A peer-reviewed version of this preprint was published in PeerJ on 27 June 2017.

View the peer-reviewed version (peerj.com/articles/3505), which is the preferred citable publication unless you specifically need to cite this preprint.

Lindsey ARI, Stouthamer R. 2017. Penetrance of symbiont-mediated parthenogenesis is driven by reproductive rate in a parasitoid wasp. PeerJ 5:e3505 https://doi.org/10.7717/peerj.3505
Penetrance of symbiont-mediated parthenogenesis is driven by reproductive rate in a parasitoid wasp

Amelia R.I. Lindsey, Richard Stouthamer

1 Department of Entomology, University of California, Riverside, Riverside, California, United States

Corresponding Author: Richard Stouthamer
Email address: richard.stouthamer@ucr.edu

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. The conditions that reduce penetrance of the parthenogenesis phenotype are not well understood. We hypothesize that more ecologically relevant conditions of limited host access will sustain female-biased sex ratios. By restricting access to host eggs, we see a strong relationship between reproductive rate and sex ratio. We show that reproductive output in the first 24 hours is critical to the total sex ratio of the entire brood, and limiting oviposition in that period results in near-complete parthenogenesis that can be sustained for long periods, without any significant impact on total fecundity. Our data suggest that this phenomenon may be due to the depletion of Wolbachia when oviposition occurs relatively constantly, and that 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.
Penetrance of symbiont-mediated parthenogenesis is driven by reproductive rate in a parasitoid wasp

Amelia R. I. Lindsey1 and Richard Stouthamer1*

1Department of Entomology, University of California Riverside, Riverside, California, 92521, USA

*Corresponding Author:

Richard Stouthamer

Email: richard.stouthamer@ucr.edu
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. The conditions that reduce penetrance of the parthenogenesis phenotype are not well understood. We hypothesize that more ecologically relevant conditions of limited host access will sustain female-biased sex ratios. By restricting access to host eggs, we see a strong relationship between reproductive rate and sex ratio. We show that reproductive output in the first 24 hours is critical to the total sex ratio of the entire brood, and limiting oviposition in that period results in near-complete parthenogenesis that can be sustained for long periods, without any significant impact on total fecundity. Our data suggest that this phenomenon may be due to the depletion of *Wolbachia* when oviposition occurs relatively constantly, and that *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.

Key Words

*Wolbachia, Trichogramma*, sex ratio, asexual, reproductive modification, symbiosis
1. 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 (CI), male-killing, feminization, and parthenogenesis-induction (PI) (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 & Hoffmann 1991). CI-*Wolbachia* modifies sperm such that crosses between an infected male and an uninfected female do not produce viable offspring. In these cases, infected females have an advantage as their infections “rescue” the fatal CI-modification in the sperm (Beckmann et al. 2017; Breeuwer & Werren 1990; LePage et al. 2017; Werren 1997). PI-*Wolbachia* infect haplodiploid species and result in the production of females without the need for a mate. This is accomplished through converting unfertilized eggs (which would normally develop as males) to diploid eggs, which then develop as females (Gottlieb et al. 2002; Pannebakker et al. 2004; Stouthamer & Kazmer 1994).

There is a large body of research indicating that the phenotypes *Wolbachia* induces are very much context dependent, with a range of genetic and environmental factors influencing the penetrance of the manipulation. These are important considerations for several reasons. Firstly, the persistence of a symbiont in a host population, and expression of resulting phenotypes will affect the potential for host-symbiont co-evolution. Secondly, with symbionts under exploration for the control of target pest species (Bourtzis et al. 2014; Hoffmann et al. 2011; Hoffmann et al. 2015; Walker 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. We know that 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; Hoffmann et al. 1990; Turelli & Hoffmann 1995).

