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

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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

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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 abberant 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

104 early fecundity has the largest effect on expression of the parthenogenesis phenotype. qPCR data
105 suggest this might be due to high levels of offspring production depleting *Wolbachia* titers and
106 resulting in incomplete parthenogenesis-induction for offspring produced later on. We discuss
107 these findings in the context of ecological and evolutionary consequences for the symbiotic
108 relationship.
109
110 **2. Materials and Methods**
111 (a) *Trichogramma* Colonies
112 Isofemale lines of *Trichogramma pretiosum* are maintained in 12 x 75 mm glass culture tubes
113 stopped with cotton and incubated at 24^{o}C, L:D = 16:8. Every 11 days colonies are given honey
114 and egg cards made of irradiated *Ephestia kuehniella* host eggs (Beneficial Insectary, Guelph,
115 Canada) adhered to card stock with double-sided tape. Species identification was confirmed by
116 molecular protocols from Stouthamer et al. (1999). We used the "Insectary" line, collected from
117 the Puira Valley of Peru, which has been maintained in a commercial insectary since 1966
118 (Beneficial Insectary, Guelph, Ontario, Canada). The Insectary line exhibits thelytokous
119 reproduction: females hatch from unfertilized eggs, indicating infection with *Wolbachia.*
120 Infection status was confirmed by PCR following Werren and Windsor (2000).
121
122 (b) Host Access Experiments
123 Individual Insectary line wasps from a single generation were isolated during the pupal stage to
124 ensure virginity. Darkened *Ephestia* eggs (indicating a developing *Trichogramma* pupa) were
125 removed from cards using a paintbrush and water, and isolated in 12 x 75 mm glass culture tubes
126 stopped with cotton. Upon emergence, wasps were subjected to one of four treatments to

127 determine how access to host eggs, and resultant offspring production, affects *Wolbachia* titers
128 and sex ratio (here defined as percentage females among all offspring). Only wasps that emerged
129 on day one were included, ensuring that experiments were carried out on age-matched wasps.
130 Only wasps that singly hatched from an *Ephestia* egg were used in trials, ensuring size-matched,
131 virgin wasps. Twenty wasps were used for each of the following treatments: 1) a surplus of fresh
132 host eggs every 24 hours for seven days, 2) a surplus of fresh host eggs for 24 hours every other
133 day, for seven days, 3) a surplus of fresh host eggs for only one hour a day, for seven days, or 4)
134 immediate collection into 100% ethanol upon adult emergence (Figure 1). For treatment three,
135 exposure to the fresh egg card was performed at the same time each day, from 10:45AM –
136 11:45AM. Egg cards were isolated in individual tubes after the exposure period, ensuring no
137 further parasitization. All mothers, regardless of treatment, were provided with a streak of fresh
138 honey every 24 hours. On day eight, all mothers from the first three treatments were collected
139 into 100% ethanol. All offspring from each isolated egg card were allowed to develop, and
140 collected into 100% ethanol within 24 hours of adult emergence. Offspring were counted and
141 identified as male, female, or intersex based on antennal morphology. *Wolbachia* quantification
142 (see below) was performed on mothers and select progeny.
143
144 (c) Limiting Host Access in the First 24 Hours
145 Given the results of the initial host access treatments, we set up a second trial to determine the
146 impact of oviposition in the first 24-hour period. Wasps were isolated from a single generation of
147 the Insectary line, and were age and size matched, as before. 12 Wasps were subjected to each of
148 the following treatments: 1) constant access to fresh host eggs every 24 hours (same as treatment
149 1 in the first experiments), or, 2) one-hour access to an egg card on day one (10:45 – 11:45AM),

