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# WITHIN- AND BETWEEN-POPULATION VARIATION FOR *WOLBACHIA*-INDUCED REPRODUCTIVE INCOMPATIBILITY IN A HAPLODIPLOID MITE

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*Abstract.*—*Wolbachia pipientis* is a bacterium that induces cytoplasmic incompatibility (CI), the phenomenon in which infected males are reproductively incompatible with uninfected females. CI spreads in a population of hosts because it reduces the fitness of uninfected females relative to infected females. CI encompasses two steps: modification (mod) of sperm of infected males and rescuing (resc) of these chromosomes by *Wolbachia* in the egg. Infections associated with CI have mod$^+$resc$^+$ phenotypes. However, mod$^-$resc$^+$ phenotypes also exist; these do not result in CI. Assuming mod/resc phenotypes are properties of the symbiont, theory predicts that mod$^-$resc$^+$ infections can only spread in a host population where a mod$^+$resc$^+$ infection already occurs. A mod$^-$resc$^+$ infection spreads if the cost it imposes on the infected females is lower than the cost inflicted by the resident (mod$^+$resc$^+$) infection. Furthermore, introduction of a mod$^-$ *Wolbachia* eventually drives infection to extinction. The uninfected population that results can be recolonized by a CI-causing *Wolbachia*. Here, we investigated whether variability for induction of CI was present in two *Tetranychus urticae* populations. In one population all isofemale lines tested were mod$^-$. In the other, mod$^+$resc$^+$ and mod$^-$resc$^+$ isofemale lines coexisted. We found no evidence for a cost difference to females expressing either type (mod$^{+/-}$). Infections in the two populations could not be distinguished based on sequences of two *Wolbachia* genes. We consider the possibility that mod$^-$ is a host effect through a population dynamics model. A mod$^-$ host allele leads to infection extinction in the absence of fecundity differences. Furthermore, the uninfected population that results is immune to reestablishment of the (same) CI-causing *Wolbachia*.

*Key words.*—Cytoplasmic incompatibility, modification and rescue, resistance, spider mite, theoretical model, *Wolbachia*.

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*Wolbachia pipientis* is a cytoplasmically transmitted bacterium that infects several arthropod and nematode hosts. In the two-spotted spider mite, *Tetranychus urticae* Koch, a phytophagous haplodiploid arthropod, *Wolbachia* can induce both cytoplasmic incompatibility (CI; Breeuwer 1997; Vala et al. 2000) and hybrid breakdown (Vala et al. 2000).

CI is expressed in crosses between uninfected (U) females and infected (W) males (reviewed by Hoffmann and Turelli 1997; Stouthamer et al. 1999). CI is not induced if the same *Wolbachia* strain that was present in the male is also present in the fertilized egg (i.e., ♀W × ♂W). CI reduces the fitness of uninfected females (which are incompatible with W males) relative to infected females (which are compatible with both W and U males). As a result, the frequency of infected females (and the CI trait) increases in the host population.

Cytological studies of CI in *Nasonia* wasps (Reed and Werren 1995) and *Drosophila simulans* (Callaini et al. 1997) showed that in ♀U × ♂W crosses the paternal set of chromosomes does not segregate properly in mitotic divisions early in embryonic development. This results in haploid or aneuploid embryos. In diploid species haploid and aneuploid embryos abort, thus CI is expressed as increased $F_1$ mortality. In haplodiploids, females are diploid and males are haploid, so haplodized eggs develop as males. Depending on the degree of aneuploidy, eggs may: develop as a male, if eggs revert to the haploid state; die, if haplodization is not complete but insufficient to develop as a female; or develop as an aneuploid female (see Breeuwer 1997; Vala et al. 2000). Mortality of aneuploid embryos would explain why, in haplodiploids, CI is expressed as a bias of $F_1$ sex ratio toward males associated with an increase in mortality (cf. Breeuwer 1997; Vala et al. 2000; Vavre et al. 2000).

### *The Modification and Rescuing Model*

Although the molecular details of CI are not known, the phenomenon is thought to involve a *Wolbachia*-produced toxin and antitoxin (Hurst 1991; Rousset and Raymond 1991) or, similarly, *Wolbachia*-mediated modification and rescuing steps (Hoffmann and Turelli 1997; Werren 1997). Chromosomes from infected males are modified by *Wolbachia* and become unable to respond properly to cell-cycle cues in uninfected eggs. If the fertilized egg is infected with the same bacterial strain that was present in the father, paternal chromosomes are rescued, that is, they segregate properly during mitosis.

An infection in which *Wolbachia* induces CI is denoted mod$^+$resc$^+$ (Werren 1997). However, if CI is not induced, the infection phenotype may be either mod$^-$resc$^-$, an infection that can neither modify sperm nor rescue modified chromosomes, or mod$^-$resc$^+$, an infection that cannot modify sperm but can rescue modified chromosomes. The test to

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distinguish between these alternatives is to mate females harboring a mod$^-$resc$^?$ infection to males with a mod$^+$resc$^+$ *Wolbachia* (Bourtzis et al. 1998; Merçot and Poinsot 1998a). If CI is observed, then the infection (in the female) is mod$^-$resc$^-$. Of course, this test is valid only if females and males are reproductively compatible in the absence of *Wolbachia*. Note that this test does not indicate whether mod$^-$ is a property of the symbiont or of the host.

Mod$^-$ is typically assumed to be a property of the symbiont. Theory predicts that in a population where a mod$^+$resc$^+$ *Wolbachia* is fixed, a mod$^-$resc$^+$ infection increases when rare, if it inflicts a cost to the infected female that is lower than the cost imposed by the resident type of infection (Prout 1994; Turelli 1994; Hurst and McVean 1996). However, if there are uninfected individuals in the population (e.g., because not all progeny of an infected mother is infected), mod$^-$resc$^+$ infections cannot spread or persist without the mod$^+$resc$^+$ type (Hurst and McVean 1996). Therefore, mod$^-$resc$^+$ phenotype is expected to coexist with a mod$^+$resc$^+$ phenotype.