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 (Bordenstein et al. 2003; Grenier et al. 1998; Huigens et al. 2004; McGraw et al. 2001; Reynolds et al. 2003), reduced maternal transmission, and the eventual loss of the symbiont from a population (Huigens et al. 2004). Additionally, there are well-established relationships between several environmental factors and the penetrance of Wolbachia-mediated phenotypes. High temperatures will reduce Wolbachia titers and result in poor host manipulation (Bordenstein & Bordenstein 2011; Hurst et al. 2000; 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 CI-Wolbachia, this means heat treated male offspring of are incapable of inducing CI, or only do so weakly (Clancy & Hoffmann 1998). In the case of PI-Wolbachia, antibiotic treated mothers produce increasingly more sons as Wolbachia titers decrease (Stouthamer & Mak 2002; Zchori-Fein et al. 2000). Many of these studies point to a “threshold” level of infection that is critical for host-manipulation (Bordenstein & Bordenstein 2011; Hurst et al. 2000; Ma et al. 2015), and a positive correlation between Wolbachia titers and expression of the manipulation (Bourtzis et al. 1996; Breeuwer & Werren 1993; Ikeda et al. 2003; Pascal et al. 2004; Zchori-Fein et al. 2000).
Trichogramma are minute parasitoid wasps in the superfamily Chalcidoidea, frequently infected with PI-Wolbachia (Stouthamer et al. 1993; Stouthamer et al. 1990a; Stouthamer et al. 1990b). Like other hymenopterans, Trichogramma are haplodiploid: unfertilized eggs typically develop into males, and fertilized eggs into females (Stouthamer et al. 1990a). Trichogramma-PI-Wolbachia restore diploidy of unfertilized eggs through via a failed anaphase in which chromosomes do not separate during the egg’s first mitotic division (Stouthamer & Kazmer 1994). For Trichogramma, increased doses of heat will reduce bacterial titers and lead to the production of increasingly more males and sexually aberrant individuals (Pascal et al. 2004; Stouthamer 1997; Tulgetske & Stouthamer 2012). It is not clear however, why occasional males are produced in the absence of antibiotics or increased temperature regimes (Hohmann et al. 2001; Stouthamer & Luck 1993).

We might exploit the production of these males to determine what factors control the expression of the symbiont phenotype. A few preliminary studies that show limited access to host eggs will improve female-biased sex ratios (Hohmann et al. 2001; Legner 1985; Stouthamer & Luck 1993). 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 had an effect on the resulting sex ratio in Muscidifurax uniraptor (Legner 1985). We know now that Muscidifurax uniraptor is infected with parthenogenesis-inducing Wolbachia, and that Wolbachia titers positively correlate 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. We find that
early fecundity has the largest effect on expression of the parthenogenesis phenotype. qPCR data suggest this might be due to high levels of offspring production depleting *Wolbachia* titers and resulting in incomplete parthenogenesis-induction for offspring produced later on. We discuss these findings in the context of ecological and evolutionary consequences for the symbiotic relationship.

2. Materials and Methods

(a) *Trichogramma* Colonies

Isofemale lines of *Trichogramma pretiosum* 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 egg cards made of irradiated *Ephestia kuehniella* host eggs (Beneficial Insectary, Guelph, Canada) adhered 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).

(b) 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. 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). Only wasps that emerged on day one were included, ensuring that experiments were carried out on age-matched wasps. Only wasps that singly hatched from an *Ephestia* egg were used in trials, ensuring size-matched, virgin wasps. Twenty 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 1). 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 male, female, or intersex based on antennal morphology. *Wolbachia* quantification (see below) was performed on mothers and select progeny.

(c) Limiting Host Access in the First 24 Hours

Given the results of the initial host access treatments, 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 Wasps were subjected to each 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. 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.

(e) 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)). Specific primers (Table 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 MgCl₂, 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 & 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).

