Wolbachia and host intrinsic reproductive barriers contribute additively to post-mating isolation in spider mites

Miguel A. Cruz[1], Sara Magalhães[1,2], Élio Sucena[2,3], Flore Zélé[1,2*]

*Correspondence: fezele@fc.ul.pt

[1] Centre for Ecology, Evolution and Environmental Changes (cE3c), Faculdade de Ciências da Universidade de Lisboa, Edificio C2, 3º Piso Campo Grande, 1749-016 Lisboa, Portugal

[2] Departamento de Biologia Animal, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, Edifício C2, 1749-016 Lisboa, Portugal

[3] Instituto Gulbenkian de Ciência, Apartado 14, 2781-901 Oeiras, Portugal

Abstract

Wolbachia are widespread maternally-inherited bacteria, suggested to play a role in arthropod host speciation through induction of cytoplasmic incompatibility, but this hypothesis remains controversial. However, most studies focus on closely-related populations of single species, failing to consider the variable degrees of intrinsic reproductive isolation between most natural populations. Here, we dissected the interactions between *Wolbachia*-induced and host-associated incompatibilities in the haplodiploid spider mite *Tetranychus urticae*. We assessed their relative contribution to post-mating isolation between three populations of two genetically differentiated colour forms. We found that these two sources of incompatibility act through different mechanisms in an additive fashion. Host-associated incompatibility contributes 1.5 to 2 times more than *Wolbachia*-induced incompatibility, the former through an overproduction of haploid sons and the latter by increasing the embryonic mortality of daughters. Furthermore, regardless of cross direction, we observed near-complete hybrid sterility and complete F2 hybrid breakdown between populations of the two forms, but that *Wolbachia* did not contribute to this outcome. This study identifies the mechanistic independence and additive nature of these two sources of isolation, and suggests that *Wolbachia* could be an important driver of reproductive character displacement in this system, thereby potentially affecting host differentiation and distribution in the field.

Keywords

Reproductive manipulators; hybridization; reproductive isolation; speciation; reproductive interference; haplodiploidy.

Introduction

In the last decades, it has become increasingly clear that speciation is a continuous process (the "speciation continuum"; Hendry et al. 2000; Powell et al. 2013; Burri et al. 2015; Supple et al. 2015). Indeed, ongoing hybridization is taxonomically widespread, and ample variation in the extent and permeability of various reproductive barriers occurs both within and between species (Pinto et al. 1991; Mallet 2008; Hendry et al. 2009; Nosil et al. 2009). Moreover, theoretical studies show that stable partial reproductive isolation can be relatively common (reviewed by Servedio and Hermisson; 2020).

Partial reproductive isolation between lineages (*i.e.* differentiated populations or incipient species) can evolve in both sympatry and allopatry due to divergent (including disruptive; Rueffler et al. 2006) sexual and/or ecological selection, and/or as a result of stochastic processes (Schluter 2001; Turelli et al. 2001; Bolnick and Fitzpatrick 2007; Schluter 2009; Maan and Seehausen 2011; Nosil 2012). Additionally, in arthropods, partial (or complete) reproductive isolation between and within lineages can result from infection by different cytoplasmically-inherited bacterial reproductive manipulators (Duron et al. 2008; Engelstädter and Hurst 2009), among which *Wolbachia* is the most widespread (Weinert et al. 2015). This endosymbiont can induce various phenotypes of reproductive manipulation in its hosts, including the most common cytoplasmic incompatibility (CI; Werren et al. 2008; Engelstädter and Hurst 2009). CI is a conditional sterility phenotype resulting in increased embryonic mortality of offspring from crosses between infected males and uninfected females (or females harbouring an incompatible strain). Thus, *Wolbachia*-induced CI (wCI) can lead to substantial barriers to gene flow between individuals with different infection status, and could act as an agent of speciation (Laven 1959; Werren 1998; Bordenstein et al. 2001; Telschow et al. 2005; Jaenike et al. 2006). However, whether it plays a significant role in host speciation is still a matter of controversy, mainly because *Wolbachia* can rapidly invade host populations (*i.e.* most individuals rapidly become infected, thus immune to CI), and because wCI must produce a sufficient barrier to gene flow to allow nuclear divergence between populations (Hurst and Schilthuizen 1998; Werren 1998; Weeks et al. 2002; Bordenstein 2003). Nevertheless, stable infection polymorphs are often found in natural populations of many host species (*e.g.* Vavre et al. 2002; Keller et al. 2004; Zhang et al. 2013; Hamm et al. 2014; Zélé et al. 2018a). Moreover, whereas speciation induced by wCI may

require very specific conditions, it could play a significant role in host speciation by interacting with other (intrinsic) isolation mechanisms.

