Wolbachia strengthens the match between pre-mating and early post-mating isolation in spider mites

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Authors' contributions

MC, SM, and FZ conceived and designed the experiments. MC and MB performed the choice and no-choice tests, respectively. MC and FZ analysed the data. Funding agencies did not participate in the design or analysis of experiments. MC and FZ wrote the manuscript with input from SM. All authors read and approved the final version of the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest with the content of this article.

Data availability

All datasets and R scripts are available at Zenodo (https://doi.org/10.5281/zenodo.11160702).

Abstract

Endosymbiotic reproductive manipulators are widely studied as sources of post-zygotic isolation in arthropods, but their effect on pre-zygotic isolation between genetically differentiated populations has garnered less attention. We tested this using two partially isolated populations of the red and green colour forms of *Tetranychus urticae*, either uninfected or infected with a *Wolbachia* strain inducing or not cytoplasmic incompatibility. We first investigated male and female preferences, and found that, in absence of infection, females were not choosy but all males preferred red-form females. *Wolbachia* effects were more subtle, with only the CI-inducing strain slightly strengthening colour-form based preferences. We then performed a double-mating experiment to test how incompatible matings affect subsequent mating behaviour and offspring production, as compared to compatible mating. Females mated with an incompatible male (infected and/or heterotypic) were more attractive and/or receptive to subsequent (compatible) matings, although analyses of offspring production revealed no clear benefit for this remating behaviour (*i.e.*, apparently unaltered first male sperm precedence). Finally, by computing the relative contributions of each reproductive barrier to total isolation, we showed that pre-mating isolation matches both host-associated and *Wolbachia*-induced post-mating isolation, suggesting that *Wolbachia* could assist speciation processes in this system.

Keywords:

Haplodiploidy, mate choice, reproductive interference, sperm precedence, cytoplasmic incompatibility, reinforcement.

Introduction

Understanding the evolution of reproductive barriers between taxa has long been a major focus of evolutionary biology (Coyne and Orr 2004). While speciation research has traditionally viewed species divergence as a process inevitably leading to full reproductive isolation (biological species concept; Mayr 1942), recent evidence has shown that partial isolation occurring along the speciation continuum (Stankowski and Ravinet 2021) can be reversible (Taylor et al. 2006; Bhat et al. 2014; Kearns et al. 2018), or may even be selected in some circumstances (Servedio and Hermisson 2020). Studying population pairs for which reproductive barriers are incomplete is of great value to understand these processes, as it can provide insight into which type of reproductive barrier is more likely to evolve first, then drive the evolution of others (Baack et al. 2015; Lackey and Boughman 2017). On the one hand late post-zygotic barriers leading to costly hybridization can evolve first (*e.g.*, in allopatry), then promote the evolution of pre- and/or early post-zygotic barriers at secondary contact (*i.e.*, reinforcement following the definition of Coughlan and Matute; 2020; but see Bank et al. 2012). On the other hand, by limiting gene flow, pre-zygotic barriers should lead to faster accumulation of genetic differences between populations in sympatry, thereby promoting the evolution of greater post-zygotic barriers (*e.g.*, Lackey and Boughman 2017). In addition, reproductive isolation may be driven not only by the genetics of the organisms themselves, but also by their endosymbionts (Brucker and Bordenstein 2012). This is especially true for endosymbionts that directly manipulate the reproduction of their hosts (Duron et al. 2008; Engelstädter and Hurst 2009; Brucker and Bordenstein 2012).

Wolbachia is a widespread endosymbiotic bacterium (Weinert et al. 2015) that manipulates its host reproduction in different ways to increase its own transmission and invasiveness (Werren et al. 2008; Engelstädter and Hurst 2009). The most common of such manipulations is cytoplasmic incompatibility (CI), a conditional sterility phenotype which results in embryonic mortality of offspring from crosses between infected males and uninfected females (or females infected with an incompatible strain; Shropshire et al. 2020). Although the contribution of *Wolbachia* to post-zygotic isolation has been extensively studied in different systems, its contribution to pre-zygotic isolation (both pre- and post-mating) between hosts has received comparatively less attention (see Shropshire and Bordenstein 2016; Bi and Wang 2020; Kaur et al. 2021), especially when acting alongside host genetic incompatibilities.

Theory predicts that *Wolbachia* could drive reinforcement between undifferentiated host populations (*i.e.*, females may evolve avoidance of incompatible males to escape CI; Champion de Crespigny et al. 2005; Telschow et al. 2005), but empirical studies have produced contrasting results, most of them showing no (or weak) evidence for CI-driven assortative mating (reviewed by Shropshire and Bordenstein 2016; Bi and Wang 2020). Such discrepancy could be explained by uneven abilities of hosts to detect *Wolbachia* infection in their mates (*e.g.*, *Wolbachia* may alter the chemical profiles of some host species only; Richard 2017; Fortin et al. 2018; Schneider et al. 2019), or because avoidance of CI might be more likely when the infection is associated with pre-existing host traits that can be used for mate recognition (Engelstädter and Telschow 2009). If such is the case, CI avoidance should be more commonly found between already differentiated populations. In line with this, the rare studies focusing on genetically differentiated hosts showed that pre-mating isolation was strengthened (possibly even caused) by *Wolbachia* infection (*e.g.*, Jaenike et al. 2006; Koukou et al. 2006; Miller et al. 2010); but see Shoemaker et al. 1999). Finally, *Wolbachia* infection may also

drive other forms of pre-zygotic isolation, including those occurring after mating. For instance, *Wolbachia* infection can have deleterious effects on the production of sperm (Snook et al. 2000), the transfer of fertile sperm (Lewis et al. 2011), the fertilization success (Bruzzese et al. 2021), and the receptivity to and/or the effectiveness of re-mating; (De Crespigny and Wedell 2006; Champion De Crespigny et al. 2008; Liu et al. 2014; He et al. 2018). However, to our knowledge, no study has specifically disentangled the relative role of *Wolbachia* from that of host genetic factors on different types of post-mating pre-zygotic barriers.

Tetranychus spider mites are an excellent system to address the interplay between host-associated and symbiont-induced incompatibilities (Cruz et al. 2021). Indeed, *Wolbachia* is ubiquitous in this genus (Breeuwer and Jacobs 1996; Gotoh et al. 2003; Xie et al. 2006; Zhang et al. 2013, 2016; Zélé et al. 2018a), and its effects have been widely studied in the two-spotted spider mite *T. urticae*. In this host species, the bacterium induces highly variable degrees of different types of CI (mortality or development as male of fertilized eggs in incompatible crosses; *e.g.*, Breeuwer 1997; Perrot-Minnot et al. 2002; Vala et al. 2002; Gotoh et al. 2007; Suh et al. 2015; Zélé et al. 2020; Wybouw et al. 2023), and has variable effects on pre-mating isolation (either no effect: Zhao et al. 2013b; Rodrigues et al. 2022; or avoidance of infected males by uninfected females: Vala et al. 2004). However, in spider mites, as in many other arthropod species, its contribution to post-mating pre-zygotic isolation has seldom been studied, which is at odds with the critical role that this symbiont may play in the speciation processes currently ongoing in this group.

Given the wide and overlapping distribution of many spider mite species (Migeon and Dorkeld 2023), as well as the high variability in genetic distances both between and within species (*e.g.*, Matsuda et al. 2018; Villacis-Perez et al. 2021), spider mites commonly suffer various degrees of reproductive isolation. In particular, there is ample evidence of variation in all possible post-zygotic reproductive barriers (zygote and juvenile hybrid mortality, hybrid sterility, hybrid breakdown), both between (Keh 1952; Helle and Van de Bund 1962; Hill and O'Donnell 1991) and within spider mite species (*e.g.*, Van de Bund and Helle 1960; de Boer 1982a,b; Sugasawa et al. 2002; Knegt et al. 2017; Cruz et al. 2021). Several studies also revealed variable post-mating pre-zygotic isolation in this group (*e.g.*, fertilization failure resulting from gametic or mechanical incompatibilities), as evidenced by a reduction in the production of female offspring in this system, because spider mites are arrhenotokous haplodiploids (haploid males develop from unfertilized eggs and diploid females from fertilized eggs; Helle and Bolland 1967). Hence, whereas no female offspring are produced in crosses between well-formed species (*e.g.*, Helle and Van de Bund 1962; Hill and O'Donnell 1991; Chain-Ing and Sheuan-Ping 1995; Clemente et al. 2016, 2018), male-biased sex ratios are often reported in crosses between genetically differentiated 'forms' of the same species or even between genotypes of the same form (*e.g.*, Gotoh et al. 1993; Navajas et al. 2000; Sugasawa et al. 2002; Auger et al. 2013; Cruz et al. 2021; Villacis-Perez et al. 2021). In addition, because spider mites exhibit first male sperm precedence (only the first male that mates with a female sires all the offspring; Helle 1967; Rodrigues et al. 2020), females usually cannot restore their fitness through re-mating. Therefore, post-mating incompatibilities are particularly costly and should select for earlier pre-zygotic barriers through reinforcement. Yet, highly variable degrees of pre-mating isolation can be found both between (Sato et al. 2014, 2016; Clemente et al. 2016; Sato and Alba 2020) and within species (*e.g.*, Murtaugh and Wrensch 1978; Gotoh et al. 1993).

