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

Running title: *Wolbachia* strengthens host mate choice

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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 different *Wolbachia* strains, one inducing cytoplasmic incompatibility and the other not. 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 matings. 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 re-mating 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 contribute to reproductive isolation 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 for 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 post-zygotic barriers (*e.g.*, Lackey and Boughman 2017). In addition, previous work suggested that reproductive isolation may be driven not only by the genetics of the organisms themselves but also by their endosymbionts, especially those 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 (Werren et al. 2008; Engelstädter and Hurst 2009). The most common of such manipulations is cytoplasmic incompatibility (CI), a conditional sterility phenotype that results in embryonic

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mortality of offspring from crosses between infected males and uninfected females (or females infected with an incompatible strain; Breeuwer and Werren 1990; 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 so, CI avoidance should be more commonly found between already differentiated populations. In line with this prediction, 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 post-mating pre-zygotic isolation. For instance, *Wolbachia* infection can have deleterious effects on sperm production or transfer (Snook et al. 2000; Lewis et al. 2011), fertilization success (Bruzzese et al. 2021), or 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

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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). *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, especially in the two-spotted spider mite *T. urticae*. Natural populations of this species can be infected with highly variable prevalence (ranging from 0 to 100%; Gotoh et al. 2003, 2007; Zhang et al. 2016; Zélé et al. 2018a,b) of different *Wolbachia* strains, mostly belonging to the *Ori* subgroup of supergroup B (Gotoh et al. 2003, 2007; Zhang et al. 2013; Suh et al. 2015; Pina et al. 2020; although some strains belonging to the *Con* subgroup have also been found; Xie et al. 2006). In this host, the bacterium also induces highly variable degrees of different types of CI (from 0 to 100% of either FM- or MD-type CI, which correspond, respectively, to 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. 2003; Suh et al. 2015; Zélé et al. 2020; Wybouw et al. 2022), and has variable effects on pre-mating isolation (Zhao et al. 2013b; Rodrigues et al. 2022; Vala et al. 2004). However, in spider mites, as in many other arthropod species, the contribution of *Wolbachia* to post-mating pre-zygotic isolation has seldom been studied (though see Cooper et al. 2017 for the *Drosophila yakuba* complex), 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, given that 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 contributions that *Wolbachia* can make to reproductive isolation among 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 closely-related colour forms, green and red, of the two spotted spider mite *T. urticae*, which are sometimes also referred to as separate species: *T. urticae* and *T. cinnabarinus* (Xie et al. 2006; Auger et al. 2013; Lu et al. 2017, 2018). Indeed, although a

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recent study showed very high differentiation between populations of these two forms at the genomic level (Xue et al. 2023), post-zygotic isolation was found to range from full to only partial (Murtaugh and Wrensch 1978; Dupont 1979; de Boer 1982a,b; Sugasawa et al. 2002), and they do not seem to differ in the prevalence or type of *Wolbachia* strains they carry, nor, overall, in CI level or pattern they induce (*e.g.*, Perrot-Minnot et al. 2002; Vala et al. 2002; Xie et al. 2006; Gotoh et al. 2007; Zélé et al. 2020; Wybouw et al. 2022). Moreover, the two forms have an overlapping worldwide distribution (Hinomoto et al. 2001; Lu et al. 2017; Godinho et al. 2020; Migeon and Dorkeld 2023; Xue et al. 2023), they share the same host plant range (Auger et al. 2013) and sometimes the same individual plant (Lu et al. 2017; Zélé et al. 2018b). 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 in detriment of diploid daughters ('male development' or MD-type incompatibility, likely due to fertilization failure) in crosses between red-form males and green-form females, whereas the reciprocal cross yields no change in sex ratio. Moreover, whereas *Wolbachia* infection in green-form males was not associated with CI induction of any type, *Wolbachia* infection in red-form males led to an increased embryonic mortality of their 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).

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Here, we significantly build upon previous work by investigating pre- and post-mating pre-zygotic reproductive barriers between these spider mite populations. We first performed male and female choice tests to determine preference for infected or uninfected mates from different colour-form populations (*i.e.*, test for pre-mating isolation; Table 1). Second, we used a no-choice test to investigate the effect of female mating history (virgin or previously-mated with a compatible *vs* incompatible male) on mating behaviour, and to test whether eggs are more likely fertilized by compatible than incompatible sperm (*i.e.*, test for 'homotypic' sperm precedence; Table 2). 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, fully described in the Supplementary Box S1, were previously used to assess post-mating isolation caused by both host-associated incompatibilities (HI) and *Wolbachia*-induced reproductive barriers (Cruz et al. 2021). 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 original Ri and Gi populations, both fully and stably infected with *Wolbachia* at the time of the experiments, were collected from locations *ca.* 34 km apart in the region of Lisbon (Portugal; see Box S1). In this region, the prevalence of *Wolbachia* is very high in red form populations (40 to 100%, *ca.* 94%