The fact that natural populations of many organisms often display variable degrees of reproductive isolation (Scopece et al. 2010; Jennings et al. 2011; Corbett-Detig et al. 2013; Harrison and Larson 2014) offers an excellent opportunity to address the role of wCI in ongoing speciation processes. Still, only few studies have done this, with very contrasting results. Indeed, these studies showed either no wCI in interspecific crosses (Maroja et al. 2008; Gazla and Carracedo 2009; Cooper et al. 2017), that wCI caused complete post-mating isolation between species (Bordenstein et al. 2001), or either a complementarity (Shoemaker et al. 1999; Dean and Dobson 2004; see also Gebiola et al. 2016 for CI induced by *Cardinium*), or a synergy (Gotoh et al. 2005) between wCI and host genetic factors in establishing post-mating isolation. However, in the latter scenarios, *i.e.* when both sources of incompatibility jointly reduce gene flow between genetically differentiated host populations and incipient species, whether they have additive or interacting effects, and precise quantification of their relative contribution to post-mating isolation, have not been addressed. This is at odds with the insight it would provide to better understanding the exact contribution of *Wolbachia* to ongoing processes of speciation in arthropods.

Tetranychus spider mites constitute an excellent system to address the interplay between symbiont-induced and host intrinsic reproductive incompatibilities. Indeed, they are arrhenotokous haplodiploids (*i.e.* males arise from unfertilized eggs and females from fertilized eggs Helle and Bolland 1967), which allows assessing fertilization failure by measuring sex-ratios. Moreover, variable degrees of reproductive isolation have been found both between and within species of this genus (*e.g.* Takafuji and Fujimoto 1985; Navajas et al. 2000; Sato et al. 2015; Clemente et al. 2016; Knegt et al. 2017). This is the case for the well-studied *Tetranychus urticae*, in which two genetically differentiated colour forms have recently diverged (Navajas et al. 1998; Hinomoto et al. 2001; Chen et al. 2014; Matsuda et al. 2018). Due to complete reproductive isolation among some populations of the two forms, they were historically described as separate species (*T. urticae* and *T. cinnabarinus*, for the 'green' and the 'red' form, respectively; Boudreaux 1956; Van de Bund and Helle 1960; Helle and Van de Bund 1962; Smith 1975). However, subsequent studies reclassified them as semi-species (Goka et al. 1996) or members of the same species (Dupont 1979; Fry 1989; Auger et al. 2013), given that many populations of the two forms are almost completely compatible

(Keh 1952; Saba 1975; Murtaugh and Wrensch 1978; Dupont 1979; de Boer 1982b,a; Sugasawa et al. 2002), suggesting that a speciation process is currently ongoing in this species.

Populations of the two forms of *T. urticae* are also often naturally infected with different *Wolbachia* strains (Gotoh et al. 2003, 2007; Zhang et al. 2016; Zélé et al. 2018a), which induce variable levels of wCI (Perrot-Minnot et al. 2002; Vala et al. 2002; Gotoh et al. 2007; Xie et al. 2010; Suh et al. 2015; Zélé et al. 2020b). In spider mites, as in some other haplodiploid species (*e.g.* Breeuwer and Werren 1990; Vavre et al. 2001), diploid zygotes arising from incompatible crosses between an infected male and an uninfected female may survive as haploid males after complete elimination of the paternal set of chromosomes (Male development - MD-type incompatibility; Vala et al. 2000; Perrot-Minnot et al. 2002; Gotoh et al. 2003). In most cases, however, fertilized eggs in incompatible crosses fail to hatch as in diploid species, leading to embryonic mortality of female offspring (Female mortality - FM-CI type incompatibility; Breeuwer 1997; Perrot-Minnot et al. 2002; Vala et al. 2002; Gotoh et al. 2003, 2007; Xie et al. 2010; Suh et al. 2015; Bing et al. 2019; Zélé et al. 2020b).

In this study, we assessed the interplay and the relative contribution of *Wolbachia*-induced CI (wCI) and host-associated incompatibilities (HI) on post-mating isolation between three naturally *Wolbachia*-infected populations, two from the red form and one from the green form of *T. urticae*. We performed all possible crosses between *Wolbachia*-infected and *Wolbachia*-free populations in a full-factorial design and measured the embryonic and juvenile mortality of the offspring, as well as the proportion of males and females produced from each cross, over two generations.

Methods

Spider mite populations

Three different populations of spider mites, all collected in Portugal and naturally infected with *Wolbachia*, were used in this study. Two populations, 'Ri1' and 'Ri2', belong to the red form of *T. urticae* and share the same *ITS2* rDNA and *COI* mtDNA sequences. The third population, 'Gi', belongs to the green form of *T. urticae* and differs from the former two populations in both *ITS2* rDNA and *COI* mtDNA (*cf.* detailed information in Box S1). The

Wolbachia strains infecting Ri1 and Ri2 are mutually compatible but induce different levels of cytoplasmic incompatibility despite identical MLST profiles (Zélé et al. 2020b). The *Wolbachia* strain infecting Gi, however, slightly differs from the former two based on MLST and whether it induces CI in this population was heretofore unknown. Since field collection (*cf.* Box S1), these populations were reared in the laboratory under standard conditions (24±2ºC, 16/8h L/D) at very high numbers (*ca.* 500-1000 females per population) in insect-proof cages containing bean plants (*Phaseolus vulgaris*, cv. Contender seedlings obtained from Germisem, Oliveira do Hospital, Portugal).

