***Wolbachia*-induced 'hybrid breakdown' in the two-spotted spider mite *Tetranychus urticae* Koch**

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The most common post-zygotic isolation mechanism between populations of the phytophagous mite *Tetranychus urticae* is 'hybrid breakdown', 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 in their hosts: uninfected (U) females become reproductively incompatible with infected (W) males. We report on the effect of *Wolbachia* infections in intra- and interstrain crosses on (i) F₁ mortality and sex ratios (a test for cytoplasmic incompatibility), and (ii) the number of haploid offspring and mortality in clutches of F₁ virgins (a test for hybrid breakdown). U × W crosses within the rose strain exhibited partial cytoplasmic incompatibility. More interestingly, F₂ males suffered increased mortality, a result identical to the hybrid breakdown phenomenon. The experiments were repeated using females from the cucumber strain. In interstrain U × W and U × U crosses, hybrid breakdown was much stronger in the former (80 versus 26%). This is the first report of a *Wolbachia* infection causing a hybrid breakdown phenotype. Our results show that *Wolbachia* infections can contribute to reproductive incompatibility between populations of *T. urticae*.

Keywords: *Wolbachia*; hybrid breakdown; spider mites; reproductive isolation; cytoplasmic incompatibility; sympatric speciation

1. INTRODUCTION

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, 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 harbouring *Wolbachia* of a different type or strain. Cytoplasmic incompatibility 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 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 & Tokioka 1996). *Tetranychus 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, which is termed 'hybrid breakdown', is the most common. A test of hybrid breakdown consists of scoring the mortality of broods of F₁ virgin females. Post-zygotic isolation may be bidirectional but unidirectional incompatibilities are more frequently reported. This has led several authors to hypothesize about the role of nucleocytoplasmic 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 diploaneuploid embryos in explaining the appearance of F₁ females in U × W crosses:

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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 cytoplasmic incompatibility in *D. simulans* (Callaini *et al.* 1997).

One way of testing this hypothesis is to allow F_1 virgin females from $U \times W$ crosses to oviposit and compare the mortality among their F_2 offspring with the F_2 mortality of F_1 virgins from crosses between uninfected females and uninfected males (hereafter $U \times U$ crosses). If females produced in $U \times W$ matings are surviving aneuploids then they will produce both normal and aneuploid eggs, leading to increased F_2 mortality. This 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 in both the F_1 (typical cytoplasmic incompatibility) and among the haploid F_2 (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 ('intrastrain' crosses). Crosses were set up in all combinations between infected and uninfected individuals and resulting F_1 mortality and sex ratios were analysed. Furthermore, F_1 virgin females from all crosses were collected and tested for hybrid breakdown (i.e. they oviposited and the subsequent mortality of their F_2 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 cytoplasmic incompatibility and hybrid breakdown.

2. MATERIAL AND METHODS

(a) 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 in our laboratory on detached leaves of the common bean (*Phaseolus vulgaris*, variety 'Arena') in climate rooms (23 °C, 60–80% relative humidity and 16 L: 8 D photoperiod). At the time of these experiments both strains had been in the laboratory for more than two 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 eight months (*ca.* 16 generations) until the crossing experiments.

Wolbachia infection in individual adult females was determined with a PCR using *ftsZ* *Wolbachia*-specific primers (Holden *et al.* 1993). 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.

(b) 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 *ca.* 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.

Males and females were collected as teleiochrysalids (in order 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 (diameter 1.5 cm). Intrastrain $RU \times RU$, $RU \times RW$, $RW \times RW$ and $RW \times RU$ and interstrain $CU \times RU$, $CU \times RW$, $CW \times RW$ and $CW \times RU$ matings were set up. Males were removed after 24 h and individual females were transferred to clean bean leaf discs (diameter 1.0 cm). Oviposition was scored for the first six days. The numbers of emerging adult females and males, and dead stages, were scored per leaf disc per female. Next, F_1 females were collected as teleiochrysalids and placed on clean bean leaf discs (diameter 1.5 cm) in groups of five or six sisters. Upon emergence, five sisters per parental female were individually transferred to fresh leaf discs. Females oviposited for six days. The numbers of dead stages and adult offspring were scored per leaf disc per female.

(c) Statistical analysis

The following variables were analysed for the F_1 results: clutch size (number of F_1 females + F_1 males + aborted eggs + other dead stages), F_1 mortality ((number of aborted eggs + other dead)/(clutch size)), F_1 sex ratio (number of males/(number of females + number of males)) and number of F_1 males and females. Analyses were conducted separately for intra- and interstrain crosses and were aimed at detecting differences between crosses with different combinations of infected and uninfected individuals. Clutch size, number of F_2 males and F_2 mortality were analysed for the F_2 results. Analyses were conducted separately for intra- and interstrain crosses, as above.