To improve our understanding of the role that *Wolbachia* can play in ongoing speciation processes of its hosts, we aimed at disentangling the relative contributions of *Wolbachia* and host genetic factors to the strength of both pre- and post-mating pre-zygotic barriers between two colour forms, green and red, of the two spotted spider mite *T. urticae* (sometimes also referred to as *T. urticae* and *T. cinnabarinus*; Xie et al. 2006; Auger et al. 2013; Lu et al. 2017, 2018). A previous study focusing on the joint effects of *Wolbachia*-induced and host-associated post-mating incompatibilities between populations of these two forms revealed full reproductive isolation due to late post-zygotic barriers (hybrid sterility and hybrid breakdown) that were independent of *Wolbachia* infection (Cruz et al. 2021). However, this study also revealed partial and asymmetrical earlier post-mating barriers (pre- and/or post-zygotic), resulting from a combination of host-associated and *Wolbachia*-induced incompatibilities (Cruz et al. 2021). Host genetic incompatibilities led to an increased proportion of haploid sons to the detriment of diploid daughters ('male development' or MD-type incompatibility, likely due to fertilization failure), whereas incompatibilities attributed to *Wolbachia* infection in males were expressed as an increased embryonic mortality of daughters ('female mortality' or FM-type CI). Furthermore, both types of incompatibility had additive effects and acted in the same direction of crosses (Cruz et al. 2021), which hinted at a possible role of *Wolbachia*-induced incompatibilities in host population divergence and subsequent evolution of intrinsic reproductive barriers, as found in *Nasonia* wasps (Bordenstein et al. 2001).

Here, we first performed male and female choice tests to determine their preference for infected or uninfected mates from their own or a different colour-form population (*i.e.*, test for pre-mating isolation). Second, we used a no-choice test to determine the effect of female mating history (virgin or previously-mated with a compatible *vs* incompatible male) on their mating behaviour, and to investigate whether eggs are more likely fertilized by compatible than incompatible sperm (*i.e.*, test for 'homotypic' sperm precedence). Finally, we used data gathered throughout all experiments stemming from this study and the previous one (Cruz et al. 2021) to estimate the relative contribution of each measured host-associated or *Wolbachia*-induced individual barrier to total reproductive isolation in this system.

Materials and Methods

Spider mite populations

Two populations of spider mites, each belonging to a different colour form of *T. urticae* ('red' or 'green'), and either infected or uninfected with *Wolbachia*, were used in this study. These populations were previously used to assess post-mating isolation caused by both host-associated incompatibilities (HI) and *Wolbachia*-induced reproductive barriers (Cruz et al. 2021; see also Zélé et al. 2018 for field collection, and Zélé et al. 2020 for the effects of *Wolbachia* following laboratory maintenance). Briefly, the *Wolbachia*-infected population 'Ri' and its uninfected counterpart 'Ru' ('Ri1' and 'Ru1' in Cruz et al. 2021) belong to the red form of *T. urticae*, whereas the *Wolbachia*-infected population 'Gi' and the uninfected population that derived from it, 'Gu', belong to the green form of *T. urticae*. The Ru and Gu populations used in the present study were obtained from the antibiotic treatments performed for Experiments 2 and 1 in Cruz et al. (2021), respectively. All populations were subsequently reared under the same standard laboratory conditions (24±2ºC, 16/8h L/D) at high

numbers (>1000 females per population) in mite-proof cages containing bean plants (*Phaseolus vulgaris*, cv. Contender seedlings obtained from Germisem, Oliveira do Hospital, Portugal). All behavioural observations were conducted during daytime at constant room temperature (25 ± 2°C).

Mate preference and behaviour of males and females in choice tests

To determine whether spider mites discriminate between mates to avoid *Wolbachia*-induced and/or host-associated incompatibilities, individual males and females were provided two mates from different populations and/or infection statuses. All combinations of choice tests performed are described in Table 1. To obtain a large number of individuals of similar age, age cohorts were created for each population twelve to fourteen days prior to the onset of each mate choice observation (*i.e.*, each cohort was used for two to three sequential days of observation). To this aim, 50 mated females or 50 virgin females (to obtain cohorts of females or males, respectively) from each population laid eggs during 3 days on detached bean leaves placed on water-soaked cotton in petri dishes under standard laboratory conditions (24±2ºC, 16/8h L/D). Ten to twelve days later, female and male deutonymphs undergoing their last moulting stage (*i.e.*, teleiochrysalids) were randomly collected from each of the female and male cohorts, respectively, and placed separately on bean leaf fragments (*ca.* 9cm^2) to obtain virgin adult females and males of similar age two days later. As conversely to females, males cannot easily be identified based on their body colouration, they were painted before each observation with either blue or white water-based paint (randomized across treatments) using a fine brush. Previous experiments showed no effect of this paint on spider mite mate choice and behaviour (Rodrigues et al. 2017, 2022). Subsequently, a pair of virgin mates was installed on a 0.4cm^2 leaf disc (called 'arena' hereafter) and each observation started when the focal individual (a virgin female or a virgin male) was introduced to the arena. The colour of the mate that first copulated with each focal individual was registered, and later assigned to the corresponding treatment (thus ensuring that the observer was blind to the treatment to which mites belonged). Simultaneously, the time until the beginning of copulation ('latency to copulation') and its duration ('copulation duration') were recorded using an online chronometer (http://online-stopwatch.chronme.com/). Each observation lasted until the end of a first copulation or for 30 minutes if no mating occurred. Male and female choice tests were performed separately, each with one replicate of each treatment observed simultaneously per session and four sessions of observations carried out per day. In total, 60 replicates per treatment were obtained over the course of 15 days for each of the two tests.

(Re)mating behaviour and offspring production in the no-choice test

Mating behaviour in the first mating event

Spider mites may possess pre-zygotic mechanisms other than mate discrimination to avoid and/or reduce the cost of incompatibilities. For instance, an incompatible mate could be rejected after a copulation has started (*e.g.*, in *Littorina* snails; Rolán-Alvarez et al. 1999). Moreover, as copulations lasting for less than 30 seconds can be insufficient to fully fertilize a spider mite female (Potter and Wrensch 1978; Satoh et al. 2001), shorter copulations might explain the excessive production of male offspring (*i.e.*, arising from unfertilized eggs) to the detriment of female offspring (*i.e.*, arising from fertilized eggs) previously observed in crosses between green females and red males (Cruz et al.

2021). To test whether such post-copulatory mechanisms of avoidance of incompatibilities occur in spider mites, we performed a no-choice test, where the mating behaviour of virgin females placed with a single male was observed. Given the workload involved in this experiment, we only performed the crosses allowing to test for the single and combined effects of host-associated and *Wolbachia*-induced incompatibility, along with their respective controls (*cf.* Table 2). Also, because only few individuals could be tested per day in this experiment, producing age cohorts was not necessary. Instead, male and female teleiochrysalids were directly sampled from the base populations two days prior to observation, and isolated on bean leaf fragments to ensure their virginity. For each treatment, one male and one female were installed together on a 0.5cm^2 bean leaf disc and observed for 60 minutes. During that time, multiple mating could occur. Thus, in addition to the mating propensity (*i.e.*, the probability that mating occurred at least once), copulation frequency (*i.e.*, the number of copulations during the observation period) was also registered. The latency to the first copulation and the duration of each copulation occurring during the observation period were recorded as in the mate choice experiment. The cumulative copulation duration of each couple was subsequently computed. At the end of the observation period, females for which at least one copulation occurred were individually placed on a 2cm^2 bean leaf disc and kept for the next step (*cf.* below), while non-mated females and all males were discarded.

Mating behaviour in the second mating event

In species with first-male sperm precedence such as *T. urticae* (*i.e.*, the first male that had mated with a female sires all of her offspring; Rodrigues et al., 2020), females usually have low receptivity to a second mate (Clemente et al. 2016). However, if the first copulation is interrupted or (at least partially) ineffective, females may show increased receptivity to second matings that could effectively contribute to fertilization (Helle 1967; Clemente et al. 2016; Costa et al. 2023). To test this, females for which at least one copulation occurred during the first mating event were placed with a second compatible male 24 hours later (*cf.* Table 2) and their mating behaviour was recorded. Behavioural observations were carried out for 60 minutes as in the first mating event. The mating propensity, mating frequency, latency to first copulation and cumulative copulation duration with the second male were simultaneously registered. At the end of the observation period, males were discarded and females were kept individually on 2 cm^2 bean leaf discs placed on water-soaked cotton in petri dishes in a climatic chamber (25 ± 2°C, 60% RH, 16/8 h L/D). Given the workload and the multiplicity of tasks involved in this experiment, only 9 couples were observed simultaneously per session of observation, corresponding to one or two replicates per treatment. Four sessions of observation were performed per day (hence 6 replicates of each treatment per day), each day corresponding to an experimental 'block'. In total, 19 blocks, each separated by 3 days, were performed to obtain *ca.* 100 replicates per treatment.

Offspring production and strength of post-mating incompatibilities

To test whether the second copulation with a compatible male could restore female offspring production, the offspring produced over 3 days of oviposition by females mated with either a single or two different males was compared, and female mortality during that period also registered. The number of unhatched eggs was counted 6 days later (day 9), and the numbers of dead juveniles, adult males and females were counted 3 and 6 days later (days 12 and 15). Then, to determine the proportion of offspring affected by host-associated MD-type incompatibility (*i.e.*, "Male Development"), and/or

Wolbachia-induced FM-type incompatibility (*i.e.*, "Female Mortality"), we computed two indexes as fully described in Cruz et al. (2021): the MD_{corr} index, which calculates the overproduction of sons in the brood, and the FM_{corr} index, which calculates the embryonic mortality of fertilized offspring (*i.e.*, only females in haplodiploids), both relative to the control crosses (thereby accounting for background variation). Finally, as in Cruz et al. (2021), we also computed the proportion of F1 females over the total number of eggs (FP) to determine the combined effect of FM- and MD-type incompatibilities on the total proportion of daughters in each cross. Raw data are shown in the Supplementary Figure S1.