Antibiotic treatments

After collection, subsets of Gi, Ri1 and Ri2 populations were treated with antibiotics to obtain the corresponding *Wolbachia*-free populations Gu, Ru1 and Ru2. For logistic reasons, the populations Gu and Ru2 used in each of the two experiments reported here were created from two different antibiotic treatments. For Experiment 1, Gu was obtained from a treatment performed in November 2013, and Ru1 and Ru2 from treatments performed in February 2014. Briefly, 100 Gi and 30 Ri1 or Ri2 adult females were installed in petri dishes containing bean leaf fragments, placed on cotton soaked in a tetracycline solution (0.1%, w/v) for three successive generations (Breeuwer 1997; Zélé et al. 2020b). For Experiment 2, Ru1 came from the previous antibiotic treatment but Gu and Ru2 were obtained from new treatments performed in September 2016 and January 2017, respectively. In this case, 300 Gi or Ri2 adult females were installed in petri dishes containing fragments of bean leaves placed on cotton soaked in a rifampicin solution (0.05%, w/v) for one generation (Gotoh et al. 2005; Zélé et al. 2020a). All antibiotic treatments were performed in the same standard conditions as population rearing (24±2ºC, 16/8h L/D). After treatment, *Wolbachia*-free populations were maintained without antibiotics in the same mass-rearing conditions as the *Wolbachia*-infected populations for a minimum of three generations to avoid potential side effects of antibiotics (Ballard and Melvin 2007; Zeh et al. 2012; O'Shea and Singh 2015). Subsequently, pools of 100 females from each population were checked by multiplex PCR as described by Zélé et al. (2018b) to confirm their *Wolbachia* infection status before performing the experiments.

122 **Experiment 1: F1 production and viability**

123 The combined effect of *Wolbachia*- and host-associated incompatibilities (wCI and HI,
124 respectively) on offspring production was investigated by performing all crosses between
125 *Wolbachia*-infected and uninfected individuals from all populations in a full factorial design.
126 These crosses were organized into 5 different categories, each with a different purpose (*cf.*
127 Table 1).

Table 1. Description of the five categories of crosses performed in this study.

Category	**Type of crosses**	**Crosses** (♀ x ♂)
1 - Controls	intra-population crosses using ♀ and ♂ with the same infection status	Ru1 x Ru1 and Ri1 x Ri1 Ru2 x Ru2 and Ri2 x Ri2 Gu x Gu and Gi x Gi
2 – Test for wCI only	intra-population crosses using uninfected ♀ and infected ♂	Ru1 x Ri1 Ru2 x Ri2 Gu x Gi
3 – Test for HI only (without *Wolbachia*)	inter-population crosses using uninfected ♀ and uninfected ♂	Ru1 x Ru2 or Gu Ru2 x Ru1 or Gu Gu x Ru1 or Ru2
4 – Test for wCI-HI interaction	inter-population crosses using (un)infected ♀ and infected ♂	Ru1 or Ri1 x Ri2 or Gi Ru2 or Ri2 x Ri1 or Gi Gu or Gi x Ri1 or Ri2
5 – Test for HI only (with *Wolbachia*, to verify that infection itself, in absence of wCI, does not affect HI)*	inter-population crosses using infected ♀ and uninfected ♂ (+ intra-population controls)	Ri1 x Ru2 or Gu Ri2 x Ru1 or Gu Gi x Ru1 or Ru2 (Ri1 x Ru1, Ri2 x Ru2, Gi x Gu)

*crosses not performed simultaneously with the others in Experiment 1. The corresponding results were thus analysed separately and are presented in the supplementary materials (Table S1; Figures S1 and S2).

128 Ten days prior to the onset of the experiment (day -10), age cohorts were created for
129 each infected and uninfected population, by allowing 3*100 mated females (*i.e.* 'female
130 cohorts') and 4*25 quiescent females (*i.e.* 'male cohorts') to lay eggs during 3 days on
131 detached bean leaves placed on water-soaked cotton. Eight days later (day -2), quiescent
132 females with similar age were randomly collected from each female cohort and placed
133 separately on bean leaf fragments (*ca.* 9 cm^2) where they completed their last moult, thus
134 becoming virgin adult females. Virgin males used in the experiment were directly obtained
135 from the male cohorts. On the first day of the experiment (day 0), 1 virgin female and 1
136 virgin male were installed together on 2.5 cm^2 bean leaf discs for 3 days before being

discarded (day 3). The number of unhatched eggs was counted 5 days later (day 8), and the numbers of dead juveniles, adult males and females were counted 12 days later (day 15).

