrotus purpuratus, Nematostella vectensis, Branchiostoma floridae, Ciona intestinalis, Ciona savignyi, Homo sapiens, Mus musculus, Capitella teleta, Helobdella robusta, Crassostrea gigas, Lottia gigantea,* and *Schistosoma mansoni*) leading to a final nr peptide evidence set of 1.03 million peptides. RNA-seq from *Trichogramma pretiosum* adult males and

females was used judiciously to identify exon-intron boundaries but with a heuristic script to identify and split erroneously joined gene models. We used CEGMA models for QC purposes: for *Trichogramma pretiosum*, of 1,977 CEGMA single copy ortholog gene models, 1,929 were found in the assembly, and 1,887 in the final predicted gene set. Additionally, genome completeness was assessed with BUSCO (Simão et al. 2015) using coding sequences (-m OGS), and comparisons to the arthropod dataset. Finally, the pipeline uses a nine-way homology prediction with human, *Drosophila* and *C. elegans*, and InterPro Scan5 to allocate gene names. The automated gene set is available from the BCM-HGSC website (https://www.hgsc.bcm.edu/arthropods/parasitic-wasp-t-pretiosum-genome-project) and at the National Agricultural Library (https://i5k.nal.usda.gov**).**

Hymenopteran phylogenetics

A species phylogeny based on 107 protein sequences was reconstructed to determine the evolutionary relationships between 21 hymenopterans. The 107 proteins were selected from the official peptide sets of the nine species with available genomes and *de novo* assembled adult female transcriptomes for the remaining 12 species (Table 2.1). The protein set from each species was searched against the official peptide set of the *Nasonia vitripennis* genome (OGSv2.0 http://arthropods.eugenes.org/genes2/nasonia/genes/) using BLASTp. A significant E value cut-off $\leq 1e^{-5}$ was applied and only genes that had a single hit across all twenty-one species were included in further analysis. Protein sequences were aligned with MAFFT (Katoh and Standley 2013) using default settings. Alignments were trimmed using gBlocks to remove gaps (Talavera and Castresana 2007). Multiple

alignments of the 107 single-copy protein-coding genes were concatenated prior to phylogenetic reconstruction using RAxML version 8.2.8 with the PROTGAMMAWAG model, and 1,000 bootstrap replicates. The tree was rooted on *Athalia rosae*, sister to the hymenopteran species used in our analyses (Klopfstein et al. 2013; Heraty et al. 2011). The phylogeny was visualized with FigTree version 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/), and annotated in Inkscape (https://inkscape.org/en/). Co-author Dr. Ellen O. Martinson performed the construction of the aligned protein dataset at the University of Rochester, and I performed the phylogenetic reconstructions.

Other genomes and gene family clustering

Seven other Hymenopteran genomes and their annotations were used in comparative analyses (Table 2.2). All unpublished i5k genomes were scanned with the Wheeler et al. (2013) pipeline to identify potential bacterial contamination. Raw reads were re-aligned to the reference genome in CLC Genomics Workbench v6.0.2 (CLC bio, Aaarhus, Denmark) and scaffold coverage was calculated to aid in identifying bacterial contamination. Scaffolds and associated annotations identified as "bacterial" and sequenced at least at a 10-fold difference of the average coverage were removed from downstream analyses. In the *Athalia rosae* assembly, we identified 39 scaffolds with strong evidence of bacterial contamination. These scaffolds and associated annotations were removed from the comparative analyses. The *Trichogramma pretiosum* assembly

contained a near-complete *Wolbachia* genome, Scaffold 109, which was removed and published separately (Lindsey et al. 2016).

Following the removal of "bacterial" scaffolds from the seven other Hymenopteran genomes used for comparative analysis, protein-coding annotations associated with the remaining scaffolds were obtained. For genes with multiple transcript variants, only the longest isoform was used in downstream clustering. After pooling together these protein-coding genes with those of *T. pretiosum,* we used OrthoMCL (Li et al. 2003), to identify clusters of ortholgous and paralogous genes. For the initial blastp step of OrthoMCL we used the E value cut-off value of 10^{-10}. The OrthoMCL parameter 'percentMatchCutoff' and MCL's inflation parameter were set to 70 and 2.5 respectively in order to ensure that only proteins that share true orthology or paralogy relationships were clustered together. Co-author Dr. Yogeshwar D. Kelkar and I collaborated on construction of the official gene sets and clustering parameters. Dr. Yogeshwar D. Kelkar ran the clustering algorithm at the University of Rochester.

Gene ontology assignments

GO terms were mapped to the coding sequences from all genomes using Blast2GO (Conesa et al. 2005) after blastp searches against the NCBI non-redundant database, and interpro scans with the Blast2GO software. To obtain GO terms for each gene family delineated by OrthoMCL, all GO terms represented by at least 40% of the members within a gene family were recorded, as was done in Grbic et al. (2011).

Gene family expansions and contractions

We used CAFE (De Bie et al. 2006) to identify significantly expanding and contracting gene families across Hymenoptera, at a corrected significance level of $p = 0.05$. The birth-death rate parameter, λ, was determined with the -s flag that optimizes the log likelihood of data for all families. To avoid long branch length attraction, the 21-species phylogeny was pruned in R, using the ape package (Paradis et al. 2004) to obtain a phylogeny upon which we mapped gene family evolution. BiNGO (Maere et al. 2005) was used to identify significantly overrepresented GO terms in sets of expanding and contracting gene families, with. Testing was performed with hypergeometric tests and Benjamini & Hochberg FDR correction, at a corrected significance level of 0.05, with the complete set of gene families and their associated GO terms (see above) as the background (Maere et al. 2005). For species-specific genes, the same statistical methods were used, but with the GO terms for individual genes in the respective genome as the background, instead of GO terms for families.

Protein evolution

To test for differences in rates of protein evolution between *Trichogramma pretiosum* and other hymenoptera, we used Tajima's relative rate test (Tajima 1993) as implemented in the R package pegas (Paradis 2010). Due to the long branch-lengths and divergence times, we used amino acid data to avoid issues of nucleotide substitution saturation. We compared amino acid sequences from *Trichogramma pretiosum* to *Nasonia vitripennis*, using *Apis mellifera* as an outgroup. Protein sequences from all gene families where these

three species were single copy (n = 3180) were aligned with MAFFT v7.271 (Katoh and Standley 2013) using standard parameters, and Gblocks v0.91b (Talavera and Castresana 2007; Castresana 2000) was used to remove poorly aligned and especially divergent regions of the alignments. Relative rate testing was performed in R v3.3.2 using both gblocked and non-gblocked alignments for comparison. P-values were collected and Bonferroni corrections were performed for multiple testing. BiNGO (Maere et al. 2005) was used to identify significantly overrepresented GO terms in gene families with significantly elevated rates of evolution. Testing was performed with hypergeometric tests and Benjamini & Hochberg FDR correction, at a corrected significance level of 0.05, with the set of single copy gene families (n = 3180) and their associated GO terms as the background. GO terms are the same as those detailed in the "Gene Ontology Assignements" section, but only the subset that were used for rate testing were used for GO term over-representation so as to not bias results towards GO terms associated with single-copy gene families.

Availability of data and materials

The *Trichogramma pretiosum* Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession JARR00000000. The version described here is version JARR00000000.1. All associated sequences are contained within the NCBI BioProject PRJNA168121. *Trichogramma pretiosum* colony "Insectary" is available upon request.

RESULTS

The *Trichogramma* genome

The *Trichogramma pretiosum* genome assembly is a high quality draft genome, contained in 7,879 contigs that are linked into 357 scaffolds (Table 2.3). The total size of the assembly is 195,087,592 basepairs, which is approximately 21 million basepairs smaller than the estimated size of a close relative, *Trichogramma kaykai* (van Vugt et al. 2005), and 45 million basepairs smaller than the parasitoid, *Nasonia vitripennis* (Werren et al. 2010). The *Trichogramma pretiosum* assembly is relatively complete, with only 4.5% of arthropod marker genes found to be missing from the assembly (Table 2.3). 12,928 genes were annotated for *Trichogramma pretiosum*, which is more similar to the fig wasp's (*Ceratosolen solmsi*) repertoire of 11,412 genes than to *Nasonia vitripennis*'s 24,388 predicted genes (Rago et al. 2016).

Comparative genomics

We identified a near-complete *Wolbachia* genome in the *Trichogramma pretiosum* assembly, which was relocated to a separate GenBank record (Accession Number LKEQ00000000), and published separately (Lindsey et al. 2016). This scaffold (Scaffold 109) and associated annotations were removed from our analyses. Phylogenetic reconstruction of the 21 Hymenopteran species using 107 single-copy orthologs recapitulated results from studies with much more substantial taxonomic sampling (Figure 2.1) (Klopfstein et al. 2013; Heraty et al. 2011). This phylogeny was trimmed to the eight species used in genomic comparisons (Figure 2.2A). Across the eight

hymenopteran genomes, we identified 14,168 gene family clusters. On average, gene family clusters were composed of 7.61 genes. The largest gene family had 187 genes, and the second largest gene family was 175 genes, 134 of them belonging to *Apis mellifera*. For each species, we determined the number of genes in the single-copy core clusters, variable-copy number core clusters, dispensable genome clusters (non-core, non-species specific), species-specific clusters, and the number of singleton genes (Figure 2.2B). The core, single-copy genome was 1,311 gene family clusters for the Hymenoptera (yellow bars in Figure 2.2B), and 3,492 gene family clusters for the Chalcids, 100 of which were unique to the Chalcid clade. When copy number was not considered, the core genome of all the hymenopteran species was composed of 5,204 clusters, and 6,382 clusters for Chalcids, 159 of which were unique to Chalcids. All Chalcids are missing 285 gene family clusters found in the other hymenopterans. These gene families are significantly overrepresented for 12 GO terms, eight of them related to signal transduction (Table 2.4). For the Chalcid-specific gene family clusters, there were 12 overrepresented GO terms, including functions related to cellular organization as well as perception of and response to stimuli (Table 2.4).

Across Hymenoptera, *Nasonia* had the largest number of singleton genes and genes in species-specific clusters, *Apis mellifera* had the largest number of genes in the variable-copy-number core genome, and *Trichogramma pretiosum* had the fewest genes devoted to the dispensable genome (Figure 2.2B). Total numbers of unique genes (singletons plus species-specific genes) and missing clusters are summarized in Table 2.5. *Trichogramma*

pretiosum has the largest number of clusters missing that were found in all other species considered, agreeing with the finding that *Trichogramma pretiosum* has the fewest genes in dispensable clusters. Despite this, there were no significantly overrepresented GO terms in missing gene families, indicating losses across a range of functions. In comparison, there were 106 overrepresented GO terms within the set of *Trichogramma pretiosum* singleton genes. Many of these GO terms relate to nucleic acid metabolic processes, nitrogen compound metabolism, DNA recombination and integration, and regulation of transcriptional processes. Several overrepresented GO terms relate to epigenetic processes including "maintenance of DNA methylation", "DNA packaging", "nucleosome assembly", and several chromatin-associated GO terms.

Gene family expansions and contractions

The numbers of gene families that are significantly expanding and contracting along branches to in the phylogeny are represented in Figure 2.2C. As expected based on the number of genes in species-specific clusters and in the core genome, *Nasonia vitripennis* and *Apis mellifera* had the highest numbers of significantly expanding gene families (306 and 161 families, respectively). There is a clear trend of increased numbers of gene family contractions in the Chalcidoidea, both on the branch leading to Chalcidoidea, and along each lineage. There are relatively few expansions (n=3) and contractions (n=1) leading to the "parasitoid" group indicating that the contractions are a feature of Chalcid evolution specifically. While the Chalcids do show higher numbers of contracting gene families, there is still a significant amount of species-specific gene family expansion. The

only branches that experienced more contractions than expansions were those leading to the Chalcidoidea (expansions = 4, contractions = 20), and to *Trichogramma pretiosum* (expansions = 26, contractions = 41). *Nasonia vitripennis* and *Trichogramma pretiosum* had the highest number of unique genes (Table 2.5), but *Trichogramma pretiosum* had relatively few significantly expanding gene families due to its species-specific genes being singletons as opposed to occurring in species-specific clusters, as they are in *Nasonia*.

Protein evolution

Phylogenetic reconstruction of the Hymenoptera (Figure 2.1 and Figure 2.2) revealed an especially long branch leading to *Trichogramma pretiosum* when using 107 single-copy protein-coding genes. We defined a set of core genes (n = 3,180) that were present as single-copy in *Apis mellifera, Nasonia vitripennis*, and *Trichogramma pretiosum* for use in comparative analyses. We looked across this set of genes to see if protein sequences are evolving faster in particular functional categories, or if sequences are evolving faster across the genome. Tajima's relative rate tests followed by overrepresentation analyses and correction for multiple comparisons revealed a suite of GO terms associated with rapidly evolving genes leading to *Trichogramma*. Stripping alignments of low quality regions had little effect on the categories of GO terms overrepresented, but it did decrease the total number of genes found to have significantly different rates of evolution. After stripping the alignments of low quality regions, 590 genes showed elevated rates of protein evolution: 441 in *Trichogramma* and 149 in *Nasonia*. Without stripping the

alignments, 1,023 genes had evidence for elevated rates of protein evolution: 832 in *Trichogramma*, and 191 in *Nasonia*.

For genes rapidly evolving in *Nasonia*, there were no overrepresented GO terms. However, for the set of genes rapidly evolving in *Trichogramma*, 30 GO terms were overrepresented, with 23 of them overrepresented in both stripped (Table 2.6) and un-stripped (Table 2.7) versions of the analyses. Significantly overrepresented GO terms include those relating to signaling and regulatory processes, specifically, regulation of metabolic processes and gene expression. Again, regulation of nitrogen compound metabolism was a significantly overrepresented GO term indicating that this function may be especially critical in *Trichogramma.* By concatenating the alignments and performing the same relative rate test, we find that the *Trichogramma* branch is indeed significantly longer overall ($p < 0.0001$).

DISCUSSION

The *Trichogramma* genome provides a platform with which to study the evolution of parasitism and the genomic consequences of irreversible asexuality. We first identified unique features of *Trichogramma* evolution using comparative genomics across Hymenoptera, to provide the platform for future studies of how changes in reproductive mode affect genome evolution.