Strength and contribution of each reproductive barrier to total isolation

Strength of reproductive isolation for each reproductive barrier (RI_n)

To estimate the strength of pre- or post-mating reproductive barriers identified for each type of cross within and between the green- and red-form populations, we used a combination of the pre-mating data obtained in the present study and the post-mating data obtained in the previous study (Cruz et al. 2021), respectively. All reproductive barriers found to play a role in reducing gene flow among the spider mite populations were considered: mate preference (RI_1), fertilization failure (RI_2), hybrid inviability (RI_3), hybrid sterility (RI_4), and hybrid breakdown (RI_5). As we found no evidence for homotypic sperm precedence in the no-choice test (*cf.* Results), this barrier was not considered. Similarly, female choice data were not used when computing RI_1, as females overall showed no mate preferences in the choice test (*cf.* Results).

To determine the strength of pre-mating isolation (RI_1), we applied a sexual isolation index, which varies between zero and one, to the male choice data. This index, adapted from Bateman (1949) and Merrell (1950) by Malogolowkin-Cohen et al. (1965), is given by:

$$RI_{1(x)} = \frac{(n_{xx} - n_{yx})}{(n_{xx} + n_{yx})}$$

where n_{xx} is the number of copulations observed between females and males of a population x, and n_{yx} is the number of copulations observed between females of a population y and males of the population x. As RI_1 represents the degree to which a population x is isolated from a population y due to mating preferences, it was set to 0 in the case of preference for heterotypic mates (*i.e.,* no negative impact on gene flow).

To determine the strength of post-mating barriers, we used the data published in Cruz et al. (2021), as late reproductive barriers (*i.e.*, hybrid sterility and hybrid breakdown) were not measured here. Moreover, earlier post-mating barriers (fertilization failure and hybrid inviability) have been estimated more precisely in the previous study than in the current one (*i.e.*, larger sample sizes). For fertilization failure (RI_2) and hybrid inviability (RI_4), we used the median values of the MD_{corr} and FM_{corr} indexes, which correspond to the percent increase in non-fertilized eggs and in embryonic mortality of fertilized eggs, respectively (*cf. 'Offspring production and strength of post-mating incompatibilities*' above). For hybrid sterility (RI_5) and hybrid breakdown (RI_6), we computed the percent decrease in ovipositing F1 females and increase in embryonic mortality of F1 females' eggs relative to compatible crosses, respectively.

Contribution of each reproductive barrier (C_n) *to total isolation* (T)

We employed a method previously adapted from Coyne and Orr (1989, 1997) by Ramsey and colleagues (2003), in which total (cumulative) reproductive isolation between two populations or

species is computed as a multiplicative function of the strength of each reproductive barrier (RI_n; see above), so that the contribution of each barrier to reducing gene flow at a stage n in life history is calculated as:

$$C_n = RI_n \left(1 - \sum_{i=1}^{n-1} C_i\right)$$

Thus, a given reproductive barrier eliminates gene flow that has not been prevented by earlier barriers, and for m reproductive barriers, total reproductive isolation is given by:

$$T = \sum_{i=1}^{m} C_i$$

Statistical analyses

Analyses were carried out using the R statistical software (v3.6.1). The general procedure for building all statistical models is detailed in the Supplementary Tables S1 and S2. Time-to-event data (latency to copulation and copulation duration) were analysed using Cox proportional hazards mixed models (*coxme* procedure; coxme package), a non-parametric method that does not assume any particular error distribution (Crawley 2007). All other data were analysed using generalized linear mixed models (*glmmTMB* procedure; glmmTMB package; Brooks et al. 2017). Proportion data were computed either as binary response variables (*e.g.*, mated or not, chosen mate) or using a concatenated response variable binding the number of successes and failures with the function *cbind* to account for the number of eggs in the analyses of female proportion (*i.e.*, number of daughters *vs*. number of eggs – number of daughters). For the corrected variables MD_{corr} and FM_{corr}, which are continuous variables bounded between 0 and 1, we weighted each individual datum by the number of observations (*i.e.*, a "weights" argument was added to the models). These data were subsequently analysed with a binomial or (zero-inflated) betabinomial error distribution when errors were overdispersed. Count data were analysed with a Poisson error distribution, and continuous data with a Gaussian, except for daily oviposition which was Box-Cox transformed (λ=0.549) to improve normality (Crawley 2007) and analysed with a zero-inflated Gaussian.

For the analyses of each response variable of the choice tests, the 'type' of the focal individual (*i.e.*, combination of population and infection status), and either the combination of provided mates (for the analyses of mating propensity and mate preference) or the chosen mate (for the analyses of latency to copulation and copulation duration), were fit as fixed explanatory variables, whereas the day and session (nested within day) of observation, the colour with which chosen males were painted (in the female choice test), and the combination of provided mates (for the analyses of latency to copulation and copulation duration) were fit as random explanatory variables. In addition, for the analyses of mate preference, the intercept of the models was forced to zero to obtain the estimate of the fixed factor as the difference to a 0.5 probability (particularity of models with categorical factors and binomial distribution; Crawley 2007).

For the analyses of the no-choice test, mating propensity data obtained in the two mating events were analysed altogether, and the mating event was fit as fixed explanatory variable, along with the type of cross (*i.e.*, female x first male) and the interaction between these two factors, whereas the day and the session (nested within day) of observation were fit as random explanatory variables. To avoid pseudoreplication in the analyses of copulation frequency, latency to copulation and

copulation duration, data obtained in each mating event were analysed separately and only the type of cross was fit as fixed explanatory variable. Subsequently, to determine whether these response variables differed when females mated with the first or with the second male, the differences in copulation frequency, latency, and duration between 1st and 2nd mating events were computed for each individual female that mated at least once in both events. For that, the type of cross was fit as fixed explanatory variable, the day and session (nested within day) were fit as random explanatory variables, and the intercept was forced to zero to obtain the estimate of the fixed factor relative to no difference between mating events. Finally, for the analyses of offspring data, the type of cross, the mating status of the females (*i.e.*, mated with only the first or both males), and the interaction between these two factors, were fit as fixed explanatory variables, whereas the day and session (nested within day) during which they mated were fit as random explanatory variables.

For all analyses, maximal models containing the complete set of explanatory variables were subsequently simplified by sequentially eliminating non-significant terms to establish a minimal model (Crawley 2007). The significance of the explanatory variables was established using chi-square tests with the Anova function (car package; Fox et al. 2019). The significant values given in the text are for the minimal model, whereas non-significant values correspond to those obtained before deletion of the variable from the model (Crawley 2007). When explanatory variables with more than two levels were found significant in the analyses of behavioural data, *a posteriori* contrasts between factor levels were carried out by aggregating factor levels together and testing the fit of the simplified model using a likelihood ratio test (function *anova*; Crawley 2007). When interactions between two explanatory variables were significant, the two variables were concatenated *(e.g.*, crosses and mating events) and *a posteriori* contrasts between the factor levels of the concatenated variable were carried out as described above. In all cases, Holm-Bonferroni corrections (*i.e.*, classical chi-squared Wald test for testing the global hypothesis H_0; Holm 1979) were subsequently applied to account for multiple testing. Note that contrast analyses were not carried out for offspring production in the no-choice test because the results did not differ qualitatively from those of the previous study (Cruz et al. 2021). Finally, to determine the difference to random mating in the analyses of mate choice, as well as changes in mating behaviour between 1st and 2nd mating events in the no-choice test, coefficients (estimated as the difference with the zero-intercept) obtained from the maximal models for each combination of focal individual and pair of provided mates (mate choice test), or for each cross (no-choice test), were analysed with Z-tests using the function *test* (emmeans package; Lenth et al. 2018).

Results

Male and female mating behaviour in choice tests

Overall, the propensity of females to mate with one of two provided males depended on their own population ('focal': χ^2_3=10.06, p=0.018; Model 1.1 in Table S1; Figure 1a), with green females being, on average, *ca.* 20% less likely to mate than red females (Tables S3 and S4). However, their mating propensity was unaffected by the type of males they were offered ('mates': χ^2_3=2.70, p=0.44; Model 1.1), and none of them showed any clear mating preference ('focal': χ^2_4=2.91, p=0.57, and 'mates': χ^2_4=3.47, p=0.48; Model 1.2; Figure 1b and Table S5). Conversely, the mating propensity and the mate choice of males were independent of their population ('focal': χ^2_3=4.13, p=0.25 and χ^2_4=5.01, p=0.29 in Model 1.5 and 1.6, respectively), but strongly affected by the type of females provided

('mates': χ^2_3=15.72, p=0.001 and χ^2_4=50.24, p<0.0001 in Model 1.5 and 1.6, respectively; Figures 1c and 1d). Indeed, males that were given the choice between two green females were less likely to mate than those that were given the chance to mate with a red female (*ca.* 40% *vs* 68% mated males on average; Figure 1c, Tables S3 and S4), and males of either colour form showed a preference for red females (*ca.* 60% to 80% preference; Tables S3 and S4).

In contrast to the mite colour form, *Wolbachia* infection had no effect on mite mating propensity and only a small effect on their mate preference (*cf.* Figure 1, Table S3 and contrasts in Table S4). Neither uninfected females nor infected males showed any preference between infected or uninfected mates of the same colour (*cf.* category I in Figures 1b and 1d), but *Wolbachia* infection in red males (CI-inducing *Wolbachia* strain; Cruz et al. 2021) strengthened their preferences for red females. Indeed, although the mate preference of Ru and Ri males did not differ significantly, Ru males showed no significant difference from random mating (*cf.* category II in Figure 1d) whereas Ri males significantly preferred red females over green females (*cf.* categories III and IV; see also Table S5). In addition, whereas red females overall did not differ in their mate preference, and showed no preference between males of either colour form when these were from the same infection status as themselves (*cf.* categories II and III in Figure 1b), Ru females preferentially mated with Gi males over Ri males, suggesting avoidance of the CI induced by *Wolbachia* infection in red males (*cf.* category IV; see also Table S5). Conversely, the non-CI-inducing *Wolbachia* strain infecting green males (Cruz et al. 2021) had no effect on the strength of mate preference of both males and females.