150 followed by constant access to fresh egg cards every 24 hours starting day two. Trials were
151 carried out for seven days. Again, mothers received fresh honey every 24 hours, and egg cards
152 were isolated after the exposure period. Offspring were allowed to emerge, then counted and
153 identified as female, male, or intersex.
154
155 (e) Quantification of *Wolbachia* Titers
156 Total DNA was extracted from wasps using a Chelex method (Walsh et al. 1991) as
157 implemented by Stouthamer et al (Stouthamer et al. 1999). Gene sequences from the single-copy
158 *Trichogramma pretiosum* gene *wingless*, and the *Wolbachia* 16S gene were identified from the
159 genome assemblies (GenBank Accession Numbers: JARR00000000 and LKEQ01000000,
160 (Lindsey et al. 2016)). Specific primers (Table 1) were designed to amplify variable regions of
161 these two genes, using primer3 (Untergasser et al. 2012). Primer specificity was checked
162 computationally with Primer-BLAST (Ye et al. 2012), and against extractions of the moth host
163 eggs*, E. kuehniella,* which has an orthologous copy of *wingless*, and is infected with its own
164 strain of *Wolbachia*. qPCR was performed in 20µl reactions containing 1x ThermoPol™ buffer
165 (New England Biolabs), 0.4 µM each primer, 200nM each of dATP, dCTP, and dGTP, 400nM
166 dUTP, 1 mM $MgCl_2$, 0.5x EvaGreen® (Biotium), 1 U *Taq* polymerase (New England Biolabs),
167 and 2µl of sample. Reactions were denatured at 95 °C for 3 minutes, followed by 35 cycles of 95
168 °C for 20 seconds, 58 °C for 20 seconds, and 72 °C for 20 seconds. All samples were run in
169 triplicate alongside calibration standards and negative controls on a Rotor-Gene® Q (QIAGEN).
170 Relative *Wolbachia* titers were determined with the ΔΔCt method (Livak & Schmittgen 2001)
171 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 asses 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* Titers

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.

241 (d) *Wolbachia* Titers in Offspring

242 We quantified *Wolbachia* titers of three female offspring and three male offspring, from each of
243 three mothers from treatment one. *Wolbachia* titer was much higher in females than in males
244 (Figure 5B; ANOVA: $F_{1,16} = 8.428$, $p = 0.010$), even when accounting for different mothers.

245

246 **4. Discussion**

247 Based on the established relationship between *Wolbachia* titers and the parthenogenesis-
248 phenotype (Pascal et al. 2004; Stouthamer 1997; Tulgetske & Stouthamer 2012; Zchori-Fein et
249 al. 2000), and previous research on *Muscidifurax uniraptor* that showed sex ratios changed with
250 reproductive patterns (Legner 1985), we hypothesized that reproductive rate might mediate the
251 level of male production in an asexual line of *Trichogramma*. Restricted access to hosts is likely
252 the more ecologically relevant condition, so the males produced under high host availability
253 conditions in the lab would not be produced under field conditions. In natural settings, host
254 resources are often patchy and limited: fluctuations in environmental conditions and the
255 requirement to physically re-locate to find suitable host eggs pose barriers to constant
256 oviposition. Through experimentally manipulating *Trichogramma* oviposition rates by limiting
257 access to host eggs, we saw that patterns of offspring production had a significant effect on total
258 sex ratio. When wasps were not able to parasitize host eggs continuously, either by alternating
259 days with access to eggs, or limiting the time per day with egg access, sex ratios were maintained
260 at higher levels (Figure 2C). In fact, for wasps that had access to host eggs for only one hour a
261 day, the near-complete parthenogenesis-phenotype was maintained for the duration of the trial,
262 without significant impact on total fecundity (Figure 2B). Critically, it is only in the first 24
263 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

