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2 WOLBACHIA-INDUCED 'HYBRID BREAKDOWN' IN THE TWO-SPOTTED SPIDER MITE *TETRANYCHUS URTICAE* KOCH

F Vala, JAJ Breeuwer & MW Sabelis

The most common post-zygotic isolation mechanism between populations of the phytophagous mite *Tetranychus urticae* is 'hybrid breakdown' (HB), i.e. when individuals from two different populations are crossed F1 hybrid females are produced, but F2 recombinant-male offspring suffer increased mortality. Two-spotted spider mites collected from two populations, one on rose and the other on cucumber plants were infected with Wolbachia bacteria. These bacteria may induce cytoplasmic incompatibility (CI) in their hosts: uninfected (U) females become reproductively incompatible with infected (W) males. We report on the effect of Wolbachia infections in intra- and inter-strain crosses on (i) F1 mortality and sex ratios (test for CI), and (ii) number of haploid offspring and mortality in clutches of F1 virgins (test for HB). Within the rose strain, U × W crosses exhibited partial CI. More interestingly, F2 males suffered increased mortality – a result identical to the HB phenomenon. The experiments were repeated using females from the cucumber strain. In inter-strain, U × W and U × U crosses, HB was much stronger in the former (80% vs. 26%). This is the first report of a Wolbachia infection causing a HB phenotype. Our results show that Wolbachia infections can contribute to reproductive incompatibility between populations of *T. urticae*.

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The vertically transmitted intracellular bacteria Wolbachia manipulate host reproduction in ways that result in population replacement: an infected population of hosts replaces an originally uninfected one. Cytoplasmic incompatibility (CI), the most common effect associated with *Wolbachia* bacteria, is the phenomenon where infected (W) males become reproductively incompatible with uninfected (U) females or with females harboring Wolbachia of a different type or strain. CI has been described in several species of insects, three species of mites and one isopod (reviewed by Stouthamer *et al.* 1999).

Cytological analyses in *Nasonia* wasps (Ryan & Saul 1968; Breeuwer & Werren 1990; Reed & Werren 1995) and *Drosophila simulans* (O'Neill & Karr

1990; Callaini *et al.* 1994, 1997) have suggested that there is a common mechanism operating across species: in uninfected eggs fertilized by *Wolbachia*-imprinted sperm from infected males, abnormal mitosis develops following syngamy, which results in improper condensation and segregation of paternal chromosomes. During anaphase, maternal chromosomes migrate to the opposite poles, whereas paternal chromosomes remain at the spindle's equator (Callaini *et al.* 1997). This results in the formation of aneuploid and haploid nuclei (Callaini *et al.* 1997). Consequently, these matings yield reduced numbers of diploid individuals: in diplo-diploid species few or no offspring are produced, and in haplodiploid species (where females are diploid and males are haploid) male biased or all-male sex ratios result. The latter suggests that egg restoration to the haploid state is complete, since normal males are produced.

Several authors have reported on reproductive incompatibilities in crosses between populations of the two-spotted spider mite, *Tetranychus urticae* Koch (*e.g.*, Helle & Pieterse 1965; De Boer & Veerman 1983; Young *et al.* 1985; Gotoh & Takioka 1996). *T. urticae* is a polyphagous herbivore with a haplodiploid reproductive system. Post-zygotic isolation between populations of this species is common and takes different forms (reviewed by De Boer 1985): (i) few or no hybrids (*i.e.* females) are produced (thus all-male or male-biased sex ratios result), (ii) hybrids are produced but are infertile, or (iii) hybrids are produced but high F₂ recombinant male mortality is observed. The latter phenomenon, termed 'hybrid breakdown', is more common. A test for hybrid breakdown consists of scoring the mortality of broods of F₁ virgin females. Post-zygotic isolation may be bi-directional but unidirectional incompatibilities are more frequently reported. This has led several authors to hypothesize on the role of nucleo-cytoplasmic interactions on reproductive compatibility in crosses between strains (*e.g.* Overmeer & Van Zon 1976; De Boer 1982; Fry 1989; Gotoh *et al.* 1995).