Finally, latencies to copulation did not differ significantly among focal females or chosen males in the female choice test (χ^2_3=6.76, p=0.08 and χ^2_3=1.35, p=0.72, respectively; Model 1.3), nor among focal males or chosen females in the male choice test (χ^2_3=1.33, p=0.72 and χ^2_3=1.03, p=0.79, respectively; Model 1.7, Figure 2a,b), but copulation duration differed between males of different colours (Figure 2c) and between females of different infection status (Figure 2d). Regardless of *Wolbachia* infection (although Ru males showed intermediate copulation durations in the female choice test; Figure 2c; Table S6 and S4), green males copulated on average 37 and 40 seconds longer than red males in the female and male choice test, respectively ('chosen': χ^2_3=7.92, p=0.048, and 'focal': χ^2_3=27.09, p<0.0001; Model 1.4 and 1.8, respectively). Conversely, the copulation duration of females was not affected by their colour form (although Gi females showed intermediate copulation durations in the female choice test; Figure 2d and Table S6; *cf.* contrasts in Table S4), but that of infected females was, on average, *ca.* 29 and 34 seconds shorter than that of uninfected females in the female and male choice test, respectively ('focal': χ^2_3=10.64, p=0.014, and 'chosen': χ^2_3=24.70, p<0.0001 in Model 1.4 and 1.8, respectively).

(Re)mating behaviour in the no-choice test

On average, 58% of the virgin females placed on a leaf disc with a single male mated within 1 hour, whereas less than 20% of those mated females re-mated when placed with another male 24 hours later. In line with this, a reduced copulation frequency (1.6±0.1 *vs* 2.1±0.1 copulations per couple) and copulation duration (118±13 *vs* 252±8 seconds) and an increased latency to copulation (1582±108 *vs* 986±46 seconds), were observed, on average, between the first and second mating events for couples that mated at least once (Figures 3,4 and Table S7). However, this reduction in the willingness to mate varied across types of crosses for all behavioural traits tested but copulation frequency (no statistically significant differences found among crosses for either or among the two mating events; *cf.* models 2.2 to 2.4 in Table S2).

For the mating propensities observed during the first mating event, we found the same tendencies as in the choice tests: Gu females were less likely to mate than Ru females (except when paired with Ru males here), and *Wolbachia* infection in red males seemed to promote mate discrimination (Ri males were less willing to mate with Gu females than Ru males, whereas both types of males mated as much with Ru females; Figure 3; Tables S7 and S8). Then, although no statistically significant differences were found among crosses in the second event, not all crosses led to the same reduction in mating propensity between the two mating events (*cross x mating event interaction*: χ^2_5=16.56, p=0.005; model 2.1; Figure 3; Table S8): Gu females showed a lower reduction in their tendency to re-mate than Ru females, especially when they were previously mated with an incompatible Ri male (hence when both types of incompatibilities were at play; Figure 3; Tables S7 and S8).

In this experiment, conversely to the previous experiment in which virgin individuals could choose their mate and were given only half an hour to mate, we found significant differences among latencies to copulation of couples that mated at least once during the first mating event (χ^2_5=13.19, p=0.02; model 2.5). Gu females took, on average, 5 more minutes than Ru females to engage in copulation with their first partner, regardless of the form or infection status of the latter (although Ru x Ru crosses had intermediate latencies to copulation; *cf.* circles in Figure 4a; Tables S7 and S9). Also, in this first mating event, as in the previous experiment (choice tests), the cumulative time spent copulating was longer for green males than for red males regardless of their infection status and the female they mated with (*ca.* 39 seconds difference; χ^2_5=21.19, p<0.001; model 2.4; Figures 2c,d and 4c; Tables S7 and S9). Then, when females that mated during the first mating event were placed with a second male 24 hours later, their latency to copulation increased by almost 10 minutes, and their copulation duration was more than 2 minutes shorter, than when they were virgin (*cf.* diamonds in Figure 4a,c; Table S7). Despite no significant differences being found among types of crosses for both latency to copulation and cumulative copulation duration in the second mating event (χ^2_5=5.16, p=0.40; model 2.6; Figure 4a, and χ^2_5=2.78, p=0.73; model 2.9; Figure 4c, respectively), behavioural changes between the two mating events at the female level (for those who mated in both events) varied depending on the type of cross (χ^2_6=12.47, p=0.05; model 2.7; Figure 4b, and χ^2_6=43.73, p<0.0001; model 2.10; Figure 4d, for latency to copulation and cumulative copulation duration, respectively). Thus, in line with the mating propensity observations, differences in latency to copulation and copulation duration between mating events tended to disappear for females that had first mated with an incompatible male (except for the copulation duration of Gu females mated with Ri males; Table S10).

Effect of remating on offspring production in the no-choice experiment

The pattern of offspring production for females that mated only with one male (Figure 5a) was consistent with that described in our previous study (Cruz et al. 2021). Briefly, (i) we found an overproduction of males (MD-type incompatibility) in crosses between green females (Gu) and red males (Ru or Ri) as compared to the other crosses (χ^2_5=76.30, p<0.0001; model 2.12; Figure 5b). Moreover, because copulations were observed in the present study, it further unambiguously revealed a high variability for this barrier: among the 66 Gu females that mated only with a Ru or Ri mate and oviposited (*i.e.*, 85 Gu females mated with a Ru or Ri male subsequently refused to mate with a second male; Table S7, and 19 of these females did not lay a single egg), 20 produced only sons (*i.e.*, full incompatibility), whereas 18 did not produce a more male-biased sex ratio than the controls (*i.e.*, no incompatibility); (ii) we found an increased female embryonic mortality (FM-type CI quantified

as a decreased hatching rate of fertilized eggs) in crosses between uninfected females (Gu or Ru) and males infected with a CI-inducing *Wolbachia* strain (Ri males), as compared to the other crosses (χ^2_5=76.78, p<0.0001; model 2.13; Figure 5c); and (iii) we found a reduction in the proportion of daughters (FP) in crosses affected by either (or both) type(s) of incompatibility (*i.e.*, Ru x Ri, Gu x Ru and Gu x Ri, female x male crosses) as compared to compatible crosses (χ^2_5=87.65, p<0.0001; model 2.14; Figure 5d). However, no difference in offspring production was found between females that mated with only one or two different males (daily fecundity: χ^2_1=3.19, p=0.07; model 2.11; MD_{corr}: χ^2_1=0.63, p=0.43; model 2.12; FM_{corr}: χ^2_1=0.14, p=0.71; model 2.13; FP: χ^2_1=0.17, p=0.68; model 2.14), regardless of whether the first male was compatible or not (*i.e.*, no significant interactions between the type of cross and whether females mated with one or two males; daily fecundity: χ^2_5=4.57, p=0.47; model 2.11; MD_{corr}: χ^2_5=0.89, p=0.97; model 2.12; FM_{corr}: χ^2_5=9.33, p=0.10; model 2.13; FP: χ^2_5=7.17, p=0.21; model 2.14; Figure 5; see also Figure S1 and Table S7).

Contribution of intrinsic and *Wolbachia*-induced reproductive barriers to reducing gene flow

Although hybrid breakdown is the strongest reproductive barrier in both directions of crosses between the two studied spider mite populations (100% F2 embryonic mortality; Cruz et al. 2021), it ultimately contributes very little to total isolation due to the occurrence of earlier barriers (Figure 6 and Table S11). Red females and green males are mainly isolated due to hybrid sterility (98 to 100% isolation regardless of *Wolbachia* infection), as no other reproductive barrier exists in this cross direction. However, despite having the same strength in both directions of heterotypic crosses, hybrid sterility acts along with other reproductive barriers in crosses between green females and red males, which strongly reduced its contribution to total isolation (*ca.* 12% and 29% in crosses with infected and uninfected males, respectively). In this cross direction, our estimations revealed that assortative mating and fertilization failure are in fact the main sources of reproductive isolation, contributing to 27-61% and 23-71% of total isolation, respectively. Moreover, although hybrid inviability caused by the CI-inducing *Wolbachia* strain infecting red males only has a weak contribution to total isolation in heterotypic crosses (as compared to homotypic crosses: *ca.* 5.5 to 6.4% in crosses between green females and Ri males *vs* 32% in crosses between Ru females and Ri males; Table S11), infection of males with this *Wolbachia* strain clearly potentiates pre-mating isolation (Figure 6). Indeed, the strength of assortative mating increases from *ca.* 27% in crosses between Gu females and Ru males (non-significantly different from random mating; *cf.* Figure 1 and above) to *ca.* 61% in crosses between Gu females and Ri males (Figure 6) and to *ca.* 49% in crosses between Gi females and Ri males (Table S11).