287 collection; whereas mothers from treatment three had 23 hours of recovery prior to collection,
288 resulting in *Wolbachia* titers similar to those that had yet to oviposit. We propose that the
289 recovery periods built in to our host access treatments are critical to maintaining *Wolbachia* titers
290 high enough to ensure effective parthenogenesis induction. This would be in line with previous
291 studies that showed a positive relationship between *Wolbachia* titers and sex ratios in PI-
292 *Wolbachia* (Pascal et al. 2004; Stouthamer & Mak 2002; Zchori-Fein et al. 2000).
293
294 Additional support for this hypothesis comes from finding of lower *Wolbachia* titers in males
295 compared to their sisters (Figure 5B). While we appreciate that adult titers may or may not be
296 reflective of the number of *Wolbachia* deposited into the egg, we argue this is preliminary
297 evidence for titers being important for proper parthenogenesis-induction. Within a set of siblings,
298 males had lower titers than their sisters, with the exception of one male. There is the chance that
299 some of the phenotypic males with higher *Wolbachia* titer could be of female karyotype, which
300 has been shown to occur in related *Trichogramma* species and other PI-*Wolbachia* infected
301 wasps (Ma et al. 2015; Tulgetske 2010). We would expect these individuals to have high enough
302 *Wolbachia* titers to induce gamete duplication, but not high enough to result in the hypothesized
303 epigenetic feminization that occurs afterward (Tulgetske 2010).
304
305 It is likely that *Wolbachia* titers in the egg may not be the final determinant of successful
306 parthenogenesis induction, but instead it is a *Wolbachia*-secreted factor that needs to be at
307 sufficient levels. This has been hypothesized as a mechanism for the previously mentioned sex-
308 ratio changes in *Muscidifurax* (Zchori-Fein et al. 2000), and is the mechanism for CI-induction,
309 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.

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References

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.

375 Breeuwer JAJ, and Werren JH. 1990. Microorganisms associated with chromosome destruction
376 and reproductive isolation between two insect species. *Nature* 346:558-560.
377 doi:10.1038/346558a0
378 Clancy DJ, and Hoffmann AA. 1998. Environmental effects on cytoplasmic incompatibility and
379 bacterial load in *Wolbachia*‐infected *Drosophila simulans*. *Entomologia Experimentalis*
380 *et Applicata* 86:13-24.
381 Gottlieb Y, Zchori-Fein E, Werren JH, and Karr TL. 2002. Diploidy restoration in *Wolbachia*-
382 infected *Muscidifurax uniraptor* (Hymenoptera: Pteromalidae). *Journal of Invertebrate*
383 *Pathology* 81:166-174.
384 Grenier S, Bernard P, Heddi A, Lassabliére F, Jager C, Louis C, and Khatchadourian C. 1998.
385 Successful horizontal transfer of *Wolbachia* symbionts between *Trichogramma* wasps.
386 *Proceedings of the Royal Society B-Biological Sciences* 265:1441-1445.
387 10.1098/rspb.1998.0455
388 Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, Muzzi F,
389 Greenfield M, Durkan M, Leong YS, Dong Y, Cook H, Axford J, Callahan AG, Kenny
390 N, Omodei C, McGraw EA, Ryan PA, Ritchie SA, Turelli M, and O'Neill SL. 2011.
391 Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue
392 transmission. *Nature* 476:454-U107. 10.1038/nature10356
393 Hoffmann AA, Ross PA, and Rasic G. 2015. *Wolbachia* strains for disease control: ecological
394 and evolutionary considerations. *Ecology and Evolution* 8:751-768. 10.1111/eva.12286
395 Hoffmann AA, Turelli M, and Harshman LG. 1990. Factors affecting the distribution of
396 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 *Wolbachia*-infected and uninfected *Trichogramma kaykai* Pinto & Stouthamer (Hymenoptera: Trichogrammatidae). *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 *Wolbachia* in *Trichogramma* wasps. *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 *Wolbachia* in *Drosophila*: a temperature-sensitive trait with a threshold bacterial density. *Genetics* 156:699-709.