Following detection of *Wolbachia* in the two-spotted spider mite (Breeuwer & Jacobs 1996; Tsagkarakou *et al.* 1996), Breeuwer (1997) investigated the effect of this symbiont in crosses between uninfected females and *Wolbachia*-infected males (hereafter 'U × W' crosses) within a strain of *T. urticae* collected from tomato plants. His results contrasted with results in hymenopteran haplodiploid species in that incompatibility was not expressed as increased male production but, rather, as increased mortality and reduced F₁ female production. Breeuwer (1997) proposed incomplete destruction of paternal chromosomes and production of diplo-aneuploid embryos in explaining the appearance of F₁ females in U × W crosses: some of the aneuploid individuals produced were non-viable and died (accounting for the increased mortality), whereas others developed into apparently normal females. This hypothesis is consistent with the cytological details of CI in *D. simulans* (Callaini *et al.* 1997).

One way of testing this hypothesis is to allow F₁ virgin females from U × W crosses to oviposit and contrast mortality among their F₂ with the F₂ mortality of F₁ virgins from crosses between uninfected females and uninfected males (hereafter 'U × U' crosses). If females produced in U × W matings are indeed surviving aneuploids then they will produce both normal and aneuploid eggs, leading to increased F₂ mortality. The result is

indistinguishable from hybrid breakdown, although in fact not related to the production of genotypic hybrids, but to the presence of *Wolbachia* in parental males.

Wolbachia infections have been reported in two other strains of two-spotted spider mites (Breeuwer & Jacobs 1996): mites collected from rose plants (hereafter 'R strain'), and mites collected from cucumber plants (hereafter 'C strain'). Here we focus on the effect of *Wolbachia* infections in males of the R-strain, on reproductive incompatibility expressed both in the F1 (typical CI) and among the haploid F2 (hybrid breakdown).

First, we investigated whether the symbiont had an effect on cross compatibility between infected males and uninfected (cured) females of the R-strain ('intra-strain' crosses). Crosses were set up in all combinations between infected and uninfected individuals and resulting F1 mortality and sex ratios were analyzed. Furthermore, F1 virgin females from all crosses were collected and tested for hybrid breakdown (HB) (*i.e.* they oviposited and subsequent mortality of their F2 haploid offspring was scored). Second, we asked whether the *Wolbachia* infection in the R-strain could affect cross compatibility between this and another strain of mites. Mites collected from cucumber plants were used as a test strain. Infected and uninfected R-males were mated to infected and uninfected (cured) C-females and tested for CI and HB.

MATERIAL AND METHODS

Mite strains

Two strains of *T. urticae* were used: mites collected from rose plants in a greenhouse in Aalsmeer, the Netherlands; and mites collected from cucumber plants obtained from the Institute for Horticultural Plant Breeding in Wageningen, the Netherlands. Since collection the spider mites have been mass reared at our laboratory on detached leaves of the common bean (*Phaseolus vulgaris*, variety 'Arena') in climate rooms (23°C, RH = 60–80%, 16L:8D photoperiod). At the time of these experiments both strains had been in the lab for more than 2 years and could be effectively considered laboratory strains. Both were infected with *Wolbachia* based on a polymerase chain reaction (PCR) assay with *Wolbachia* specific primers (Breeuwer & Jacobs 1996).

Uninfected populations from the C and R strains were established by curing with tetracycline antibiotic as described by Breeuwer (1997). The strains remained uninfected and were kept without further antibiotic treatment for 8 months (*ca.* 16 generations) until the crossing experiments.