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on average; Zélé et al. 2018a,b) and CI levels are moderate to high (*ca*. 27 to 65%; Zélé et al. 2020), as for the Ri population used here (naturally fully infected and *ca*. 57 ± 3% CI; Zélé et al. 2018a, 2020). The incidence of *Wolbachia* in green form populations of this same region seems comparatively much lower (only 1 out of 4 populations were found carrying the symbiont, as reported in Godinho et al. 2020), perhaps due to weak or no CI induction as previously found for the Gu population used here (unknown prevalence but no CI induction; Cruz et al. 2021). The Ru and Gu populations were then obtained from antibiotic treatments as detailed in Box S1. All populations were reared 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) under the same standard laboratory conditions (24±2ºC, 16/8h L/D). 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 (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 the cohorts and placed separately on bean leaf fragments (*ca.* 9cm^2) to obtain virgin adult females and males of similar

age two days later. As opposed to females, males cannot easily be identified based on their body colouration, hence 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 placed on a 0.4cm^2 leaf disc (called 'arena' hereafter), then the observation started when the focal individual was also 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). 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, such as rejecting a mate after a copulation has started. Moreover, copulations lasting less than 30 seconds can be insufficient to fully fertilize a spider mite female (Potter and Wrensch 1978; Satoh et al. 2001), which might explain the excessive production of males to the detriment of females (*i.e.*, arising from unfertilized and fertilized eggs, respectively) previously observed in the brood of green females mated with red males (Cruz et al. 2021). To test whether such post-copulatory mechanisms of avoidance of

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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 (see Table 2). Also, because only few individuals could be tested per day, 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 placed 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) and latency to the first copulation, the copulation frequency (*i.e.*, the number of copulations during the observation period) and the duration of each copulation, to compute the cumulative copulation duration of each couple, were also recorded. 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 (see 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*, 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 (see Table 2) and their mating behaviour was recorded for 60 minutes as in the first mating event. At the end of the observation period, males were discarded and females were kept individually on $2 cm^2$ bean

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leaf discs placed on water-soaked cotton in petri dishes in a climatic chamber (25 ± 2°C, 60% RH, 16/8 h L/D).

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 indices as fully described in Cruz et al. (2021): the MD_{corr} index, which calculates the overproduction of sons in the brood (using the number of adult sons and the total number of offspring), and the FM_{corr} index, which calculates the embryonic mortality of fertilized offspring (using the number of unhatched eggs and the number of adult daughters), both relative to the control crosses to account for background variation. Hence, higher values of MD_{corr} indicate a greater proportion of sons in the brood to the detriment of daughters, while higher values of FM_{corr} indicate a greater mortality of female embryos. 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.

Given the workload and the multiplicity of tasks involved in the entire experiment (first and second mating events, as well as offspring production), only 9 couples were observed simultaneously for each mating event, corresponding to one or two replicates per treatment.

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Four sessions of observation were performed per day (hence 6 replicates of each cross category 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 cross category (regardless of whether the females mated during the first and/or second mating events).

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 the pre-mating data obtained here and the post-mating data from Cruz et al. (2021), respectively. Only reproductive barriers found to play a role in reducing gene flow among the spider mite populations were considered (see. Figure S2): mate preference (RI_1), fertilization failure (RI_2), hybrid inviability (RI_3), hybrid sterility (RI_4), and hybrid breakdown (RI_5), with homotypic sperm precedence and female choice not being included (see 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 from Cruz et al. (2021), as late reproductive barriers (*i.e.*, hybrid sterility and hybrid breakdown) were not measured here.

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Moreover, earlier post-mating barriers (fertilization failure and hybrid inviability) have been estimated in all possible crosses and more precisely in the previous study that focused specifically on post-mating isolation (*i.e.*, experiments were done with larger sample sizes, as they only included single mating treatments). For fertilization failure (*RI₂*) and hybrid inviability (*RI₃*) we used the median values of the MD_{corr} and FM_{corr} indices in Cruz et al. (2021), which correspond to the percent increase in non-fertilized eggs and in embryonic mortality of fertilized eggs, respectively (see *'Offspring production and strength of post-mating incompatibilities'* above). For hybrid sterility (*RI₄*) and hybrid breakdown (*RI₅*), 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

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All analyses were carried out using Mixed Models with the R statistical software (v3.6.1). The general procedure used for building all statistical models (R packages, datasets and sample size, coding of the response variables, choice of error structures, usage of random vs. fixed explanatory variables and their interactions); for the simplification of maximal models (containing the complete set of explanatory variables) into minimal models to establish the significance of the explanatory variables (Crawley 2007); and to determine significant changes relative to the intercept as well as to perform contrasts analyses between factor levels, was always substantially the same. It is fully detailed in supplementary materials Box S2, and Tables S1 and S2.