Discussion

In this study, we sought to shed light on the potential role played by *Wolbachia* as an agent of pre-zygotic isolation between genetically differentiated colour forms of the spider mite *Tetranychus urticae*. Our results revealed that *Wolbachia* infection had no effect on the mating preference of both males and females in homotypic crosses, but the CI-inducing strain infecting the red form exacerbated pre-existing colour-based mate preferences. Whereas both types of males showed a preference for red females, this preference seemingly disappeared when red males were cured from *Wolbachia* infection. In line with this, females showed no mate preferences in the absence of *Wolbachia* infection, but uninfected red females showed a preference for green infected males (which do not

carry a CI-inducing *Wolbachia* strain) over red infected ones (which do carry a CI-inducing *Wolbachia* strain). Then, we also found that (i) females that had engaged in matings where both types of incompatibility occurred (*Wolbachia*-induced and host-associated) were more likely to re-mate with a compatible male, and (ii) females exposed to either type of incompatibility did not significantly increase their latency to re-mate, nor reduce their copulation duration when re-mating, as compared to their first matings. Yet, the significance of such behaviour is puzzling, as we found no evidence of sperm contribution by the second (compatible) males following copulations with incompatible mates (*i.e.*, adult offspring production and sex-ratios were not more similar to the controls). This further indicated that 'homotypic sperm precedence' was not a reproductive barrier at play in our experiment. Finally, our estimations of the relative contribution of each reproductive barrier to reproductive isolation between the studied populations clearly illustrate the strong asymmetries that occur in this system: red females are isolated from green males due to late intrinsic post-zygotic barriers (hybrid sterility and hybrid breakdown), whereas green females are isolated from red males by a combination of early and late reproductive barriers (pre-mating, post-mating pre-zygotic, and post-zygotic), either directly caused (hybrid inviability due to CI) or strengthened (assortative mating) by *Wolbachia* infection in males.

A system driven by male rather than female mate preferences

In most tested scenarios, *T. urticae* females did not choose between mates with different colour forms. This corroborates earlier results, where females from different morphs or infection status did not discriminate between different types of males (Murtaugh and Wrensch 1978, Zhao et al. 2013b; Rodrigues et al. 2022, but see Vala et al. 2004; Rohrscheib et al. 2015), and indicates no differences in male competitive ability as well (Murtaugh and Wrensch 1978; Wagner 1998). In fact, several other studies revealed an absence of mate choice in spider mite females (*e.g.*, Magalhães et al. 2009; Zhou et al. 2020). This is surprising, as females invest more energy than males in their reproduction (Kokko et al. 2006), and spider mites have first-male sperm precedence (Helle 1967; Satoh et al. 2001; Rodrigues et al. 2020), hence the choice of the first male has enormous consequences for females (Wittenberger and Tilson 1980; Howlett 1988; Griffith et al. 2011). Possibly, this weak female choice is a consequence of male guarding of females just before their emergence as virgin adults (Potter et al. 1976), leading to little opportunity for females to choose their mate (Everson and Addicott 1982; Oku 2014). In contrast, we found strong mate preferences in males, which is also in line with earlier studies on spider mites (*e.g.*, Everson and Addicott 1982; Rodrigues et al. 2017), and in other arthropods in which males invest time and energy in pre- and/or post-copulatory guarding (reviewed in Bonduriansky 2001).

Asymmetric reinforcement could explain the match between pre- and early post-mating barriers

In this system, one might expect assortative mating (*i.e.*, homotypic preference in both cross directions) to be selected for due to severe costs of hybridization in both cross directions (Cruz et al. 2021). Instead, our results revealed an asymmetry in pre-mating isolation (only red males prefer homotypic females). Possibly, post-mating pre-zygotic barriers (*e.g.*, fertilization failure due to cytonuclear incompatibility) first evolved incidentally between green-form females and red-form males in allopatric populations. The resulting asymmetrical maladaptive hybridization may have subsequently (*i.e.*, at secondary contact) led to asymmetrical levels of reinforcement in areas of sympatry (Noor 1999; Servedio and Noor 2003; Coyne and Orr 2004), thereby driving the evolution

of homotypic mate preferences by red males only (*e.g.*, as observed between *Drosophila recens* and *D. subquinaria* populations due to unidirectional CI induced by *Wolbachia*; Jaenike et al. 2006). This may explain the match between pre-mating and early-acting post-mating barriers in this system (sex ratio distortion likely due to fertilization failure in crosses between red males and green females; Cruz et al. 2021), as found in other systems (reviewed in Ortiz-Barrientos et al. 2009; see also Giesbers et al. 2013; Yukilevich et al. 2018). Alternatively, asymmetric barriers acting in the same cross direction could as well be due to genetic linkage between barriers (*e.g.*, Merrill et al. 2011), a possibility not yet investigated in spider mites. Subsequently, the two forms might have further diverged due to limited gene flow, leading to the establishment of strong late post-zygotic barriers in both directions (Servedio and Sætre 2003). In line with this, previous work has shown that barriers acting early in reproduction tend to evolve faster than those acting later (Coyne and Orr 1989; Servedio 2001; Turissini et al. 2018).

Aside from pre-mating isolation, reinforcement could also drive the evolution of other types of pre-zygotic barriers, including those occurring after mating, such as conspecific sperm precedence (Coughlan and Matute 2020). Although preferential use of sperm from conspecific (or 'homotypic') males would reduce the negative effects of mating with heterospecifics (*e.g.*, Price 1997; Fricke and Arnqvist 2004; Noriyuki et al. 2012), we did not find any evidence for such reproductive barrier. Nevertheless, we observed that the latencies to copulation and copulation durations of green-form females previously mated with red-form males tended to remain the same as when they were virgins. This contrasts with the behaviour of females mated with fully-compatible males, which become less receptive to subsequent males (increased latency and reduced copulation duration), in line with the first-male sperm precedence pattern (Helle 1967; Rodrigues et al. 2020). Other studies also found that spider mites first mated with (fully or partially) incompatible males behave as virgins in subsequent matings (Clemente et al. 2016; Costa et al. 2023). Moreover, when the incompatibilities are not caused by fertilization failure (as in the case of *Wolbachia*-induced CI), a reduced latency to copulation and increased copulation duration with a second male could increase the likelihood that the sperm of the latter outcompete the sperm of the first male (Potter and Wrensch 1978; Satoh et al. 2001). However, contrarily to these earlier studies, the results obtained here do not indicate any use of the sperm from second males, which could rescue limited offspring production resulting from incompatible matings. Yet, this pattern may be jeopardized under other conditions than those tested in the current study. Here, we allowed for several copulations with the first male, and mated females were exposed to a second male only 24 hours later. This was done to detect potential issues with sperm transfer or storage when an excess of male offspring is found (*i.e.*, in crosses between green females and red males). If this was the case, significant effects of double mating on offspring production could not be unambiguously attributed to changes in the sperm precedence patterns (García-González 2004). However, the timing used might have been excessive to enable sperm competition/cryptic sperm choice (Potter and Wrensch 1978; Satoh et al. 2001), and future studies are necessary to uncover potential benefits of the behaviours observed here.

Wolbachia-induced CI might strengthen asymmetrical reinforcement

Our results concerning the effect of *Wolbachia* infection on mate preferences between mite colour forms corroborate those of previous studies showing that *Wolbachia* infection strengthens assortative mating between genetically differentiated hosts (*e.g.*, Jaenike et al. 2006; Koukou et al. 2006; Miller et al. 2010). In particular, the fact that *Wolbachia* infection alone (*i.e.*, in homotypic crosses) has no significant effect on mate choice (as in Rodrigues et al. 2022), supports the hypothesis that avoidance

of CI might evolve more readily when the infection is associated with pre-existing host traits that can be used for mate recognition (Engelstädter and Telschow 2009). Moreover, the fact that mating preferences contribute more to total reproductive isolation when infected red males, which carry a CI-inducing *Wolbachia* strain, were involved (*cf.* fig. 6), suggests that CI could be a mechanism driving asymmetrical reinforcement between spider-mite colour forms. Consistent with a previous study on incompatibilities between different geographic strains of green-form *T. urticae*, in which the only females receptive to a second mate were those previously mated with a genetically incompatible male carrying a CI-inducing *Wolbachia* strain (Navajas et al. 2000), we also found that only uninfected green females previously mated with a red infected male (hence carrying a CI-inducing *Wolbachia* strain) were as likely to mate with a second male as when they were virgins. In line with this, only when uninfected females (both red and green) had mated with an infected red male (with the CI-inducing strain) did their latency to copulation and copulation duration remain as when they were virgin. Hence, together these findings revealed that *Wolbachia* can affect other mating behaviours beyond mating preferences (as in other systems; reviewed in Bi and Wang 2020), and suggest that *Wolbachia*-induced CI could assist reinforcement processes in this system.

Not just a missing barrier: Heterotypic mate preference may be an adaptive strategy

Although reinforcement is a seductive hypothesis to explain why red-form males prefer red females, it does not explain why green-form males also prefer these females. The occurrence of such seemingly maladaptive behaviour suggests that other, or additional, evolutionary forces are at play.

One possibility could be that heterotypic mating preference is a by-product of inbreeding avoidance in the green-form population. Spider mites effectively avoid related individuals (Tien et al. 2011; Bitume et al. 2013; Yoshioka and Yano 2014), but it is not clear whether this extends to more distantly-related individuals. For instance, males of both *T. evansi* and *T. urticae* preferentially mate with *T. urticae* females (Sato et al. 2016; but see (Clemente et al. 2016), but this occurs even when *T. evansi* females are non-kin (Sato et al. 2016). In line with this, evidence that inbreeding avoidance can drive the evolution of disassortative mating is also lacking in other systems (van den Berg et al. 1984; Juola and Dearborn 2012; Huchard et al. 2013; Galaverni et al. 2016).

Another possibility could be that preference of both types of males for red-form females is due to these females being more attractive. For instance, a new trait (*e.g.*, a pheromone profile) may have evolved in red females in response to intense female competition (*i.e.*, their sex ratio is more female-biased than that of green mites when they oviposit in groups; unpublished data), and this trait may then be fortuitously preferred by green males if it stimulates the same coding system as the ancestral trait (Endler and Basolo 1998). Alternatively, both types of male may have conserved an ancestral preference for a trait that has been lost or diverged in green-form females (Endler and Basolo 1998). This could occur if the rate of evolution of male preference is slower than that of the female trait. The observed male preferences may also be caused by differences in female reluctance and male vigour (*e.g.*, van den Berg et al. 1984) in response to stronger sexual conflicts in the green-form population. This hypothesis is supported by the fact that green females are less likely to mate than red females even in the absence of choice, whereas green males spend longer periods of time copulating than red males do (suggesting longer post-copulatory guarding; (Satoh et al. 2001). In line with this, theory predicts that sexual conflicts can drive the evolution of mate preferences, increasing reproductive isolation and, consequently, the rates of speciation (Parker and Partridge 1998).