The mod$^-$resc$^+$ *Wolbachia* type (initially) increases in frequency within the infected subpopulation because it reduces host fecundity less than the resident mod$^+$resc$^+$ *Wolbachia*. However, mod$^-$resc$^+$ *Wolbachia* rely on the sterilizing effect of the mod$^+$resc$^+$ infection to reduce the fitness of uninfected individuals. As mod$^-$resc$^+$ *Wolbachia* increase within the infected subpopulation, the mod$^+$resc$^+$ phenotype decreases in frequency and, consequently, the frequency of uninfected hosts increases. Thus, spread of a mod$^-$ *Wolbachia* eventually leads to infection extinction (Hurst and McVean 1996). Both mod$^-$ and mod$^+$ infection types disappear, and the population returns to the uninfected state. Because potentially any uninfected population may be (re)colonized by a CI-causing *Wolbachia* and again revert to the uninfected state, in a sense, reversible or cyclic evolution is possible (Hurst and McVean 1996).

Mod$^-$resc$^+$ infection phenotypes have been described in *Drosophila* (Bourtzis et al. 1998; Merçot and Poinsot 1998a). However, it was not investigated whether these mod$^-$ infections coexist with mod$^+$resc$^+$ infections as predicted by theory. In the present study we investigate whether variability for mod and resc phenotypes is present between and/or within two spider mite populations.

## Materials and Methods

### *Base Populations*

Two populations of *T. urticae* spider mites were established in the laboratory, one from mites collected from rose plants (the R strain, hereafter) in a greenhouse at Aalsmeer, The Netherlands and another from mites collected from cucumber plants (the C strain, hereafter) obtained from the Institute for Horticultural Plant Breeding in Wageningen, The Netherlands. Since collection, spider mites have been reared on detached leaves of *Phaseolus vulgaris* 'Arena'. Cultures were maintained, and experiments were performed in one climate room, at 23°C, 60–80% relative humidity, and 16L:8D photoperiod. Both strains were infected with *Wolbachia* based on a polymerase chain reaction (PCR) assay with *Wolbachia*-specific primers (Breeuwer and Jacobs 1996). DNA isolation and PCR were as in Breeuwer (1997). The R and C strains are the same as those described in Vala et al. (2000).

### *Isofemale Lines*

Isofemale lines of the two strains were created by taking virgin females from the infected base populations and performing mother × son matings for four consecutive generations; for arrenotokous haplodiploid organisms this gives an expected inbreeding coefficient of 0.98 (Hartl and Clark 1997). From each inbred isofemale line an uninfected counterpart was created either by tetracycline curing as described by Breeuwer (1997) or by heat treatment as described by Van Opijnen and Breeuwer (1999; whatever method worked first). For tetracycline treatment, 20–30 females were placed on arenas and fed an antibiotic solution (described by Breeuwer 1997). For heat treatment, 20–30 females were placed at 32°C (Van Opijnen and Breeuwer 1999). Mites were reared as a culture at this temperature for eight to nine generations to maximize the number of uninfected females at the end of treatment. Isofemale lines cured by tetracycline are labeled TET, and isofemale lines cured by heat treatment are labeled HT. For one isofemale line (R3), two uninfected sublines were established, one by curing with tetracycline the other by curing with heat treatment. The TET and HT R3 sublines were compared to control for treatment effects. To establish the uninfected sublines, 10–15 mated females per isofemale line were placed alone on leaf discs to oviposit for three days and subsequently collected for PCR. For each isofemale line, offspring from females that did not give amplification products on a PCR with *Wolbachia*-specific primers were kept. Offspring from females positive in the PCR was discarded. This process was repeated twice. Finally, $F_3$ females were pooled to establish the uninfected sublines. PCR assays with *Wolbachia*-specific primers and DNA isolation were as in Breeuwer (1997).

Because lines were inbred prior to curing and thus are expected to be nearly homozygous, differences between the infected and uninfected sublines of each isofemale line are most likely due to presence or absence of *Wolbachia*. Assuming nuclear genetic variation in the base population, differences between isofemale lines are likely to be due to genetic differences at the nuclear level (the genetic similarity of *Wolbachia* in the two populations is discussed below).

To detect variation in *Wolbachia*-induced reproductive incompatibility, several isofemale lines from each population were tested for CI. The test for aneuploidy of $F_1$ females, or hybrid breakdown test, is presented in a separate paper. To test for CI, all possible crosses between infected (W) and uninfected (U) individuals were performed (♀ × ♂: W × W, W × U, U × U, U × W). In haplodiploids, CI may result in a male bias of $F_1$ sex ratio associated with an increase in mortality in ♀U × ♂W crosses compared to U × U crosses. If *Wolbachia* is present in the female and/or absent in the male (thus, ♀ × ♂: W × W and W × U), crosses should be compatible. To distinguish between mod$^-$resc$^-$ and mod$^-$resc$^+$, crosses were performed between mod$^-$resc$^?$ females and mod$^+$resc$^+$ males of another isofemale line.

### *Procedures for All Experiments*

Twenty-five to 30 females of each line laid eggs on detached bean leaves (*P. vulgaris*) placed on water-soaked cotton balls. These females were transferred at three-day intervals to produce age cohorts. Offspring from these cohorts were used in the experiments to ensure that all mites tested were of the same age. All experiments were performed on bean leaf discs (1.5 cm in diameter). Leaf discs were placed on water-soaked cotton sheets stretched on sponges. Sponges were placed on plastic trays, and water was added regularly to prevent the leaf discs from drying. In all experiments, crosses and spider mite lines were randomized across sponges.