Ikeda T, Ishikawa H, and Sasaki T. 2003. Infection density of *Wolbachia* and level of cytoplasmic incompatibility in the Mediterranean flour moth, *Ephestia kuehniella*. *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. *Journal of biosciences* 22:593-603. 10.1007/BF02703397

Legner E. 1985. Natural and induced sex ratio changes in populations of thelytokous *Muscidifurax uniraptor* (Hymenoptera: Pteromalidae). *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

419 recapitulate and enhance *Wolbachia*-induced cytoplasmic incompatibility. *Nature*
420 543:243-247. 10.1038/nature21391
421 Lindsey ARI, Werren JH, Richards S, and Stouthamer R. 2016. Comparative genomics of a
422 parthenogenesis-inducing *Wolbachia* symbiont. *G3: Genes|Genomes|Genetics*.
423 10.1534/g3.116.028449
424 Livak KJ, and Schmittgen TD. 2001. Analysis of relative gene expression data using real-time
425 quantitative PCR and the 2− ΔΔCT method. *methods* 25:402-408.
426 10.1006/meth.2001.1262
427 Ma WJ, Pannebakker BA, van de Zande L, Schwander T, Wertheim B, and Beukeboom LW.
428 2015. Diploid males support a two-step mechanism of endosymbiont-induced thelytoky
429 in a parasitoid wasp. *BMC Evolutionary Biology* 15:84. 10.1186/s12862-015-0370-9
430 McGraw E, Merritt D, Droller J, and O'Neill S. 2001. *Wolbachia*-mediated sperm modification is
431 dependent on the host genotype in *Drosophila*. *Proceedings of the Royal Society of*
432 *London B: Biological Sciences* 268:2565-2570.
433 Mouton L, Henri H, Charif D, Boulétreau M, and Vavre F. 2007. Interaction between host
434 genotype and environmental conditions affects bacterial density in *Wolbachia* symbiosis.
435 *Biology Letters* 3:210-213.
436 Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara R, Simpson GL, Solymos P,
437 Stevens M, and Wagner H. 2015. vegan: Community Ecology Package. R package
438 version 2.0-1. http://CRANR-projectorg/package=vegan.
439 Pannebakker BA, Pijnacker LP, Zwaan BJ, and Beukeboom LW. 2004. Cytology of *Wolbachia*-
440 induced parthenogenesis in *Leptopilina clavipes* (Hymenoptera: Figitidae). *Genome*
441 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. 10.1093/nar/gks596

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. 10.1046/j.1365-2486.2003.00621.x

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. 10.1007/BF02373035

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 *w*Mel *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

509 Werren JH. 1997. Biology of *Wolbachia*. *Annual Review of Entomology* 42:587-609.
510 10.1146/annurev.ento.42.1.587
511 Werren JH, Baldo L, and Clark ME. 2008. *Wolbachia*: master manipulators of invertebrate
512 biology. *Nature Reviews Microbiology* 6:741-751. 10.1038/nrmicro1969
513 Werren JH, and Windsor DM. 2000. *Wolbachia* infection frequencies in insects: Evidence of a
514 global equilibrium? *Proceedings of the Royal Society B-Biological Sciences* 267:1277-
515 1285. 10.1098/rspb.2000.1139
516 Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, and Madden TL. 2012. Primer-BLAST:
517 a tool to design target-specific primers for polymerase chain reaction. *BMC*
518 *Bioinformatics* 13:1. 10.1186/1471-2105-13-134
519 Zchori-Fein E, Gottlieb Y, and Coll M. 2000. *Wolbachia* density and host fitness components in
520 *Muscidifurax uniraptor* (Hymenoptera: Pteromalidae). *Journal of Invertebrate Pathology*
521 75:267-272. 10.1006/jipa.2000.4927
522
523

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.