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 faced a choice that involved 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 (see 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 (see 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 (see category II in Figure 1d) whereas Ri males significantly preferred red females over green females (see categories III and IV; see also Table S5). In addition, whereas red females showed no preference between males of either colour form when these were from the same infection status as themselves (see 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 (see 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

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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; see 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 (Figures 3,4 and Table S7). However, this reduction in the willingness to mate varied across types of crosses for all behavioural traits tested except for copulation frequency (no statistically significant differences found among crosses for either or among the two mating events; see 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 were, 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

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events (*cross x mating event interaction*: $\chi^2_5=16.56$, $p=0.005$; 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).

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 ($\chi^2_5=13.19$, $p=0.02$; 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; see circles in Figure 4a; Tables S7 and S9). Also, 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; $\chi^2_5=21.19$, $p<0.001$; 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 (see 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 ($\chi^2_5=5.16$, $p=0.40$; model 2.6; Figure 4a, and $\chi^2_5=2.78$, $p=0.73$; 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 ($\chi^2_6=12.47$, $p=0.05$; model 2.7; Figure 4b, and $\chi^2_6=43.73$, $p<0.0001$; 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

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with an incompatible male (except for the copulation duration of Gu females mated with Ri males; Figures 4b,d; Table S10).

Effect of re-mating 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

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two males; daily fecundity: $\chi^2_5=4.57$, $p=0.47$; model 2.11; MD_{corr}: $\chi^2_5=0.89$, $p=0.97$; model 2.12; FM_{corr}: $\chi^2_5=9.33$, $p=0.10$; model 2.13; FP: $\chi^2_5=7.17$, $p=0.21$; 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). The strength of assortative mating increases from *ca.* 27% in crosses between Gu females and Ru males (non-significantly different from random mating; see 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*. To this aim, we assessed the relative contribution of *Wolbachia*-induced and host-related (pre- and post-mating) pre-zygotic barriers to total reproductive isolation. 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 enhanced 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). 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 mating. Yet, we found no evidence of sperm contribution by the second (compatible) males following copulations with incompatible mates, which indicates 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

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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 red 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 or infection status. This corroborates earlier results (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

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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 incompatibilities, such as mitochondria-nucleus interactions; see Hill 2015) 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 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 (Castillo and Moyle 2019; Coughlan and Matute 2020). Although preferential use of sperm from conspecific (or 'homotypic') males would reduce the negative effects of mating

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with heterospecifics (*e.g.*, Price 1997; Fricke and Arnqvist 2004; Noriyuki et al. 2012), we did not find any evidence for such reproductive barrier. Yet, green-form females previously mated with red-form males remained as receptive as when they were virgins, conversely to females mated with fully-compatible males, which became 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). Similar results were also found for spider mite females first mated with (fully or partially) incompatible males of the same or different species (Clemente et al. 2016; Costa et al. 2023), but contrarily to these earlier studies, the results obtained here do not indicate any use of the sperm from second males. However, this pattern may be jeopardized under other conditions than those tested in the current study. For instance, 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), in which 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 the use of the sperm from the second male (Potter and Wrensch 1978; Satoh et al. 2001). Moreover, given the reduced receptivity of females to second mating, the sample sizes for females mated with two different males were sometimes very low (see Table S7), which may have masked small changes in offspring production. Future studies are thus necessary to uncover potential benefits of the behaviours observed here.

Wolbachia-induced CI might strengthen asymmetrical reinforcement

We show that *Wolbachia* infection strengthens assortative mating between genetically differentiated hosts, corroborating earlier findings in other systems (*e.g.*, Jaenike et al. 2006;

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Koukou et al. 2006; Miller et al. 2010). In addition, *Wolbachia* infection alone (*i.e.*, in homotypic crosses) has no significant effect on mate choice (as found in Rodrigues et al. 2022). Avoidance of CI might evolve more readily in structured populations, where the infection may become associated with pre-existing host traits that can be used for mate recognition (Engelstädter and Telschow 2009). Although no study has specifically addressed the population structure of spider mite populations in the field, their reliance of cultivated annual crops and several indirect lines of evidence suggest that these populations are highly structured (Navajas et al. 2000; Uesugi et al. 2009). Moreover, the fact that mating preferences contributed more to total reproductive isolation when infected red males, which carry a CI-inducing *Wolbachia* strain, were involved (see Figure 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. Together these findings thus revealed that *Wolbachia* can affect other mating behaviours beyond mating preferences (as in other systems; reviewed in Bi and Wang 2020), and raised the possibility that *Wolbachia*-induced CI could assist reinforcement processes in this system.