Wolbachia infection in individual adult females was determined with PCR using *ftsZ* *Wolbachia* specific primers (Holden *et al.* 1999). PCR assay and DNA isolation procedures were as described by Breeuwer (1997). All individuals from tetracycline-treated strains were PCR negative when tested before and after the experiments with *ftsZ* *Wolbachia*-specific primers. Conversely, all individuals from infected (non-treated) strains yielded amplification products with the same primers.

Effect of *Wolbachia* on reproductive compatibility

Experiments were performed using spider mites from age cohorts produced from mass cultures of each strain. Cohorts were produced by *ca.* 100 females per strain, laying eggs in groups of approximately 25 females on detached bean leaves, placed on a water-soaked cotton wool ball. Offspring from these cohorts were used in the experiments. These were performed in the climate room as above.

Females and males were collected as teleiochrysalids (to ensure they were virgins) and kept separately until emergence. Upon emergence, groups of five females and three males were placed on bean leaf discs ($\varnothing = 1.5$ cm). Intra-strain, ♀♂RU × ♂RU, ♀RU × ♂RW, ♀RW × ♂RW, ♀RW × ♂RU, and inter-strain, ♀CU × ♂RU, ♀CU × ♂RW, ♀CW × ♂RW, ♀CW × ♂RU, matings were set up. Males were removed after 24 hrs, and individual females were transferred to clean bean leaf discs ($\varnothing = 1.0$ cm). Oviposition was scored for the first six days. Numbers of emerging adult females and males, and of dead stages, were scored per leaf disc per female. Next, F1 females were collected as teleiochrysalids and placed on clean bean leaf discs ($\varnothing = 1.5$ cm), in groups of five or six sisters. Upon emergence five sisters per parental female were transferred individually to fresh leaf discs. Females oviposited for six days. The number of dead stages and number of adult offspring were scored per leaf disc per female.

Statistical analysis

For F1 results, the following variables were analyzed: clutch sizes (CS) = (number of F1 females + F1males + aborted eggs + other dead stages), F1 mortality = [(number of aborted eggs + other dead)/CS]; F1 sex ratio = [number males/(number of females + number of males)]; and number of F1 females and of F1 males. Analyses were conducted separately for intra- and inter-strain crosses, and aimed at detecting differences between crosses with different combinations of infected and uninfected individuals. For F2 results, CS, number of F2 males, and F2 mortality were analyzed.

The normality of data was estimated graphically by means of quartile plots and histograms. Mortality data were transformed: $\arcsin\sqrt{\text{mortality}}$, for data from crosses within the R-strain, or $\arcsin\sqrt{\{(total\ dead + 3/8)/(clutch\ size + 3/4)\}}$, for data from C × R crosses] (Zar 1996).

The effect of crossing treatment was first investigated by MANOVA on derived variables, *i.e.* variables computed from what was actually measured in the experiments (clutch size, mortality, and sex ratio) since these variables are not truly independent from each other. Variables for which MANOVAs detected a significant effect of crossing ($P \leq 0.005$) were further investigated by univariate ANOVAs, followed by pairwise comparisons between crosses using Tukey *post hoc* tests. This allowed us to identify those crosses responsible for the significant effects detected in the overall MANOVA.

Sex ratio was always significantly affected by cross type. However, differences in sex ratio can arise due to changes in the number of females, males, or both. Therefore, the mean values of the numbers of F1 females and males obtained were analyzed by univariate ANOVAs followed by Tukey *post*

hoc pairwise comparison tests, so that the changes underlying shifts in the sex ratio can be identified.

Finally, the number of sons produced by virgin F1 females is listed as number of F2 males in Tables 3 and 4. These values have been included because they provide estimates of actual numbers of surviving individuals. However, they have not been analyzed statistically because their effect on total variance has already been taken into account in the overall MANOVAs.