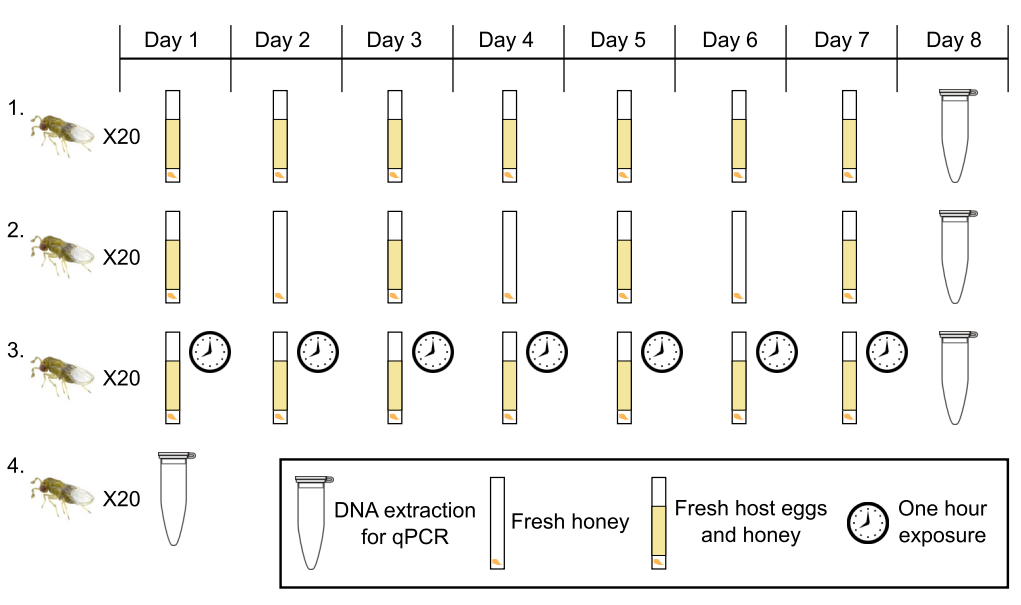
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
1.
X20
2.
X20
3.
X20
4.
X20
DNA extraction for qPCR
Fresh honey
Fresh host eggs and honey
One hour exposure

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.

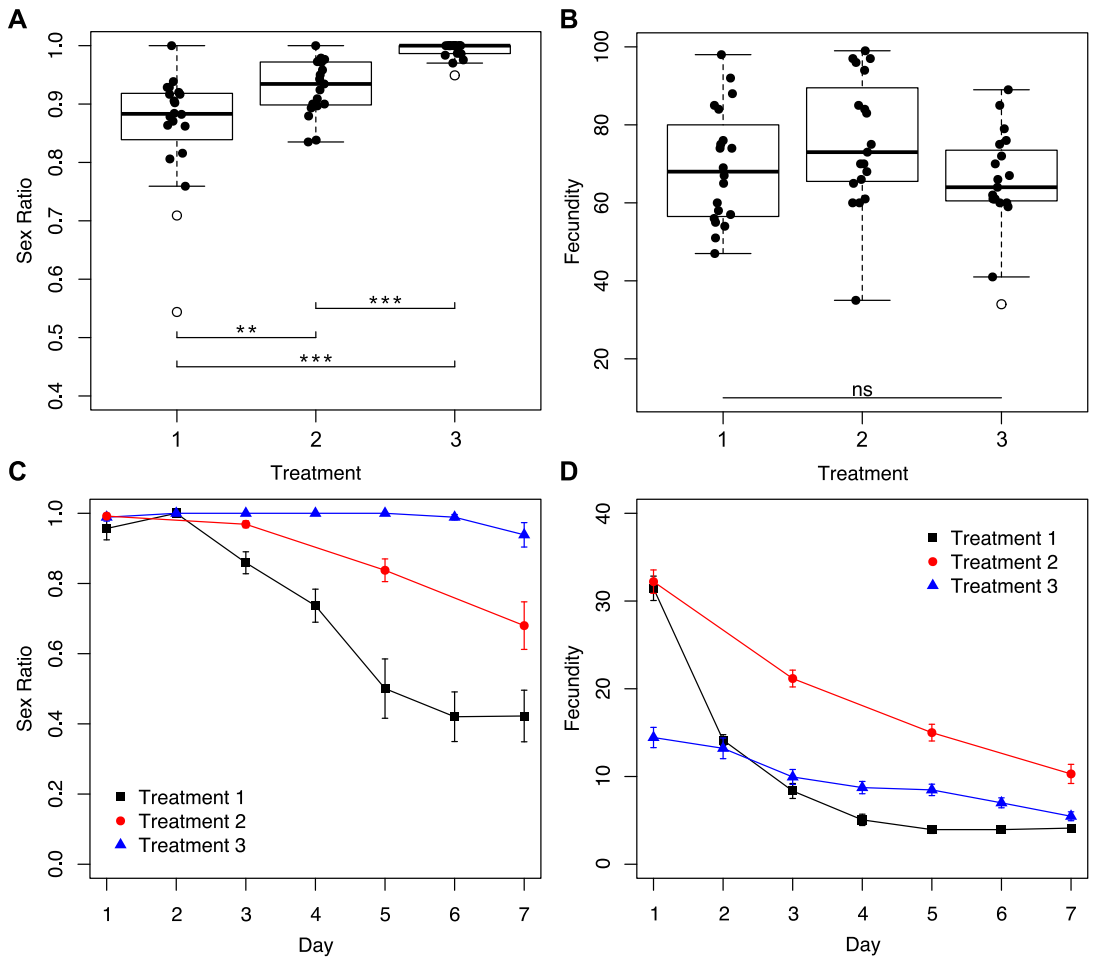
A
Sex Ratio
0.4
0.5
0.6
0.7
0.8
0.9
1.0
**
***
***
1
2
3
Treatment
B
Fecundity
20
40
60
80
100
ns
1
2
3
Treatment
C
Sex Ratio
0.0
0.2
0.4
0.6
0.8
1.0
Treatment 1
Treatment 2
Treatment 3
1
2
3
4
5
6
7
Day
D
Fecundity
0
10
20
30
40
Treatment 1
Treatment 2
Treatment 3
1
2
3
4
5
6
7
Day