Whether the pattern observed in our study is only incidental or the result of *Wolbachia*-assisted reinforcement remains elusive. Indeed, while the latter hypothesis necessarily hinges upon a common *Wolbachia*-host evolutionary history, hence stable and long-lasting *Wolbachia*

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infection, we do not have sufficient knowledge about the past evolutionary history of the populations studied here to adjudicate which hypothesis holds true, if any. Nevertheless, previous work has shown a lack of congruence between the phylogenies of *Wolbachia* and its spider mite hosts, irrespective of their colour forms (Xie et al. 2006), or even species (Zhang et al. 2013). This suggests that *Wolbachia* infections were acquired after the forms diverged, which would preclude the bacteria from playing a role in the evolution of host reproductive barriers. In line with this, no link was found between CI induction by *Wolbachia* (neither in whether or not strains induce CI, nor in the level of CI) and the colour form of the spider mites hosting them (Gotoh et al. 2007). Moreover, the occurrence, strength, and direction of asymmetries in genetic incompatibilities between colour forms also varies depending on the host population (*e.g.*, Xue et al. 2023 vs. Cruz et al. 2021). This indicates that the pattern observed in our study is population-specific, and that *Wolbachia* is unlikely to have played a role in the establishment of incompatibilities between *T. urticae* colour forms. However, it is still possible for *Wolbachia* to be involved in the reinforcement of reproductive barriers between particular populations. Although *Wolbachia* infections can be labile in some spider mite populations, others could last much longer, depending on the transmission rate, fitness effect on host, and CI levels induced by *Wolbachia* (Zélé et al. 2020). In line with this, two subsequent field surveys conducted in the region of Lisbon, where our populations were collected, show that the prevalence of *Wolbachia* infection in red-form populations remains very high through time and across different host plant species (Zélé et al. 2018a,b), although studies on the stability of specific *Wolbachia* strains are still lacking. Alternatively, even if *Wolbachia* infections are transient, the hosts themselves may be involved in expressing the pattern of asymmetrical *Wolbachia*-induced CI observed in this study, regardless of which specific strain is infecting them. In particular, a recent study showed direct evidence that the expression of CI has a strong host-specific component in spider mites, with the host genotypes

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strongly modulating the expression (level and pattern) of CI induced by a same *Wolbachia* strain (Wybouw et al. 2022). This should be of particular relevance for host populations living in areas where *Wolbachia* infections are highly prevalent, as in the case of spider mites from the Iberian Peninsula (Zélé et al. 2018a,b; Pina et al. 2020).

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). Moreover, this supposes that the green population suffers more from inbreeding than the red one, a possibility that could be tested in the future.

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

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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 multiple times (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;

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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 (see Cruz et al. 2021) to promote the exclusion of the red form population (see 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

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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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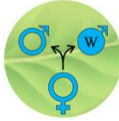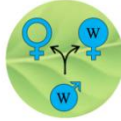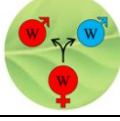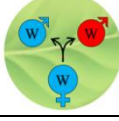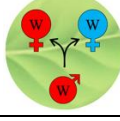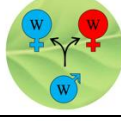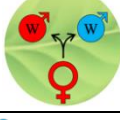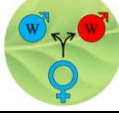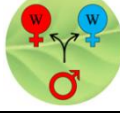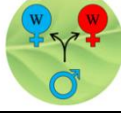
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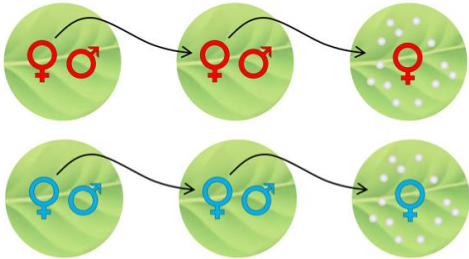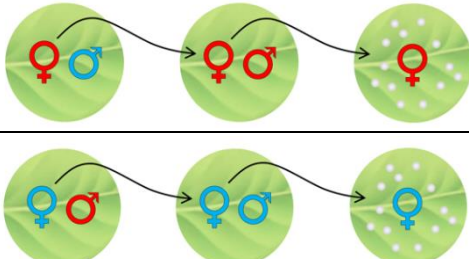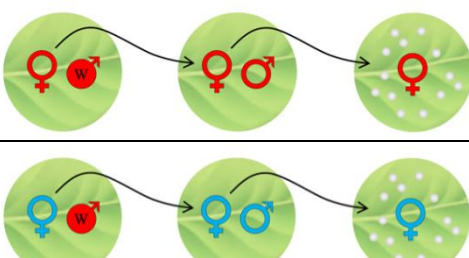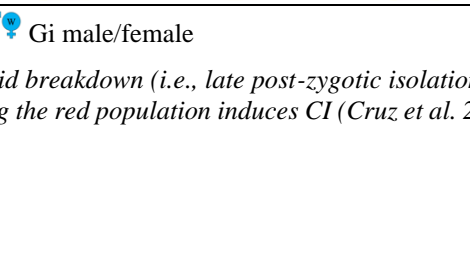
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Tables