Finally, building upon the recent idea that partial reproductive isolation may be an adaptive optimum (Servedio and Hermisson 2020), we considered the possibility that heterotypic mating

preference might be selected for under reproductive interference (Gröning and Hochkirch 2008), as the two colour forms have overlapping distribution and host plant range (Migeon and Dorkeld 2023), and often co-occur on the same individual host plant (Lu et al. 2017, 2018; Zélé et al. 2018b). Although most conditions that have been theoretically considered to promote the evolution of 'disassortative mating', such as a heterozygote advantage (*e.g.*, Maisonneuve et al. 2021), are not met in our system (hybrids are sterile or suffer breakdown; Cruz et al. 2021), heterotypic mating preference may still confer higher benefits than costs to the green-form population in the presence of red-form competitors. Indeed, this behaviour should be highly costly for red females due to first male sperm precedence, but green males may only pay relatively small costs as they can mate multiply (Krainacker and Carey 1989). Thus, similarly to how CI induced by *Wolbachia* increases the relative fitness of infected females, the 'spiteful' behaviour of green males might be selected for as it confers an indirect fitness advantage to their green sisters (Hamilton 1970; Gardner and West 2004; Engelstädter and Charlat 2006). Disassortative mating may thus act synergistically with sex-ratio distortion (*i.e.*, the overproduction of sons) in crosses between green females and red males (*cf.* Cruz et al. 2021) to promote the exclusion of the red form population (*cf.* Grether et al. 2017; Cruz et al. 2023). Conversely, homotypic mating preference by red males should decrease the strength of reproductive interference for the red population, as it reduces the prevalence of crosses between green females and red males (hence the overproduction of green males stemming from these crosses) and should prevent (non-choosy) red females from having a higher chance to mate with a green male. Following this hypothesis, the CI-inducing *Wolbachia* strain naturally infecting the red-form population seems to favour its own host population by increasing the likelihood that red males mate with compatible (red) females, whereas it has no control over heterotypic mating preference by green males. Testing whether such an 'escalating arms race' could indeed occur in response to reproductive interference (involving or not *Wolbachia*-induced CI) is of high relevance for future speciation studies.

Conclusion

In this study, we identified a mechanism through which *Wolbachia* could assist host speciation processes. Our results show that *Wolbachia* infection in *T. urticae* males indirectly contributes to pre-mating isolation between genetically differentiated *T. urticae* colour forms by strengthening pre-existing preferences. These preferences match early post-mating barriers in the system, as crosses that are affected both by host-associated and *Wolbachia*-induced incompatibilities are generally avoided. Our results also further highlight the importance of pre-mating isolation in this system, as they revealed that, in our experimental conditions, females of either form are unable to compensate for incompatible crosses by re-mating. Overall, our comprehensive study of pre- and post-zygotic reproductive barriers allowed identifying asymmetries in patterns of isolation between the two populations, hinting at a possible history of reinforcement followed by an interruption of gene flow. These findings also open new research avenues, such as to study the impact of complex patterns of isolation on population dynamics, and of the resulting selection pressures on the evolutionary trajectories of populations.

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Table 1. Choice tests to assess the mating behaviour and preference of males or females that were given the choice between two mates of different colour forms and/or *Wolbachia* infection status. CI: cytoplasmic incompatibility; HI: host-associated incompatibility.

Category	Choice	Female tests	Male tests
I – Avoidance of *Wolbachia*-induced CI	Mates with different infection statuses	Ru♀ x (Ri♂ *vs* Ru♂) Gu♀ x (Gi♂ *vs* Gu♂)	Ri♂ x (Ri♀ *vs* Ru♀) Gi♂ x (Gi♀ *vs* Gu♀)
II – Avoidance of HI in absence of *Wolbachia*	Uninfected mates from different populations	Ru♀ x (Ru♂ *vs* Gu♂) Gu♀ x (Ru♂ *vs* Gu♂)	Ru♂ x (Ru♀ *vs* Gu♀) Gu♂ x (Ru♀ *vs* Gu♀)
III – Avoidance of HI in presence of *Wolbachia*	Infected mates from different populations	Ri♀ x (Ri♂ *vs* Gi♂) Gi♀ x (Ri♂ *vs* Gi♂)	Ri♂ x (Ri♀ *vs* Gi♀) Gi♂ x (Ri♀ *vs* Gi♀)
IV – Avoidance of HI, *Wolbachia*-induced CI, or both	Mates both with different infection statuses and from different populations	Ru♀ x (Ri♂ *vs* Gi♂) Gu♀ x (Ri♂ *vs* Gi♂)	Ri♂ x (Ru♀ *vs* Gu♀) Gi♂ x (Ru♀ *vs* Gu♀)

Table 2. No-choice tests to assess the behaviour and offspring production of virgin females (♀) placed with a compatible or incompatible male (1st ♂ of a similar or different colour form and/or *Wolbachia* infection status, respectively), then (for those that had mated with the first male) with a second compatible male (2nd ♂ from their own population). CI: cytoplasmic incompatibility; HI: host-associated incompatibility.

Category	First crosses	♀	1st ♂	2nd ♂
A – Controls	Intra-population crosses between uninfected ♀ and ♂	Ru	Ru	Ru
		Gu	Gu	Gu
B – HI but no effect on F1 production[1]	Inter-population crosses between uninfected red ♀ and green ♂	Ru	Gu	Ru
C – HI with reduced F1 production	Inter-population crosses between uninfected green ♀ and red ♂	Gu	Ru	Gu
D – *Wolbachia*-induced CI[2]	Intra-population crosses between red uninfected ♀ and infected ♂	Ru	Ri	Ru
E – HI and *Wolbachia*-induced CI[2]	Inter-population crosses between green uninfected ♀ and red infected ♂	Gu	Ri	Gu

[1]*HI in this cross direction leads to F1 female sterility and hybrid breakdown (i.e., late post-zygotic isolation).*
[2]*Only Ri males were used as only the Wolbachia strain infecting the red population induces CI (Cruz et al. 2021).*

Figure 1. Mating propensity and mate choice of spider mites of different colour forms and/or *Wolbachia* infection status. For each type of choice test, bars represent mean (± s.e.) proportion of mated (a) females and (c) males, and of mates chosen by (b) females and (d) males (dotted: *Wolbachia*-infected mates; plain: uninfected mates; orange: red mates; blue: green mates). Identical or absent superscripts indicate non-significant differences at the 5% level among treatments, asterisks indicate a difference to random mating (white dotted line).