The experiment was conducted in a growth chamber with standard conditions (24±2°C, 60% RH, 16/8 h L/D). All types of crosses were performed simultaneously, each with 50 independent replicates distributed within two experimental blocks performed one day apart (*i.e.* 25 replicates per block). However, given the high number of possible types of crosses (*i.e.* 36 combinations) and associated workload, the crosses of category 5 were performed *ca*. 23 months later with minor differences in the methodology (*cf.* details in Box S2). Therefore, data obtained with this latter category were analysed separately and are provided in the supplementary materials (Table S1, Figures S1 and S2).

To calculate the overproduction of F1 males in the brood (MD-type incompatibility; *e.g.* Breeuwer and Werren 1990; Navajas et al. 2000; Vala et al. 2000; Vavre et al. 2001) or embryonic mortality of fertilized offspring (*i.e.* only females in haplodiploids, hence FM-type incompatibility; Vavre et al. 2000; Vala et al. 2002; Gotoh et al. 2007; Suh et al. 2015; Zélé et al. 2020b), we used indexes adapted from Poinsot et al (1998; see also Cattel et al. 2018; Zélé et al. 2020). MD-type incompatibility was computed as the proportion of sons produced in each cross relative to the control crosses:

$$MD_{corr} = \frac{MD_{obs} - CCMD}{1 - CCMD}$$

where MD_{obs} = number of F1 males/total number of eggs, and $CCMD$ (calculated as MD_{obs}) is the mean proportion of F1 males observed in control crosses (*i.e.* between uninfected individuals of the same maternal population).

Similarly, FM-type incompatibility was computed as the proportion of embryonic death of daughters produced in each cross relative to the control crosses (hence accounting for variation in background embryonic mortality of both F1 males and females):

$$FM_{corr} = \frac{FM_{obs} - CCFM}{1 - CCFM}$$

where FM_{obs} = number of unhatched eggs/[number of unhatched eggs + number of F1 females], and $CCFM$ (calculated as FM_{obs}) is the mean embryonic mortality observed in the control crosses. To avoid biases arising from very low numbers of F1 females produced in some inter-population crosses due to MD-type incompatibilities (*cf.* above and results), all females that produced less than two daughters were removed from statistical analyses of FM_{corr} (*cf.* final sample sizes in Table S1).

Subsequently, to control for potential incompatibilities affecting juvenile survival, we estimated the proportion of dead juveniles in the brood accounting for variation in background juvenile mortality (hence including juvenile mortality of both F1 males and females):

$$JM_{corr} = \frac{JM_{obs} - CCJM}{1 - CCJM}$$

where JM_{obs} = number of dead juveniles/total number of eggs, and $CCJM$ (calculated as JM_{obs}) is the mean juvenile mortality observed in control crosses.

Finally, as both FM- and MD-type incompatibilities affect the proportion of F1 (hybrid) females, their combined effect was determined by assessing the proportion of F1 females (FP) resulting from each type of cross:

$$FP = \frac{number\ of\ F1\ females}{total\ number\ of\ eggs}$$

Moreover, to determine the interplay between FM- and MD-type incompatibilities on hybrid production, we predicted the proportion of F1 females that should be produced in crosses affected by both incompatibilities, assuming that their effects are independent (H_0 hypothesis):

$$FP_{pred} = \frac{FP_{md} \times FP_{fm}}{FP_{comp}}$$

where FP_{comp}, FP_{md} and FP_{fm} are the mean proportions of F1 females observed in the corresponding compatible crosses, in crosses affected only by MD-type incompatibility, and in crosses affected only by FM-type incompatibility. Thus, this formula assumes that the decrease in female production due to FM-type incompatibility in crosses already affected by MD-type incompatibility is the same as that observed between compatible crosses and crosses affected by FM-type incompatibility only (and inversely for MD-type incompatibility). Deviations from this prediction indicate that the two types of incompatibility interfere with each other, that is, they are not independent.

To compare, for each cross, observed and predicted values, we used a Goodness-of-fit Test, with the Pearson goodness-of-fit statistic calculated as follows:

$$\chi^2_{df} = \sum_j \frac{(FP_{obs_j} - FP_{pred})^2}{FP_{pred}}$$

where FP_{obs_j} is the proportion of F1 females observed in each replicate j, and FP_{pred}