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		
II – Avoidance of HI in absence of *Wolbachia*	Uninfected mates from different populations		
III – Avoidance of HI in presence of *Wolbachia*	Infected mates from different populations		
IV – Avoidance of HI, *Wolbachia*-induced CI, or both	Mates both with different infection statuses and from different populations		

Ru male/female   Gu male/female   Ri male/female   Gi male/female

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

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

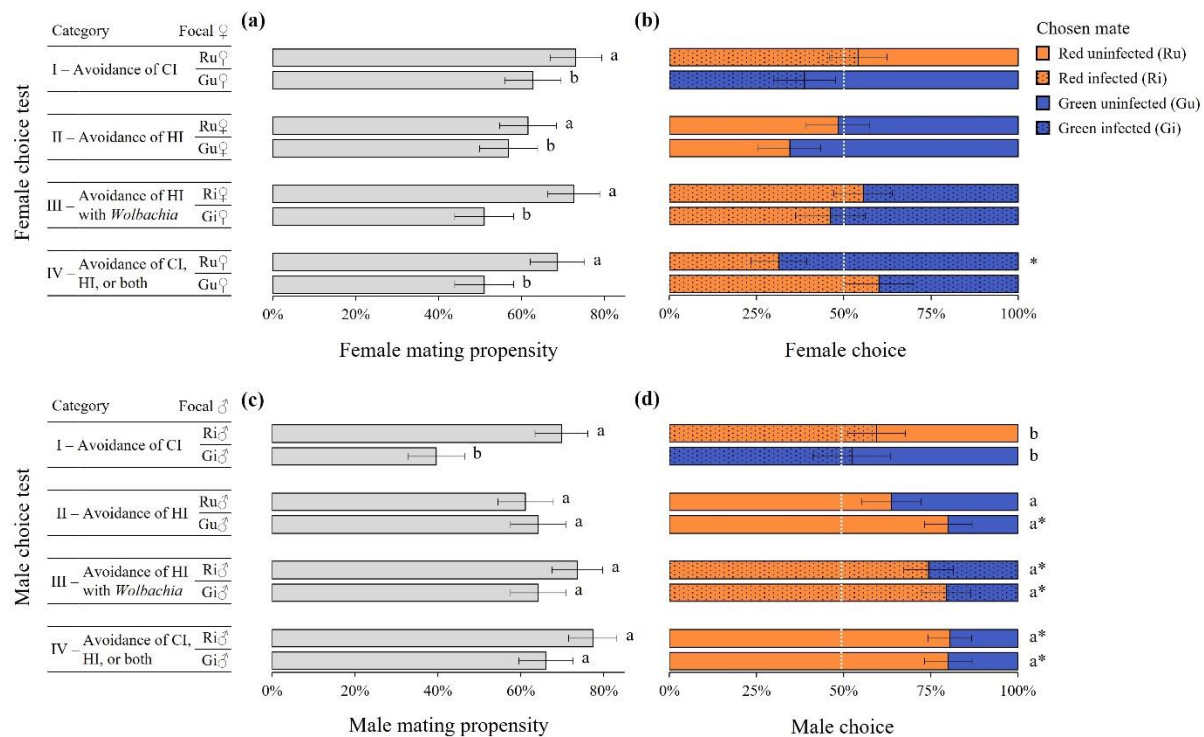♂♀ Ru male/female ♂♀ Gu male/female ♂♀ Ri male/female ♂♀ Gi male/female