(f) Statistics

Statistical analyses and data visualization were performed in R version 3.1.2. While proportions
of female, male, and intersex offspring were used for significance testing, only the proportions of
female offspring were plotted, as this represents successful Wolbachia-mediated
parthenogenesis. We used permutational multivariate analysis of variance with adonis from the R
vegan package (Oksanen et al. 2015) to assess variation in sex ratios between treatments using
Euclidean distance, 1,000 permutations, treatment by day of the trial as a fixed effect, and
individual wasp as a random effect to account for repeated measures. We assessed differences in
total sex ratios in a separate analysis with adonis, using Euclidean distance, 1,000 permutations,
and treatment as a fixed effect. Pairwise comparisons were performed with Bonferroni
corrections for multiple testing. To assess variation in fecundity among treatments, we used a
generalized linear model (GLM) with treatment by day of the trial as a fixed effect, individual
wasp 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 with adonis, using Euclidean distance, 1,000
permutations, cumulative fecundity and treatment as fixed effects, and individual wasp as a
random effect. Differences in Wolbachia titers between host access treatments were assessed
with a one-way ANOVA. Differences in Wolbachia titer between offspring were determined
with a one-way ANOVA, adding mother as a random effect. Tukey Honest Significant
Difference was used for post hoc testing after ANOVAs.
3. Results

(a) Host Access Experiments

Overall brood sex ratio was significantly different between treatments (Figure 2A; adonis: $F_{2,55} = 17.388, p < 0.001$). Wasps in treatment three, where access to host eggs was for only one hour a day, produced the most female biased sex ratios. In contrast to sex ratio, there was no significant difference in total fecundity over the seven-day period between treatments (Figure 2B; GLM: $df = 2,55, p = 0.140$). Daily sex ratio differed by treatment (Figure 2C; adonis: $F_{2,326} = 67.214, p < 0.001$) and over time (Figure 2C; adonis: $F_{1,326} = 125.061, p < 0.001$). Levels of daily fecundity differed by treatment (Figure 2D; GLM: $df = 2,331, p < 0.001$), and over time (Figure 2D; GLM: $df = 1,331, p < 0.001$). For both sex ratios, and fecundity, there was a significant effect of the interaction between treatment and day of trial (Figure 2C; adonis: $F_{2,326} = 40.762, p < 0.001$, and Figure 2D; GLM: $df = 2,331, p < 0.001$, respectively). 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 see a significant effect of treatment on cumulative sex ratio (Figure 3; adonis: $F_{2,328} = 24.699, p < 0.001$).

(b) Limiting Host Access in the First 24 Hours

Given the finding that the most significant difference in fecundity between treatments one and three was during the first 24 hours, we set up a second set of experiments in which wasps’ access to egg cards was only restricted on day one. By comparing this experimental treatment to wasps that had constant access to egg cards for one week, we see that only one day of restricted host access results in significant differences in total sex ratios (Figure 4A; $F_{1,22} = 4.140$, adonis: $p =$
0.029) without a significant effect on total fecundity (Figure 4B; GLM: df = 1,22, p = 0.176). For
sex ratios, there were significant effects of treatment (Figure 4C; adonis: F_{1,154} = 7.706, p =
0.007) and day (Figure 4C; adonis: F_{1,54} = 74.700, p < 0.001), but no interactive effect of
treatment by day (Figure 4C; adonis: F_{1,154} = 2.169, p = 0.125). There were significant effects of
treatment (Figure 4D; GLM: df = 1,154, p < 0.001) and day (Figure 4D; GLM: df = 1,154, p <
0.001) on fecundity, as well as an interactive effect of treatment by day (Figure 4D; GLM: df =
1,154, p < 0.001). In the first day, we see the same fecundity pattern as treatments one and three
in the previous trial. The experimental treatment did see more of a drop in sex ratios starting day
three (Figure 4C), and this is likely related to the spike in offspring production on day two
(Figure 4D), at which point wasps were switched from one hour a day access to egg cards to
constant access.

(c) Maternal Wolbachia Titors
We determined Wolbachia titers in mothers from the first four treatment regimes, and detected
significant differences between treatments (Figure 5A; ANOVA: 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) (Tukey HSD: p = 0.280). Treatments one and two (constant access, and
constant access every other day, respectively) resulted in mothers with significantly lower
Wolbachia titers relative to immediately collected wasps (Tukey HSD: p = 0.033, and p = 0.003
respectively). However, there was no significant difference between treatments one and two
(Tukey HSD: p = 0.805), even though egg card access was restricted in treatment two.
We quantified *Wolbachia* titers of three female offspring and three male offspring, from each of three mothers from treatment one. *Wolbachia* titer was much higher in females than in males (Figure 5B; ANOVA: $F_{1,16} = 8.428, p = 0.010$), even when accounting for different mothers.