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.

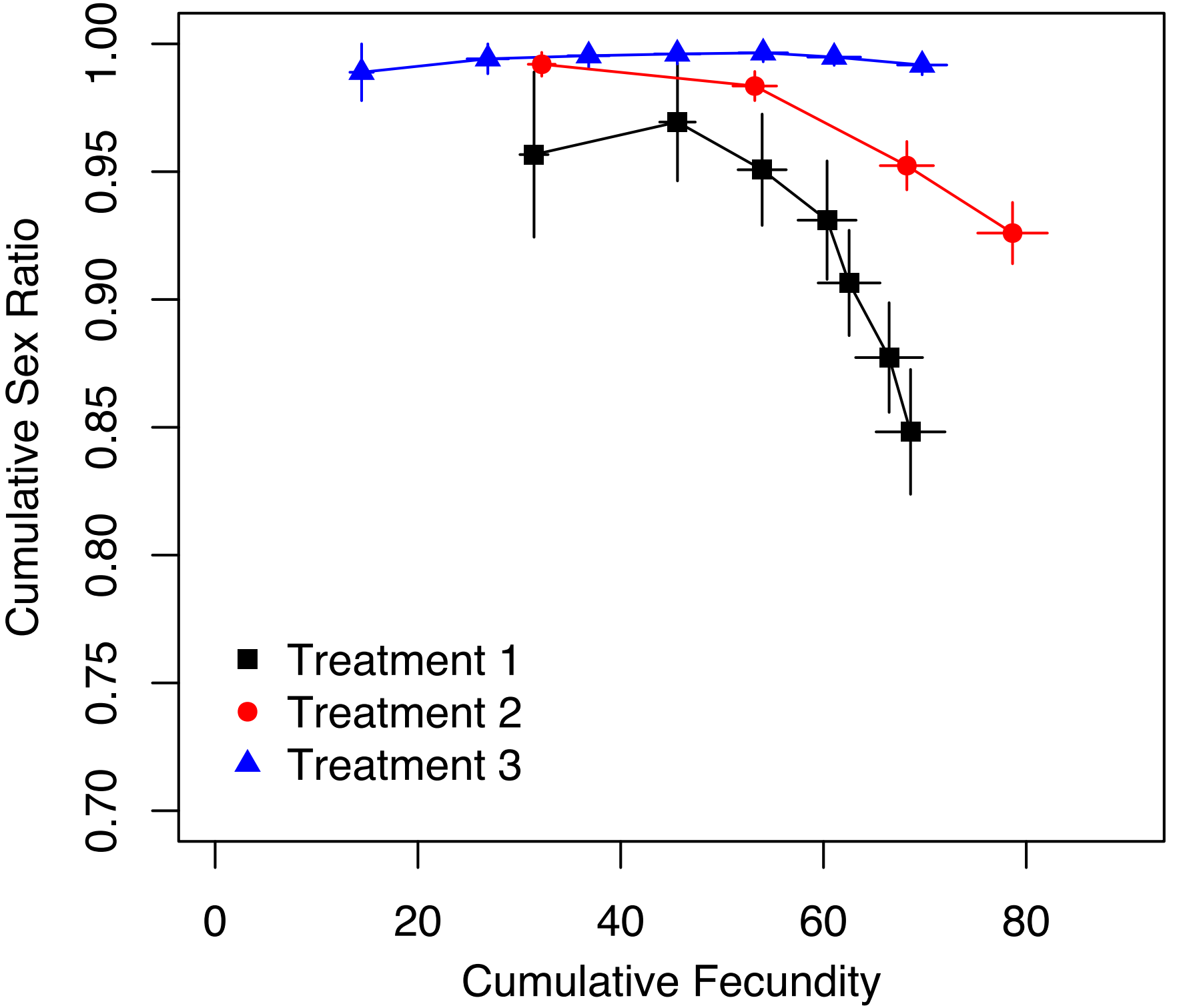
Cumulative Sex Ratio
1.00
0.95
0.90
0.85
0.80
0.75
0.70
Treatment 1
Treatment 2
Treatment 3
0
20
40
60
80
Cumulative Fecundity