[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 legends

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 the 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 (see Table S4), and asterisks indicate a difference to random mating (white dotted line; see Table S5). CI: cytoplasmic incompatibility; HI: host-associated incompatibility.
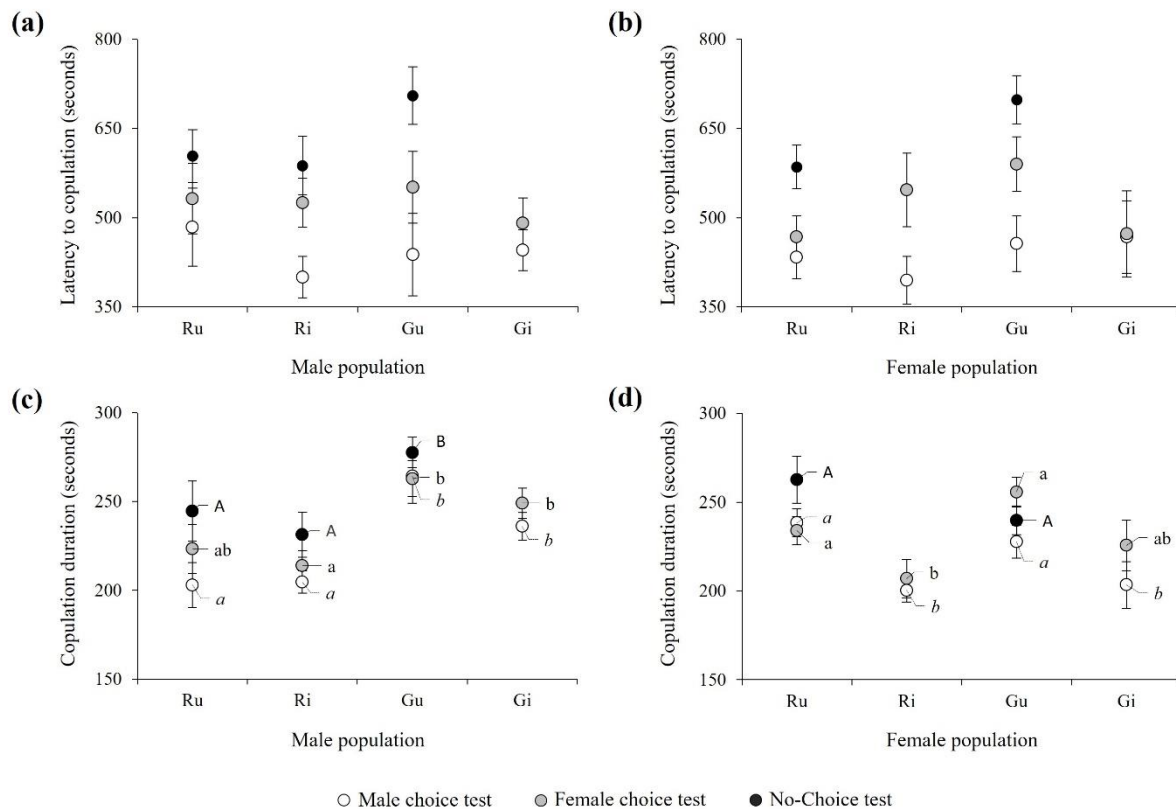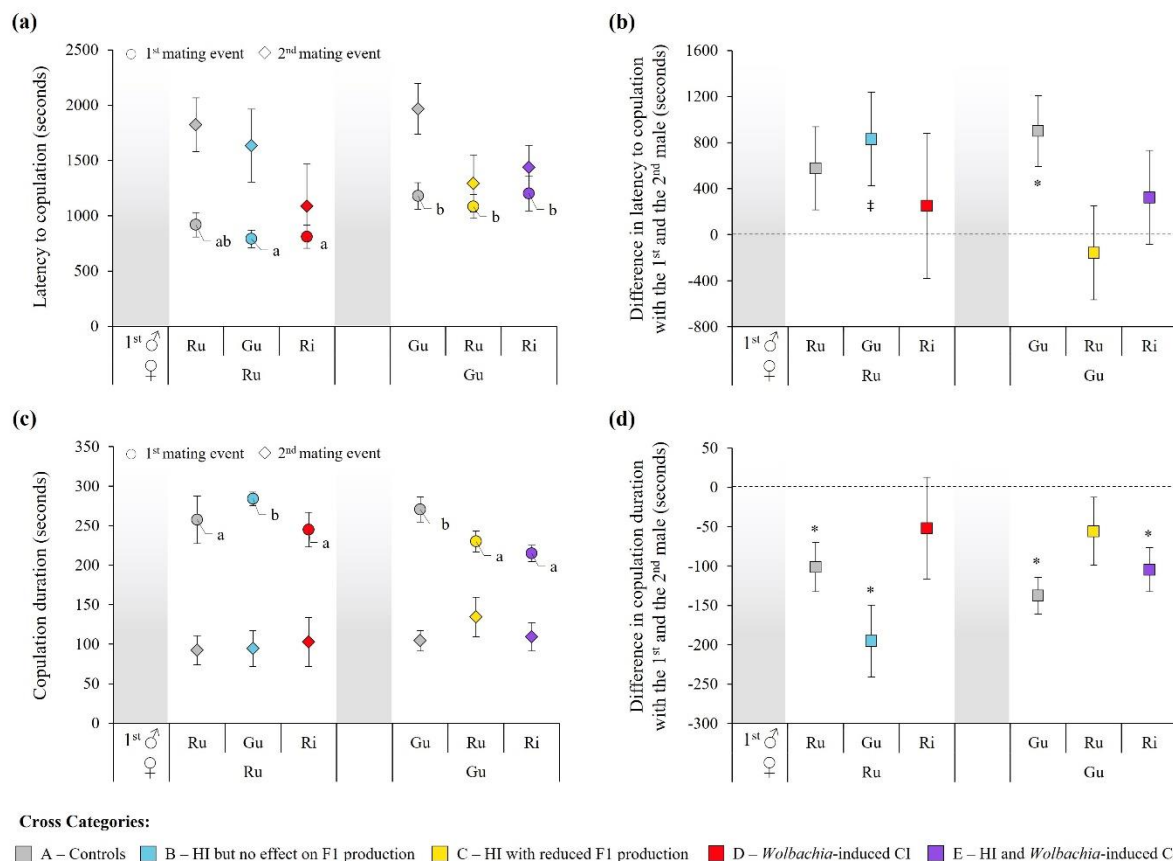
Figure 2. Latency to copulation (a, b) and copulation duration (c, d) of virgin mites during the choice and no-choice tests. Dots represent mean time ($\pm$ s.e.) in seconds observed for males (a, c) and females (b, d) in the male and female choice tests (white and grey dots, respectively), and in the first mating event of the no-choice test (black dots), regardless of the identity of their mate. The panels (a, c) thus display results obtained for focal males in the male choice test, or male mates in the female choice and no-choice tests, whereas panels (b, d) display