4. Discussion

Based on the established relationship between *Wolbachia* titers and the parthenogenesis-phenotype (Pascal et al. 2004; Stouthamer 1997; Tulgetske & Stouthamer 2012; 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 resources are 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 experimentally manipulating *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 2C). 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 2B). Critically, it is only in the first 24 hours where treatment one wasps show drastically different fecundity than the treatment three
wasps. 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 2D). 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). This corroborates the finding that there is no significant difference in total fecundity between treatments. We see that sex ratios start to 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 4).

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 germline, which would be responsible for symbiont provisioning to the egg. Despite this, Wolbachia titers were highest in immediately collected wasps, which is congruent with our expectations (Figure 5A). 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 5A). 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 5A). 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. We propose that the recovery periods built in to our host access treatments are critical to maintaining *Wolbachia* titers high enough to ensure effective parthenogenesis induction. This would be in line with previous studies that showed a positive relationship between *Wolbachia* titers and sex ratios in PI-*Wolbachia* (Pascal et al. 2004; Stouthamer & Mak 2002; Zchori-Fein et al. 2000).

Additional support for this hypothesis comes from finding of lower *Wolbachia* titers in males compared to their sisters (Figure 5B). While we appreciate that adult titers may or may not be reflective of the number of *Wolbachia* deposited into the egg, we argue this is preliminary evidence for titers being important for proper parthenogenesis-induction. Within a set of siblings, males had lower titers than their sisters, with the exception of one male. There is the 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 PI-*Wolbachia* infected wasps (Ma et al. 2015; Tulgetske 2010). 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 previously mentioned sex-ratio changes in *Muscidifurax* (Zchori-Fein et al. 2000), and is the mechanism for CI-induction, as sperm do not contain *Wolbachia* cells, but do contain *Wolbachia*-derived proteins (Beckmann
& Fallon 2013; 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 & 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 & 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 host eggs is likely the norm, lepidopteran populations do fluctuate, with abundance peaking during certain seasons or in response to particular weather patterns (Kunte 1997; Pollard 1988; Roy et al. 2001; van den Bosch 2003). Environmental conditions could have direct effects on *Wolbachia* titers (such as high temperatures decreasing bacterial titers (Pintureau et al. 2002; Stouthamer et al. 1990a)), and indirect effects through availability of host eggs. More host resources would lead to an increase in offspring production, and if high enough, a decrease in sex ratio. Males produced under these circumstances would provide a mechanism for gene flow between asexual lineages.

The higher penetrance of parthenogenesis induction under host limited conditions as found in our study can in part explain the common coexistence of infected and uninfected females in
Trichogramma field populations (Huigens et al. 2004; Stouthamer 1997; Stouthamer et al. 1990a). How these populations can coexist has been somewhat in question because laboratory experiments with infected and uninfected lines from these field populations often showed that under unlimited host availability, the daughter production of infected females was lower than that of mated uninfected females (Silva et al. 2000; Stouthamer & 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 & Hoffmann 1991), it is especially 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.

Acknowledgments

We thank Barbara Baker and Christina Luu for their assistance in collecting many tiny wasps into ethanol, Sarah Lillian for her statistical advice, and Paul Rugman-Jones and Eric Smith for helpful discussions and feedback on drafts of the manuscript.
Aron S, de Menten L, Van Bockstaele DR, Blank SM, and Roisin Y. 2005. When hymenopteran males reinvented diploidy. *Current Biology* 15:824-827. 10.1016/j.cub.2005.03.017

Beckmann JF, and Fallon AM. 2013. Detection of the *Wolbachia* protein WPIP0282 in mosquito spermathecae: implications for cytoplasmic incompatibility. *Insect Biochemistry and Molecular Biology* 43:867-878. 10.1016/j.ibmb.2013.07.002