For $F_1$ analysis (test for CI), experimental females were collected at the last molting stage from age cohorts (to ensure they were virgin) and placed in groups of five females and three males for 48 h to mate. Subsequently, females were transferred individually to fresh leaf discs for oviposition. In total six days of oviposition were scored, and females were transferred to a fresh leaf disc after three days. Offspring ($F_1$ female, $F_1$ male, unhatched eggs, and dead individuals) were counted 10–12 days later and used to compute clutch size (CS = number unhatched eggs + number dead + number $F_1$ females + number $F_1$ males), $F_1$ sex ratio (SR = number $F_1$ males/[number $F_1$ females + number $F_1$ males]), and $F_1$ mortality (mortality = [number unhatched eggs + number of dead]/CS).

### *Statistical Analysis*

Effect of factors was analyzed by MANOVAs on derived variables (i.e., variables computed from what was actually measured in the experiments: clutch size, sex ratio, and mortality) because these variables will generally not be independent. We report the MANOVA Wilk's $\lambda$ test statistic. Normality of data was tested graphically and significance was examined using the Shapiro-Wilk test. Homocedasticity (equality of group variances) was tested using Levine's test. In MANOVAs, equality of covariance matrices was tested using the Box's test. Nonparametric tests (Kruskal-Wallis) were used when assumptions of normality and homocedasticity were violated (provided they could not be solved by transformation). When (M)ANOVA were performed, sex ratio and mortality were arcsine-square-root transformed. Statistic analysis was performed using SPSS (Chicago, IL). MANOVAs were followed by a series of univariate ANOVAs. The significance $\alpha$-level of these ANOVAs ($P = 0.05$) was adjusted following the Bonferroni procedure to correct for multiple analysis (Field 2000). Pairwise comparisons were performed using Tukey post hoc tests.

### *Cloning and Sequencing of Wolbachia ftsZ and wsp Genes*

Two *Wolbachia* genes (*wsp* and *ftsZ*) from both the cucumber and rose populations of *T. urticae* were cloned and sequenced to determine the relatedness of their *Wolbachia* strains. Ten individual female mites from each mass-bred population of cucumber and rose were pooled separately. DNA was extracted using the CTAB method adapted for mites from Breeuwer (1997). The primers *ftsZ*491F and *ftsZ*1262R, which amplify 730 base pairs (bp) of the cell-division gene (Holden et al. 1993), and the primer pairs *wsp*81F and *wsp*619R (Zhou et al. 1998), which amplify 590–632 bp of the *wsp* gene, were used in separate PCR amplifications. PCR reaction mixes and amplification conditions were the same as described in Weeks and Breeuwer (2001). PCR products were then cleaned using Geneclean® (BIO 101, Inc., Bingham, Nottingham, U.K.) and cloned into a pGEM®-T vector (Promega, Madison, WI). We extracted five vectors from recombinant colonies for each gene from each strain using the alkaline-lysis method (Sambrook et al. 1989). After extraction, 1 $\mu$g of vector DNA was used as template for a cycle sequencing reaction (Thermosequenase kit, Amersham/Pharmacia, Piscataway, NJ) using fluorescent-labeled primer (IRD 700/800, Biolegio, Malden, The Netherlands) and subsequently run on an NEN Global IR2 DNA analyzer (LI-COR, Lincoln, NE).

## RESULTS

Eight isofemale lines of two spotted spider mites were established through mother $\times$ son mating. One reason why it may be easy to establish these lines is haplodiplody: Recessive deleterious mutations are mostly eliminated through male mortality (Crozier 1985). Thus, in a sense, mothers usually mated to good sons.

Lines established from PCR-negative females in an assay with *Wolbachia*-specific primers remained negative without further treatment. For isofemale line 3, two cured sublines were established by curing with tetracycline (TET) and by curing with heat treatment (HT). No differences were found in crosses with mites cured by one or the other method (Table 1, R3). Therefore, for assessment of reproductive compatibility, we conclude that method of curing had no effect other than removal of the symbiont.

### *The Effect of Wolbachia on Cytoplasmic Incompatibility*

Variability for *Wolbachia*-induced reproductive incompatibility was found among isofemale lines of the rose strain. Presence of *Wolbachia* in males of isofemale line R1 and R2 resulted in induction of CI when mated with R1 and R2 uninfected females, respectively. CI was expressed as increased $F_1$ mortality and a sex-ratio bias toward males (Table 1). However, in crosses of infected R3 males with uninfected R3 females CI was not observed (Table 1). PCR with *Wolbachia* primers confirmed that this result was not due to a change in infection status of the uninfected sublines (or of the infected one). Finally, absence of CI was stable over time; the same result was obtained in later experiments (cf. Table 3).

Although in isofemale line C3 higher $F_1$ mortality was observed in U $\times$ W crosses (Table 2), this effects was not statistically significant. Thus, the cucumber isofemale lines tested did not show CI associated with presence of *Wolbachia* in males—all infections in C lines were associated with mod$^-$ phenotypes. In C5 a significant effect in sex ratio was found: Infected females produced more female-biased sex ratios. A

TABLE 1. Test for induction of cytoplasmic incompatibility: crosses within isofemale lines from the Rose population. W, Wolbachia infected; U, uninfected (cured); TET, cured by tetracycline; HT, cured by heat treatment; variables significant (after Bonferroni correction) according to univariate ANOVAs are marked with an asterisk; identical superscripts (a, b, c, d) within columns indicate nonsignificant differences between crosses at the 5% level (Tukey test).