Not only does *Trichogramma* show signatures of genome reduction, the Chalcid lineage as a whole has a strong signature of gene loss, in addition to the presence of unique gene families. Both gene loss and gain are adaptive processes (Albalat and Canestro 2016). As such, the changes unique to the Chalcids may underlie the transition to a parasitic lifestyle and the subsequent adaptive radiation of the superfamily. There are very few lineage-specific contractions in the branch leading to *Microplitis demolitor* (n=1), a species that represents a transition to parasitism independent from the Chalcids (Klopfstein et al. 2013). This implies that the especially high levels of gene loss are a unique feature of Chalcid evolution, and not necessarily parasitic wasps or parasitism in general.

Trichogramma has a suite of unique genes that are overrepresented for a large number of GO terms, many related to nucleic acid metabolic processes, nitrogen compound metabolism, DNA recombination and integration, and regulation of transcriptional processes. Nitrogen compound metabolism is likely especially important for *Trichogramma* as endoparasitoids, because wasps stay in close contact with their waste until eclosing as an adult. The over-represented epigenetic processes are particularly interesting. These include "maintenance of DNA methylation", "DNA packaging", "nucleosome assembly", and several chromatin-associated GO terms. Sex determination is not well understood in parasitic hymenoptera. In *Nasonia*, female development depends upon a properly imprinted paternal genome contribution: being diploid is not sufficient (Dobson and Tanouye 1998). Methylation likely plays a critical role in this

process. High levels of methylation are seen at the sex-determining locus in *Nasonia* (Park et al. 2011), and DNMTs are maternally deposited into developing embryos (Zwier et al. 2012). In *Trichogramma,* females can successfully develop from diploid eggs that contain two maternal copies of the genome, as mediated by *Wolbachia*-induced gamete duplication (Stouthamer and Kazmer 1994). In *Trichogramma*, either sex-determination occurs through a different mechanism, or *Wolbachia* has the ability to sculpt host epigenetic patterns on top of inducing gamete duplication. The reference genome we generated will provide a framework to study sex determination in *Trichogramma*, and the mechanism of *Wolbachia*-mediated parthenogenesis.

We identified a large number of *Trichogramma* unique genes as compared to other Hymenoptera, as well as a high number of genes showing rapid evolution at the protein level. The *Trichogramma* branch is evolving much more rapidly than any of the other hymenopterans we studied. At this point, we do not know whether these are features of *Trichogramma* evolution more broadly, or if this is a result of the transition to irreversible asexuality. Having a high quality reference genome will make it possible to make comparisons between sexual and asexual lines of *Trichogramma*, with the goal of determining how *Wolbachia*-mediated reproductive switches affect genome evolution.

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TABLES AND FIGURES

Table 2.1 Genomes and transcriptomes used in phylogenetic reconstruction.

Species	Sequence Source	Accession	Reference
Athalia rosae	Genome	GCA_000344095.1	I5k, unpublished
Orussus abietinus	Genome	GCA_000612105.1	I5k, unpublished
Apis mellifera	Genome	GCA_000002195.1	Consortium (2006)
Microplitis demolitor	Genome	GCA_000572035.2	Burke et al. (2014)
Trichogramma pretiosum	Genome	GCA_000599845.2	This study
Copidosoma floridanum	Genome	GCA_000648655.1	I5k, unpublished
Tachinaephagus zealandicus	Transcriptome	N/A	Martinson and Werren, unpublished
Melittobia spp.	Transcriptome	N/A	Martinson and Werren, unpublished
Ceratosolen solmsi	Genome	GCA_000503995.1	Xiao et al. (2013)
Spalangia endius	Transcriptome	N/A	Martinson and Werren, unpublished
Spalangia cameronii	Transcriptome	N/A	Martinson and Werren, unpublished
Muscidifurax raptor	Transcriptome	N/A	Martinson and Werren, unpublished
Muscidifurax raptorellus	Transcriptome	N/A	Martinson and Werren, unpublished
Muscidifurax uniraptor	Transcriptome	N/A	Martinson and Werren, unpublished
Pteromalus puparum	Genome	N/A	Martinson and Werren, unpublished
Urolepis rufipes	Transcriptome	N/A	Martinson and Werren, unpublished
Trichomalopsis sarcophagae	Transcriptome	N/A	Martinson and Werren, unpublished
Nasonia vitripennis	Genome	GCA_000002325.2	Rago et al. (2016)
Nasonia longicornis	Transcriptome	N/A	Martinson and Werren, unpublished
Nasonia giraulti	Transcriptome	N/A	Martinson and Werren, unpublished
Nasonia oneida	Transcriptome	N/A	Martinson and Werren, unpublished

Table 2.2 Genomes used in comparative analyses.

Species	Common Name	Family	Assembly	Reference
Apis mellifera	Honey Bee	Apidae	AADG00000000	(Consortium 2006)
Athalia rosae	Sawfly	Tenthredinidae	AOFN00000000	I5k, Unpublished
Ceratosolen solmsi	Fig Wasp	Agaonidae	ATAC00000000	(Xiao et al. 2013)
Copidosoma floridanum	Polyembryonic Wasp	Encyrtidae	JBOX00000000	I5k, Unpublished
Microplitis demolitor	Braconid Parasitoid Wasp	Braconidae	AZMT00000000	(Burke et al. 2014)
Nasonia vitripennis	Jewel Wasp	Pteromalidae	AAZX00000000	(Werren et al. 2010)
Orussus abietinus	Wood Wasp	Orussidae	AZGP00000000	I5k, Unpublished
Trichogramma pretiosum	*Trichogramma* Wasp	Trichogrammatidae	JARR00000000	This study

Table 2.3 Genome assembly statistics for *Trichogramma pretiosum*.

Statistic	***Trichogramma pretiosum***
Scaffolds	357
Total length of scaffolds	195,087,592
Total ungapped length	180,028,424
Scaffold N50	3,706,225
Contigs	7,879
Contig N50	78,655
Predicted genes	12,928
BUSCO score[a]	C:91.8%[D:11.6%],F:3.5%,M:4.5%,n:2675

Table 2.4 Overrepresented Gene Ontology (GO) terms for genes missing from and unique to Chalcid wasps.

Gene Family Set	GO ID	p-value	corr p-value	Description
Chalcid Unique	32501	3.18E-05	1.09E-02	multicellular organismal process
Chalcid Unique	6996	3.44E-05	1.09E-02	organelle organization
Chalcid Unique	51276	4.88E-05	1.09E-02	chromosome organization
Chalcid Unique	7600	8.01E-05	1.35E-02	sensory perception
Chalcid Unique	50890	1.07E-04	1.44E-02	cognition
Chalcid Unique	16043	2.79E-04	3.13E-02	cellular component organization
Chalcid Unique	30178	3.58E-04	3.44E-02	negative regulation of Wnt receptor signaling
Chalcid Unique	50877	5.44E-04	4.29E-02	neurological system process
Chalcid Unique	50896	6.61E-04	4.29E-02	response to stimulus
Chalcid Unique	3008	6.94E-04	4.29E-02	system process
Chalcid Unique	48523	7.01E-04	4.29E-02	negative regulation of cellular process
Chalcid Unique	22402	8.09E-04	4.54E-02	cell cycle process
Chalcid Missing	7165	1.06E-04	2.06E-02	signal transduction
Chalcid Missing	16311	2.41E-04	2.06E-02	dephosphorylation
Chalcid Missing	50794	2.50E-04	2.06E-02	regulation of cellular process
Chalcid Missing	23034	3.38E-04	2.06E-02	intracellular signaling pathway
Chalcid Missing	23046	3.55E-04	2.06E-02	signaling process
Chalcid Missing	23060	3.55E-04	2.06E-02	signal transmission
Chalcid Missing	50789	3.97E-04	2.06E-02	regulation of biological process
Chalcid Missing	7264	6.61E-04	3.00E-02	small GTPase mediated signal transduction
Chalcid Missing	23033	8.42E-04	3.39E-02	signaling pathway
Chalcid Missing	23052	9.92E-04	3.60E-02	signaling
Chalcid Missing	65007	1.41E-03	4.40E-02	biological regulation
Chalcid Missing	35556	1.45E-03	4.40E-02	intracellular signal transduction

Table 2.5 Total numbers of species-specific and missing genes for each genome.

Genome	Species-Specific Genes[a]	Missing Gene Families[b]
Athalia rosae	2,933	267
Orussus abietinus	1,148	139
Apis mellifera	2,932	172
Microplitis demolitor	4,085	169
Trichogramma pretiosum	4,203	403
Copidosoma floridanum	2,704	125
Ceratosolen solmsi	1,225	110
Nasonia vitripennis	11,809	51

[a]Species-specific genes include singletons and genes within species-specific clusters.

[b]Number of gene family clusters for which the species in question is the only species to not have at least one representative gene for that family.

Table 2.6 Overrepresented Gene Ontology (GO) terms for rapidly evolving *Trichogramma* proteins, based off of stripped protein alignments.

GO-ID	p-value	corr p-value	Description
50794	4.82E-13	4.28E-10	regulation of cellular process
65007	1.17E-12	5.10E-10	biological regulation
50789	1.73E-12	5.10E-10	regulation of biological process
19219	2.63E-10	4.67E-08	regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process
51171	2.63E-10	4.67E-08	regulation of nitrogen compound metabolic process
31323	1.07E-09	1.58E-07	regulation of cellular metabolic process
23052	1.31E-09	1.66E-07	signaling
45449	1.57E-09	1.74E-07	regulation of transcription
80090	2.52E-09	2.48E-07	regulation of primary metabolic process
19222	3.79E-09	3.36E-07	regulation of metabolic process
6355	4.16E-09	3.36E-07	regulation of transcription, DNA-dependent
51252	7.10E-09	5.25E-07	regulation of RNA metabolic process
31326	1.22E-08	7.72E-07	regulation of cellular biosynthetic process
9889	1.22E-08	7.72E-07	regulation of biosynthetic process
10556	1.87E-08	1.10E-06	regulation of macromolecule biosynthetic process
10468	7.32E-08	4.06E-06	regulation of gene expression
60255	9.23E-08	4.81E-06	regulation of macromolecule metabolic process
23046	3.92E-07	1.83E-05	signaling process
23060	3.92E-07	1.83E-05	signal transmission
23033	5.69E-07	2.52E-05	signaling pathway
7165	1.61E-06	6.82E-05	signal transduction
7166	5.47E-06	2.21E-04	cell surface receptor linked signaling pathway
7186	7.33E-06	2.83E-04	G-protein coupled receptor protein signaling pathway
43687	1.80E-04	6.64E-03	post-translational protein modification
7610	4.58E-04	1.63E-02	behavior
43085	1.09E-03	3.72E-02	positive regulation of catalytic activity
6468	1.17E-03	3.83E-02	protein amino acid phosphorylation
44093	1.45E-03	4.59E-02	positive regulation of molecular function

Table 2.7 Overrepresented Gene Ontology (GO) terms for rapidly evolving *Trichogramma* proteins, based off of un-stripped protein alignments.

GO-ID	p-value	corr p-value	Description
50794	1.85E-09	2.47E-06	regulation of cellular process
50789	3.99E-09	2.68E-06	regulation of biological process
65007	1.03E-08	4.58E-06	biological regulation
23046	6.27E-07	1.68E-04	signaling process
23060	6.27E-07	1.68E-04	signal transmission
23052	1.01E-06	2.25E-04	signaling
7165	2.06E-06	3.95E-04	signal transduction
10468	2.70E-05	3.90E-03	regulation of gene expression
45449	2.73E-05	3.90E-03	regulation of transcription
10556	2.91E-05	3.90E-03	regulation of macromolecule biosynthetic process
19222	3.41E-05	3.94E-03	regulation of metabolic process
6468	3.61E-05	3.94E-03	protein amino acid phosphorylation
31323	3.82E-05	3.94E-03	regulation of cellular metabolic process
6355	4.66E-05	4.24E-03	regulation of transcription, DNA-dependent
43687	5.03E-05	4.24E-03	post-translational protein modification
60255	5.13E-05	4.24E-03	regulation of macromolecule metabolic process
31326	5.85E-05	4.24E-03	regulation of cellular biosynthetic process
9889	5.85E-05	4.24E-03	regulation of biosynthetic process
43412	6.02E-05	4.24E-03	macromolecule modification
51252	7.62E-05	5.06E-03	regulation of RNA metabolic process
80090	7.93E-05	5.06E-03	regulation of primary metabolic process
19219	1.21E-04	7.04E-03	regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process
51171	1.21E-04	7.04E-03	regulation of nitrogen compound metabolic process
6464	2.75E-04	1.54E-02	protein modification process
23033	3.24E-04	1.74E-02	signaling pathway

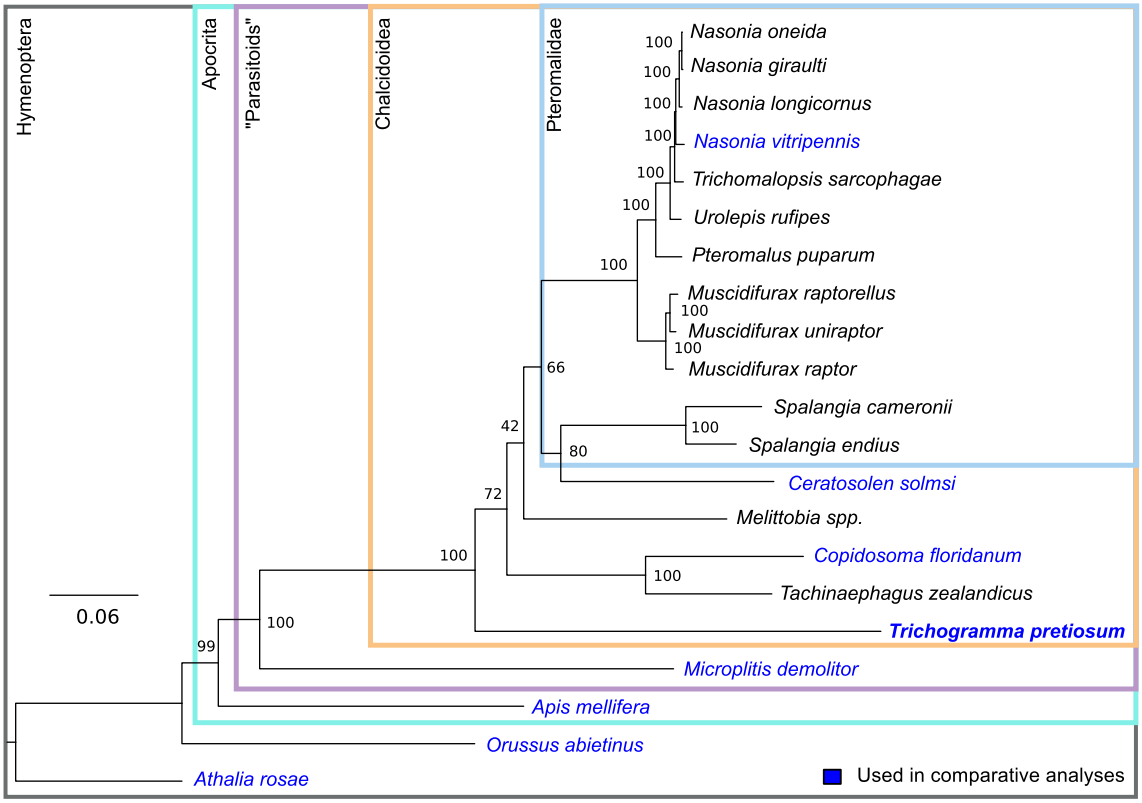

Figure 2.1 Hymenopteran phylogeny showing relationships of species for which there is transcriptomic or genomic data available. Taxa in blue have genomes available and were used in further comparative analyses. *Trichogramma pretiosum* is in bold.