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.

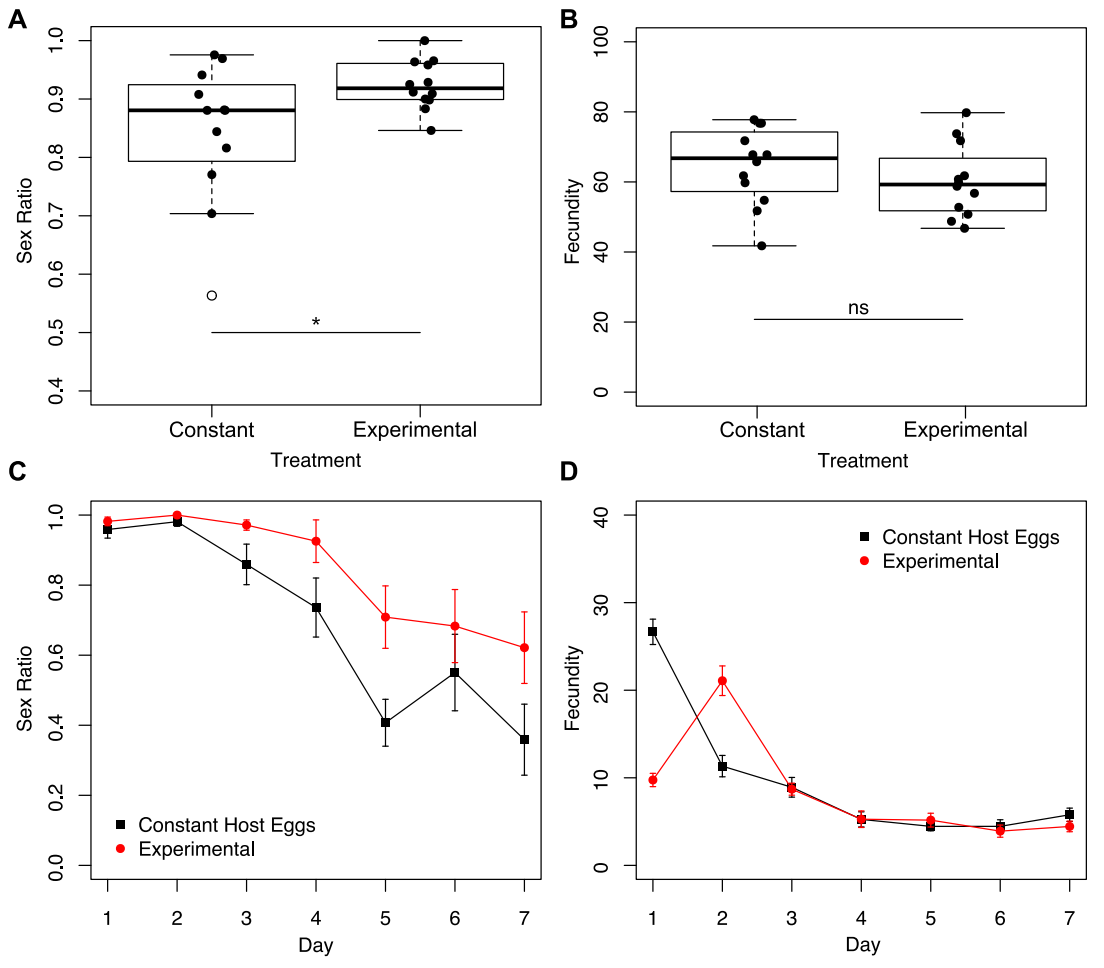
A
Sex Ratio
1.0
0.9
0.8
0.7
0.6
0.5
0.4
*
Constant
Experimental
Treatment
B
Fecundity
100
80
60
40
20
0
ns
Constant
Experimental
Treatment
C
Sex Ratio
1.0
0.8
0.6
0.4
0.2
0.0
Constant Host Eggs
Experimental
1
2
3
4
5
6
7
Day
D
Fecundity
40
30
20
10
0
Constant Host Eggs
Experimental
1
2
3
4
5
6
7
Day