| | | Rose 1 (HT) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Clutch size (cs) | | Mortality* | | Sex ratio* | |
| Cross | *N* | Mean | SE | Mean | SE | Mean | SE |
| U × U | 33 | 42.21 | 1.50 | $0.41^{a}$ | 0.03 | $0.53^{a}$ | 0.03 |
| U × W | 39 | 39.98 | 2.16 | $0.64^{b}$ | 0.03 | $0.93^{b}$ | 0.02 |
| W × W | 31 | 40.79 | 2.20 | $0.21^{c}$ | 0.02 | $0.51^{a}$ | 0.03 |
| W × U | 24 | 43.97 | 1.68 | $0.28^{c}$ | 0.03 | $0.44^{a}$ | 0.03 |
| MANOVA | | | | $F_{9,295} = 29.09$, Wilk's $\lambda = 0.21$, $P < 0.001$ | | | |
| ANOVAS | | $F_{3,126} = 0.47$, ns | | $F_{3,126} = 3.70$, $P < 0.001$ | | $F_{3,126} = 4.13$, $P < 0.01$ | |
| | | Rose 2 (TET) | | | | | |
| | | Clutch size | | Mortality* | | Sex ratio* | |
| Cross | *N* | Mean | SE | Mean | SE | Mean | SE |
| U × U | 37 | 46.32 | 2.19 | $0.15^{a}$ | 0.02 | $0.28^{a}$ | 0.02 |
| U × W | 47 | 40.49 | 2.04 | $0.48^{b}$ | 0.02 | $0.58^{b}$ | 0.02 |
| W × W | 36 | 40.56 | 2.44 | $0.14^{a}$ | 0.02 | $0.27^{a}$ | 0.02 |
| W × U | 45 | 42.57 | 2.01 | $0.17^{a}$ | 0.02 | $0.26^{a}$ | 0.02 |
| MANOVA | | | | $F_{9,385} = 37.06$, Wilk's $\lambda = 0.22$, $P < 0.001$ | | | |
| ANOVAS | | $F_{3,163} = 1.46$, ns | | $F_{3,163} = 3.70$, $P < 0.001$ | | $F_{3,163} = 4.13$, $P < 0.001$ | |
| | | Rose 3 (HT and TET) | | | | | |
| | | Clutch size* | | Mortality* | | Sex ratio* | |
| Cross | *N* | Mean | SE | Mean | SE | Mean | SE |
| $U_{HT} \times U_{HT}$ | 33 | $46.12^{b,d}$ | 2.44 | $0.25^{a,b}$ | 0.03 | $0.26^{a,b}$ | 0.04 |
| $U_{HT} \times W$ | 27 | $44.41^{b,c}$ | 1.86 | $0.20^{a,b}$ | 0.03 | $0.28^{a,b}$ | 0.03 |
| W × W | 26 | $29.35^{a}$ | 3.00 | $0.22^{a,b}$ | 0.03 | $0.24^{a}$ | 0.03 |
| $W \times U_{HT}$ | 33 | $41.76^{b,c}$ | 2.45 | $0.27^{a,c}$ | 0.03 | $0.39^{b}$ | 0.04 |
| $U_{TET} \times U_{TET}$ | 33 | $36.61^{a,c}$ | 2.04 | $0.25^{a}$ | 0.02 | $0.23^{a,b}$ | 0.03 |
| $U_{TET} \times W$ | 37 | $37.79^{a,c}$ | 2.16 | $0.31^{a,c}$ | 0.03 | $0.29^{a}$ | 0.03 |
| W × W | 39 | $43.79^{b,c}$ | 1.54 | $0.15^{b}$ | 0.02 | $0.25^{a,b}$ | 0.02 |
| $W \times U_{TET}$ | 35 | $39.42^{b,c}$ | 2.53 | $0.18^{b,a}$ | 0.01 | $0.26^{a,b}$ | 0.02 |
| MANOVA | | | | $F_{21,721} = 3.96$, Wilk's $\lambda = 0.81$, $P < 0.001$ | | | |
| ANOVAS | | $F_{7,260} = 5.64$, $P < 0.001$ | | $F_{7,260} = 3.70$, $P = 0.001$ | | $F_{7,260} = 2.54$, $P = 0.015$ | |

sex-ratio shift toward females provides a spreading mechanism for *Wolbachia* (Egas et al. 2002) and is consistent with results obtained previously in the base population (Vala et al. 2000, 2002).

### *Mod⁻resc⁻ or mod⁻resc⁺?*

To test whether infected R3 females retained the property of rescuing modified sperm, despite the fact that sperm from infected R3 males is not modified, R3 and R1 mites were crossed. Results are presented in Table 3. First, as for previous experiments (Tables 1, 2), infected R1 males induced CI in uninfected R1 females, but infected R3 males did not induce CI in uninfected R3 females. Second, infected R1 males induced CI in uninfected R3 females, whereas infected R3 males did not induce CI in uninfected R1 females. Third, presence of *Wolbachia* in R3 females eliminated incompatibility in crosses with infected R1 males. In other words, mortality and sex ratio of ♀R3W × ♂R1W was not significantly different from mortality and sex ratio of ♀R3U × ♂R1U crosses, whereas both differed from ♀R3U × ♂R1W crosses. Thus, we conclude that the phenotype of the *Wolbachia*-host association in isofemale line R3 is of type mod⁻resc⁺.

### *Wsp and ftsZ Sequence Variation for the Two Strains of Tetranychus urticae*

No differences were found in either of the *ftsZ* or *wsp* sequences within or between the cucumber and rose mass-bred populations of *T. urticae*. All 10 inserts sequenced (five from cucumber and five from the rose populations) for both *ftsZ* and *wsp* were identical (Genbank accession numbers are *ftsZ*-AF404763 and *wsp*-AF404765 for cucumber and *ftsZ*-AF404764 and *wsp*-AF404766 for rose).

## DISCUSSION

This is the first report of within-population variation for *Wolbachia*-induced phenotypes. The variation reported here is likely to reflect genetic differences because environmental conditions were constant throughout experiments. The question is whether these differences are due to genetic variation at the host, at the symbiont level, or both.

### *Is mod⁻ a Property of Wolbachia or of the Host?*

Three genes are commonly used to infer *Wolbachia* phylogeny, 16S rDNA, *wsp*, and *ftsZ*. The latter two evolve faster and *wsp* is the most variable and informative (Zhou et al.