results obtained for focal females in the female choice and no-choice tests, or female mates in the male choice test. No significant differences between latencies to copulation were found among the different types of males or females 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 within each test (Italic: male choice test, see Table S4; lowercase: female choice test, see Table S4; uppercase: 1st mating event of the no-choice test, see Table S9). 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).
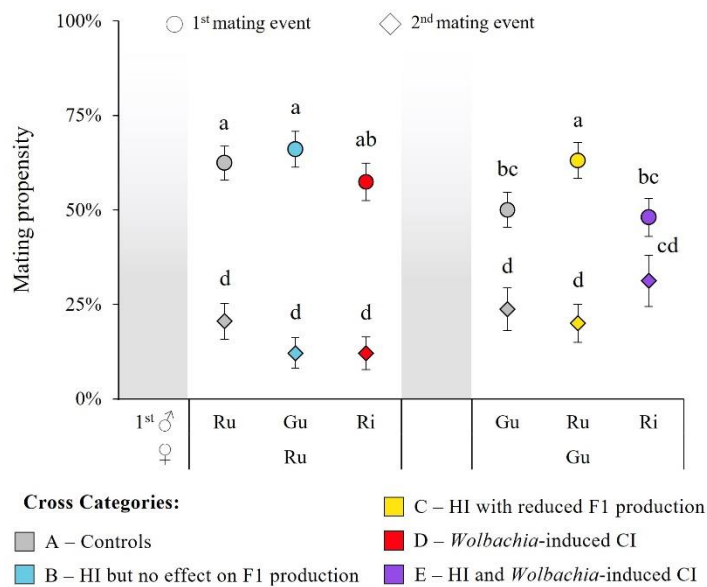
Figure 3. Mating propensity observed in two successive mating events in the no-choice test. For each cross category, circles and diamonds represent mean (± s.e.) proportion of females that mated during the first and the second mating event, respectively. The population of the female is displayed at the 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 (see Table S8). CI: cytoplasmic incompatibility; HI: host-associated incompatibility.