Beckmann JF, Ronau JA, and Hochstrasser M. 2017. A *Wolbachia* deubiquitylating enzyme induces cytoplasmic incompatibility. *Nature Microbiology* 2:17007. 10.1038/nmicrobiol.2017.7

http://www.nature.com/articles/nmicrobiol20177 - supplementary-information

Bordenstein SR, and Bordenstein SR. 2011. Temperature affects the tripartite interactions between bacteriophage WO, *Wolbachia*, and cytoplasmic incompatibility. *PLoS One* 6:e29106.

Bordenstein SR, Uy JJ, and Werren JH. 2003. Host genotype determines cytoplasmic incompatibility type in the haplodiploid genus *Nasonia*. *Genetics* 164:223-233.

Bourtzis K, Dobson SL, Xi Z, Rasgon JL, Calvitti M, Moreira LA, Bossin HC, Moretti R, Baton LA, Hughes GL, Mavingui P, and Gilles JRL. 2014. Harnessing mosquito–*Wolbachia* symbiosis for vector and disease control. *Acta Tropica* 132, Supplement:S150-S163. http://dx.doi.org/10.1016/j.actatropica.2013.11.004

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

Breeuwer J, and Werren JH. 1993. Cytoplasmic incompatibility and bacterial density in *Nasonia vitripennis*. *Genetics* 135:565-574.
Breeuwer JAJ, and Werren JH. 1990. Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. *Nature* 346:558-560. doi:10.1038/346558a0

Clancy DJ, and Hoffmann AA. 1998. Environmental effects on cytoplasmic incompatibility and bacterial load in *Wolbachia*-infected *Drosophila simulans*. *Entomologia Experimentalis et Applicata* 86:13-24.

Gottlieb Y, Zchori-Fein E, Werren JH, and Karr TL. 2002. Diploidy restoration in *Wolbachia*-infected *Muscidifurax uniraptor* (Hymenoptera: Pteromalidae). *Journal of Invertebrate Pathology* 81:166-174.

Grenier S, Bernard P, Heddi A, Lassablière F, Jager C, Louis C, and Khatchadourian C. 1998. Successful horizontal transfer of *Wolbachia* symbionts between *Trichogramma* wasps. *Proceedings of the Royal Society B-Biological Sciences* 265:1441-1445. 10.1098/rspb.1998.0455

Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, Muzzi F, Greenfield M, Durkan M, Leong YS, Dong Y, Cook H, Axford J, Callahan AG, Kenny N, Omodei C, McGraw EA, Ryan PA, Ritchie SA, Turelli M, and O'Neill SL. 2011. Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. *Nature* 476:454-U107. 10.1038/nature10356

Hoffmann AA, Ross PA, and Rasic G. 2015. *Wolbachia* strains for disease control: ecological and evolutionary considerations. *Ecology and Evolution* 8:751-768. 10.1111/eva.12286

Hoffmann AA, Turelli M, and Harshman LG. 1990. Factors affecting the distribution of cytoplasmic incompatibility in *Drosophila simulans*. *Genetics* 126:933-948.
Hohmann CL, Luck RF, and Stouthamer R. 2001. Host deprivation effect on reproduction and survival of \emph{Wolbachia}-infected and uninfected \emph{Trichogramma kaykai} Pinto & Stouthamer (Hymenoptera: Trichogrammatidae). \textit{Neotropical Entomology} 30:601-605. 10.1590/S1519-566X2001000400014

Huigens ME, de Almeida RP, Boons PAH, Luck RF, and Stouthamer R. 2004. Natural interspecific and intraspecific horizontal transfer of parthenogenesis-inducing \emph{Wolbachia} in \emph{Trichogramma} wasps. \textit{Proceedings of the Royal Society B-Biological Sciences} 271:509-515. 10.1098/rspb.2003.2640

Hurst GD, Johnson AP, vd Schulenburg JHG, and Fuyama Y. 2000. Male-killing \emph{Wolbachia} in \emph{Drosophila}: a temperature-sensitive trait with a threshold bacterial density. \textit{Genetics} 156:699-709.