Table 1 F1 female and male production, clutch sizes, mortality and sex ratio for intra-strain crosses (Rose-strain female \times Rose-strain male).

cross	clutch size*		mortality (frequency)		sex ratio* (proportion $\sigma\sigma$)		number of F1 females		number of F1 males	
	$\mu \pm se$	N	$\mu \pm se$	N	$\mu \pm se$	N	$\mu \pm se$	N	$\mu \pm se$	N
U \times U	55.5 \pm 0.9 ^b	62	0.10 \pm 0.02	62	0.31 \pm 0.01 ^b	62	33.8 \pm 1.1 ^b	62	15.1 \pm 0.7 ^b	62
U \times W	52.9 \pm 1.0 ^b	79	0.14 \pm 0.02	79	0.41 \pm 0.02 ^c	79	26.9 \pm 1.0 ^a	79	19.2 \pm 1.0 ^c	79
W \times W	41.8 \pm 1.4 ^a	63	0.11 \pm 0.01	61	0.30 \pm 0.01 ^{ab}	61	26.3 \pm 1.1 ^a	63	10.8 \pm 0.6 ^a	63
W \times U	42.4 \pm 0.8 ^a	59	0.10 \pm 0.02	58	0.24 \pm 0.02 ^a	59	29.0 \pm 1.0 ^a	59	9.1 \pm 0.6 ^a	59

W: Wolbachia-infected; U: uninfected (cured); $\mu \pm se$: mean \pm standard error; N: sample size. Clutch size, mortality and sex ratio were included in an overall MANOVA; variables marked with * are those for which a significant effect of crossing was detected in this analysis. Entries within columns marked with the same superscript (^{a,b,c}) are not significantly different ($P > 0.005$) on a pairwise comparison with Tukey *post hoc* test.

RESULTS

Effects of Wolbachia on reproductive incompatibility for crosses within the R strain

The results of crosses between uninfected and infected R mites are presented in Table 1. The MANOVA analysis detected a significant effect of crossing treatment (Wilks' $\lambda = 0.514$, $F_{9,623} = 21.756$, $p < 0.001$) on the observed variance. The variables significantly affected were clutch size and sex ratio ($F_{3,258} = 45.556$, $p < 0.001$, and $F_{3,258} = 20.882$, $p < 0.001$, respectively) (Table 1). A Tukey *post hoc* pairwise comparison test following univariate ANOVA on sex ratio ($F_{3,256} = 20.478$, $p < 0.001$) revealed that the least female-biased sex ratio is that produced by the U \times W cross, the potential incompatible cross, suggesting that the presence of Wolbachia in R males results in partial cytoplasmic incompatibility. This result is associated with an increase in male production, but not with an increase in F1 mortality (Table 1).

With respect to the F2 results (Table 2), the MANOVA revealed a significant effect of treatment on the observed variance (Wilks' $\lambda = 0.557$, $F_{6,392} = 22.215$, $p < 0.001$) and this effect was explained by mortality alone ($F_{3,197} = 47.902$, $p < 0.001$). Female virgins from W \times W crosses showed the

lowest mortality among their F₂ haploid broods, which was associated with some increase in the number of males produced (Table 2). However, the most striking effect is that of virgins from U × W crosses: their clutches had the highest F₂ mortality in association with a dramatic decrease in the number of F₂ males (Table 2). Thus, the presence of *Wolbachia* in *R*-males resulted in hybrid breakdown if female mates were uninfected.

Table 2 F₂ recombinant haploid production, clutch sizes and mortality of F₁ virgin females from intra-strain crosses (Rose-strain female × Rose-strain male).