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 (see Table S9). 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*≤0.05; see Table S10). 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). CI: cytoplasmic incompatibility; HI: host-associated incompatibility.

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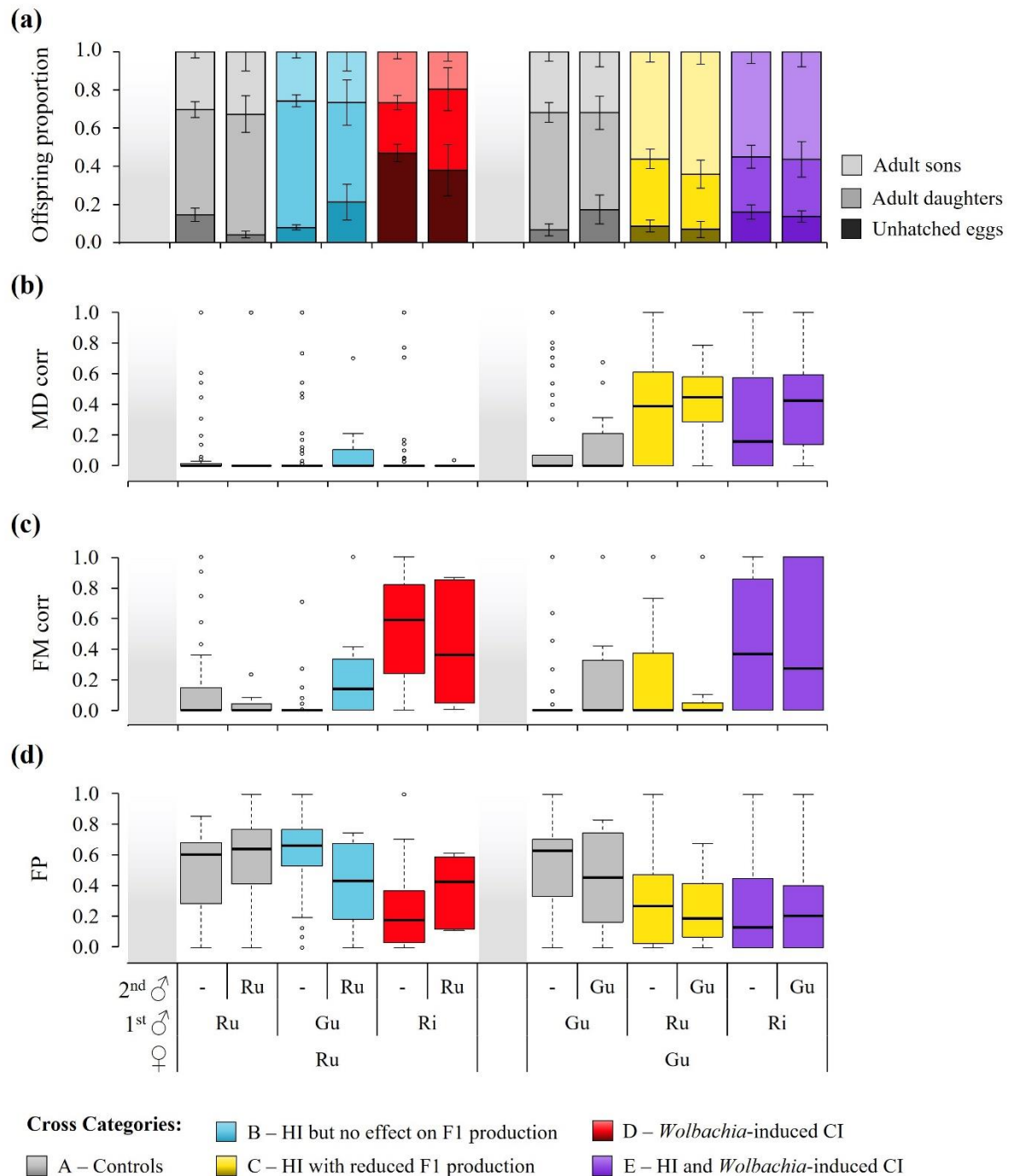
Figure 5. Effect of re-mating on offspring production in crosses affected by *Wolbachia*-induced CI and/or HI. (a) Outcome of egg development from each cross category, with bars representing the mean (± s.e.) relative proportions of unhatched eggs (*i.e.* embryonic mortality), adult daughters and sons. (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). CI: cytoplasmic incompatibility; HI: host-associated incompatibility.
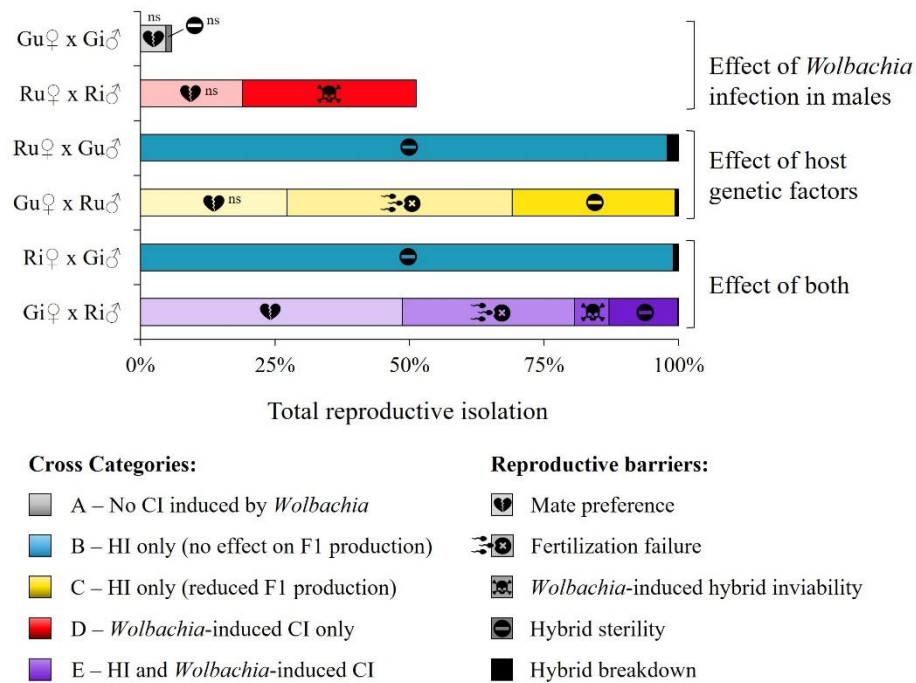
Figure 6. Contribution of the different reproductive barriers to reducing gene flow within and between populations. Percent contributions to total reproductive isolation were computed based on the estimated strength of reproductive isolation (*RI*) caused by a given reproductive barrier. They are shown for the six most representative types of cross in this system (see Table S11 for all crosses). 'ns' indicates no significant difference to zero at the 5% level. CI: cytoplasmic incompatibility; HI: host-associated incompatibility.