Ikeda T, Ishikawa H, and Sasaki T. 2003. Infection density of \emph{Wolbachia} and level of cytoplasmic incompatibility in the Mediterranean flour moth, \emph{Ephestia kuehniella}. \textit{Journal of Invertebrate Pathology} 84:1-5.

Kunte KJ. 1997. Seasonal patterns in butterfly abundance and species diversity in four tropical habitats in northern Western Ghats. \textit{Journal of biosciences} 22:593-603. 10.1007/BF02703397

Legner E. 1985. Natural and induced sex ratio changes in populations of thelytokous \emph{Muscidifurax uniraptor} (Hymenoptera: Pteromalidae). \textit{Annals of the Entomological Society of America} 78:398-402. 10.1093/aesa/78.3.398

LePage DP, Metcalf JA, Bordenstein SR, On J, Perlmutter JI, Shropshire JD, Layton EM, Funkhouser-Jones LJ, Beckmann JF, and Bordenstein SR. 2017. Prophage WO genes
recapitulate and enhance Wolbachia-induced cytoplasmic incompatibility. *Nature* 543:243-247. 10.1038/nature21391

Lindsey ARI, Werren JH, Richards S, and Stouthamer R. 2016. Comparative genomics of a parthenogenesis-inducing *Wolbachia* symbiont. *G3: Genes|Genomes|Genetics*. 10.1534/g3.116.028449

Livak KJ, and Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. *methods* 25:402-408. 10.1006/meth.2001.1262

Ma WJ, Pannebakker BA, van de Zande L, Schwander T, Wertheim B, and Beukeboom LW. 2015. Diploid males support a two-step mechanism of endosymbiont-induced thelytoky in a parasitoid wasp. *BMC Evolutionary Biology* 15:84. 10.1186/s12862-015-0370-9

McGraw E, Merritt D, Droller J, and O’Neill S. 2001. *Wolbachia*-mediated sperm modification is dependent on the host genotype in *Drosophila*. *Proceedings of the Royal Society of London B: Biological Sciences* 268:2565-2570.

Mouton L, Henri H, Charif D, Boulétreau M, and Vavre F. 2007. Interaction between host genotype and environmental conditions affects bacterial density in *Wolbachia* symbiosis. *Biology Letters* 3:210-213.

Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O’Hara R, Simpson GL, Solymos P, Stevens M, and Wagner H. 2015. vegan: Community Ecology Package. R package version 2.0-1. [http://CRAN.R-project.org/package=vegan](http://CRAN.R-project.org/package=vegan).

Pannebakker BA, Pijnacker LP, Zwaan BJ, and Beukeboom LW. 2004. Cytology of *Wolbachia*-induced parthenogenesis in *Leptopilina clavipes* (Hymenoptera: Figitidae). *Genome* 47:299-303. 10.1139/g03-137
Pascal C, Pintureau B, Charles H, Katchadourian C, Grenier S, Bolland P, and Robin C. 2004. Relationship between Wolbachia density and sex-ratio in a Trichogramma strain. Agrociencia 8:11-22.
Pintureau B, Lassabliere F, Daumal J, and Grenier S. 2002. Does a cyclic natural thermal cure occur in Wolbachia-infected Trichogramma species? Ecological Entomology 27:366-372.
Pollard E. 1988. Temperature, rainfall and butterfly numbers. Journal of Applied Ecology 819-828. 10.2307/2403748
Reynolds KT, Thomson LJ, and Hoffmann AA. 2003. The effects of host age, host nuclear background and temperature on phenotypic effects of the virulent Wolbachia strain popcorn in Drosophila melanogaster. Genetics 164:1027-1034.
Roy DB, Rothery P, Moss D, Pollard E, and Thomas J. 2001. Butterfly numbers and weather: predicting historical trends in abundance and the future effects of climate change. Journal of Animal Ecology 70:201-217. 10.1111/j.1365-2656.2001.00480.x
Silva I, Van Meer MMM, Roskam MM, Hoogenboom A, Gort G, and Stouthamer R. 2000. Biological control potential of Wolbachia-infected versus uninfected wasps: Laboratory and greenhouse evaluation of Trichogramma cordubensis and T. deion strains. Biocontrol Science and Technology 10:223-238. 10.1080/09583150050044501
Stouthamer R. 1997. Wolbachia-induced parthenogenesis.
Stouthamer R, Breeuwer JAJ, Luck RF, and Werren JH. 1993. Molecular-identification of microorganisms associated with parthenogenesis. Nature 361:66-68. 10.1038/361066a0
Stouthamer R, Hu JG, van Kan F, Platner GR, and Pinto JD. 1999. The utility of internally transcribed spacer 2 DNA sequences of the nuclear ribosomal gene for distinguishing sibling species of Trichogramma. BioControl 43:421-440. 10.1023/a:1009937108715