F ₁ female's parents (♀ × ♂)	clutch size		mortality* (frequency)		number of F ₂ males	
	μ±se	N	μ±se	N	μ±se	N
(U × U)	44.2±1.2	56	0.24±0.04 ^b	56	33.3±1.8	56
(U × W)	41.1±1.2	60	0.57±0.02 ^c	59	17.8±1.0	60
(W × W)	42.2±1.2	38	0.07±0.01 ^a	38	39.3±1.3	39
(W × U)	45.3±1.2	48	0.24±0.04 ^b	48	33.9±1.8	48

W: *Wolbachia*-infected; U: uninfected (cured); μ±se: mean ± standard error; N: sample size. Clutch size, mortality and sex ratio were included in an overall MANOVA; variables marked with * are those for which a significant effect of crossing was detected in this analysis. Entries within columns marked with the same superscript (^{a,b,c}) are not significantly different ($P > 0.005$) on a pairwise comparison with Tukey *post hoc* test.

Table 3 F₁ female and male production, clutch sizes, mortality and sex ratio for inter-strain crosses (Cucumber-strain female × Rose-strain male).

♀ × ♂	clutch size*		mortality (frequency)		sex ratio* proportion ♂♂		number of F ₁ females		number of F ₁ males	
	μ±se	N	μ±se	N	μ±se	N	μ±se	N	μ±se	N
U × U	59.0±1.4 ^b	29	0.12±0.02	29	0.62±0.03 ^c	29	20.0±1.7 ^a	29	31.6±1.6 ^b	29
U × W	57.4±1.5 ^b	28	0.16±0.03	28	0.66±0.03 ^c	28	16.8±1.5 ^a	28	31.8±1.9 ^b	28
W × W	50.0±1.0 ^a	72	0.07±0.01	72	0.43±0.01 ^b	72	26.4±0.8 ^b	72	20.2±0.9 ^a	72
W × U	49.0±1.1 ^a	63	0.06±0.01	63	0.34±0.02 ^a	63	30.3±1.0 ^b	63	15.3±0.8 ^a	63

W: *Wolbachia*-infected; U: uninfected (cured); μ±se: mean ± standard error; N: sample size. Clutch size, mortality and sex ratio were included in an overall MANOVA; variables marked with * are those for which a significant effect of crossing was detected in this analysis. Entries within columns marked with the same superscript (^{a,b,c}) are not significantly different ($P > 0.005$) on a pairwise comparison with Tukey *post hoc* test.

Table 4 F2 recombinant haploid production, clutch sizes and mortality of F1 virgin females from inter-strain crosses (Cucumber-strain female \times Rose-strain male).

F1 female's parents (♀ \times ♂)	clutch size		mortality* (frequency)		number of F2 males	
	$\mu \pm se$	N	$\mu \pm se$	N	$\mu \pm se$	N
(U \times U)	42.4 \pm 1.6 ^b	37	0.21 \pm 0.04 ^a	37	34.6 \pm 2.3	38
(U \times W)	28.4 \pm 2.5 ^a	29	0.80 \pm 0.03 ^b	28	6.1 \pm 0.9	29
(W \times W)	45.8 \pm 1.4 ^b	58	0.67 \pm 0.02 ^b	58	15.2 \pm 1.0	58
(W \times U)	47.7 \pm 2.2 ^b	52	0.26 \pm 0.03 ^a	52	37.4 \pm 2.2	52

W: Wolbachia-infected; U: uninfected (cured); $\mu \pm se$: mean \pm standard error; N: sample size. Clutch size, mortality and sex ratio were included in an overall MANOVA; variables marked with * are those for which a significant effect of crossing was detected in this analysis. Entries within columns marked with the same superscript (^{a,b}) are not significantly different ($P > 0.005$) on a pairwise comparison with Tukey *post hoc* test.