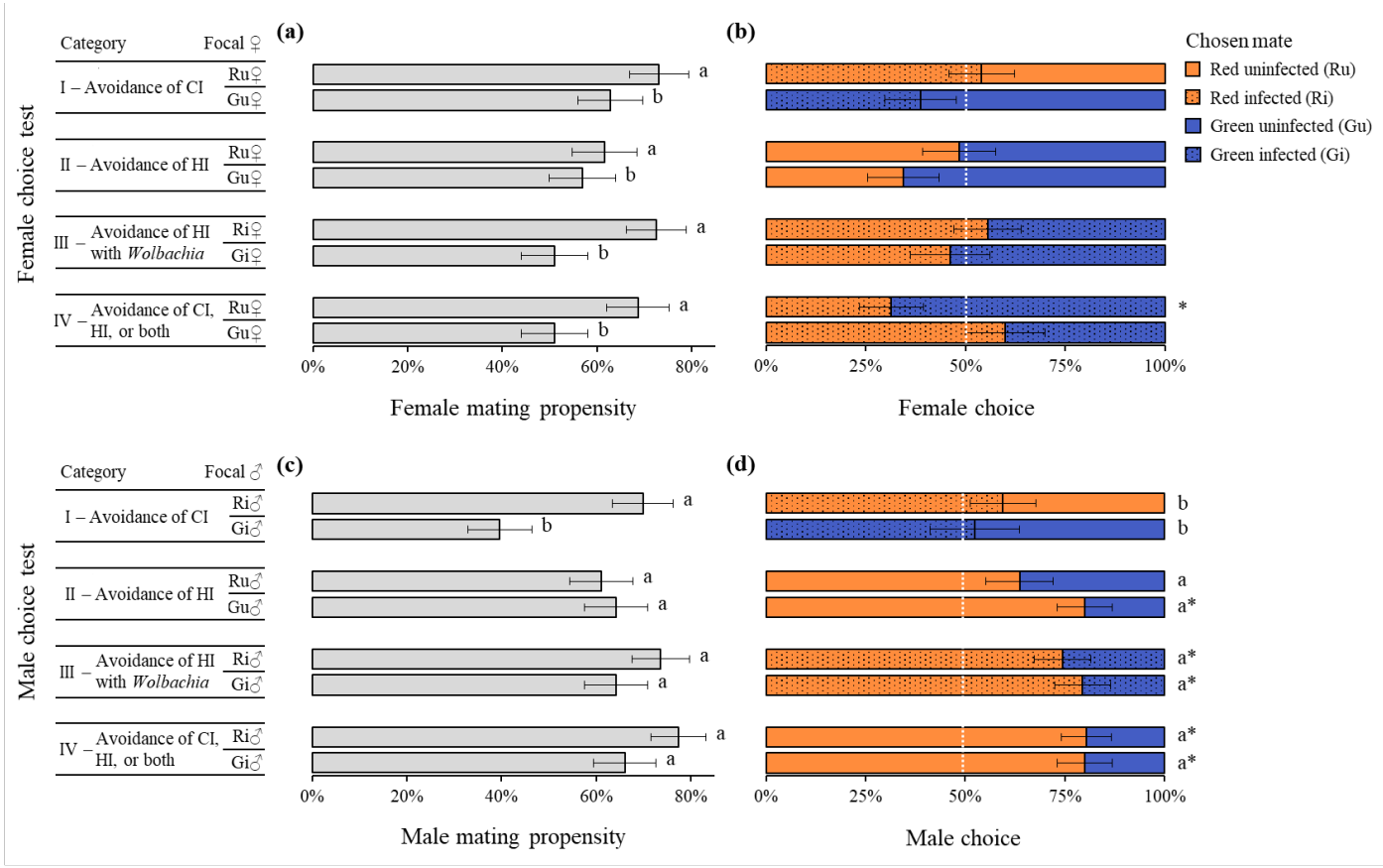

Figure 2. Latency to copulation (a, b) and copulation duration (c, d) of virgin mites during the choice and no-choice tests. Dots represent mean time (± s.e.) in seconds observed for males (a, c) and females (b, d) in the male and female choice tests (white and grey dots, respectively), and in the first mating event of the no-choice test (black dots). Overall, no significant differences were found among males or females for latency to copulation in the choice tests (statistical results are not given for the no-choice test as latencies to copulation exceeding 30 minutes were excluded from the means displayed in this figure to allow comparisons across experiments). For copulation duration, identical superscripts indicate non-significant differences at the 5% level among crosses within each test (Italic: male choice test, lowercase: female choice test, uppercase: 1st mating event of the no-choice test). Note that infected females were not used in the no-choice test (hence, black dots are not displayed for Ri and Gi females in panel d).

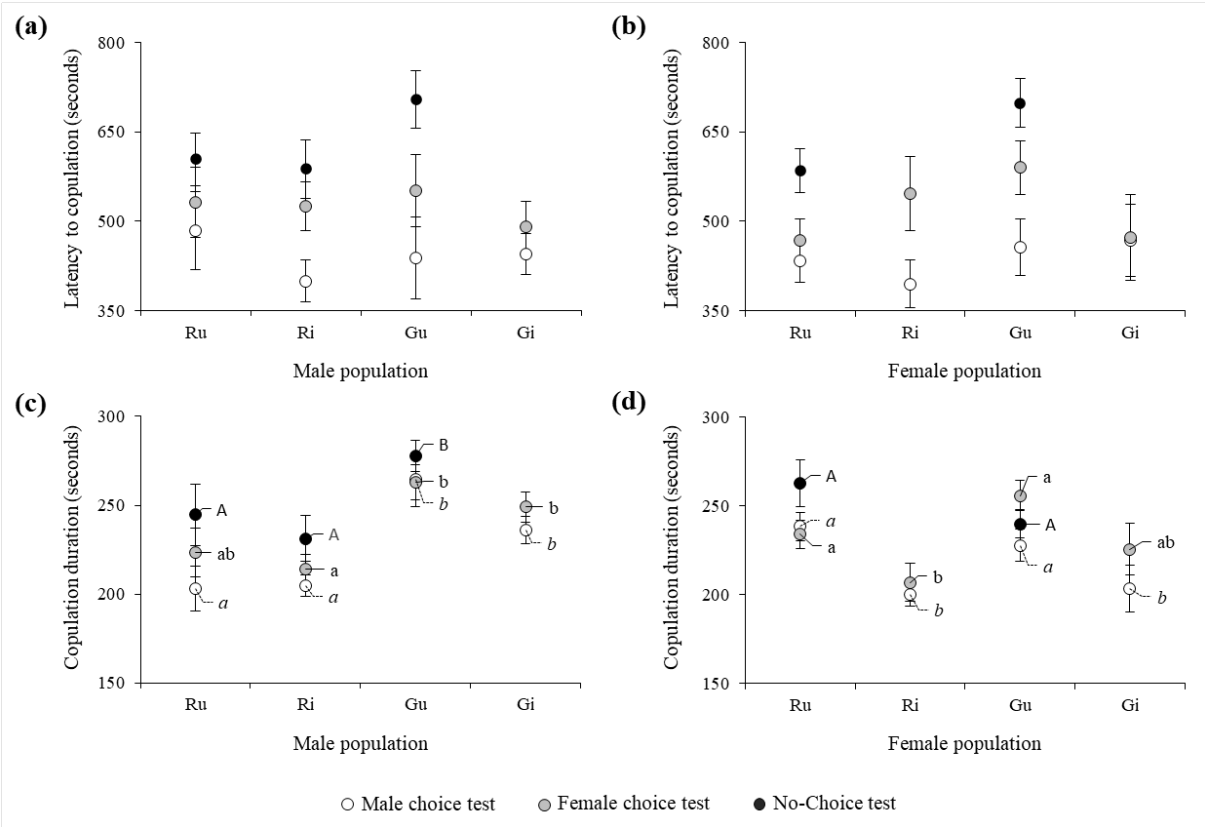

Figure 3. Mating propensity observed in two successive mating events in the no-choice test. For each cross category, circles and diamonds represent mean (± s.e.) proportion of females that mated during the first and the second mating event, respectively. The population of the female is displayed at the bottom level of the x-axis and the population of the first male at the top level (the population of the second male is always the same as that of the female). Identical superscripts indicate non-significant differences at the 5% level among crosses across mating events.

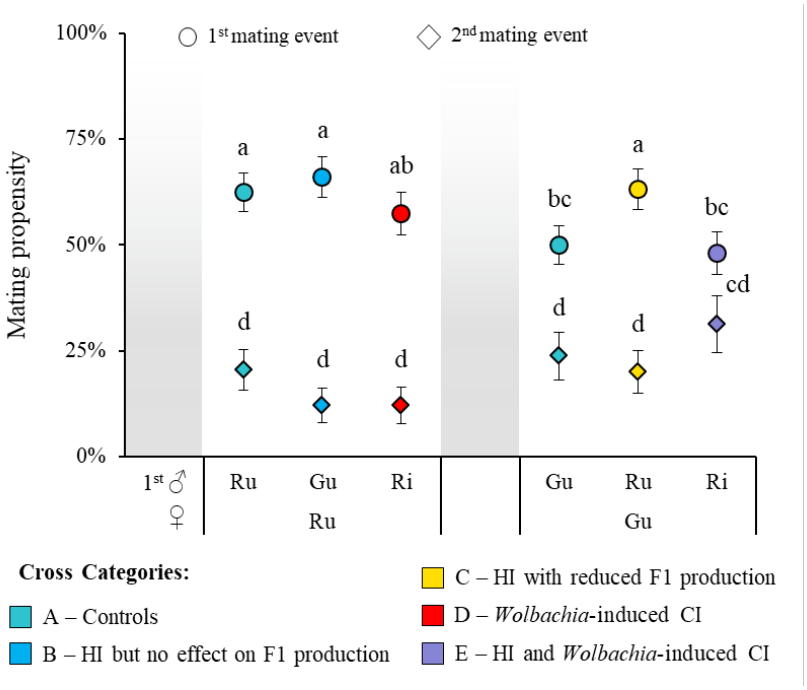

Figure 4. Latency to copulation (a, b) and copulation duration (c, d) observed in two successive mating events in the no-choice test. In (a) and (c), circles and diamonds represent mean time (± s.e.) in seconds for each cross category during the first and the second mating event, respectively. Identical or absent superscripts indicate non-significant differences at the 5% level among crosses within each mating event. In (b) and (d), squares represent the mean time difference (± s.e.) observed between the two mating events for each female that mated with both males (*i.e.* [time spent for the second mating] – [time spent for the first mating]). Superscripts indicate significant differences from zero at the 10% level (‡: $p<0.10$) and at the 5% level (*: $p\leq0.05$). In all panels, the population of the female is displayed at the bottom of the x-axis and the population of the first male at the top (the population of the second male is always the same as that of the female).