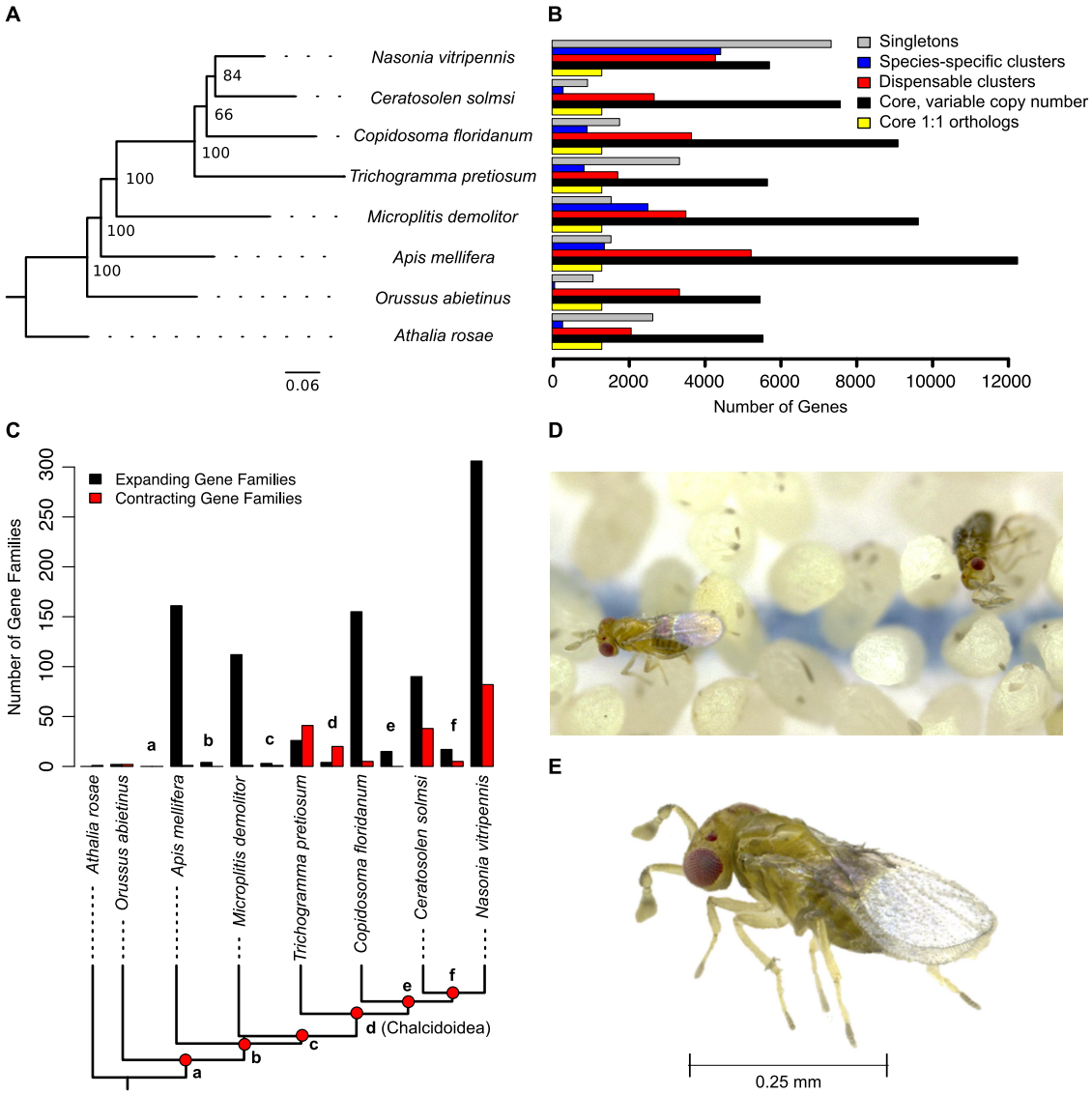

Figure 2.2 *Trichogramma pretiosum* comparative genomics. (A) Trimmed phylogeny representing relationships of species used in genomic comparisons. (B) For each species, we determined the number of genes in the single-copy core clusters, variable-copy number core clusters, dispensable genome clusters (non-core, non-species specific), species-specific clusters, and singleton categories. (C) Numbers of gene family clusters that have significantly expanded or contracted at branches across the phylogeny. Lowercase letters above pairs of bars refer to branches leading to internal nodes of the tree and corresponding points on the phylogeny in red. (D) Female (left) and male (right) *Trichogramma pretiosum* from a sexual line (CA-29) sitting on an egg card of host eggs (*Ephestia kuehniella*). (E) Female *Trichogramma pretiosum* from the asexual Insectary line. Her body is approximately three tenths of a millimeter long.

CHAPTER 3

Penetrance of symbiont-mediated parthenogenesis is driven by reproductive rate in a parasitoid wasp

Amelia R. I. Lindsey[1] and Richard Stouthamer[1]

[1]Department of Entomology, University of California Riverside, Riverside, California, 92521

ABSTRACT

Trichogramma wasps are tiny parasitoids of lepidopteran eggs, used extensively for biological control. They are often infected with the bacterial symbiont *Wolbachia,* which converts *Trichogramma* to an asexual mode of reproduction, whereby females develop from unfertilized eggs. However, this *Wolbachia*-induced parthenogenesis is not always complete, and previous studies have noted that infected females will produce occasional males in the lab. The conditions that reduce penetrance of the parthenogenesis phenotype are not well understood. We hypothesized that more ecologically relevant conditions of limited host access will sustain female-biased sex ratios. After restricting access to host eggs, we found a strong relationship between reproductive rate and sex ratio. By limiting reproduction to one hour a day, wasps could sustain up to 100% effective parthenogenesis for one week, with no significant impact on total fecundity. Reproductive output in the first 24-hours appears to be critical to the total sex ratio of the entire brood. Limiting oviposition in that period resulted in more effective parthenogenesis after one week, again without any significant impact on total fecundity. Our data suggest that this phenomenon may be due to the depletion of *Wolbachia* when oviposition occurs continuously, whereas *Wolbachia* titers may recover when offspring production is limited. In addition to the potential to improve mass rearing of *Trichogramma* for biological control, findings from this study help elucidate the context-dependent nature of a pervasive symbiotic relationship.

INTRODUCTION

Wolbachia is a maternally transmitted, symbiotic bacterium that inhabits numerous arthropods and nematodes. Its ubiquity can be attributed to both fitness advantages for the host, and reproductive modifications of the host. Known reproductive modifications include cytoplasmic incompatibility, male-killing, feminization, and parthenogenesis-induction (Werren et al. 2008), all of which increase the relative fitness of infected females, thus allowing *Wolbachia* to spread through a population (Hoffmann et al. 2011; Turelli and Hoffmann 1991). Parthenogenesis-inducing *Wolbachia* infect haplodiploid species and result in the production of females without the need for a mate. This is accomplished through converting unfertilized eggs (that would otherwise develop as males) to diploid eggs, which then develop as females (Pannebakker et al. 2004; Gottlieb et al. 2002; Stouthamer and Kazmer 1994).

There is a large body of research indicating that the phenotypes *Wolbachia* induces are context dependent, with a range of genetic and environmental factors influencing the penetrance of the manipulation (proportion of individuals displaying the phenotype)(Mouton et al. 2007; Wiwatanaratanabutr and Grandjean 2016; Pascal et al. 2004; Serbus et al. 2015). These are important considerations for several reasons. Firstly, with symbionts under exploration for the control of target pest species (Bourtzis et al. 2014; Hoffmann et al. 2015; Walker et al. 2011; Hoffmann et al. 2011), it is critical that we understand the dynamics that result in the desired host-symbiont extended phenotype, and the persistence of the infection in the target population. Secondly, the persistence of a

symbiont in a host population, and expression of resulting phenotypes will affect the potential for host-symbiont co-evolution. The levels of maternal transmission, penetrance of the reproductive modification or manipulation, relative fitness costs or benefits for the host, and the proportion of infected individuals in the population all play into the ability of *Wolbachia* to spread and maintain itself in a population (Hoffmann et al. 2011; Turelli and Hoffmann 1995; Hoffmann et al. 1990).

Changes in host genotype or the introduction to a novel host can result in altered *Wolbachia* titers (Mouton et al. 2007; Watanabe et al. 2013), failure to induce the anticipated phenotype (McGraw et al. 2001; Bordenstein et al. 2003; Reynolds et al. 2003; Huigens et al. 2004; Grenier et al. 1998), reduced maternal transmission, and the eventual loss of the symbiont from a population (Huigens et al. 2004; Newton et al. 2015). Additionally, there are well-characterized relationships between several environmental factors and the penetrance of *Wolbachia*-mediated phenotypes. High temperatures will reduce *Wolbachia* titers and result in poor host manipulation (Hurst et al. 2000; Bordenstein and Bordenstein 2011; Pascal et al. 2004). The same result has been found for antibiotic treatments: the higher the antibiotic dose, the lower the symbiont titer, and the lower the penetrance of the reproductive manipulation (Zchori-Fein et al. 2000). In the case of cytoplasmic incompatibility-inducing *Wolbachia*, this means heat-treated male offspring of are incapable of inducing cytoplasmic incompatibility, or only do so weakly (Clancy and Hoffmann 1998). In the case of parthenogenesis-inducing *Wolbachia*, antibiotic treated mothers produce increasingly

more sons at lower *Wolbachia* titers (Zchori-Fein et al. 2000; Stouthamer and Mak 2002). Many of these studies point to a "threshold" level of infection that is critical for host-manipulation (Ma et al. 2015; Hurst et al. 2000; Bordenstein and Bordenstein 2011), and a positive correlation between *Wolbachia* titers and expression of the manipulation (Bourtzis et al. 1996; Pascal et al. 2004; Breeuwer and Werren 1993; Zchori-Fein et al. 2000; Ikeda et al. 2003).

The effect of removing or reducing *Wolbachia* titers is well documented for wasps in the genus *Trichogramma* (Tulgetske and Stouthamer 2012; Stouthamer et al. 1990a; Pintureau et al. 1999). *Trichogramma* are minute parasitoid wasps in the superfamily Chalcidoidea, frequently infected with parthenogenesis-inducing *Wolbachia* (Stouthamer et al. 1990b; Stouthamer et al. 1990a; Stouthamer et al. 1993). Like other hymenopterans, *Trichogramma* are haplodiploid: unfertilized eggs typically develop into males, and fertilized eggs into females (Stouthamer et al. 1990a). In *Trichogramma*, parthenogenesis-inducing *Wolbachia* restores diploidy of unfertilized eggs through via a failed anaphase in which chromosomes do not separate during the egg's first mitotic division (Stouthamer and Kazmer 1994). For *Trichogramma*, increased doses of heat reduce bacterial titers and lead to the production of increasingly more males and sexually aberrant individuals (Pascal et al. 2004; Tulgetske and Stouthamer 2012; Stouthamer 1997). It is not clear however, why males are occasionally produced in the absence of antibiotics or increased temperature regimes (Hohmann et al. 2001; Stouthamer and Luck 1993).

The production of males could be useful in determining what factors control the expression of the symbiont phenotype. Preliminary studies that suggest that limited access to host eggs results in more female-biased sex ratios (Hohmann et al. 2001; Stouthamer and Luck 1993; Legner 1985). However, the relationship between access to host eggs and progeny sex ratio has not been teased apart. Prior to the discovery of *Wolbachia* as a parthenogenesis-inducer, fecundity patterns were suggested to affect the resulting sex ratio in the parasitoid wasp *Muscidifurax uniraptor* (Legner 1985). It was later discovered that *Muscidifurax uniraptor* is a host for parthenogenesis-inducing *Wolbachia,* and that *Wolbachia* titers are positively correlated with the proportion of females produced (Zchori-Fein et al. 2000). Here, we use a line of *Trichogramma pretiosum* fixed for *Wolbachia* infection to explore the relationship between patterns of offspring production and sex ratios.

MATERIALS AND METHODS

Trichogramma colonies

Isofemale lines of *Trichogramma pretiosum* are maintained in 12 x 75 mm glass culture tubes stopped with cotton and incubated at 24^{o}C, L:D = 16:8. Every 11 days colonies are given honey and egg cards made of irradiated *Ephestia kuehniella* host eggs (Beneficial Insectary, Guelph, Ontario, Canada) afixed to card stock with double-sided tape. Species identification was confirmed by molecular protocols from Stouthamer et al. (1999). We used the "Insectary" line, collected from the Puira Valley of Peru, which has been

maintained in a commercial insectary since 1966 (Beneficial Insectary, Guelph, Ontario, Canada). The Insectary line exhibits thelytokous reproduction: females hatch from unfertilized eggs, indicating infection with *Wolbachia.* Infection status was confirmed by PCR following Werren and Windsor (2000).