196 is the predicted proportion of F1 females under the assumption that the null hypothesis is
197 true. P-values were defined as the proportion of times the observed values were equal or
198 lower than the predicted one (Fragata et al. 2014).
199
200 **Experiment 2: F1 fertility and F2 viability**
201 To assess the fertility of F1 offspring obtained from inter-population crosses and potential
202 unviability of F2 offspring (*i.e.* hybrid breakdown; de Boer 1982b; Sugasawa et al. 2002), all
203 crosses performed in Experiment 1, except those involving Ru2 and Ri2 (because they
204 yielded results similar to Ru1 and Ri1), were repeated in panmixia to obtain large numbers of
205 individuals 13 days prior to the onset of the experiment (day -13). For each cross, 100 virgin
206 females were placed with 100 males (obtained from age cohorts as described for Experiment
207 1) on an entire bean leaf to produce F1 offspring of the same age. These offspring were used
208 separately to test for F1 female fertility and viability of their offspring (test 1 below) and F1
209 male fertility and viability of their offspring (test 2 below).
210 The experiment was conducted in a growth chamber with standard conditions
211 (24±2°C, 16/8 h L/D). In the first test, F1 females from all types of cross were tested
212 simultaneously within four experimental blocks (with a maximum of 25 females per cross
213 tested in each block), while in the second test, uninfected and infected F1 males (*i.e.* sons of
214 uninfected or infected females, respectively, independently of the male mated with these
215 females) were tested (and thus analysed) separately. Uninfected F1 males were tested
216 within 3 experimental blocks (with a maximum of 30 males per cross tested in each block);
217 and infected F1 males within 2 experimental blocks (with a maximum of 24 males per cross
218 tested in each block). The number of replicates in each test was limited to the number of F1
219 offspring that could be obtained from the crosses performed in panmixia (*cf.* final sample
220 sizes in Table S2).
221
222 *Test 1: F1 female fertility and F2 viability*
223 Quiescent F1 females were collected from each cross performed in panmixia and installed on
224 9 cm^2 bean leaf fragments 2 days prior to the beginning of experiment (day -2) to emerge as
225 adults while remaining virgin. They were then isolated on 2.5 cm^2 bean leaf discs on the first
226 experimental day (day 0), and allowed to lay eggs for 4 days, after which they were
227 discarded and the number of eggs laid was counted (day 4). The number of unhatched eggs

was counted 5 days later (day 9), and the numbers of dead juveniles and adult males were counted 12 days later (day 16; as mothers were virgin, they could only produce sons).

As F1 female fertility corresponds to their ability to lay at least one egg, and/or a normal number of eggs (Navajas et al. 2000), we estimated both the proportion of ovipositing females and the daily oviposition of these females, taking into account their daily mortality (*i.e.* total number of eggs laid by each female/total number of days each female was alive). Hybrid breakdown was assessed as male embryonic and juvenile mortality accounting for variation in background mortality (*i.e.* not related to hybrid breakdown). The corresponding mEM_{corr} and mJM_{corr} indexes were calculated as follows:

$$mEM_{corr} = \frac{mEM_{obs} - CCmEM}{1 - CCmEM}$$

where mEM_{obs} = number of unhatched eggs/total number of eggs, and $CCmEM$ (calculated as mEM_{obs}) is the mean embryonic mortality observed in control crosses (*i.e.* category 1);

$$mJM_{corr} = \frac{mJM_{obs} - CCmJM}{1 - CCmJM}$$

where mJM_{obs} = number of dead juveniles/total number of eggs, and $CCmJM$ (calculated as mJM_{obs}) is the mean juvenile mortality observed in control crosses (*i.e.* category 1).

Test 2: F1 male fertility and F2 viability

As in haplodiploids sons are hemiclones of their mothers, they inherit a single maternal chromosome. Thus, in absence of reproductive anomalies they should be fully compatible with females from their maternal population, whereas the expression of an incompatibility may indicate that these males are aneuploid. To test this, adult F1 males were collected from each cross performed in panmixia and placed on 9 cm^2 bean leaf fragments 2 days prior to the beginning of experiment (day -2) to avoid sperm depletion. On the first experimental day (day 0), each male was installed with one virgin female (obtained from age cohorts created as in Experiment 1) from the same population as its mother on a 2.5 cm^2 bean leaf disc. Four days were given for males to mate and for the females to lay eggs before both were discarded (day 4). The number of unhatched eggs was counted 5 days later (day 9), and the numbers of dead juveniles, adult males and adult females were counted 12 days later (day 16).

As F1 male fertility corresponds to their ability to sire at least one daughter and/or a normal proportion of offspring (*i.e.* F2 females), we estimated both the proportion of males siring daughter(s) and the sex ratio (SR; here calculated as the ratio of females to males because haploid males only sire daughters) in the adult offspring of the females they mated with. Hybrid breakdown was assessed as F2 female embryonic and juvenile mortality accounting for variation in background mortality. As above, fEM_{corr} and fJM_{corr} indexes were calculated as:

$$fEM_{corr} = \frac{fEM_{obs} - CCfEM}{1 - CCfEM}$$

where fEM_{obs} = number of unhatched eggs/[number of unhatched eggs + number of F2 females] and $CCfEM$ (calculated as fEM_{obs}) is the mean embryonic mortality observed in control crosses (*i.e.* category 1);

$$fJM_{corr} = \frac{fJM_{obs} - CCfJM}{1 - CCfJM}$$

where fJM_{obs} = number of dead juveniles/[number of dead juveniles + number of F2 females] and $CCfJM$ (calculated as fJM_{obs}) is the mean juvenile mortality observed in control crosses (*i.e.* category 1).