TABLE 2. Test for induction of cytoplasmic incompatibility: crosses within isofemale lines from the cucumber population (for table legend, see Table 1).

| | | Cucumber 1 (TET) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Clutch size | | Mortality | | Sex ratio | |
| Cross | *N* | Mean | SE | Mean | SE | Mean | SE |
| U × U | 33 | 43.84 | 1.50 | 0.20 | 0.03 | 0.57 | 0.03 |
| U × W | 43 | 43.33 | 1.85 | 0.22 | 0.02 | 0.58 | 0.04 |
| W × W | 43 | 44.16 | 1.50 | 0.14 | 0.03 | 0.55 | 0.03 |
| W × U | 24 | 47.57 | 2.36 | 0.16 | 0.02 | 0.54 | 0.03 |
| MANOVA | | | | $F_{9,435} = 1.85$, Wilk's $\lambda = 0.89$, ns | | | |
| | | Cucumber 2 (TET) | | | | | |
| | | Clutch size | | Mortality* | | Sex ratio* | |
| Cross | *N* | Mean | SE | Mean | SE | Mean | SE |
| U × U | 24 | 33.88 | 2.74 | 0.32[a] | 0.04 | 0.45[a,b] | 0.05 |
| U × W | 19 | 33.55 | 3.50 | 0.30[a] | 0.03 | 0.50[a] | 0.05 |
| W × W | 40 | 34.32 | 2.08 | 0.21[b] | 0.03 | 0.35[b] | 0.03 |
| W × U | 34 | 37.91 | 2.20 | 0.23[b,a] | 0.03 | 0.33[b] | 0.03 |
| MANOVA | | | | $F_{9,270} = 2.61$, Wilk's $\lambda = 0.81$, $P = 0.007$ | | | |
| ANOVAS | | $F_{3,116} = 0.72$, ns | | $F_{3,116} = 3.70$, $P = 0.014$ | | $F_{3,116} = 4.13$, $P = 0.008$ | |
| | | Cucumber 3 (HT) | | | | | |
| | | Clutch size* | | Mortality* | | Sex ratio | |
| Cross | *N* | Mean | SE | Mean | SE | Mean | SE |
| U × U | 38 | 53.17[a] | 2.17 | 0.16[a] | 0.03 | 0.36 | 0.03 |
| U × W | 34 | 38.67[b] | 2.67 | 0.28[b] | 0.04 | 0.44 | 0.05 |
| W × W | 34 | 61.23[c] | 1.49 | 0.17[a] | 0.03 | 0.35 | 0.02 |
| W × U | 33 | 43.54[b] | 2.97 | 0.17[a,b] | 0.03 | 0.36 | 0.04 |
| MANOVA | | | | $F_{9,334} = 6.49$, Wilk's $\lambda = 0.68$, $P < 0.001$ | | | |
| ANOVAS | | $F_{3,142} = 16.75$, $P < 0.001$ | | $F_{3,142} = 5.75$, $P = 0.001$ | | $F_{3,142} = 0.81$, ns | |
| | | Cucumber 4 (TET) | | | | | |
| | | Clutch size* | | Mortality | | Sex ratio* | |
| Cross | *N* | Mean | SE | Mean | SE | Mean | SE |
| U × U | 38 | 43.66[a] | 1.35 | 0.12 | 0.02 | 0.31[a] | 0.02 |
| U × W | 20 | 48.60[a] | 1.61 | 0.21 | 0.02 | 0.22[b] | 0.02 |
| W × W | 39 | 48.79[a] | 0.99 | 0.19 | 0.01 | 0.24[a,b] | 0.02 |
| W × U | 43 | 51.14[b] | 1.15 | 0.17 | 0.01 | 0.20[b] | 0.02 |
| MANOVA | | | | $F_{9,302} = 3.79$, Wilk's $\lambda = 0.77$, $P < 0.001$ | | | |
| ANOVAS | | $F_{3,129} = 4.54$, $P = 0.005$ | | $F_{3,129} = 4.54$, ns | | $F_{3,129} = 4.54$, $P < 0.004$ | |
| | | Cucumber 5 (HT) | | | | | |
| | | Clutch size* | | Mortality | | Sex ratio* | |
| Cross | *N* | Mean | SE | Mean | SE | Mean | SE |
| U × U | 44 | 46.45[a,b] | 2.00 | 0.10 | 0.01 | 0.35[a] | 0.01 |
| U × W | 48 | 40.58[a] | 2.23 | 0.12 | 0.02 | 0.34[a] | 0.02 |
| W × W | 38 | 43.66[a] | 1.35 | 0.12 | 0.02 | 0.23[b] | 0.02 |
| W × U | 41 | 48.88[b] | 1.40 | 0.13 | 0.01 | 0.28[a,b] | 0.02 |
| MANOVA | | | | $F_{9,402} = 4.74$, Wilk's $\lambda = 0.78$, $P < 0.001$ | | | |
| ANOVAS | | $F_{3,171} = 4.17$, $P = 0.007$ | | $F_{3,171} = 1.53$, ns | | $F_{3,171} = 7.70$, $P < 0.001$ | |

1998; Jiggins et al. 2001). In our study, *wsp* and *ftsZ* sequences did not correlate with infection phenotype. Sequences were identical in the rose and cucumber strains, and reproductive incompatibility was induced in isofemale lines of the first, but not of the second strain. Fialho and Stevens (2000) report a similar result. In *Tribolium madens Wolbachia* is associated with male killing and in *T. confusum* the infection results in CI. However, based on *ftsZ* and *wsp* sequences the two bacteria cannot be distinguished (Fialho and Stevens 2000). Of course, it is possible that the *Wolbachia* present in these pairs of strains and species differs in genes other than the two we have sequenced. The two strains of *Wolbachia* may have acquired similar copies of *wsp* and *ftsZ* genes through recombination (Jiggins et al. 2001; Werren and Bartos 2001). Alternatively, the phenotypes associated with these infections may be influenced by host effects.