Host access experiments

Individual Insectary line wasps from a single generation were isolated during the pupal stage to ensure virginity. Darkened *Ephestia* eggs (indicating a developing *Trichogramma* pupa) were removed from cards using a paintbrush and water, and isolated in 12 x 75 mm glass culture tubes stopped with cotton. Only wasps that hatched singly from an *Ephestia* egg on day one were used in trials, ensuring size-matched, age-matched, virgin wasps. Upon emergence, wasps were subjected to one of four treatments to determine how access to host eggs, and resultant offspring production, affects *Wolbachia* titers and sex ratio (here defined as percentage females among all offspring). Twenty replicate individual wasps were used for each of the following treatments: 1) a surplus of fresh host eggs every 24 hours for seven days, 2) a surplus of fresh host eggs for 24 hours every other day, for seven days, 3) a surplus of fresh host eggs for only one hour a day, for seven days, or 4) immediate collection into 100% ethanol upon adult emergence (Figure 3.1A). Treatments two and three were designed to restrict access to host eggs, and treatment four was designed to collect data on *Wolbachia* titers in newly emerged wasps. For treatment three, exposure to the fresh egg card was performed at the same time each day, from 10:45AM – 11:45AM. Egg cards were isolated in individual

tubes after the exposure period, ensuring no further parasitization. All mothers, regardless of treatment, were provided with a streak of fresh honey every 24 hours. On day eight, all mothers from the first three treatments were collected into 100% ethanol. All offspring from each isolated egg card were allowed to develop, and collected into 100% ethanol within 24 hours of adult emergence. Offspring were counted and identified as female, male, or intersex based on antennal morphology. *Wolbachia* quantification (see below) was performed on mothers and select progeny.

Quantification of *Wolbachia* titers

Total DNA was extracted from wasps using a Chelex method (Walsh et al. 1991) as implemented by Stouthamer et al (Stouthamer et al. 1999). Gene sequences from the single-copy *Trichogramma pretiosum* gene *wingless*, and the *Wolbachia* 16S gene were identified from the genome assemblies (GenBank Accession Numbers: JARR00000000 and LKEQ01000000, (Lindsey et al. 2016)). *Trichogramma pretiosum wingless* was identified through BLAST searches of the genome, using the *Trichogramma evanescens* homolog as a query (GenBank Accession Number: GQ368153.1). Specific primers (Table 3.1) were designed to amplify variable regions of these two genes, using primer3 (Untergasser et al. 2012). Primer specificity was checked computationally with Primer-BLAST (Ye et al. 2012), and against extractions of the moth host eggs*, E. kuehniella,* which has an orthologous copy of *wingless*, and is infected with its own strain of *Wolbachia*. qPCR was performed in 20µl reactions containing 1x ThermoPol™ buffer (New England Biolabs), 0.4 µM each primer, 200nM each of dATP, dCTP, and dGTP,

400nM dUTP, 1 mM $MgCl_2$, 0.5x EvaGreen® (Biotium), 1 U *Taq* polymerase (New England Biolabs), and 2µl of sample. Reactions were denatured at 95 °C for 3 minutes, followed by 35 cycles of 95 °C for 20 seconds, 58 °C for 20 seconds, and 72 °C for 20 seconds. All samples were run in triplicate alongside calibration standards and negative controls on a Rotor-Gene® Q (QIAGEN). Relative *Wolbachia* titers were determined with the ΔΔCt method (Livak and Schmittgen 2001) with normalization to *wingless*. When testing titers in offspring, we did not correct *wingless* quantification for ploidy levels between males and females as there is evidence that most of the somatic tissues in males are diploid (Aron et al. 2005).

Limiting host access in the first 24-hours

Given the results of the initial host access treatments (Figure 3.1A), we set up a second trial to determine the impact of oviposition in the first 24-hour period. Wasps were isolated from a single generation of the Insectary line, and were age and size matched, as before. 12 replicate individual wasps were subjected to either of the following treatments: 1) constant access to fresh host eggs every 24 hours (same as treatment 1 in the first experiments), or, 2) one-hour access to an egg card on day one (10:45 – 11:45AM), followed by constant access to fresh egg cards every 24 hours starting day two (Figure 3.1B). Trials were carried out for seven days. Again, mothers received fresh honey every 24 hours, and egg cards were isolated after the exposure period. Offspring were allowed to emerge, then counted and identified as female, male, or intersex.

Statistics

Statistical analyses and data visualization were performed in R version 3.1.2. We used a generalized linear mixed-effects model (GLMM) to assess variation in sex ratios (proportions female and non-female offspring) between treatments using treatment and day of the trial as fixed effects, individual wasp identity as a random effect to account for repeated measures, and a binomial error distribution (Bates et al. 2014). We followed up significant effects of treatment with pairwise GLMs, followed by Bonferroni corrections for multiple testing. To assess variation in fecundity among treatments, we used a GLMM with treatment and day of the trial as fixed effects, individual wasp identity as a random effect, and a Poisson error distribution. Here too, we separately assessed variation in total fecundity with a GLM using treatment as a fixed effect, and a Poisson error distribution. We assessed variation in cumulative sex ratio with a GLMM, using cumulative fecundity and treatment as fixed effects, and individual wasp as a random effect. Differences in *Wolbachia* titers between mothers of different host access treatments were assessed with a one-way ANOVA including treatment as a fixed effect, followed by Tukey Honest Significant Difference for post hoc testing. Differences in *Wolbachia* titer between offspring were determined with a linear mixed-effects model, with offspring sex as a fixed effect and mother's identity as a random effect (Bates et al. 2014).

RESULTS

Sex ratios are more female biased when host access is systematically restricted

To determine how reproductive rate affects sex ratio, we subjected wasps to treatments with different degrees of accessibility to host eggs for seven days. We found a significant effect of the interaction between treatment and day of trial for sex ratio (Figure 3.2A; $\chi^2 = 7.38$, $p = 0.0250$). Daily sex ratio significantly differed by treatment (Figure 3.2A; $\chi^2 = 77.38$, $p < 0.001$) and over time (Figure 3.2A; $\chi^2 = 326.07$, $p < 0.001$). Wasps in treatment three, where access to host eggs was for only one hour a day, consistently produced the most female-biased sex ratios. Treatment one, where wasps had constant access to host eggs, produced the least female-biased sex ratios, with male-bias increasing over time. Wasps subjected to treatment two (hosts every other day) produced intermediate sex ratios. These patterns resulted in overall brood sex ratios that were significantly different between treatments (Figure 3.2B; $\chi^2 = 59.72$, $p < 0.001$).

Similarly, we found a significant effect of the interaction between treatment and day of trial for fecundity (Figure 3.2C; $\chi^2 = 181.81$, $p < 0.001$). Levels of daily fecundity significantly differed by treatment (Figure 3.2C; $\chi^2 = 154.65$, $p < 0.001$), and over time (Figure 3.2C; $\chi^2 = 817.83$, $p < 0.001$). Treatment one wasps produced very high numbers of offspring on day one, and fecundity for the remainder of the trial decayed exponentially. In contrast, reproduction by treatment three wasps was initially lower, but did not drop exponentially as did the reproduction of treatment one wasps. Treatment two wasps had an intermediate level of reproductive output throughout the trial. Unlike

total sex ratios, overall fecundity was not significantly different between treatments (Figure 3.2D; $\chi^2 = 4.43$, $p = 0.1091$).

Periodicity of host access, not cumulative fecundity drives sex ratios

To show that prior offspring production alone was not the driver of sex ratio, we tracked cumulative fecundity and cumulative sex ratios for the duration of the trial, and found a significant effect of treatment on cumulative sex ratio (Figure 3.3; $\chi^2 = 38.795$, $p < 0.001$). The most restrictive treatment (three) results in the production of almost all female offspring. At the same point in total reproductive output, wasps in treatments one and two are producing significantly fewer females (Figure 3.3). This shows that it is the restricted access to hosts, and not the total number of offspring produced up to that point that is maintaining the production of more female offspring in treatments two and three.

***Wolbachia* titers are higher in wasps that have not recently oviposited**

We determined *Wolbachia* titers in mothers from the first four treatments (Figure 3.1A), and detected significant differences between treatments (Figure 3.4A; $F_{3,70} = 5.559$, $p = 0.002$). The wasps from treatment four that were collected immediately upon emergence had the highest average *Wolbachia* titers, but they were not significantly different from wasps in treatment three (one hour a day access) ($p = 0.280$). Treatments one and two (constant access, and constant access every other day, respectively) resulted in mothers, on day eight, with significantly lower *Wolbachia* titers relative to immediately collected wasps ($p = 0.033$, and $p = 0.003$ respectively). However, there was no significant

difference between treatments one and two ($p = 0.805$), even though egg card access was restricted in treatment two.

Female wasps have higher *Wolbachia* titers than males

We hypothesized that male offspring would have lower *Wolbachia* titers, as a consequence of having received an insufficient quantity of *Wolbachia* from their mother to induce gamete duplication. We quantified *Wolbachia* titers of three female offspring and three male offspring, from each of three mothers from treatment one (Figure 3.1A). After accounting for differences among mothers, *Wolbachia* titer differed significantly between male and female progeny, with female titers that were more than three times higher than in males (Figure 3.4B; $\chi^2 = 22.22$, $p < 0.001$). With the exception of one male, all female offspring had higher titers than their male siblings.

Reproductive output in the first 24-hours significantly affects sex ratios after one week

Given the finding that the largest difference in fecundity between treatments one and three was during the first 24 hours, we set up a second set of experiments in which one group of wasps' access to egg cards was restricted only on day one, and a second group of wasps had constant access to host eggs (Figure 3.1B). Under these conditions, we found no interactive effect of treatment and day on sex ratio (Figure 3.5A; $\chi^2 = 1.90$, $p = 0.1677$). However, we found a significant effect of treatment (Figure 3.5A; $\chi^2 = 8.75$, $p = 0.0031$), and a significant effect of day (Figure 3.5A; $\chi^2 = 201.46$, $p < 0.001$) on sex

ratio. The experimental treatment (only one hour with an egg card on day one) maintained higher female-biased sex ratios for the duration of the trial. The overall sex ratio of the experimental treatment was significantly more female-biased than the control (Figure 3.5B; $\chi^2 = 24.40$ $p < 0.001$). In contrast to the sex-ratio results for these trials, we found a significant interactive effect of treatment and day on fecundity (Figure 3.5C; $\chi^2 = 7.79$, $p = 0.0053$), as well as a significant effect of day alone (Figure 3.5C; $\chi^2 = 395.74$, $p < 0.001$). Importantly, there was no significant difference in total fecundity between treatments (Figure 3.5D; $\chi^2 = 1.83$, $p = 0.1761$).

DISCUSSION

Based on the established relationship between *Wolbachia* titers and the parthenogenesis-phenotype (Pascal et al. 2004; Tulgetske and Stouthamer 2012; Stouthamer 1997; Zchori-Fein et al. 2000), and previous research on *Muscidifurax uniraptor* that showed sex ratios changed with reproductive patterns (Legner 1985), we hypothesized that reproductive rate might mediate the level of male production in an asexual line of *Trichogramma*. Restricted access to hosts is likely the more ecologically relevant condition, so the males produced under high host availability conditions in the lab would not be produced under field conditions. In natural settings, host availability is often patchy and limited: fluctuations in environmental conditions and the requirement to physically re-locate to find suitable host eggs pose barriers to constant oviposition. Through manipulation of *Trichogramma* oviposition rates, by limiting access to host eggs, we saw that patterns of offspring production had a significant effect on total sex ratio. When wasps were not able

to parasitize host eggs continuously, either by alternating days with access to eggs, or limiting the time per day with egg access, sex ratios were maintained at higher levels (Figure 3.2A). In fact, for wasps that had access to host eggs for only one hour a day, the near-complete parthenogenesis phenotype was maintained for the duration of the trial, without significant impact on total fecundity (Figure 3.2D). Critically, it is only in the first 24-hours where treatment one wasps (constant host eggs) show drastically different fecundity than the treatment three wasps (host eggs for one hour a day). On day two, mothers of these two treatments produced nearly the same number of offspring, and for the remainder of the trial the treatment three wasps produced higher numbers of offspring (Figure 3.2C). High fecundity within the first 24 hours had a lasting effect on the sex ratio of progeny produced for the remainder of the trial.

We show that it is not cumulative fecundity alone that determines the likelihood of the next offspring being feminized (Figure 3.3). This corroborates the finding that there is no significant difference in total fecundity between treatments. We see that sex ratios drop precipitously in treatment one when approximately 45 offspring had been produced, significantly diverging from the host-limited treatments. Even restricting access to hosts on only the first day has a prolonged effect on the sex ratio of the offspring (Figure 3.5A).

Results from qPCR analysis of *Wolbachia* titers were mixed. It is worth noting that whole-body extractions, which are necessary for the minute *Trichogramma*, likely do not

provide the most resolved look at *Wolbachia* titers in the ovaries, which could be responsible for symbiont provisioning to the egg (Ferree et al. 2005). Regardless, *Wolbachia* titers were highest in immediately collected wasps, which is congruent with our expectation (Figure 3.4A). The most restrictive egg card access treatment maintained *Wolbachia* titers at a level comparable to those of wasps who had yet to reproduce, indicating that *Wolbachia* titers had been sustained (Figure 3.4A). However, treatment two, which produced intermediate sex ratios, resulted in *Wolbachia* titers that were indistinguishable from treatment one wasps that oviposited constantly, albeit significantly lower than the immediately collected and treatment three wasps (Figure 3.4A). We predict that this is reflective of the fact that wasps from both of those treatments were able to oviposit up until their collection; whereas mothers from treatment three had 23 hours of recovery prior to collection, resulting in *Wolbachia* titers similar to those that had yet to oviposit.