Stouthamer R, and Kazmer DJ. 1994. Cytogenetics of microbe-associated parthenogenesis and its consequences for gene flow in Trichogramma wasps. Heredity 73:317-327. 10.1038/hdy.1994.139

Stouthamer R, and Luck R. 1993. Influence of microbe-associated parthenogenesis on the fecundity of Trichogramma deion and T. pretiosum. Entomologia Experimentalis et Applicata 67:183-192. 10.1111/j.1570-7458.1993.tb01667.x

Stouthamer R, Luck RF, and Hamilton WD. 1990a. Antibiotics cause parthenogenetic Trichogramma (Hymenoptera, Trichogrammatidae) to revert to sex. Proceedings of the National Academy of Sciences 87:2424-2427. 10.1073/pnas.87.7.2424

Stouthamer R, and Mak F. 2002. Influence of antibiotics on the offspring production of the Wolbachia-infected parthenogenetic parasitoid Encarsia formosa. Journal of Invertebrate Pathology 80:41-45.

Stouthamer R, Pinto JD, Platner GR, and Luck RF. 1990b. Taxonomic status of thelytokous forms of Trichogramma (Hymenoptera: Trichogrammatidae). Annals of the Entomological Society of America 83:475-481. 10.1093/aesa/83.3.475

Tulgetske GM. 2010. Investigations into the mechanisms of Wolbachia induced parthenogenesis and sex determination in the parasitoid wasp, Trichogramma.

Tulgetske GM, and Stouthamer R. 2012. Characterization of intersex production in Trichogramma kaykai infected with parthenogenesis-inducing Wolbachia. Naturwissenschaften 99:143-152. 10.1007/s00114-011-0880-2
Turelli M, and Hoffmann AA. 1991. Rapid spread of an inherited incompatibility factor in California Drosophila. Nature 353:440-442.

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

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, and Rozen SG. 2012. Primer3—new capabilities and interfaces. Nucleic Acids Research 40:e115-e115.

van den Bosch R. 2003. Fluctuations of Vanessa cardui butterfly abundance with El Nino and Pacific Decadal Oscillation climatic variables. Global Change Biology 9:785-790.

Volkoff A, and Daumal J. 1994. Ovarian cycle in immature and adult stages of Trichogramma cacoeciae and T. brassicae (Hym.: Trichogrammatidae). BioControl 39:303-312.

Walker T, Johnson PH, Moreira LA, Iturbe-Ormaetxe I, Frentiu FD, McMeniman CJ, Leong YS, Dong Y, Axford J, Kriesner P, Lloyd AL, Ritchie SA, O'Neill SL, and Hoffmann AA. 2011. The wMel Wolbachia strain blocks dengue and invades caged Aedes aegypti populations. Nature 476:450-U101. 10.1038/nature10355

Walsh PS, Metzger DA, and Higuchi R. 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. BioTechniques 10:506-513.