Crosses within isofemale lines of the cucumber strain revealed that infection phenotype was mod$^-$resc$^?$ in all C isofemale lines (Tables 2). The same result is observed in crosses within the cucumber base population (Vala et al. 2002). Previous results suggest that C infections can, to some extent, rescue R-modified sperm (cf. Vala et al. 2000). However, the two strains are reproductively isolated even in the absence of *Wolbachia* (Vala et al. 2000). Thus, it is difficult to conclusively determine whether infected C females can rescue sperm from infected R males. In any case, how does an in-

TABLE 3. Crosses between R1 and R3 isofemale lines for resc$^-$ or resc$^+$ status (for table legend, see Table 1).

| Cross ♀ × ♂ | *N* | Mortality* Mean | SE | Sex ratio* Mean | SE |
|---|---|---|---|---|---|
| Does R1W induce CI in R1U? Yes. | | | | | |
| R1U × R1U | 58 | 0.25$^{a}$ | 0.03 | 0.35$^{a,b}$ | 0.04 |
| R1U × R1W | 31 | 0.51$^{c}$ | 0.05 | 0.60$^{d,e}$ | 0.05 |
| Does R1W induce CI in R3U? Yes. | | | | | |
| R3U × R1U | 35 | 0.09$^{b}$ | 0.02 | 0.32$^{a,b}$ | 0.02 |
| R3U × R1W | 41 | 0.49$^{c}$ | 0.03 | 0.44$^{e}$ | 0.03 |
| Can R3W rescue R1W? Yes. | | | | | |
| R3W × R1W | 51 | 0.15$^{a,b}$ | 0.02 | 0.26$^{a}$ | 0.02 |
| Does R3W induce CI in R3U? No. | | | | | |
| R3U × R3U | 36 | 0.24$^{a}$ | 0.04 | 0.46$^{b,c,d}$ | 0.03 |
| R3U × R3W | 34 | 0.14$^{a,b}$ | 0.03 | 0.55$^{c,d}$ | 0.04 |
| Does R3W induce CI in R1U? No. | | | | | |
| R1U × R3U | 48 | 0.15$^{a,b}$ | 0.03 | 0.44$^{b,c}$ | 0.03 |
| R1U × R3W | 40 | 0.20$^{a,b}$ | 0.03 | 0.56$^{c,d}$ | 0.04 |
| MANOVA | $F_{16,728} = 18.74$, Wilk's $\lambda = 0.48$, $P < 0.001$ | | | | |
| ANOVAS | $F_{8,373} = 23.24$, $P < 0.001$ $F_{8,373} = 21.11$, $P < 0.001$ | | | | |

fection that does not induce CI maintain itself in the host population? A separate study suggests that C-*Wolbachia* induces a sex-ratio bias toward females (Vala et al. 2002). Mathematical analysis shows that such an effect provides a spreading mechanism for the bacteria (Egas et al. 2002).

Mod$^+$resc$^+$ and mod$^-$resc$^+$ infection phenotypes were isolated from the rose strain. Crosses within isofemale line R3 did not show induction of CI (Table 1), whereas CI was induced in crosses within isofemale lines R1 and R2. Additional crossing experiments demonstrated that the infection in R3 exhibits a mod$^-$resc$^+$ phenotype (Table 3). Thus, mod$^+$resc$^+$ and mod$^-$resc$^+$ phenotypes co-exist within a single host population.

Theory predicts that if a resident mod$^+$resc$^+$ *Wolbachia* is present and the mod$^-$resc$^+$ type is more deleterious than the resident mod$^+$, then mod$^-$ will be excluded by selection. If the mod$^-$ and mod$^+$ infections are equally harmful, then mod$^-$ is essentially neutral (Hurst and McVean 1996). Its frequency may drift around the level at which it was introduced and, assuming the mutation that causes the shift in mod is not very common, disappear. Thus, the probability of finding a neutral mod$^-$ symbiont seems low. A mod$^-$resc$^+$ *Wolbachia* increases in frequency when rare if it entails a lower cost to infected females than the resident mod$^+$ (Prout 1994; Turelli 1994; Hurst and McVean 1996). If uninfected individuals are present in the population, spread of the mod$^-$resc$^+$ type leads both infections to extinction (Hurst and McVean 1996). If uninfected individuals are not present, like in our infected laboratory populations, a less costly mod$^-$ symbiont type would spread to fixation.

Our results do not indicate any correlated differences between fecundity costs to infected females and CI induction. Infected R1 and R3 females generally produce similar clutch sizes (Table 1). Average $F_1$ mortality in W × W and W × U crosses is 0.25 for R1, 0.16 for R2, and 0.21 for R3. Therefore, the mod$^+$resc$^+$ infection in R2 incurs lower mortality in broods of W females than the mod$^-$resc$^+$ infection in R3. If we repeat these calculations for sex ratio, then R1 has the least female-biased sex ratio (0.48), whereas R2 and R3 have similar sex ratios (0.27 and 0.26). Thus, infections in R1 or R2 do not appear more costly to females than the infection in R3. Absence of a difference in cost to infected R1, R2, and R3 females is expected if the *Wolbachia* infecting these lines is the same. This hypothesis cannot be refuted based on our sequence data. The phenotypic differences observed may be due to genetic differences between hosts.

Like a symbiont mod$^-$ allele, a host mod$^-$ allele cannot invade a population unless there is a resident mod$^+$. CI provides a spreading mechanism to the symbiont, but for nuclear host genes CI means that not all crosses between infected and uninfected individuals will produce viable offspring. In a population with infected and uninfected hosts, males that possess a nuclear allele conferring resistance to sperm modification are compatible with all females in the population. Therefore, such a host allele invades (Turelli 1994), even if the mod$^-$ trait is not associated with a lower cost to the infected (mod$^-$) host. The allele spreads in the absence of other fitness differences because, being a nuclear allele, it is transmitted by both sexes. If uninfected individuals are not present in the population, like in our laboratory population, a mod$^-$ host allele is, like a symbiont mod$^-$ allele, a neutral trait.