Statistical analyses

Analyses were carried out using the R statistical package (v3.6.1). The different statistical models built to analyse the data are described in the Supplementary Materials Table S3. The general procedure to analyse all response variables was as follows: the type of cross was fit as fixed explanatory variable and block was fit as a random explanatory variable. In addition, for the analyses of the proportion of fertile F1 females (*i.e.* females that produced at least one egg) and F1 males (*i.e.* males that sired at least one daughter), their daily mortality over the 4-day oviposition period was added to the models as it significantly improved their fit. Proportion data were computed as binary response variables (fertile or sterile F1 females and males), or using the function cbind (*i.e.* female proportion and sex-ratio, respectively), except for all corrected variables (*e.g.* FM_{corr}, MD_{corr}, etc.), which are continuous variables bounded between 0 and 1, and for which a "weights" argument was added to the models to account for the number of observations on which they are based. All data were subsequently analysed using generalized linear mixed models with the glmmTMB procedure

(glmmTMB package), which allows using a wide range of error distributions that are not implemented in the glmer procedure (Brooks et al. 2017). Proportion data were analysed with a binomial error distribution, or a (zero-inflated) betabinomial error distribution to account for overdispersed errors, and F1 female daily oviposition in experiment 2 was analysed using a log-linked Gaussian error distribution. For all analyses, the significance of the explanatory variable 'cross' was established using chi-square tests (Bolker et al. 2009). When explanatory variables were found to be significant, *a posteriori* contrasts were carried out between crosses by aggregating factor levels together and testing the fit of the simplified model using ANOVA (Crawley 2007). Holm-Bonferroni corrections were applied to account for multiple testing (*i.e.* classical chi-square Wald test for testing the global hypothesis H_0; Holm 1979).

Results

F1 offspring production and viability

Reciprocal crosses performed between naturally *Wolbachia*-infected or *Wolbachia*-free populations of the red ('Ri1' and 'Ri2') and green ('Gi') form of *T. urticae* allowed testing for the effects of wCI only, HI only, and the combined effect of both sources of incompatibility (*cf. Methods* and Table 1). Overall, we found no significant differences in juvenile mortality among crosses (see Figure 1, Tables S1 and S3), but ample variation in embryonic mortality (*i.e.* proportion of unhatched eggs) and/or in male production, both leading to an important decrease in female production (Figures 1 and S1). To dissect, in each type of crosses, the sources of such variation (*i.e.* wCI and/or HI), we determined incompatibilities of the MD-type (male development; overproduction of males resulting from fertilization failure and/or paternal genome elimination) and FM-type (female embryonic mortality resulting from paternal genome fragmentation), and measured the resulting proportion of F1 hybrids (only females in haplodiploids).

Overproduction of males (MD-type incompatibility)

Overall, we found an overproduction of males (*i.e.* higher values of the MD_{corr} index; *cf. Methods*) in all inter-population crosses involving females from the green population (*ca.* 46.4 to 64.3%) relative to all other crosses (*ca.* 5.6 to 21.5%; *Main cross effect*: χ^2_{26}=460.70,