Wasps that oviposit relatively constantly may be maintaining a rate of germ line stem cell turnover such that *Wolbachia* cannot keep up, resulting in fewer *Wolbachia* deposited into eggs, and the subsequent production of males. This is consistent with the finding that a large number of *Wolbachia* are transferred from nurse cells into the developing oocyte (Ferree et al. 2005; Serbus et al. 2008). Alternatively, *Wolbachia* from somatic tissues could continually invade the germ line to ensure a high proportion of infected eggs (Toomey et al. 2013; Fast et al. 2011). Under these circumstances, constant oviposition may draw *Wolbachia* from the soma resulting in lower whole-body titers. We propose

that the recovery periods built into our host access treatments are critical to maintaining *Wolbachia* titers high enough in developing eggs to ensure effective parthenogenesis induction. This is also in line with previous studies that showed a positive relationship between *Wolbachia* titers and sex ratios in parthenogenesis-inducing *Wolbachia* (Zchori-Fein et al. 2000; Pascal et al. 2004; Stouthamer and Mak 2002).

Additional support for the hypothesis that *Wolbachia* titer is essential for parthenogenesis induction comes from finding of lower *Wolbachia* titers in males compared to their sisters (Figure 3.4B). While it is possible that adult titers may not be reflective of the number of *Wolbachia* deposited into the egg, arguably, our data point to titers being important for proper parthenogenesis-induction, which is again consistent with previous findings on the effect of heat and antibiotics (Pintureau et al. 1999; Stouthamer et al. 1990a; Pascal et al. 2004), and the importance of maternal loading of *Wolbachia* into eggs (Serbus et al. 2008; Ferree et al. 2005). There is a chance that some of the phenotypic males with higher *Wolbachia* titer could be of female karyotype, which has been shown to occur in related *Trichogramma* species and other parthenogenesis-inducing *Wolbachia* infected wasps (Tulgetske 2010; Ma et al. 2015). We would expect these individuals to have high enough *Wolbachia* titers to induce gamete duplication, but not high enough to result in the hypothesized epigenetic feminization that occurs afterward (Tulgetske 2010).

It is likely that *Wolbachia* titers in the egg may not be the final determinant of successful parthenogenesis induction, but instead it is a *Wolbachia*-secreted factor that needs to be at sufficient levels. This has been hypothesized as a mechanism for the sex-ratio changes in *Muscidifurax* (Zchori-Fein et al. 2000), and is the mechanism for inducing cytoplasmic incompatibility, as sperm do not contain *Wolbachia* cells, but have been modified by *Wolbachia*-derived proteins (Beckmann et al. 2017; LePage et al. 2017). Females from other closely related species of *Trichogramma* hatch with a set of fully developed eggs, but will mature new eggs over the course of their adult life (Volkoff and Daumal 1994). The newly matured eggs may need a longer "incubation time" in order to accumulate the appropriate concentration of *Wolbachia* or a *Wolbachia*-derived parthenogenesis factor. More resolved studies of *Wolbachia* densities, *Wolbachia*-protein densities, and the time that eggs spend in the mother, would aid in identifying a threshold level of infection critical for effective parthenogenesis induction.

There is evidence for gene flow between populations of *Trichogramma* in the field, and that *Wolbachia*-infected females can mate with males and fertilize their eggs (Stouthamer and Kazmer 1994). Given that access to host egg resources has an impact on the likelihood of males being produced, the amount of gene flow may fluctuate with environmental conditions. While limited availability of host eggs is likely the norm, lepidopteran populations fluctuate, with abundance peaking during certain seasons or in response to particular weather patterns (Kunte 1997; van den Bosch 2003; Pollard 1988; Roy et al. 2001). Environmental conditions could have direct effects on *Wolbachia* titers

(such as high temperatures decreasing bacterial titers (Stouthamer et al. 1990a; Pintureau et al. 2002)), and indirect effects through availability of host eggs. Greater host availability may lead to an increase in offspring production, and if high enough, a decrease in sex ratio. Males produced under these circumstances would likely provide a mechanism for gene flow between asexual lineages.

The higher penetrance of parthenogenesis induction under host-limited conditions, as occurred in our study can in part explain the frequent coexistence of infected and uninfected females in *Trichogramma* field populations (Stouthamer et al. 1990a; Huigens et al. 2004; Stouthamer 1997). How these populations can coexist has been unclear, because laboratory experiments with infected and uninfected lines from these field populations often showed that under constant host availability (such as in our treatment one), the daughter production of infected females was lower than that of mated uninfected females (Silva et al. 2000; Stouthamer and Luck 1993).

In conclusion, we provide evidence for *Trichogramma* reproductive patterns mediating the parthenogenesis phenotype, likely through the depletion of *Wolbachia* titers. The males produced during times of high oviposition rates may provide an opportunity for gene flow between populations, and thus new host-symbiont combinations. Given the interest in using *Wolbachia* as a tool to control insect populations (Hoffmann et al. 2015; Turelli and Hoffmann 1991), it is critical that we understand the context-dependent

nature of *Wolbachia* phenotypes, and how this may result in different selective pressures for the host-symbiont relationship.

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TABLES AND FIGURES

Table 3.1 Sequences of primers used in this study.

Locus	Primer	Sequence (5' to 3')	Amplicon Size
16S	16S_qF	GAG GAA GGT GGG GAT GAT GTC	103bp
	16S_qR	CTT AGG CTT GCG CAC CTT G	
wingless	wg_qF	AGC TCA AGC CCT ACA ATC CG	99bp
	wg_qR	CCA GCT TGG GGT TCT TCT CG	

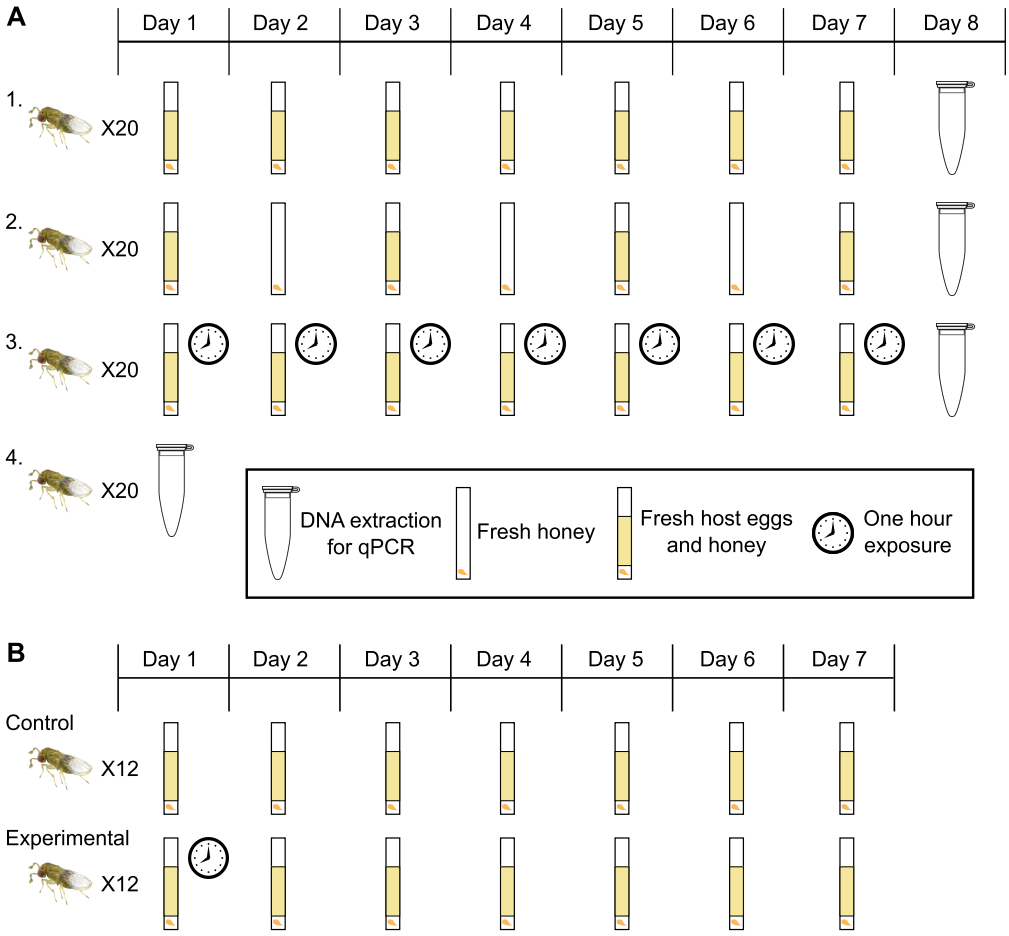

Figure 3.1 Experimental design for host access treatments. A) Treatment One: a fresh egg card every 24 hours; wasps have constant access to host eggs. Treatment Two: one day on, one day off; wasps have constant access to host eggs every other day. Treatment Three: wasps have access to a fresh egg card for only one hour a day. Treatment Four: collect adult wasps into ethanol immediately upon emergence. B) Control: a fresh egg card every 24 hours; wasps have constant access to host eggs (same as treatment one in 3.1A). Experimental: wasps have access to a fresh egg card for only one hour on day one, followed by constant access to host eggs starting day two, with a egg cards exchanged every 24 hours.

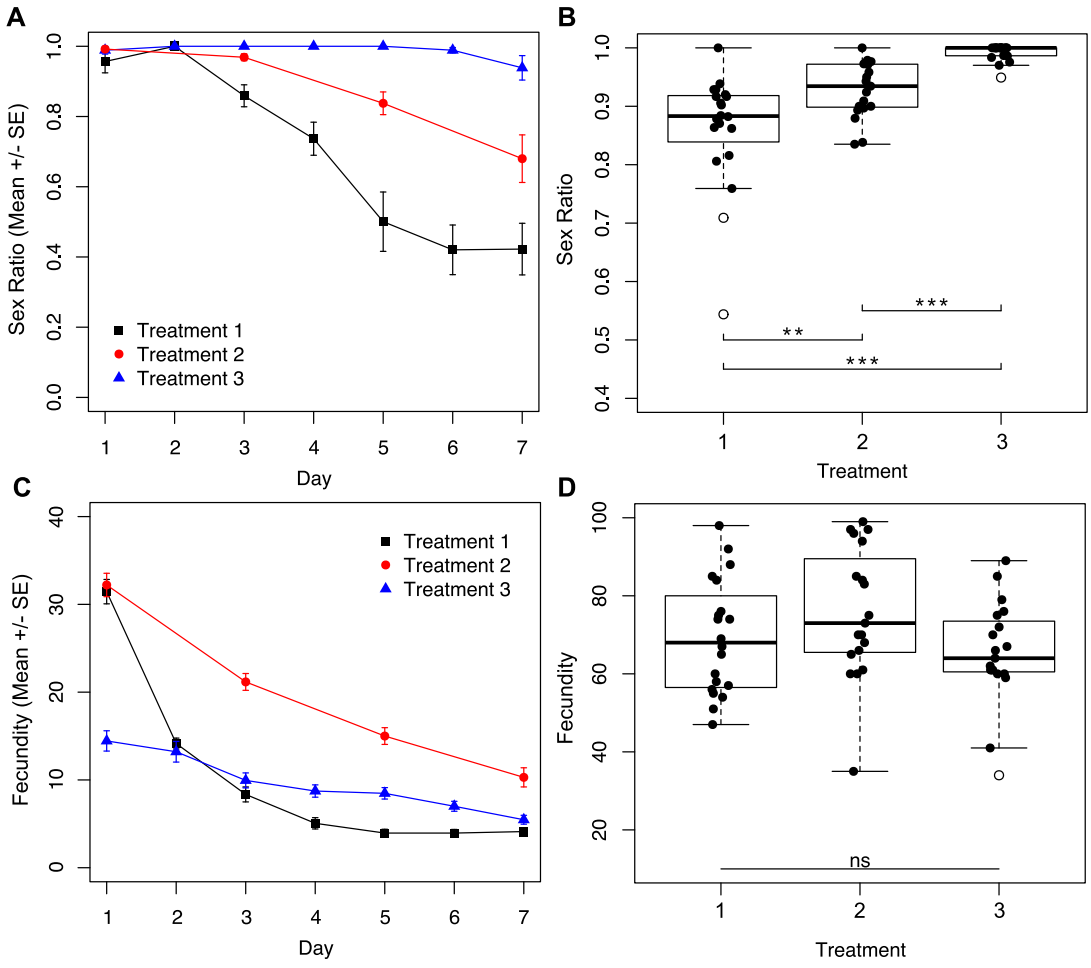

Figure 3.2 Sex ratios and fecundity for host access treatments shown in Figure 3.1A. In panels B and D, open circles represent outliers, double asterisks represent $p \leq 0.01$, and triple asterisks represent $p \leq 0.001$. A) Temporal variation in sex ratio. B) Total sex ratio for the seven-day period. C) Temporal variation in fecundity. D) Total fecundity for the seven-day period.

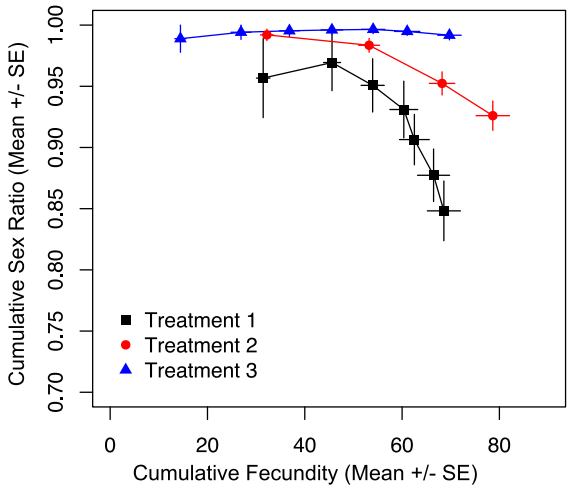

Figure 3.3 Cumulative fecundity and sex ratios for host access treatments. The left-most point of each line represents the first day of the trial, and the right-most point represents the last day of the trial.