To summarize, in a field situation, where uninfected individuals are likely to be present, a mod$^-$ host allele invades if there is a mod$^+$ allele present. Under the same circumstances a symbiont allele can only invade if it entails a lower cost to infected females than the resident mod$^+$. Our results do not support the hypothesis that mod$^-$ is less costly to infected females than mod$^+$. Thus, if mod$^-$ were a symbiont trait, it would be rare and essentially neutral in the field. Consequently, in the absence of a cost effect to infected females, the probability of picking up a host mod$^-$ in the field seems higher. Once in the laboratory, where uninfected individuals are not present, a mod$^-$ host allele is a neutral trait. The probability of it drifting to fixation or to extinction depends on its original frequencies. If introduced at intermediate frequencies it could be maintained.

### *Population Dynamics Consequences of a Host mod$^-$ Allele*

Hosts may mutate or otherwise protect the target sites of modification by *Wolbachia*, or bacterial growth in males may be prevented (Bressac and Rousset 1993; Poinsot et al. 1998; McGraw et al. 2001). Two examples suggest mod$^-$ host alleles may occur in *Drosophila* hosts. First, a *Wolbachia* strain that does not induce CI in *D. melanogaster*, because it is excluded from sperm cysts, induces CI when present *D. simulans* males (McGraw et al. 2001). Second, the observation that wAu, a symbiont type occurring in *D. simulans*, fails to induce CI in flies from Australia (Hoffmann et al. 1996; Merçot and Poinsot 1998b), but induces CI in some isofemale lines of flies collected in Florida (Ballard et al. 1996).

It is therefore interesting to ask what happens after invasion by a mod$^-$ host allele. It is conceivable that spread of a nuclear mod$^-$ would result in conditions for reestablishment of uninfected individuals, analogous to spread of a *Wolbachia* mod$^-$ (Hurst and McVean 1996). Simulations presented in

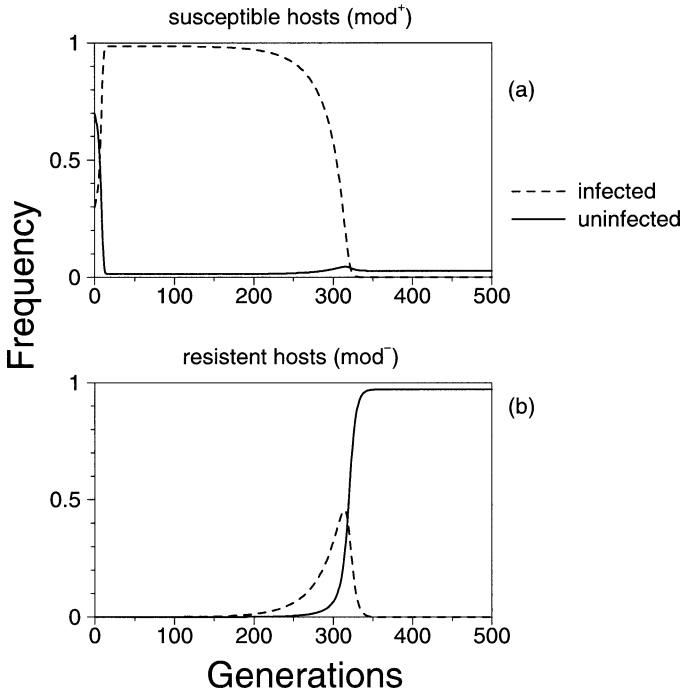


FIG. 1. The effect of a host allele resistant to modification by *Wolbachia* on the population dynamics of the infection (see Appendix and Table A1 for details). The dynamics depicted here are based on $\mu = 0.9$, $F = 0.9$, $H = 0.1$. With these parameters and in the absence of mod$^-$ ($p_r = q_r = 0$), there is an unstable equilibrium at $p_s = 0.235$, $q_s = 0.765$. With initial conditions $p_s = 0.3$, $q_s = 0.7$, $p_r = 0$, $q_r = 0$, the dynamics quickly converge to the equilibrium $p_s = 0.986$, $q_s = 0.014$. After one generation, a mod$^-$ allele was introduced ($p_r = 10^{-5}$), thus mimicking a mutation in a single infected host. (a) Frequency of types susceptible to modification by *Wolbachia* (mod$^+$): $p_s$ (dashed) and $q_s$ (solid). (b) Frequency of types resistant to modification by *Wolbachia* (mod$^-$): $p_r$ (dashed) and $q_r$ (solid).

Figure 1 show that this is indeed the case (see the Appendix for details). However, invasion by a host allele conferring resistance to sperm modification by *Wolbachia* leads to establishment of resistant uninfecteds. Spread of a host mod$^-$ allele differs from spread of a mod$^-$ *Wolbachia* because the uninfected population that results can only be recolonized by a *Wolbachia* using a novel modification site (i.e., a new incompatibility type). This could provide selective pressure for new incompatibility types. For example, in *D. simulans* five *Wolbachia* strains have been identified (wRi, wHa, wAu, wKi, wMa, and wNo), a classification that is consistent with sequence data from *wsp* and 16S rDNA (cf. James and Ballard 2000). These symbiont types correspond to four incompatibility groups. It would be interesting to test if *D. simulans* hosts resist modification by other *simulans Wolbachia* types.

The theoretical result that a host mod$^-$ allele may lead to symbiont exclusion is also interesting. For example, infections by wAu in Australia occur at zero to low frequencies in different populations (Hoffmann et al. 1996). The effect of these infections on reproductive incompatibility was tested on pooled samples from an Australian population and shown not to cause CI. However, when isofemale lines were created from Florida populations, CI was detected in some isofemale lines (Ballard et al. 1996). It is possible that populations infected with wAu in Australia constitute an example of host allele–mediated dynamics such as those simulated here.