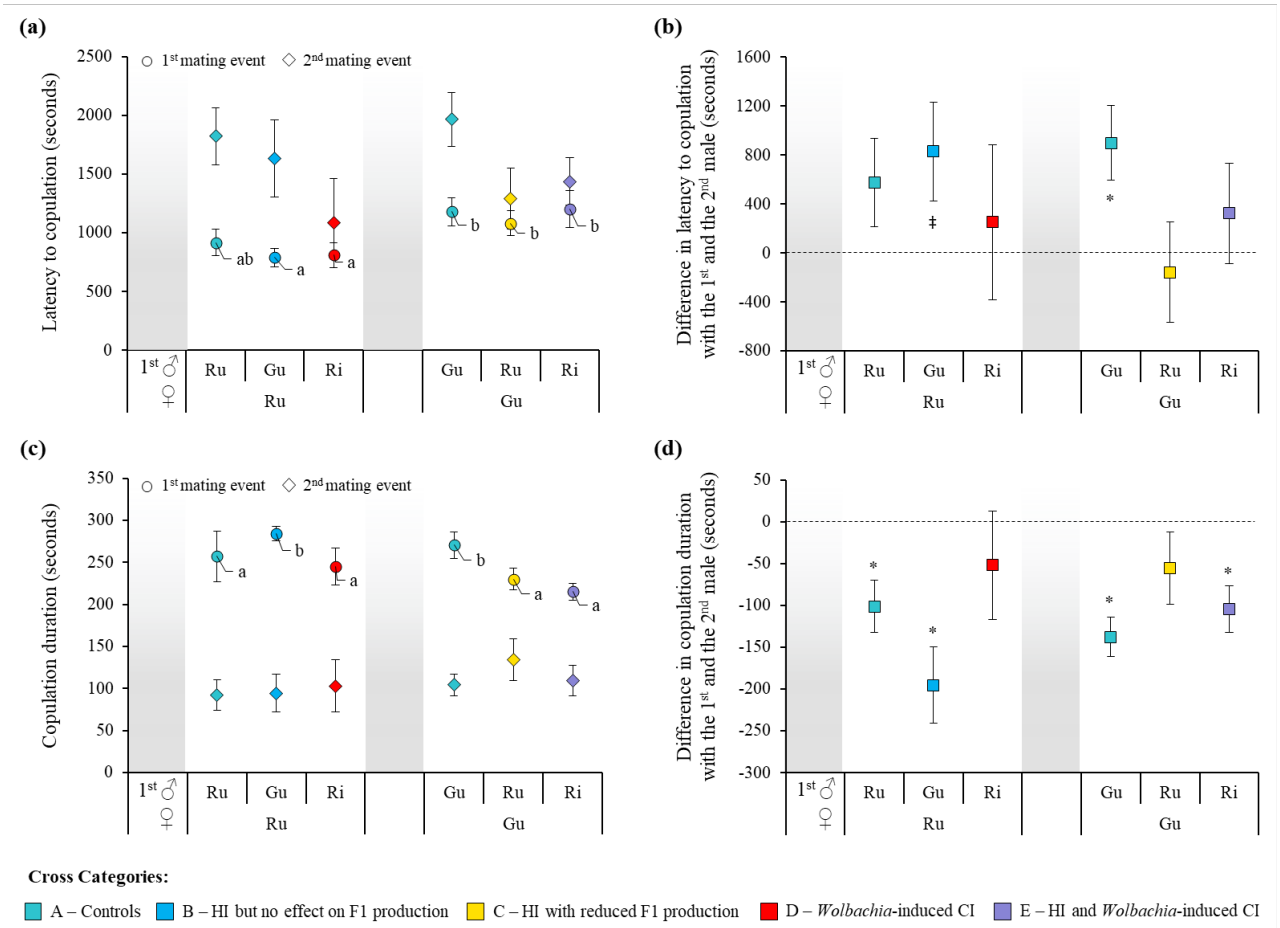

Figure 5. Effect of re-mating on offspring production in crosses affected by *Wolbachia*-induced CI and/or HI. (a) Outcome of egg development from each cross category, with bars representing the mean (± s.e.) relative proportions of unhatched eggs (*i.e.* embryonic mortality), adult daughters and sons. (b) Boxplot of the proportion of males produced in all crosses relative to control crosses (MD_{corr}). (c) Boxplot of the proportion of estimated unhatched female eggs relative to control crosses (FM_{corr}). (d) Boxplot of the proportion of F1 adult females in the brood (FP). The population of the female (♀) is displayed at the bottom level of the x-axis, that of the first male (1st ♂) at the middle level, and that of the second male (2nd ♂) at the top level ("-" indicates females that did not mate with the second male).

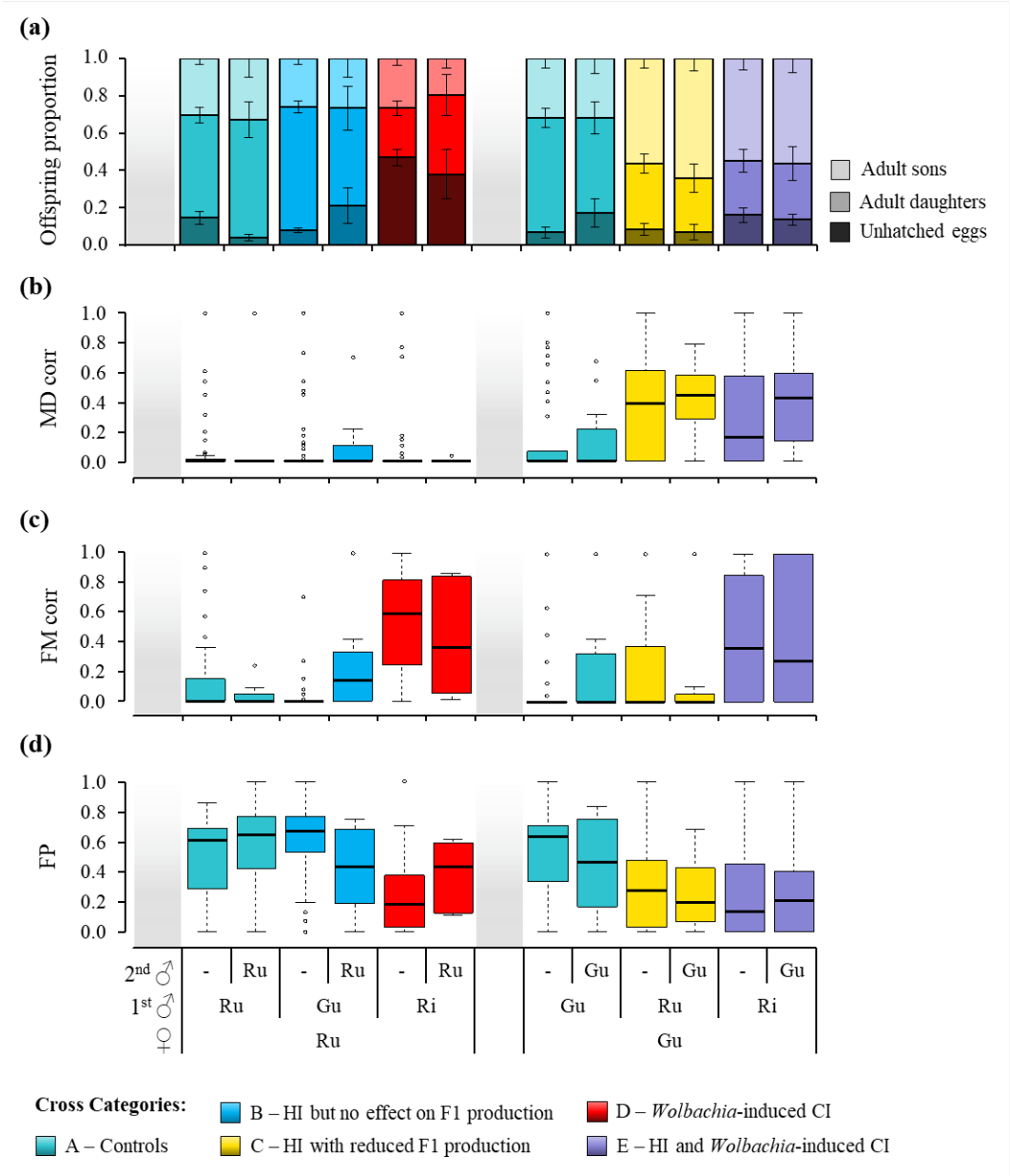
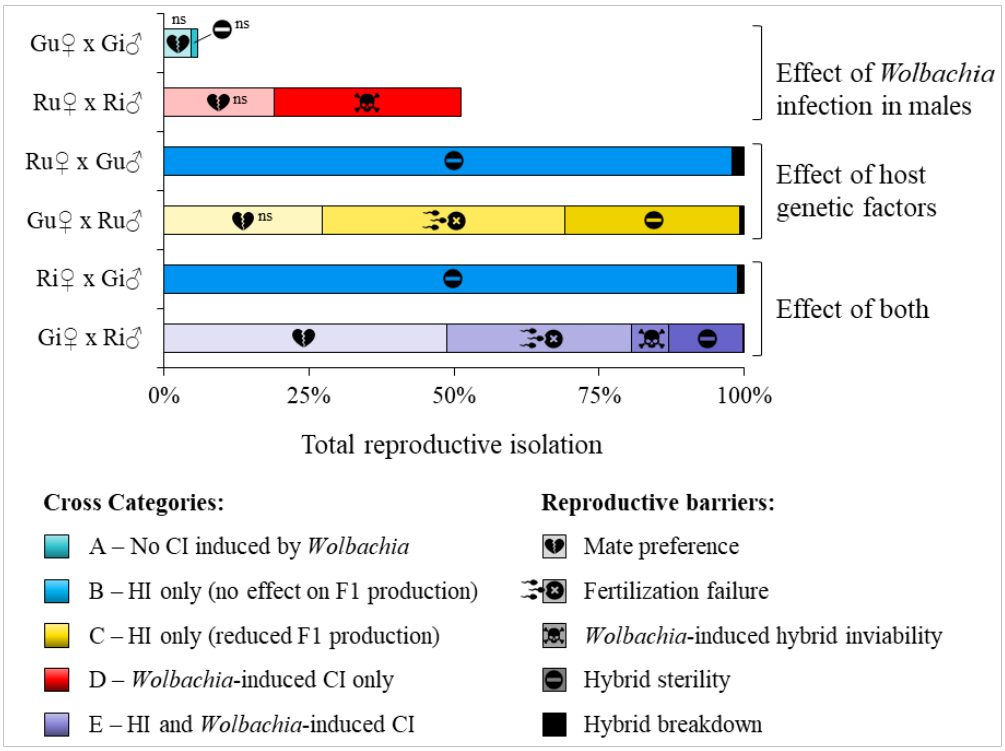
Figure 6. Contribution of the different reproductive barriers to reducing gene flow within and between populations. Percent contributions to total reproductive isolation were computed based on the estimated strength of reproductive isolation (*RI*) caused by a given reproductive barrier. They are shown for the six most representative types of cross in this system (see Table S11 for all crosses). Mate preference corresponds to male preferences only, as females overall showed no mate preferences. 'ns' indicates no significant difference to zero at the 5% level.