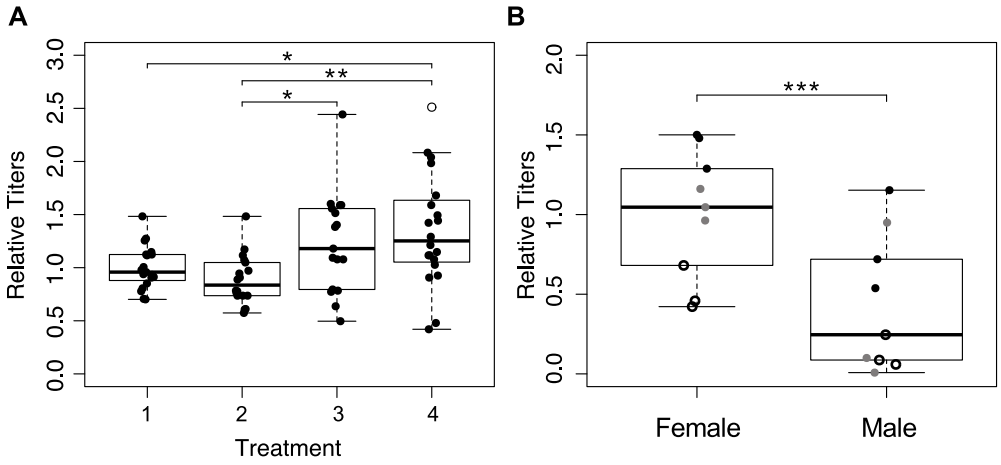

Figure 3.4 *Wolbachia* titers, as measured by quantification of 16S relative to *wingless*. Within a plot, titers have been normalized to the sample shown most left. Open circles represent outliers, a single asterisk represents $p \leq 0.05$ and double asterisks represent $p \leq 0.01$. A) *Wolbachia* titers of mothers collected after the host access treatments one through four. Only significant pairwise comparisons are denoted. B) *Wolbachia* titers of the offspring produced by mothers subjected to treatment one. Point styles denote offspring that originated from the same mother.

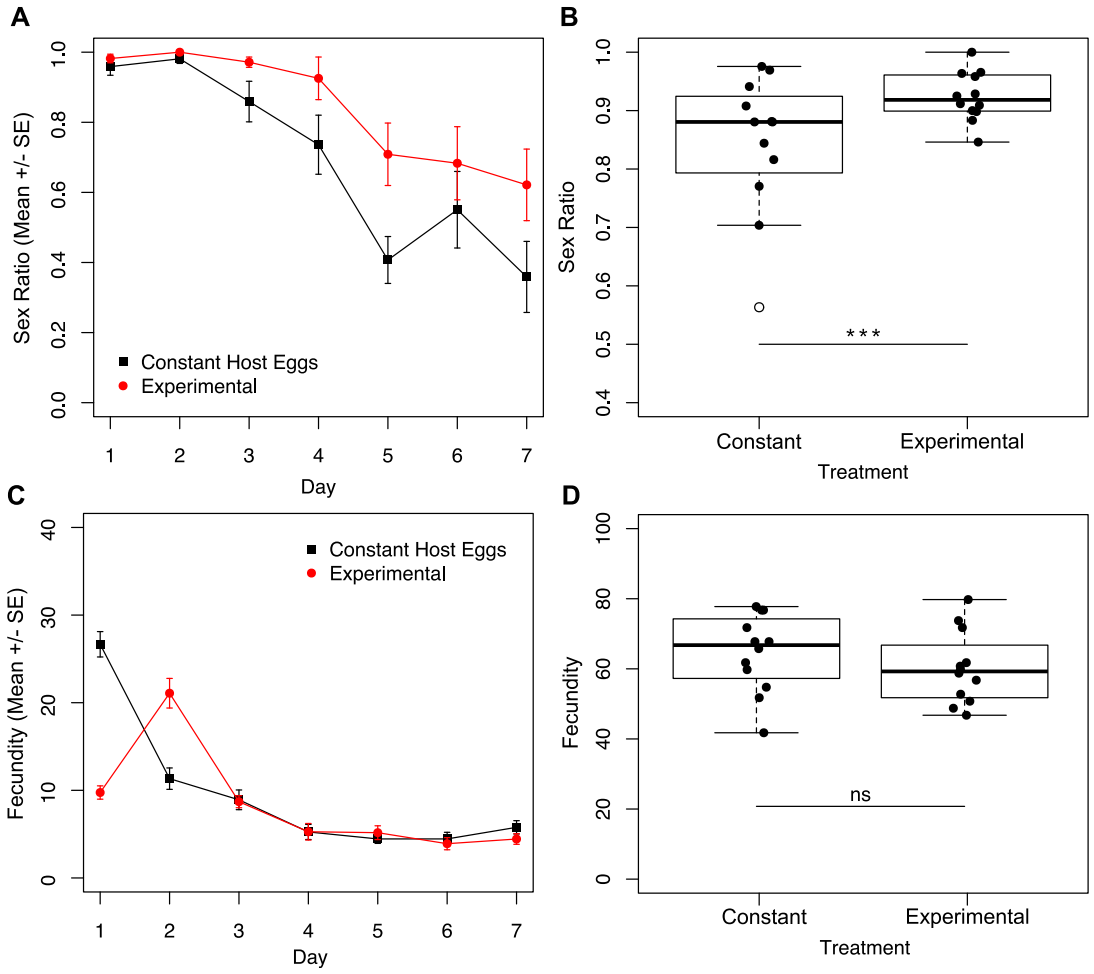

Figure 3.5 Sex ratios and fecundity for host access treatments shown in Figure 3.1B. In panels B and D, open circles represent outliers and triple asterisks represent $p \leq 0.001$. A) Temporal variation in sex ratio. B) Total sex ratio for the seven-day period. C) Temporal variation in fecundity. D) Total fecundity for the seven-day period.

CHAPTER 4

The effects of outbreeding on a parasitoid wasp fixed for infection with a parthenogenesis-inducing *Wolbachia* symbiont

Amelia R. I. Lindsey[1] and Richard Stouthamer[1]

[1]Department of Entomology, University of California Riverside, Riverside, California, 92521

ABSTRACT

Trichogramma wasps can be rendered asexual by infection with the maternally inherited symbiont *Wolbachia*. Previous studies have shown that the *Wolbachia* strains infecting *Trichogramma* wasps are host-specific, resulting in failed horizontal transfer of *Wolbachia* to novel *Trichogramma* hosts. Additionally, *Trichogramma* can become dependent upon their resident *Wolbachia* infection for the production of female offspring, leaving them irreversibly asexual, and linking host and symbiont. We hypothesized that *Wolbachia* strains infecting these irreversibly asexual, resistant to horizontal transfer *Trichogramma* would show adaptation to a particular host genetic background. To test this, we mated *Wolbachia*-dependent females with males from a *Wolbachia*-naïve population to create heterozygous wasps. We measured sex ratios and fecundity, a proxy for *Wolbachia* fitness, produced by heterozygous wasps, and by their recombinant offspring. We find a heterozygous advantage compared to control lines, resulting in higher fitness for *Wolbachia*, as wasps will produce more offspring without any reduction in the proportion of females. While recombinant wasps did not differ in total fecundity after ten days, recombinants produced fewer offspring early on, leading to an increased female-biased sex ratio for the whole brood. Despite the previously identified barriers to horizontal transfer of *Wolbachia* to and from *Trichogramma pretiosum*, there were no apparent barriers for *Wolbachia* to induce parthenogenesis in the non-native background. This implicates different mechanisms for the infection of a new host after horizontal transfer, and the induction of parthenogenesis in a new host.

INTRODUCTION

Wolbachia is a maternally inherited symbiont of arthropods and nematodes that is estimated to infect one to five million species (Werren and Windsor 2000). The success of *Wolbachia* is attributed to its ability to increase host fitness through a variety of mechanisms, including reproductive modifications such as cytoplasmic incompatibility, male-killing, feminization, and parthenogenesis-induction (Werren et al. 2008). These manipulations increase the relative fitness of infected females within a population. As such, *Wolbachia* has the ability to invade populations, and the infection can become fixed (Turelli and Hoffmann 1991; Stouthamer et al. 2010). In certain cases, the host can become dependent on its resident *Wolbachia* infection, and removal of the symbiont can result in severe fertility reduction (Kremer et al. 2009; Dedeine et al. 2001; Hoerauf et al. 1999; Hosokawa et al. 2010; Stouthamer and Mak 2002).

This phenomenon of symbiont-dependence has been documented in some populations of *Trichogramma* parasitoid wasps. *Trichogramma* are often infected with parthenogenesis-inducing *Wolbachia*, which converts the wasps to an asexual mode of reproduction. There is extensive inter- and intra-specific variation in infection frequencies across *Trichogramma*: some populations are never found with *Wolbachia* (Pinto 1998), others maintain a consistent low-level infection (Stouthamer and Kazmer 1994; Stouthamer et al. 2001), and some are completely fixed for *Wolbachia*-infection. The extremes of this variation are particularly evident in one species, *Trichogramma pretiosum*. *Wolbachia* appears to be absent from wild populations of *Trichogramma pretiosum* in California

(Pinto 1998), but in contrast, conspecific populations elsewhere have evolved complete dependency upon *Wolbachia*-induced thelytokous parthenogenesis for the production of female offspring (Russell and Stouthamer 2011; Stouthamer et al. 2010).

This dependence results from mechanism by which *Wolbachia* manipulates the haplodiploid sex determination system of *Trichogramma*. *Wolbachia* duplicates chromosomes in unfertilized *Trichogramma* eggs, turning what would have been a haploid male offspring into a diploid female offspring. The duplication occurs through a failed anaphase during the first mitotic division of the egg (Stouthamer and Kazmer 1994). Over time, *Trichogramma* become dependent on this gamete duplication for the production of female offspring. If cured of their *Wolbachia* infection, eggs are not fertilized at high enough rates (ensuring females) to sustain the population (Russell and Stouthamer 2011; Jeong and Stouthamer 2005; Stouthamer et al. 2010). As such, irreversibly asexual *Trichogramma* are now tied to their resident *Wolbachia* infection.

While *Wolbachia*'s primary form of transmission is vertical, from mother to offspring, over evolutionary time horizontal transfer across species appears to have been common (Stouthamer et al. 1999a; Zhou et al. 1998; Vavre et al. 1999). That said, experimental attempts at horizontal transfer between *Trichogramma* wasps have proved largely unsuccessful, resulting in the loss of bacterial titers or failure to express parthenogenesis (Huigens et al. 2004; Pintureau et al. 2000; Grenier et al. 1998). This is surprising given that the *Wolbachia* strains infecting *Trichogramma* wasps are closely related to each

other (Schilthuizen and Stouthamer 1997; Werren et al. 1995), and that horizontal transfers across larger phylogenetic distances are often successful with other *Wolbachia* strains and other insect hosts (Heath et al. 1999; Hoffmann et al. 2011; Kang et al. 2003). It was hypothesized that the failed transfers amongst *Trichogramma* wasps are due to a lack of co-evolution between *Wolbachia* and the novel *Trichogramma* host (Huigens et al. 2004; Watanabe et al. 2013). Here, we test this prediction by manipulating the nuclear genetic background of an irreversibly asexual line (where host and symbiont are dependent upon each other) and observing how *Wolbachia* performs in the new genetic background. We can manipulate the genetic background of *Trichogramma* by exploiting the fact that irreversibly asexual *Trichogramma* will, if mated to males derived from sexual populations, fertilize a small proportion of their eggs (Stouthamer and Kazmer 1994), resulting in a small number of heterozygous offspring. Heterozygous daughters of this cross will then produce recombinant *Wolbachia*-infected daughters that can be used to start a unique colony, in which gamete duplication renders all subsequent offspring identical and homozygous. The newly recombined host-alleles allow us to test whether or not *Wolbachia* is adapted to a particular *Trichogramma* host genetic background.

MATERIALS AND METHODS

Trichogramma **colonies**

Two separate lines of *Trichogramma pretiosum* were used in experiments. The first, referred to as the "Insectary line", was originally collected from the Piura Valley of Peru in 1966, and has since been continuously maintained in a commercial insectary

(Beneficial Insectary, Guelph, Ontario, Canada). The Insectary line exhibits thelytokous parthenogenesis with females hatching from unfertilized eggs as a result of infection with *Wolbachia.* Attempts at curing the Insectary line of *Wolbachia* have failed, indicating it is irreversibly asexual, similar to (Russell and Stouthamer 2011). The second line, CA-29, is a highly inbred line initiated from a sib-mated female collected in Irvine, California in 2008. CA-29 exhibits normal haplodiploid sex determination (arrhenotokous parthenogenesis) with males developing from unfertilized eggs, indicating no *Wolbachia*. These two lines are ~1% diverged from each other, genome wide (Lindsey et al, unpublished results). Infection status was confirmed by PCR assays following Werren and Windsor (2000). Species identifications were confirmed by molecular protocols from Stouthamer et al. (1999b). Colonies are maintained in 12 x 75 mm glass culture tubes stopped with cotton and incubated at 24°C, L:D = 16:8. Every 11 days, colonies are given honey and new hosts: a surplus of UV irradiated *Ephestia kuehniella* host eggs (Beneficial Insectary, Guelph, Ontario, Canada) affixed to card stock with double-sided tape (egg cards).

Crossing experiments

We set up crosses of *Wolbachia*-infected and *Wolbachia*-uninfected lines to look for evidence of co-adaptation between *Wolbachia* and host. The Insectary line was chosen as the maternal line, as it is *Wolbachia*-infected and irreversibly asexual. CA-29 was chosen as the paternal line due to the fact that only uninfected *Trichogramma pretiosum* have

been collected in California, (Pinto 1998) providing a nuclear background that is not co-evolved with *Wolbachia.*

Eight replicate crosses of a virgin female from the Insectary line with a virgin male from CA-29 were performed (F0). After copulation, females were allowed to parasitize an egg card for 48 hours, after which they were removed and killed in 100% ethanol. Under this mating regime, the female can produce both homozygous F1 daughters (from unfertilized eggs that undergo *Wolbachia*-induced gamete duplication), and heterozygous F1 daughters (from eggs fertilized with sperm from the CA-29 male). Figure 4.1 shows the set-up of this crossing experiment. All F1 offspring were isolated as pupae to ensure virgin status, and upon emergence, each daughter was moved to a fresh egg card every 48 hours for ten days. At the end of this period, each daughter (F1 mother) was removed, killed in 100% ethanol, and her zygosity (either homozygous or heterozygous) was determined as described below. Egg cards were maintained under colony conditions awaiting offspring emergence.