To conclude, we found within-population variation for mod. The possibility that the differences we observed are due to host effects cannot be excluded. Simple mathematical analysis of the dynamics of a host mod$^-$ allele suggests interesting evolutionary implications and novel interpretations of existing data. Clearly, more effort should be made to distinguish between *Wolbachia* and host mod$^-$ alleles.

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## Appendix

We study the dynamics of allele frequencies after invasion of the mod$^-$ host allele, with a population genetic model that follows Turelli (1994). The variables in our model are the frequency of infected hosts susceptible to modification by *Wolbachia* ($p_s$), the frequency of infected hosts resistant to modification by *Wolbachia* ($p_r$), the frequency of uninfected hosts possessing the allele for susceptibility to modification by *Wolbachia* ($q_s$), and the frequency of uninfected hosts possessing the resistant allele to modification by *Wolbachia* ($q_r$). Other notation follows Turelli (1994); $\mu$ is the proportion infected offspring produced by an infected mother, $F$ is the fecundity of infected females relative to uninfected females, and $H$ is the hatchability in incompatible crosses ($H = 0$ implies that CI results in 100% $F_1$ mortality). Note that $\mu$, $F$, and $H$ correspond to $\alpha$, $1 - U$, and $1 - k$, respectively, in Hurst and McVean (1996).

Table A1 lists all possible matings, the frequency at which they occur assuming random mating, and the expected distribution of offspring over the four different host categories. Assuming nonoverlapping generations and haploid genetics for reasons of model tractability, the dynamics of the population is described by four difference equations. For example, the frequency of infected, susceptible hosts in the next generation ($p_s'$) is obtained by summing all separate contributions to Ws individuals in Table A1, and dividing by the sum of all contributions:

$$p_s' = (p_s^2\mu F + \tfrac{1}{2}p_s p_r \mu F + \ldots + \tfrac{1}{2}p_r q_s \mu F) \div (p_s^2\mu F + \ldots + q_r^2) \quad \text{(A1)}$$

Note that because $p_s + p_r + q_s + q_r = 1$, one variable can be eliminated, leaving three difference equations.

The objective of this exercise is to investigate whether Hurst and

Table A1. List of possible crosses between host types, the frequency by which they occur, and the distribution of offspring over the four types in a population that segregates a host allele resistant to modification by *Wolbachia*. Host types are classified by infection status (W, infected; U, uninfected) and susceptibility to modification (s, susceptible or mod$^+$; r, resistant or mod$^-$). The columns Ws, Wr, Us, and Ur are expressed in units of the clutch size of an uninfected female.

| Crosses | | Offspring | | | |
|---|---|---|---|---|---|
| ♀ × ♂ | Frequency | Ws | Wr | Us | Ur |
| Ws × Ws | $p_s^2$ | $\mu F$ | — | $(1 - \mu)F\,H$ | — |
| Ws × Wr | $p_s p_r$ | $\frac{1}{2}\mu F$ | $\frac{1}{2}\mu F$ | $\frac{1}{2}(1 - \mu)F$ | $\frac{1}{2}(1 - \mu)F$ |
| Ws × Us | $p_s q_s$ | $\mu F$ | — | $(1 - \mu)F$ | — |
| Ws × Ur | $p_s q_r$ | $\frac{1}{2}\mu F$ | $\frac{1}{2}\mu F$ | $\frac{1}{2}(1 - \mu)F$ | $\frac{1}{2}(1 - \mu)F$ |
| Wr × Ws | $p_r p_s$ | $\frac{1}{2}\mu F$ | $\frac{1}{2}\mu F$ | $\frac{1}{2}(1 - \mu)F\,H$ | $\frac{1}{2}(1 - \mu)F\,H$ |
| Wr × Wr | $p_r^2$ | — | $\mu F$ | — | $(1 - \mu)\,F$ |
| Wr × Us | $p_r q_s$ | $\frac{1}{2}\mu F$ | $\frac{1}{2}\mu F$ | $\frac{1}{2}(1 - \mu)F$ | $\frac{1}{2}(1 - \mu)F$ |
| Wr × Ur | $p_r q_r$ | — | $\mu F$ | — | $(1 - \mu)F$ |
| Us × Ws | $q_s p_s$ | — | — | $H$ | — |
| Us × Wr | $q_s p_r$ | — | — | $\frac{1}{2}$ | $\frac{1}{2}$ |
| Us × Us | $q_s^2$ | — | — | 1 | — |
| Us × Ur | $q_s q_r$ | — | — | $\frac{1}{2}$ | $\frac{1}{2}$ |
| Ur × Ws | $q_r p_s$ | — | — | $\frac{1}{2}H$ | $\frac{1}{2}H$ |
| Ur × Wr | $q_r p_r$ | — | — | — | 1 |
| Ur × Us | $q_r q_s$ | — | — | $\frac{1}{2}$ | $\frac{1}{2}$ |
| Ur × Ur | $q_r^2$ | — | — | — | 1 |

McVean's (1996) predictions hold assuming mod$^-$ is a property of the host. In other words, do uninfected individuals reestablish following invasion by mod$^-$? Using the same parameter values as Hurst and McVean (1996, fig. 2), we find: (1) invasion of the mod$^-$ host allele, followed by extinction of *Wolbachia* (Fig. 1); (2) the resulting uninfected host population is immune to any *Wolbachia* that uses the same modification site; (3) results (1) and (2) hold provided that $\mu < 1$ and that the two equilibria ($p_s$, $q_s$, 0, 0) exist (i.e., the unstable, or threshold, equilibrium and the high prevalence, or polymorphic, internal equilibrium).

Note that if there were a cost to mod$^-$, the uninfected population would slowly return to the susceptible state. However, population immunity is provided by any frequency $q_r > 0$. Therefore, we argue that reinvasion of *Wolbachia* requires a new modification site.