Watanabe M, Kageyama D, and Miura K. 2013. Transfer of a parthenogenesis-inducing Wolbachia endosymbiont derived from Trichogramma dendrolimi into Trichogramma evanescens. Journal of Invertebrate Pathology 112:83-87.

http://dx.doi.org/10.1016/j.jip.2012.09.006
Werren JH. 1997. Biology of Wolbachia. *Annual Review of Entomology* 42:587-609. 10.1146/annurev.ento.42.1.587

Werren JH, Baldo L, and Clark ME. 2008. Wolbachia: master manipulators of invertebrate biology. *Nature Reviews Microbiology* 6:741-751. 10.1038/nrmicro1969

Werren JH, and Windsor DM. 2000. Wolbachia infection frequencies in insects: Evidence of a global equilibrium? *Proceedings of the Royal Society B-Biological Sciences* 267:1277-1285. 10.1098/rspb.2000.1139

Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, and Madden TL. 2012. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* 13:1. 10.1186/1471-2105-13-134

Zchori-Fein E, Gottlieb Y, and Coll M. 2000. Wolbachia density and host fitness components in *Muscidifurax uniraptor* (Hymenoptera: Pteromalidae). *Journal of Invertebrate Pathology* 75:267-272. 10.1006/jipa.2000.4927
Figure 1 (on next page)

Experimental design for host access treatments one through four.

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.
|   | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 | Day 8 |
|---|-------|-------|-------|-------|-------|-------|-------|-------|
| 1. | ![Fly](fly.png) X20 | ![Fly](fly.png) X20 | ![Fly](fly.png) X20 | ![Fly](fly.png) X20 | ![Fly](fly.png) X20 | ![Fly](fly.png) X20 | ![Fly](fly.png) X20 | ![Fly](fly.png) X20 |
| 2. | ![Fly](fly.png) X20 | ![Fly](fly.png) X20 | ![Fly](fly.png) X20 | ![Fly](fly.png) X20 | ![Fly](fly.png) X20 | ![Fly](fly.png) X20 | ![Fly](fly.png) X20 | ![Fly](fly.png) X20 |
| 3. | ![Fly](fly.png) X20 | ![Fly](fly.png) X20 | ![Fly](fly.png) X20 | ![Fly](fly.png) X20 | ![Fly](fly.png) X20 | ![Fly](fly.png) X20 | ![Fly](fly.png) X20 | ![Fly](fly.png) X20 |
| 4. | ![Fly](fly.png) X20 | ![Fly](fly.png) X20 | ![Fly](fly.png) X20 | ![Fly](fly.png) X20 | ![Fly](fly.png) X20 | ![Fly](fly.png) X20 | ![Fly](fly.png) X20 | ![Fly](fly.png) X20 |

- **DNA extraction for qPCR**
- **Fresh honey**
- **Fresh host eggs and honey**
- **One hour exposure**
Figure 2 (on next page)

Sex ratios and fecundity for host access treatments.

In panels A and B open circles represent outliers, double asterisks represent \( p \leq 0.01 \), and triple asterisks represent \( p \leq 0.001 \). In panels C and D, error bars show standard error. A) Total sex ratios for the seven-day period. B) Total fecundity for the seven-day period. C) Temporal variation in sex ratio. D) Temporal variation in fecundity.
Figure 3 (on next page)

Cumulative fecundity and sex ratios for host access treatments.

Vertical error bars show standard error for cumulative sex ratio for that time point. Horizontal error bars show standard error for cumulative fecundity at that time point.
Figure 4 (on next page)

Sex ratios and fecundity for additional host access experiments.

One cohort of wasps were given fresh egg cards every 24 hours (constant access), and a second cohort of wasps were given an egg card for only one hour on day one, and then fresh hosts every 24 hours starting day two (experimental). In panels A and B open circles represent outliers and a single asterisk represents $p \leq 0.05$. In panels C and D, error bars show standard error. A) Total sex ratios for the seven-day period. B) Total fecundity for the seven-day period. C) Temporal variation in sex ratio. D) Temporal variation in fecundity.
Relative *Wolbachia* titers.

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.
Table 1 (on next page)

Sequences of primers used in this study.
Table 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           |               |