Effects of Wolbachia on reproductive incompatibility for crosses between C females and R males

The results of crosses between uninfected and infected C females, and uninfected and infected R males, are presented in Table 3. The MANOVA showed a significant effect of crossing (Wilks' $\lambda = 0.442$, $F_{9, 452} = 20.050$, $p < 0.001$) on the observed variance. The variables significantly affected were clutch size and sex ratio ($F_{3, 188} = 13.926$, $p < 0.001$, and $F_{3, 188} = 53.192$, $p < 0.001$, respectively). In the case of sex ratio, U females produce more males and fewer females than infected females and, consequently, show a less female biased sex ratio (Table 3). These results could suggest that presence of Wolbachia in C females is associated with increased daughter production. Two known Wolbachia associated effects result in increased proportion of daughters: parthenogenesis and male killing (reviewed by Stouthamer *et al.* 1999). We can exclude (i) the possibility of a male-killer Wolbachia in these females because mortality among their broods was not higher than that of broods from uninfected females; and (ii) the possibility of parthenogenetic production of females because infected F1 virgin females did not produce daughters. The difference in the number of daughters could have been due to genetic divergence of both uninfected and infected strains since they had been separated for 16 generations. Nevertheless, these results are independent of the infection status of the R-males, which is the focus of this paper, and may actually mask any effect that the presence of Wolbachia in these males may have had on reproductive incompatibility with C-females.

The effect of the presence of Wolbachia in R-males becomes clear when the F2 results are considered (Table 4). The MANOVA of the F2 results revealed a significant effect of treatment on the observed variance (Wilks' $\lambda = 0.324$, $F_{6, 340} = 42.867$, $P < 0.001$) for both clutch size and mortality. Univariate ANOVAs and pairwise comparisons were performed on clutch size ($F_{3, 172} = 16.041$, $P < 0.001$) and on F2 mortality ($F_{3, 171} = 87.637$,

$P < 0.001$). F2 mortality among haploid clutches of the F1 hybrids increased dramatically when parental males were infected: virgin females from U \times W and W \times W crosses, show the highest F2 mortality among their offspring (Table 4). Thus, the presence of *Wolbachia* in R-males was associated with hybrid breakdown induction. Furthermore, virgin females from U \times W also laid significantly fewer eggs (Table 4), a result that was not observed in crosses within the R strain. This partial sterility effect could be another consequence of female aneuploidy.

DISCUSSION

The presence of *Wolbachia* in males of the R strain induced reproductive incompatibilities with uninfected females of this strain as well as with infected and uninfected females of the C strain of *T. urticae*. More interestingly, the incompatibility effects extended into the next generation, thereby also affecting also the haploid offspring of $\text{♀} \times \text{♂}$, RU \times RW, CU \times RW and CW \times RW females. This effect is similar to that which has been described as hybrid breakdown in the literature on spider mite.

The effect of *Wolbachia* in rose males on reproductive incompatibility: partial cytoplasmic incompatibility and hybrid breakdown

Partial cytoplasmic incompatibility in crosses within the R-strain is expressed as an increase in sex ratio (proportion males) due to an increase in male production observed in U \times W crosses, and is not associated with an increase in F1 mortality (Table 1). These results contrast with Breeuwer (1997), who found that sex ratios were more male-biased due to decreased female production in a tomato strain of *T. urticae*, but are in accordance with the CI phenotype described in *Nasonia* wasps (Breeuwer & Werren 1990).

The increase in male production in U \times W crosses (Table 1) could arise from two different processes: (i) fewer eggs are fertilized than in U \times U crosses, or (ii) a proportion of the fertilized eggs return to the haploid state due to cytoplasmic incompatibility. Since crosses of uninfected or infected males with infected females (*i.e.*, RW \times RU and RW \times RW crosses) produced similar numbers of F1 males and F1 females (Table 1), reduced fertilization ability of sperm from infected males can be rejected. Moreover, the fact that a significant increase in F2 mortality was observed among broods of F1 (U \times W) females (Table 2) indirectly supports the second hypothesis. The reasoning is similar to which was proposed by Breeuwer (1997) and consistent with the cytological phenotype of CI described by Callaini *et al.* (1997). Production of aneuploid females when haplodization of (U \times W) eggs is not complete. This process may be particularly common in spider mites due to the holokinetic structure of their chromosomes (Breeuwer 1997). Holokinetic chromosomes do not have a localized centromere and spindle fibers can attach anywhere in the chromosome. Consequently, fragments of paternal may still segregate into daughter nuclei. Resulting U \times W females will develop as apparently 'normal' because only the paternal set of chromosomes is affected (the maternal set of chromosomes is not affected).