The normality of the data was estimated graphically by means of quartile plots and histograms. Mortality data were arcsine transformed (arcsine $\sqrt{\text{mortality}}$) for data from crosses within the R strain or arcsine ($\sqrt{(\text{total dead} + 3/8)/(\text{clutch size} + 3/4)}$) for data from $C \times R$ 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), as these variables are not truly independent of 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.

The sex ratio was always significantly affected by cross type. However, differences in the sex ratio can arise due to changes in

the number of males or females or both. Therefore, the mean values of the numbers of F_1 males and females were analysed by univariate ANOVAs followed by Tukey post-hoc pairwise comparison tests so that the changes underlying shifts in the sex ratio could be identified.

Finally, the numbers of F_2 males produced by virgin F_1 females were included because they provide estimates of the actual numbers of surviving individuals. However, they were not analysed statistically because their effect on the total variance had already been taken into account in the overall MANOVAs.

3. RESULTS

(a) *Effects of Wolbachia on reproductive incompatibility within the R strain*

The results of crosses between uninfected and infected R mites are presented in table 1. The MANOVA detected a significant effect of crossing treatment (Wilk's $\lambda = 0.514$, $F_{9,623} = 21.8$ and $p < 0.001$) on the observed variance. The variables that were significantly affected were clutch size and sex ratio ($F_{3,258} = 45.6$ and $p < 0.001$ and $F_{3,258} = 20.9$ and $p < 0.001$, respectively) (table 1). A Tukey post-hoc pairwise comparison test following univariate ANOVA on the sex ratio ($F_{3,256} = 20.5$ and $p < 0.001$) revealed that the least female-biased sex ratio was 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. A less female-biased sex ratio was associated with an increase in male production, but not with an increase in F_1 mortality (table 1).

With respect to the F_2 results (presented in table 2), the MANOVA revealed a significant effect of treatment on the observed variance (Wilk's $\lambda = 0.557$, $F_{6,392} = 22.2$ and $p < 0.001$) and this effect was explained by mortality alone ($F_{3,197} = 47.9$ and $p < 0.001$). Female virgins from $W \times W$ crosses showed the lowest mortality among their F_2 haploid broods, which was associated with a slight increase in the number of males produced. However, the most striking effect was that of virgins from $U \times W$ crosses: their clutches had the highest F_2 mortality in association with a dramatic decrease in the number of F_2 males (table 2). Thus, the presence of *Wolbachia* in R males resulted in hybrid breakdown if female mates were uninfected.

(b) *Effects of Wolbachia on reproductive incompatibility 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 (Wilk's $\lambda = 0.442$, $F_{9,452} = 20.1$ and $p < 0.001$) on the observed variance. The variables which were significantly affected were clutch size and sex ratio ($F_{3,188} = 13.9$ and $p < 0.001$, and $F_{3,188} = 53.2$ and $p < 0.001$, respectively). In the case of the sex ratio, U females produced more males and fewer females than W females and, consequently, showed a less female-biased sex ratio (table 3). These results could suggest that the presence of *Wolbachia* in C females is associated with increased daughter production. Two, known, *Wolbachia*-associated effects result in an 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 no higher than that of broods from uninfected females, and (ii) the possibility of parthenogenetic production of females because infected F_1 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 F_2 results are considered (table 4). The MANOVA of the F_2 results revealed a significant effect of treatment on the observed variance (Wilk's $\lambda = 0.324$, $F_{6,340} = 42.9$ and $p < 0.001$) for both clutch size and mortality. Univariate ANOVAs and pairwise comparisons were performed on clutch size ($F_{3,172} = 16.0$ and $p < 0.001$) and on F_2 mortality ($F_{3,171} = 87.6$ and $p < 0.001$). F_2 mortality among haploid clutches of the F_1 hybrids increased dramatically when parental males were infected: virgin females from $U \times W$ and $W \times W$ crosses showed the highest F_2 mortality among their offspring (table 4). Thus, the presence of *Wolbachia* in R males was associated with hybrid breakdown induction. Furthermore, virgin females from $U \times W$ crosses 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.

4. 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 the haploid offspring of $RU \times RW$, $CU \times RW$ and $CW \times RW$ females. This effect is similar to that which has been described as hybrid breakdown in the literature on spider mites. This result suggests it may be essential to extend experimental studies to the F_2 generation before concluding on the absence of an effect of *Wolbachia* on reproductive incompatibility.