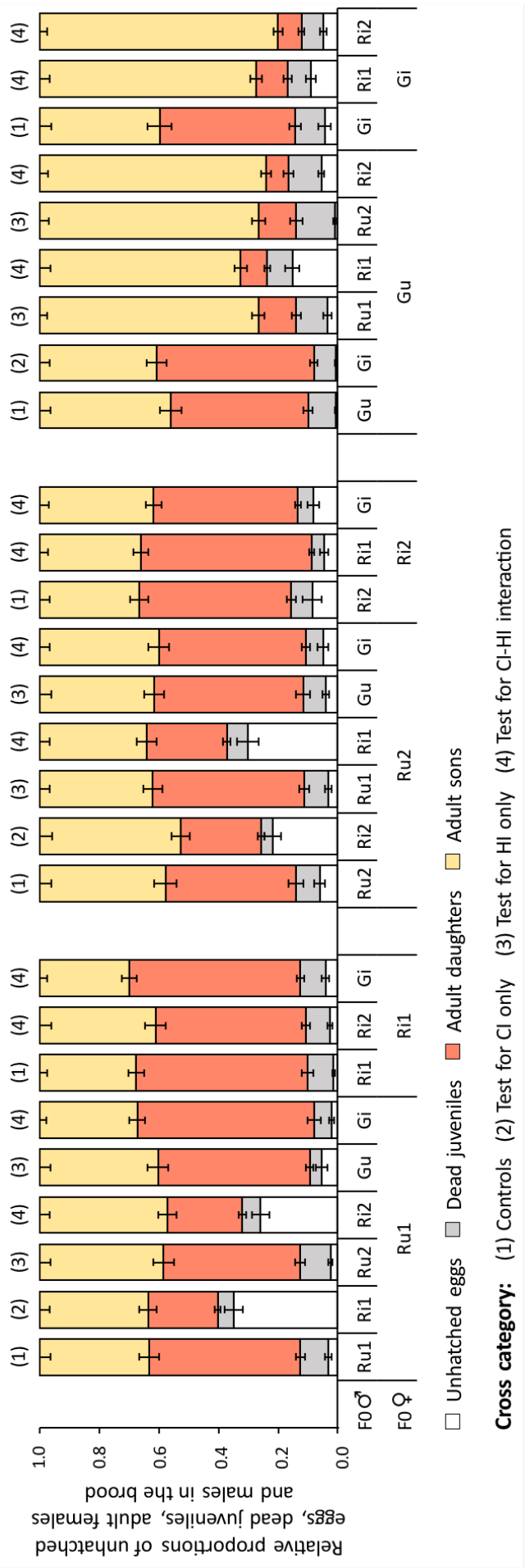

Figure 1. Summary of the development of *T. urticae* eggs resulting from intra- and inter-population crosses between *Wolbachia*-infected and -uninfected mites. Bar plots represent mean ± s.e. relative proportions of unhatched eggs (i.e. embryonic mortality), dead juveniles (i.e. juvenile mortality), adult daughters and sons for each type of cross. Mothers are displayed at the bottom level of the x-axis and fathers on the top level; Cross categories (1) to (4) are displayed above each bar.

329 p<0.0001; model 1.1, Figure 2a for crosses of categories 1 to 4). Moreover, the level of MD-
330 type incompatibility in these inter-population crosses involving green females was not
331 affected by *Wolbachia* infection in red males (*Contrasts among all inter-population crosses*
332 *using Gu or Gi*♀: χ^2_5=7.69, p=0.17). In contrast, regardless of *Wolbachia* infection, we found
333 no overproduction of males in any of the inter-population crosses involving red females
334 (*Contrasts among all crosses with low* MD_{corr} *including the controls*: χ^2_{20}=26.11, p=0.16).
335 Finally, the analysis of crosses involving *Wolbachia*-infected females and uninfected males
336 (*i.e.* crosses of category 5; Figure S2a) revealed that *Wolbachia* infection in females also does
337 not affect this pattern, as higher values of MD_{corr} were found for inter-population crosses
338 involving green females (*ca.* 57.9 to 64.5%) as compared to all other crosses (*ca.* 5.9 to
339 30.3%; *Main cross effect*: χ^2_8=174.26, p<0.0001; model 1.2; Table S2).
340
341 *Hybrid (female) embryonic mortality (FM-type incompatibility)*
342 Overall, we found higher levels of female embryonic mortality relative to controls (FM_{corr}
343 index; *cf. Methods*) in all crosses using *Wolbachia*-infected red males, either crossed with
344 uninfected red females (*i.e.* due to wCI as found by Zélé et al; 2020b), or with green females
345 independently of their *Wolbachia* infection status (from 22.2 to 42.7% on average; *Main*
346 *cross effect*: χ^2_{26}=506.20, p<0.0001; model 1.3; Figure 2b). In addition, there were no
347 significant differences among these crosses (χ^2_7=8.76, p=0.27; despite a tendency for Ri1
348 males to induce higher levels of FM-type CI than Ri2 males: 35% vs. 29% on average), which
349 shows that the *Wolbachia* strain infecting the green population did not rescue (even
350 partially) wCI induced by *Wolbachia*-infection in red males. All other crosses resulted in no
351 (or low) female embryonic mortality (from 0.2 to 10.5% on average; *Contrasts among all*
352 *these crosses with low* FM_{corr}: χ^2_{16}=19.99, p=0.22). Thus, these results restrict FM-type
353 incompatibilities between populations to *Wolbachia* infection in males from the two red
354 populations, with the same penetrance in inter-population and intra-population crosses.
355
356 *Consequences of MD- and FM-type incompatibilities for hybrid (female) production*
357 As a result of the MD- and FM-type incompatibilities described above, we also found ample
358 variation in the proportion of females (FP) produced across crosses (*Main cross effect*:
359 χ^2_{26}=966.45, p<0.0001; model 1.7; Figure 2c). Our contrast analyses further revealed four
360 statistically different proportions depending on the type of crosses: (1) *ca.* 51% daughters