After zygosity was determined, F2 wasps were isolated as pupae from the first egg card of both heterozygous and homozygous F1's. Each female F2 from a heterozygous mother is: 1) a unique mix of the Insectary and CA-29 nuclear backgrounds as a result of recombination during meiosis; 2) infected with *Wolbachia* inherited from the Insectary line; and, 3) completely homozygous (because of gamete duplication). Three such F2 females from each of five parental (F0) crosses were used to initiate a total of 15

Recombinant Isofemale Lines (RILs). Five F2 wasps from homozygous F1 mothers (from the same F1 broods used for initiating RILs) were used to initiate control isofemale lines (CONs). Remaining F2 offspring produced throughout the 10-day trial were allowed to hatch, then were counted, and identified as female, male, or intersex. Intersex *Trichogramma* are a result of incomplete feminization after gamete duplication (Tulgetske and Stouthamer 2012; Tulgetske 2010).

Experimental isofemale lines (RILs and CONs) were maintained under colony conditions, then after three generations they were assayed for sex ratio. Eighteen replicate wasps from each isofemale line were moved to fresh individual egg cards every 48 hours for ten days, and subsequent offspring were counted upon emergence and identified as female, male, or intersex based on antennal morphology. In summary, eight separate crosses were performed to generate heterozygote and homozygote wasps. We then randomly selected five of those crosses to initiate RILs and CONs (Figure 4.1).

Determination of zygosity

DNA was extracted from ethanol-preserved F1 mothers using the EDNA HiSpEx Tissue Kit (Fisher biotic, Australia). Zygosity was determined using a single microsatellite locus, arbitrarily designated A9. A PCR assay of the A9 locus, developed for use in our lab by Genetic Identification Services (Chatsworth, CA, USA), results in the production of a size-diagnostic amplicon: 310bp for the Insectary line versus 220 bp for CA-29. Thus, in our crosses between Insectary females and CA-29 males, homozygous daughters

(from unfertilized eggs that undergo *Wolbachia*-induced gamete duplication) are expected to produce a single 310 bp amplicon, while heterozygous daughters (from eggs fertilized with sperm from the CA-29 male) will produce both amplicons (Figure 4.1). Reactions were set up in 25µl volumes containing 1X ThermoPol™ buffer (New England Biolabs, Ipswich MA), 400 nM uracil, 200 nM each adenine, cytosine and guanine, 0.8 mM $MgCl_2$, 0.8 mg/ml BSA, 0.2 µM each of the primers A9F (5'-CAG CAC AAG TAC ACG ACT GTC-3') and A9R (5'-AGC GAA GCG TAT ATT AGC AAG-3'), one unit *Taq* polymerase (New England Biolabs, Ipswich MA), and 2 µl DNA template. Reaction conditions were: an initial hold for 3 min at 94°C, followed by 38 cycles of 94°C for 45 sec, 51°C for 45 sec, and 72°C for 45 sec. Reactions were terminated with a 5 min hold at 72°C after which, PCR products were electrophoresed on 1.5% agarose gels stained with ethidium bromide.

Statistics

All statistics were performed in R version 3.3.2. We assessed variation in sex ratios and fecundity with generalized linear mixed-effects models (GLMM), using the glmer function from the lme4 package (Bates et al. 2014), a Poisson error distribution for fecundity analyses, and a binomial (logit) error distribution for sex ratios. We used the number of females (successful parthenogenesis-induction events), and the number of non-female offspring (males plus intersex) as the response variable for the sex ratio analyses. Differences in survivorship, as measured by the number of wasps of each

genotype that did and did not survive the length of the trial, were determined with a Chi-Square test.

For comparisons among F1s, we assessed variation in total sex ratios or fecundity after the 10-day period (as described above) with genotype (either heterozygote vs homozygote) as a fixed effect, and initial F0 cross as a random effect. F1 sex ratios and fecundity over time (sex ratio or fecundity per egg card) were assessed with genotype and egg card number (time within trial) as fixed effects, along with their interaction, and individual wasp nested within initial F0 cross as a random effect.

For comparisons of RIL and CON reproduction, we assessed variation in total sex ratios or fecundity after the 10-day period with genotype (either RIL or CON) as a fixed effect, and unique line (ex, RIL3B or CON5) nested with initial F0 cross as a random effect. RIL/CON sex ratio and fecundity over time (sex ratio or fecundity per egg card) were assessed with genotype and egg card number as fixed effects, along with their interaction, and individual wasp nested within unique line, nested within initial F0 cross as a random effect. Correlation between sex ratio and 48-hour fecundity was tested with a Spearman's rank correlation.

RESULTS

Heterozygous *Trichogramma* have higher fecundity, with no impact on total sex ratio

We created heterozygous and homozygous *Wolbachia*-infected *Trichogramma* wasps and assayed them for fecundity and offspring sex ratio over a ten-day period. 31.5% (24/76) of eggs were fertilized by the mated Insectary line mothers, resulting in heterozygotes. This is in contrast to sexual lines of *Trichogramma* that fertilize between 60-90% of their eggs (Suzuki et al. 1984; Russell and Stouthamer 2011). Zygosity had no effect on the likelihood of wasps to survive the duration of the trial ($\chi^2 = 1.048$, $p = 0.3059$). We found a significant effect of the interaction between zygosity and time on sex ratios (Figure 4.2A; $\chi^2 = 165.5$, $p < 0.0001$). Wasp zygosity alone had a significant effect on sex ratio (Figure 4.2A; $\chi^2 = 23.69$, $p < 0.0001$), as did time (Figure 4.2A; $\chi^2 = 1080$, $p < 0.0001$). Homozygous wasps showed a more drastic change in sex ratio with a particularly low level of parthenogenesis induction for egg cards three and four (corresponding to trial days five through eight; Figure 4.2A). However, sex ratios of the entire ten-day brood were not significantly different between heterozygous and homozygous wasps (Figure 4.2B; $\chi^2 = 3.744$, $p < 0.5301$).

Similarly, we found a significant effect of the interaction between zygosity and time on fecundity across incremental 48-hour periods (Figure 4.2C; $\chi^2 = 473.8$, $p < 0.0001$). Again, wasp zygosity alone had a significant effect on fecundity (Figure 4.2C; $\chi^2 = 14.40$, $p < 0.0001$), as did time (Figure 4.2C; $\chi^2 = 2192$, $p < 0.0001$). During the first 48-

hour period the control homozygous wasps produced more offspring than the heterozygotes but for the remainder of the trial, the heterozygous wasps out-performed the controls (Figure 4.2C). In contrast to there being no significant difference between total sex ratios produced by heterozygous and homozygous wasps, there was a significant difference in total fecundity after the ten-day period (Figure 4.2D; $\chi^2 = 116.9$, $p < 0.0001$).

Recombinant *Trichogramma* produce more female-biased sex ratios with no impact on total fecundity

We took offspring from virgin heterozygous *Trichogramma*, and used them to initiate RILs (F2). Similarly, we used offspring from homozygous *Trichogramma* to initiate control (CON) lines (F2). After allowing RIL and CON colonies to propagate for three generations, we assayed fecundity and sex ratio. RIL and CON wasps were equally as likely to survive the duration of the ten-day trial ($\chi^2 = 2.431$, $p = 0.1189$). There was a significant effect of the interaction between genotype (RIL or CON) and time on the sex ratios produced across incremental 48-hour periods (Figure 4.3A; $\chi^2 = 85.00$, $p < 0.0001$). Likewise, sex ratio was significantly affected by genotype (Figure 4.3A; $\chi^2 = 10.82$, $p = 0.0010$), and time (Figure 4.3A; $\chi^2 = 1492$, $p < 0.0001$), considered individually.

Similarly, there was a significant effect of the interaction between genotype and time on fecundity (Figure 4.3B; $\chi^2 = 508.5$, $p < 0.0001$). In contrast, time alone (Figure 4.3B; χ^2

= 2836, $p < 0.0001$), but not genotype alone (Figure 4.3B; $\chi^2 = 0.5706$, $p = 0.45$) significantly affected fecundity of RIL and CON wasps. Given that fecundity of CON lines was only higher than RIL lines for the first 48 hours, we looked to see if there was a correlation between total sex ratio and early fecundity. Indeed, total sex ratio was significantly negatively correlated with fecundity in the first 48-hour period (Figure 4.3C; $r_s = -0.4916$: $p < 0.0001$).

Lastly, we looked at the total fecundity across individual RIL and CON lines, and found no significant difference in total fecundity after the ten-day period (Figure 4.4A; $\chi^2 = 0.3734$, $p = 0.5411$). There was however, a significant difference in fecundity between individual RIL and CON lines, during the first 48-hours (Figure 4.4B; $\chi^2 = 54.08$, $p < 0.0001$). Finally, total sex ratios produced after ten days are significantly higher in RIL lines, as compared to the CON lines (Figure 4.4C; $\chi^2 = 5.3575$, $p = 0.0206$).

DISCUSSION

Parthenogenesis-inducing *Wolbachia* are unique in that their parasitic, selfish host-manipulations can result in the host becoming dependent upon the infection for reproduction (Jeong and Stouthamer 2005; Russell and Stouthamer 2011; Stouthamer et al. 2010; Gottlieb and Zchori−Fein 2001; Zchori-Fein et al. 1992; Kremer et al. 2009; Dedeine et al. 2001). As host and symbiont become irreversibly linked, we might expect co-evolution of host and symbiont. Preliminary support for this hypothesis comes from finding that horizontal transfer of *Wolbachia* between *Trichogramma* hosts is rarely

successful. Either the infection wont transfer to a new host, the novel infection fails to induce the parthenogenesis phenotype, or the infection is lost after a few generations in the novel host (Huigens et al. 2004; Grenier et al. 1998; Watanabe et al. 2013). *Trichogramma pretiosum* is one such species where separate populations can either be sexual or irreversibly asexual (Pinto 1998; Russell and Stouthamer 2011), and have been shown to be resistant to horizontal transfer of *Wolbachia* (Huigens et al. 2004). Here we can combine the nuclear genomes from *Trichogramma pretiosum* lines with these two different reproductive modes, and test how *Wolbachia* performs in the new system.

In asexual, completely inbred populations, outbreeding may be advantageous as a result of hybrid vigor. However, this could come at a cost for *Wolbachia* due to the breakup of co-adapted gene complexes. In our system, we see a heterozygote advantage, with no apparent cost for *Wolbachia* (Figure 4.2), which is congruent with previous studies showing that one complete copy of the maternal genome is sufficient for proper cyto-nuclear interactions (Breeuwer and Werren 1995; Ellison and Burton 2008). Heterozygote wasps produced more offspring without a reduction in female biased sex ratio, implying *Wolbachia* has no difficulty inducing parthenogenesis in the offspring of these hybrids. Given that zygosity has no impact on the total sex ratio or likelihood to survive, but heterozygosity results in higher fecundity, *Wolbachia* had a higher fitness when infecting heterozygous wasps.

We anticipated a breakdown in the F2 recombinant lines, with respect to the induction of parthenogenesis, due to incorrect interactions of nuclear and cytoplasmic genes that would start to appear when organisms are homozygous for paternal genes (Breeuwer and Werren 1995; Ellison and Burton 2008; Burton et al. 2013). However, the recombinant lines produced similar numbers of total offspring with a more female-biased sex ratio than the control lines (Figures 4.3 and 4.4). Our data point to a high early fecundity driving this change in sex ratio. This corroborates recent findings that manipulating temporal variation in *Trichogramma* reproduction results in different sex ratios over the course of a week (Lindsey and Stouthamer, in press). By producing fewer offspring early on, it appears that wasps can maintain sufficient levels of *Wolbachia* to provision to eggs, ensuring successful gamete duplication and thus the production of females. Our data from the recombinant and control lines indicate that the high early fecundity of control lines may be rapidly depleting *Wolbachia*, resulting in the quick drop in sex ratio (Figure 4.3). It is difficult to predict how these reproductive patterns might affect fitness in the field, but the slow-to-start recombinants may maintain an advantage if it results in sustaining a more female-biased sex ratio.

In contrast to our expectations, *Wolbachia* did not appear to have any difficulties inducing parthenogenesis in the new genetic backgrounds. This is especially curious given that the California *Trichogramma pretiosum* (our paternal line) seem to be resistant to infection with *Wolbachia*. These sexually reproducing, uninfected wasps co-occur with and will super-parasitize host eggs with other *Wolbachia*-infected *Trichogramma* species

(Pinto 1998). Super-parasitization is a viable route for horizontal transfer of *Wolbachia* between *Trichogramma* species (Huigens et al. 2004; Schilthuizen and Stouthamer 1997; Huigens et al. 2000), so why the California *Trichogramma pretiosum* seem to resist infection remains unknown.

The ability to invade a new host germline after horizontal transfer, and the ability to successfully induce parthenogenesis in a new genetic background are likely quite mechanistically different (Sheehan et al. 2016; Beckmann et al. 2017; LePage et al. 2017), and our data here indicate that the *Trichogramma* resistant to infection through horizontal transmission may not be resistant to parthenogenesis induction. We can conclude that in this system there is no evidence for co-evolution between *Wolbachia* and a particular host genetic background that once broken up results in a negative effect on the ability of *Wolbachia* to induce parthenogenesis, or in a reduction in the fitness of the *Wolbachia*-host combination.