(a) *The effect of Wolbachia in R 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 the sex ratio (proportion of males) due to higher male production in $U \times W$ crosses and is not associated with an increase in F_1 mortality (table 1). These results contrast with Breeuwer (1997), who found that sex ratios were more male biased due to female mortality in a tomato strain of *T. urticae*, but are in accordance with the cytoplasmic incompatibility phenotype described in *Nasonia* wasps (Breeuwer & Werren 1990).

Table 1. *F₁ female and male production, clutch sizes, mortality and sex ratio for intrastain crosses (R female × R male)*

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

cross female × male	clutch size*		mortality (frequency)	sex ratio* (proportion of males)	number of F ₁ females	number of F ₁ males
	<i>n</i>	$\mu \pm \text{s.e.}$	$\mu \pm \text{s.e.}$	$\mu \pm \text{s.e.}$	$\mu \pm \text{s.e.}$	$\mu \pm \text{s.e.}$
U × U	62	55.5 ± 0.9 ^b	0.10 ± 0.02	0.31 ± 0.01 ^b	33.8 ± 1.1 ^b	15.1 ± 0.7 ^b
U × W	79	52.9 ± 1.0 ^b	0.14 ± 0.02	0.41 ± 0.02 ^c	26.9 ± 1.0 ^a	19.2 ± 1.0 ^c
W × W	63	41.8 ± 1.4 ^a	0.11 ± 0.01	0.30 ± 0.01 ^{ab}	26.3 ± 1.1 ^a	10.8 ± 0.6 ^a
W × U	59	42.4 ± 0.8 ^a	0.10 ± 0.02	0.24 ± 0.02 ^a	29.0 ± 1.0 ^a	9.1 ± 0.6 ^a

Table 2. *F₂ recombinant haploid production, clutch sizes and mortality of F₁ virgin females from intrastain crosses (R female × R male)*

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

F ₁ female's female × male	clutch size		mortality* (frequency)	number of F ₂ males
	<i>n</i>	$\mu \pm \text{s.e.}$	$\mu \pm \text{s.e.}$	$\mu \pm \text{s.e.}$
U × U	56	44.2 ± 1.2	0.24 ± 0.04 ^b	33.3 ± 1.8
U × W	60	41.1 ± 1.2	0.57 ± 0.02 ^c	17.8 ± 1.0
W × W	38	42.2 ± 1.2	0.07 ± 0.01 ^a	39.3 ± 1.3
W × U	48	45.3 ± 1.2	0.24 ± 0.04 ^b	33.9 ± 1.8

The increase in male production in U × W crosses (table 1) could arise from two different processes: (i) fewer eggs are fertilized than in U × 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 × RU and RW × RW crosses) produced similar numbers of F₁ males and 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 × W females (table 2) indirectly supports the second hypothesis. The reasoning is similar to that which was proposed by Breeuwer (1997) and consistent with the cytology of cytoplasmic incompatibility (Callaini *et al.* 1997). Production of aneuploid females may occur when haplodization of U × 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 fibres can attach anywhere in the chromosome. Consequently, fragments of paternal chromosomes may still segregate into daughter nuclei leading to the production of aneuploid cells. Resulting F₁ U × 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 produce 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 F₂ mortality pattern that is identical to that which has been termed hybrid breakdown in mite literature (reviewed by De Boer 1985). Cytological analysis of haploid eggs from F₁ U × W virgins is necessary to confirm or dismiss this hypothesis: in the former case, aneuploid F₂ eggs should be observed.

The similarity between the F₂ mortality pattern observed among broods of F₁ U × 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 × RW) and between infected C females and uninfected R males (CW × RU) in this analysis. These crosses tell us whether cucumber *Wolbachia* can rescue rose *Wolbachia*-imprinted sperm.

Although cytoplasmic incompatibility was not detected in crosses between CU or CW females and RW males, F₁ CU × RW and CW × 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): F₁ W × W virgins produced larger clutches and lower F₂ mortality than U × W virgins (table 4). This result suggests that cucumber *Wolbachia* may be able to rescue rose *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).

(b) Is there a relationship between cytoplasmic incompatibility and hybrid breakdown?