However, meiosis in these 'aneuploid hangover' females will result in haploid (n) and aneuploid ($n-x$) gametes. Since males develop from unfertilized eggs and, thus, have no extra set of chromosomes to compensate for the missing genome, aneuploid eggs will abort. This will result in an F_2 mortality pattern which is identical to what has been termed 'hybrid breakdown' in mite literature (reviewed by De Boer 1985). Cytological analysis of haploid eggs from F_1 ($U \times W$) virgins is necessary to confirm or dismiss this hypothesis: in the former case, aneuploid F_2 eggs should be observed.

The similarity between the F_2 mortality pattern observed among broods of F_1 $U \times W$ females, in crosses within the R strain, and the reported cases of hybrid breakdown observed between different populations of *T. urticae*, prompted us to investigate whether the presence of Wolbachia in R males could also affect the reproductive compatibility between R males and females from a C strain of mites of the same species. Because the C strain was also infected with Wolbachia we included the crosses between infected C females and infected R males ($CW \times RW$) and between infected C females and uninfected R males ($CW \times RU$) in this analysis. These crosses tell us whether cucumber-Wolbachia can rescue rose-Wolbachia imprinted sperm.

Although CI was not detected in crosses between CU or CW females and RW males, F_1 $CU \times RW$ and $CW \times RW$ females suffered severe hybrid breakdown. Does the infection status of the female play a role when the parental male is infected? It is interesting to note that, when the parental female was infected, hybrid breakdown seemed to be ameliorated (although never to the extent of broods where the parental male was also uninfected): F_1 $W \times W$ virgins produced larger clutches and lower F_2 mortality than $U \times W$ virgins (Table 4). This result suggests that C-Wolbachia may be able to rescue R-Wolbachia imprinted sperm. This could indicate that these Wolbachia are closely related (Bourtzis *et al.* 1998) or that they are the same. In the latter case, the hybrid breakdown effect observed could still be obtained if the symbiont densities in the two strains were different (the R strain having the highest density).

Is there a relationship between the cytoplasmic incompatibility and hybrid breakdown phenotypes?

Our results show that the presence of Wolbachia in R-males is associated with reproductive incompatibility induction expressed both as partial CI (less female biased sex ratios due to increased male production) and HB (increased mortality among broods of F_1 $U \times W$ virgin females). Provided a certain infection threshold is reached (Turelli 1994), HB may result in population replacement through a similar process to that of CI in that it reduces the fecundity of $U \times W$ females – at least with respect to male production. However, this hypothesis should be formally tested by means of theoretical simulations. In order to understand the evolution of HB it is also important to determine whether this phenotype is a property of the host, of the symbiont, or of the interaction.

The degree of CI expression in different populations of the same species may vary (see, for example, Hoffmann & Turelli 1988) or may not be expressed at all (see, for example, Hoffmann *et al.* 1996) – even if the

symbionts in the female retain the ability to rescue imprint from other *Wolbachia* strains (Merçot & Poinso 1998). Differential bacteria densities have been implicated in the level of CI expression (Clancy & Hoffmann 1998; Sinkins *et al.* 1995; Breeuwer & Werren 1993; Perrot-Minnot & Werren 1999). However, other host and/or bacteria factors cannot always be excluded in explaining different levels of CI (Bourtzis *et al.* 1996). Partial CI in both the tomato strain of *T. urticae* (Breeuwer 1997) and in the R-strain (Table 1) of *T. urticae* could be the result of low densities of infection, but what about HB? Could female aneuploidy be the consequence of 'leakage' at the imprinting stage of incompatibility induction due to lower densities of the symbiont? Cytological studies in *D. simulans* have shown that, in this species, reduced densities of infection result in decreased numbers of infected sperm cysts per male but not in overall reduced densities of the symbiont per sperm cyst (Bressac & Rousset 1993). If this is the general case, 'leakage' cannot explain HB: female embryos either develop from eggs fertilized by unimprinted sperm, or fail to develop because they result from eggs fertilized by imprinted sperm. Cytological studies are essential in order to elucidate the details of 'HB' and its relation with the CI phenotype.