Figure 5 (on next page)

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.

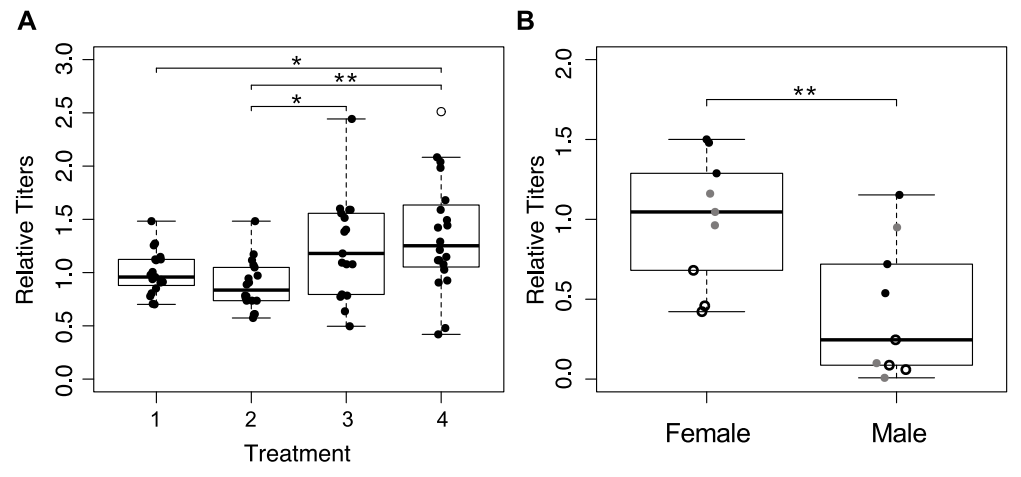
A
Relative Titers
0.0
0.5
1.0
1.5
2.0
2.5
3.0
*
**
*
1
2
3
4
Treatment
B
Relative Titers
0.0
0.5
1.0
1.5
2.0
**
Female
Male

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	