Our results show that the presence of *Wolbachia* in R males is associated with reproductive incompatibility

Table 3. *F₁ female and male production, clutch sizes, mortality and sex ratio for intrastain crosses (C female × R male)*

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

cross female × male	clutch size*		mortality (frequency)	sex ratio* (proportion of males)	number of F ₁ females	number of F ₁ males
	<i>n</i>	$\mu \pm \text{s.e.}$	$\mu \pm \text{s.e.}$	$\mu \pm \text{s.e.}$	$\mu \pm \text{s.e.}$	$\mu \pm \text{s.e.}$
U × U	29	59.0 $\pm$ 1.4 ^b	0.12 $\pm$ 0.02	0.62 $\pm$ 0.03 ^c	20.0 $\pm$ 1.7 ^a	31.6 $\pm$ 1.6 ^b
U × W	28	57.4 $\pm$ 1.5 ^b	0.16 $\pm$ 0.03	0.66 $\pm$ 0.03 ^c	16.8 $\pm$ 1.5 ^a	31.8 $\pm$ 1.9 ^c
W × W	72	50.0 $\pm$ 1.0 ^a	0.07 $\pm$ 0.01	0.43 $\pm$ 0.01 ^b	26.4 $\pm$ 0.8 ^b	20.2 $\pm$ 0.9 ^a
W × U	63	49.0 $\pm$ 1.1 ^a	0.06 $\pm$ 0.01	0.34 $\pm$ 0.02 ^a	30.3 $\pm$ 1.0 ^b	15.3 $\pm$ 0.8 ^a

Table 4. *F₂ recombinant haploid production, clutch sizes and mortality of F₁ virgin females from intrastain crosses (C female × R male)*

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

F ₁ female's parents female × male	clutch size		mortality* (frequency)	number of F ₂ males
	<i>n</i>	$\mu \pm \text{s.e.}$	$\mu \pm \text{s.e.}$	$\mu \pm \text{s.e.}$
U × U	56	42.4 $\pm$ 1.6 ^b	0.21 $\pm$ 0.04 ^a	34.6 $\pm$ 2.3
U × W	60	28.4 $\pm$ 2.5 ^a	0.80 $\pm$ 0.03 ^b	6.1 $\pm$ 0.9
W × W	38	45.8 $\pm$ 1.4 ^b	0.67 $\pm$ 0.02 ^b	15.2 $\pm$ 1.0
W × U	48	47.7 $\pm$ 2.2 ^b	0.26 $\pm$ 0.03 ^a	37.4 $\pm$ 2.2

induction expressed as both partial cytoplasmic incompatibility (less female-biased sex ratios due to increased male production) and hybrid breakdown (increased mortality among broods of F₁ U × W virgin females). Provided a certain infection threshold is reached (Turelli 1994), hybrid breakdown may result in population replacement through a process similar to that of cytoplasmic incompatibility since it reduces the fecundity of U × 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 hybrid breakdown 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 cytoplasmic incompatibility 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 symbionts in the female retain the ability to rescue an imprint from other *Wolbachia* strains (Merçot & Poinot 1998). Differential bacteria densities have been implicated in the level of cytoplasmic incompatibility expression (Breeuwer & Werren 1993; Sinkins *et al.* 1995; Clancy & Hoffmann 1998; Perrot-Minnot & Werren 1999). However, other host and/or bacteria factors cannot always be excluded in explaining different levels of cytoplasmic incompatibility (Bourtzis *et al.* 1996). Partial

cytoplasmic incompatibility in both the tomato (Breeuwer 1997) and R strains (table 1) of *T. urticae* could be the result of low densities of infection, but what about hybrid breakdown? 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 hybrid breakdown: 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 hybrid breakdown and its relationship with the cytoplasmic incompatibility phenotype.

(c) *Effect of Wolbachia on clutch size*

An important parameter when modelling 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 results showed that infected females generally produced smaller clutches than uninfected females and this was true for both the R (table 1) and C (table 3) strains. This result suggests a cost of harbouring 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 Indo-Pacific populations of *D. simulans* do not have detectable effects on host fecundity (Hoffmann *et al.* 1996; Poinot & Merçot 1997). If, in accordance with our F₁ results, there is a cost to the infected spider mite females included in this study, why is this result not repeated among broods of virgin females (tables 3 and 4)? One possible explanation is that the cost is only associated with the production of fertilized eggs (daughters), the *Wolbachia*-transmitting sex.

(d) *Wolbachia infection 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 (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 selected 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 situations where adaptation to a new habitat does not result in reproductive isolation. Adapted genomes that are reproductively isolated from non-adapted genomes, for example by being associated with new incompatibility-type *Wolbachia*, will persist. Adapted genomes that 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 favour stronger degrees of incompatibility (reinforcement) 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 were allopatric (they originated 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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