Effect of *Wolbachia* on clutch size

An important parameter when modeling the dynamics of *Wolbachia* infections in a host population is whether or not the infection carries a cost for the infected female (Turelli 1994). In this respect, our result showed that, infected females generally produce smaller clutches than uninfected females, and this is true for both the R (Table 1) and C (Table 3) strains. This result suggests a cost of harboring the symbiont. In fact, decreased fecundity of infected females has been previously reported in infections by both cytoplasmic incompatibility-inducing *Wolbachia* (*e.g.*, Hoffmann & Turelli 1988; Hoffmann *et al.* 1990; Stevens & Wade 1990) and some parthenogenetic-inducing *Wolbachia* (*e.g.* Stouthamer & Luck 1993). However, infections of Australian and of Indo-Pacific populations of *D. simulans* do not have detectable effects on host fecundity (Hoffmann *et al.* 1996; Poinso & Merçot 1997). If, in accordance with our F1 results, there is a cost to infected spider mite females included in this study, why is this result not repeated among broods of virgin females (Tables 2 and 4)? One possible explanation is that the cost is only associated with the production of fertilized eggs (daughters), the *Wolbachia*-transmitting sex.

***Wolbachia* infections as a reproductive isolating mechanism**

Reproductive incompatibility between populations or strains of spider mites is a frequent finding, and it is interesting to ask why this is so. If populations are allopatric the appearance of reproductive isolation may be incidental. However, if populations are sympatric, for example living on two different host plants, reproductive isolation probably evolved in order to maintain an adapted genome. Evolution of reproductive isolation may therefore be considered adaptive (see Dieckmann & Doebeli 1999; Kondrashov & Kondrashov 1999). So far, verbal models have argued that the contribution of *Wolbachia* to a sympatric speciation process is likely to be restrictive (Werren 1998), probably because reproductive isolation has not been

considered as a desired trait.

At least two non-mutually exclusive possibilities are conceivable for the evolution of reproductive isolation in sympatry: one is that reproductive isolation arises directly as a by-product of adaptation to the new habitat (e.g. a novel host plant); while the other is that an isolating mechanism must evolve separately. One study in spider mites investigated the former possibility (Fry 1999). In that particular case adaptation to a novel host plant did not result in reproductive isolation. However, Overmeer (1966) showed that, as a result of selection for resistance to a pesticide, selected and unselected lines became reproductively isolated, while pesticide lines remained compatible among themselves. Thus the possibility that reproductive isolation can arise as a by-product of the adaptation-selection process itself should not be excluded. *Wolbachia* symbionts may provide an isolating mechanism for the situations where adaptation itself does not result in reproductive isolation. Adapted genomes that are reproductively isolated from non-adapted genomes, for example by being associated with a new incompatibility type *Wolbachia*, will persist. Adapted genomes, which are not associated with an isolating mechanism, will be diluted through recombination (Dieckmann & Doebeli 1999; Kondrashov & Kondrashov 1999). Of course natural selection is expected to favor ever-stronger degrees of incompatibility (re-enforcement) and ultimately pre-zygotic isolating mechanisms.

In conclusion, we suggest that *Wolbachia* could provide a reproductive isolation mechanism in a sympatric speciation process. Our results provide only partial evidence for this hypothesis because the two strains used are allopatric since they originate from two different greenhouses. However, the results in this paper clearly show that *Wolbachia* can serve as an isolating mechanism in *T. urticae*.

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