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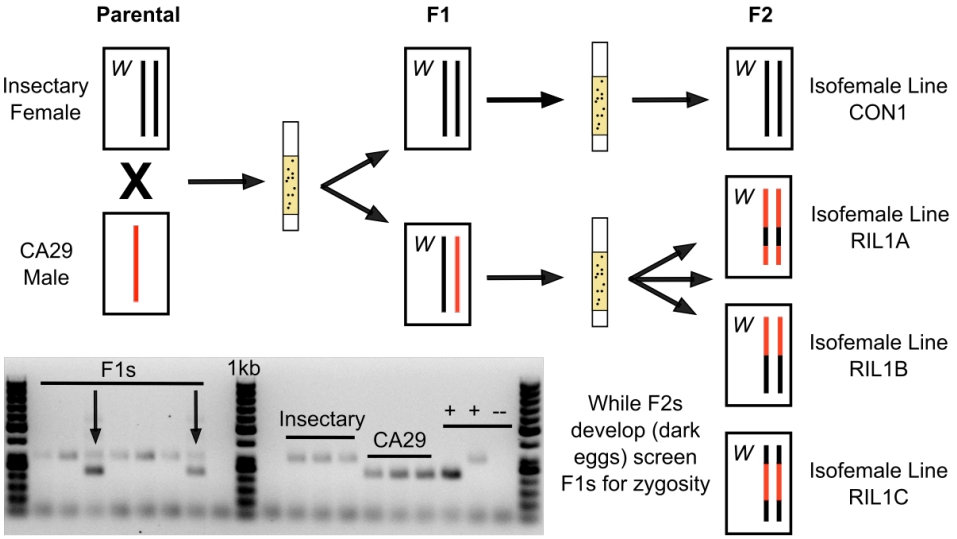

Figure 4.1 Experimental design for creating recombinant and control lines. "*W*" indicates infection with *Wolbachia*, inherited maternally. Arrows on the gel image point to heterozygote F1s, from which individual offspring were isolated to initiate Recombinant Isofemale Lines (RILs). Positive controls are previously amplified samples from the Insectary and CA-29 lines, where as the Insectary and CA-29 labels indicate exact parents used in crosses.

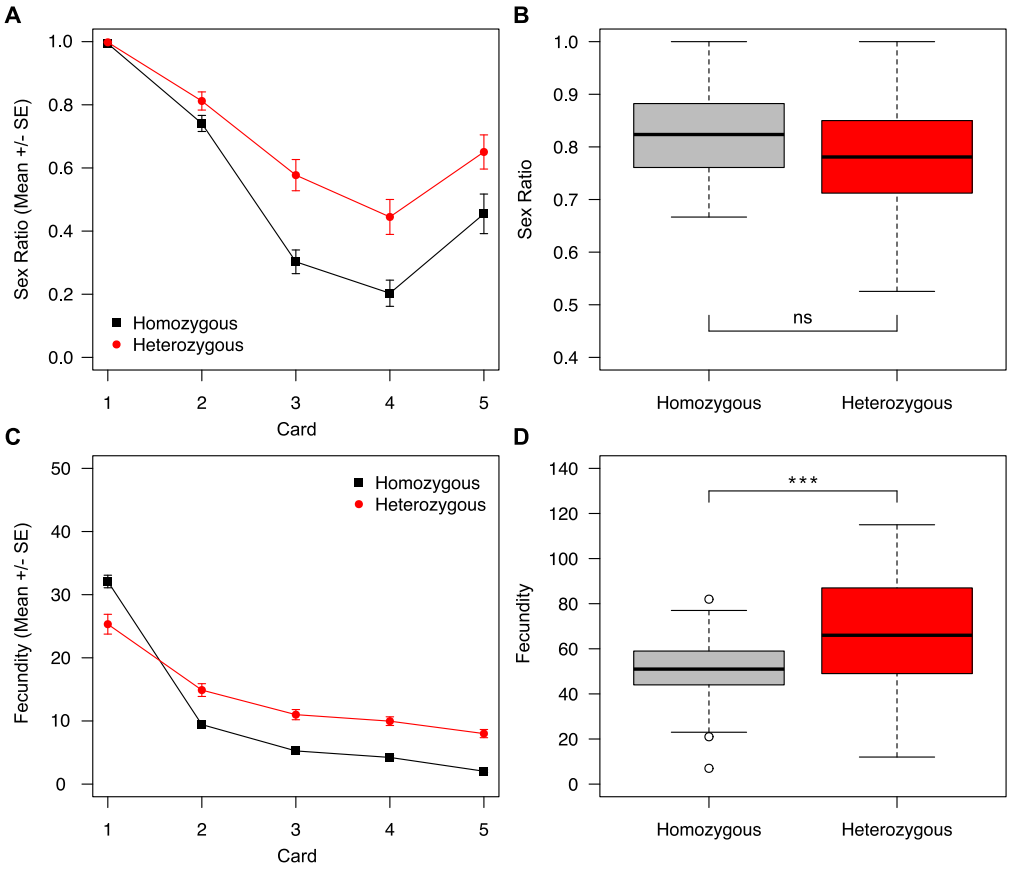

Figure 4.2 Sex ratios and fecundity of heterozygous wasps. "Card" refers to a 48-hour period. For example, card one is 0-48 hours, card two is 48-96 hours, etc. A) Variation in sex ratio across sequential 48-hour periods. B) Total sex ratio after the 10-day period. C) Variation in fecundity across sequential 48-hour periods. D) Total fecundity after the 10-day period. Three asterisks represents $p < 0.001$.

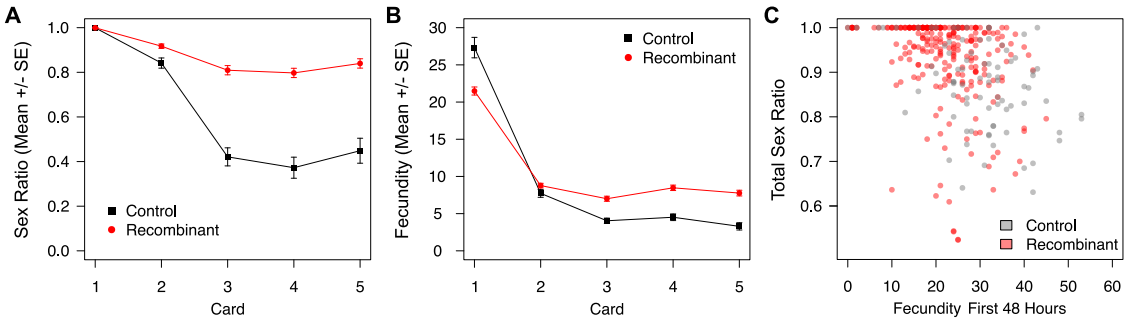

Figure 4.3 Relationship between fecundity and sex ratio of control and recombinant genotypes. "Card" refers to subsequent 48-hour periods. A) Variation in sex ratio across sequential 48-hour periods. B) Variation in fecundity across sequential 48-hour periods. C) Correlation between fecundity in the first 48-hours and total sex ratio. Points are semi-transparent, with darker color indicating more overlapping data points.

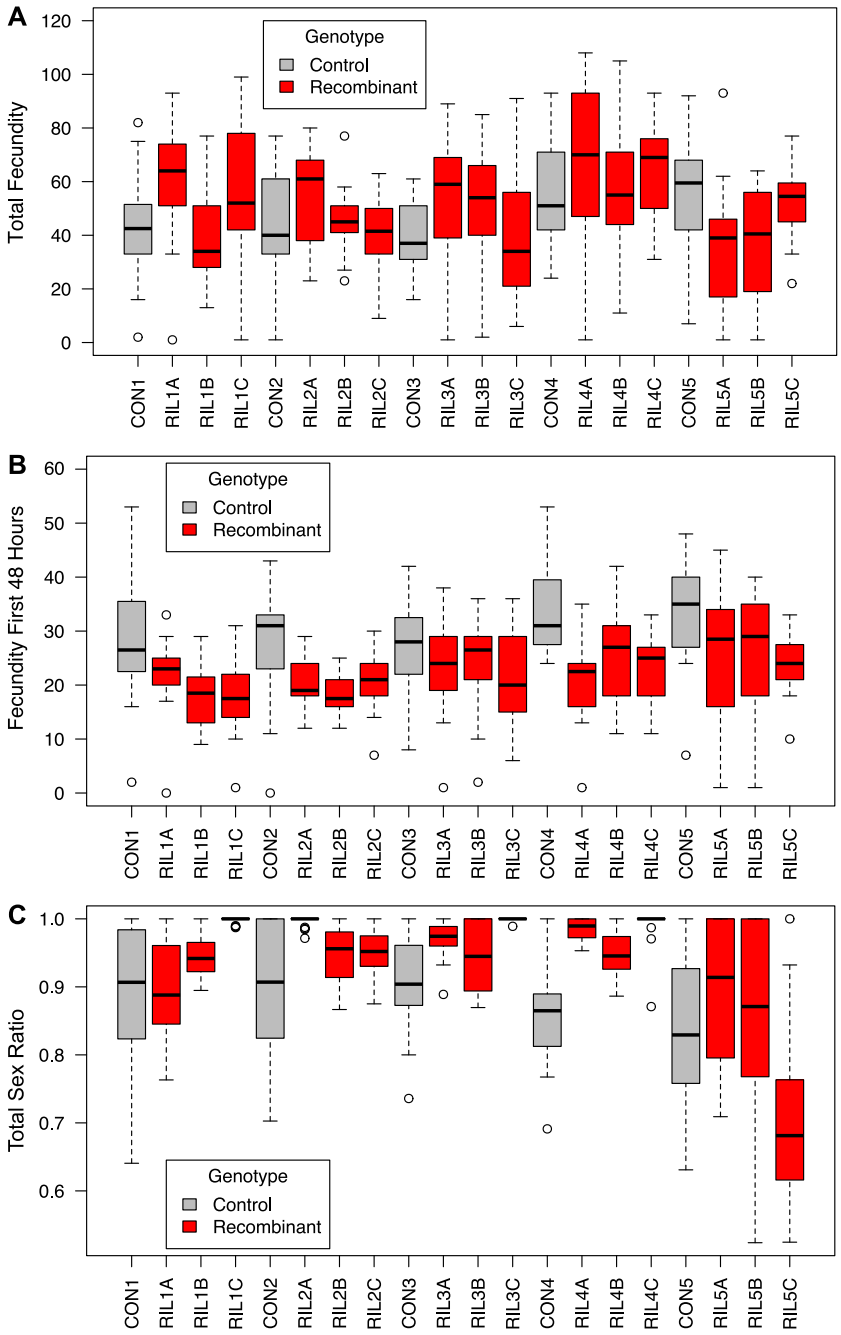

Figure 4.4 Sex ratios and fecundity for recombinant (RIL) and control (CON) isofemale lines. Open circles represent outliers. A) Total fecundity for individual CON and RIL lines. B) Fecundity in the first 48-hours for individual CON and RIL lines. C) Total sex ratios for individual CON and RIL lines.

CONCLUSIONS

Trichogramma wasps have a unique relationship with their *Wolbachia* symbionts, due to the varied levels of infection and resulting reproductive phenotypes found across populations. We aim to identify the factors that might contribute to the diversity of infection types in this system, and develop genomic resources to better understand the mechanisms and evolutionary consequences of this interaction.

The *Wolbachia* strain *w*Tpre, infecting *Trichogramma pretiosum*, is the most complete assembly of a parthenogenesis-inducing *Wolbachia* strain to date. We find large amounts of gene degradation through fragmentation and truncation of open reading frames (Chapter 1). Further work will be necessary to determine whether or not these resulting open reading frames are expressed or even functional. We hypothesize that the extensive protein coding frame truncations present in *w*Tpre reflect the change in reproductive phenotype from CI to PI. This agrees with another recently published study showing that the *w*Uni PI-*Wolbachia* strain (an independent transition to PI) also has signatures of gene degradation (Newton et al. 2016).

In *Trichogramma,* the fixation of asexual reproduction occurs through mutations in the host genome; so called virginity mutations (Russell and Stouthamer 2011; Stouthamer et al. 2010; Jeong and Stouthamer 2005). While the *w*Tpre reference genome is from a strain that does infect a host with a virginity mutation, not all *Trichogramma* have this mutually dependent relationship with their resident *Wolbachia* strain. Sequencing of

additional *Trichogramma*-infecting *Wolbachia* strains will be necessary to determine whether or not these coding frame truncations are unique to *Wolbachia* strains that infect irreversibly asexual hosts, or are a feature of parthenogenesis-inducing *Wolbachia* more generally.

The *Trichogramma* genome provides a platform with which to study the evolution of parasitism and the genomic consequences of irreversible asexuality. We identified unique features of the *Trichogramma* genome using comparative genomics across Hymenoptera, and find signatures of genome reduction and gene loss, a large number of *Trichogramma* unique genes, and that the *Trichogramma* branch is evolving much more rapidly than any of the other hymenopterans we studied (Chapter 2). Having a high quality reference genome will now make it possible to compare sexual and asexual lines of *Trichogramma*, with the goal of determining how *Wolbachia*-mediated reproductive switches affect genome evolution. Additionally, the *Trichogramma* genome will be a valuable resource for determining the mechanism of PI-induction by *Wolbachia*, and the mechanism of sex determination in *Trichogramma.*

The likelihood of *Wolbachia* fixing in a population and creating the opportunity for virginity mutations to evolve is not well understood. We first look at environmental factors that may mediate the expression of the parthenogenesis phenotype. We find that reproductive rate, as mediated by access to host eggs, has a significant effect on the penetrance of parthenogenesis-induction by *Wolbachia* (Chapter 3). We show that

reproductive rate in the first day has a lasting effect on sex ratios of the entire brood. These findings contribute to our understanding of how *Wolbachia* behaves in natural settings, and the context dependent nature of the relationship, even for a line that is fixed for *Wolbachia* infection.

In Chapter 4, we looked at genetic factors that could contribute to the expression of the *Wolbachia* phenotype. New combinations of host alleles did not affect *Wolbachia*'s ability to induce parthenogenesis, but they did result in an altered reproductive rate that in turn improved female-biased sex ratios. This is surprising given the previous studies that hypothesized host-specificity of the *Wolbachia* strains infecting *Trichogramma* wasps, due to the failed horizontal transfers of those strains amongst *Trichogramma* hosts (Watanabe et al. 2013; Huigens et al. 2004; Grenier et al. 2002; Grenier et al. 1998). The relationship between sex ratios and early fecundity in Chapter 4 further strengthens the finding from Chapter 3 that reproductive rate is an important variable in determining the expression of *Wolbachia* mediated parthenogenesis.

Given the interest in using *Wolbachia* to modify insect populations (Hoffmann et al. 2015; Turelli and Hoffmann 1991; Walker et al. 2011; Hoffmann et al. 2011), and the prevalence of *Wolbachia* across arthropods and nematodes (Zug and Hammerstein 2012; Werren et al. 1995; Hilgenboecker et al. 2008), it is important that we understand the factors contributing to *Wolbachia*'s ability to express the phenotype of interest, and spread through a population. Furthermore, identifying the long term consequences to host

and symbiont will help us better understand the evolution and maintenance of symbiotic interactions.

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