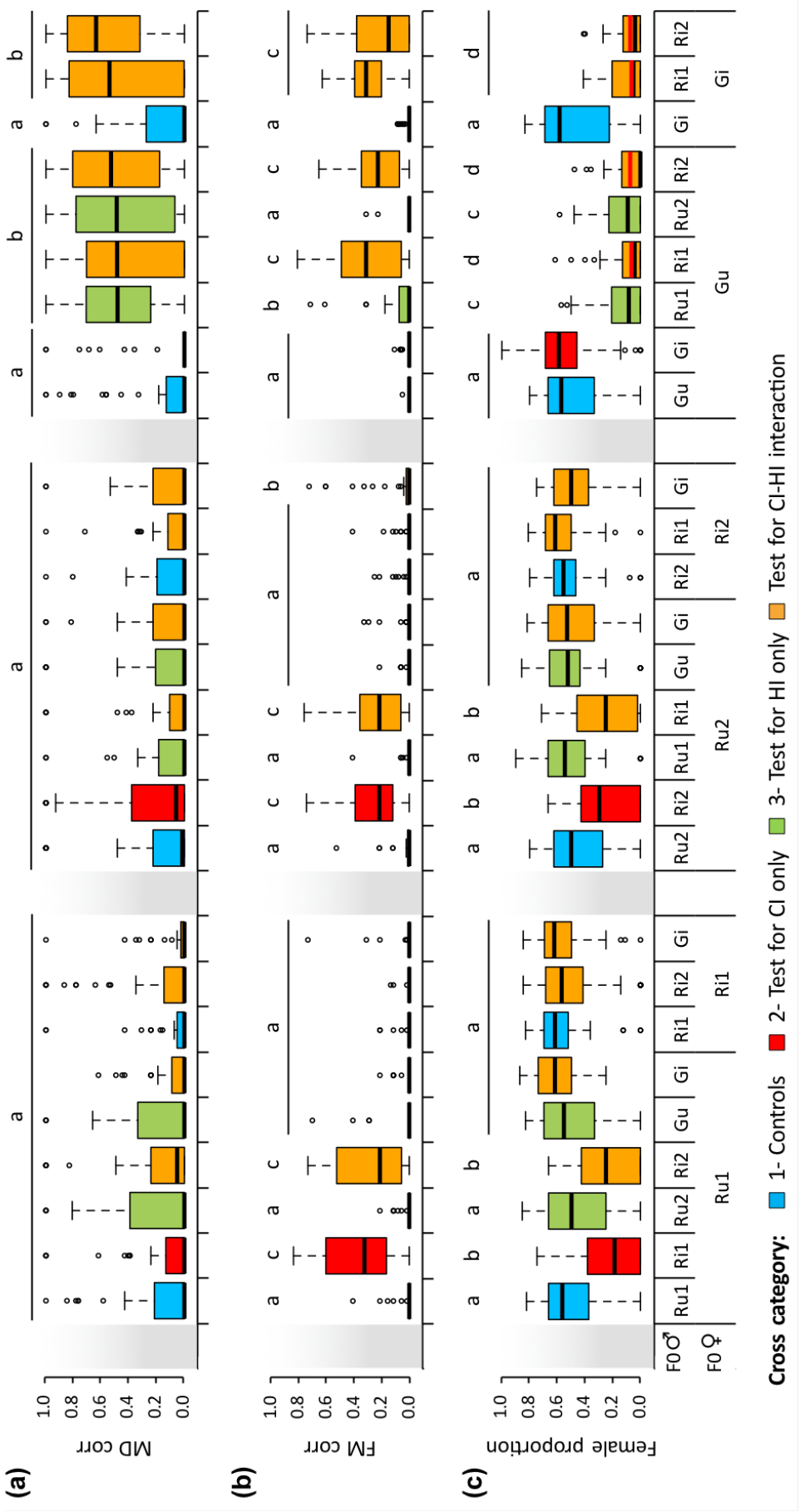

Figure 2. Overproduction of males, female embryonic mortality, and resulting hybrid production in intra- and inter-population crosses using *Wolbachia*-infected and uninfected mites. (a) Boxplot of the proportion of males produced in all crosses relative to that in control crosses (MD_{corr}). **(b)** Boxplot of the proportion of unhatched eggs relative to females, accounting for the basal level of this proportion observed in control crosses (FM_{corr}). **(c)** Proportion of F1 adult females (*i.e.* hybrids) in the brood. Horizontal red bars displayed within boxes for crosses affected by both MD- and FM-type incompatibilities indicate predicted values of female proportion (FP_{pred}) under the assumption that the two types of incompatibilities have an independent effect on hybrid production. Mothers are displayed on the bottom level of the x-axis and fathers on the top level. Identical or absent superscripts indicate nonsignificant differences at the 5% level among crosses.

produced in compatible crosses (*i.e.* unaffected by both incompatibilities; *Contrasts among these crosses*: χ^2_{16}=21.22, *p*=0.17); (2) *ca.* 26% daughters produced in crosses affected by FM-type incompatibilities only (*Contrasts among these crosses*: χ^2_3=2.98, *p*=0.40; *ca.* 50% decrease compared to compatible crosses: χ^2_1=187.67, *p*<0.0001); (3) *ca.* 13% daughters produced in crosses affected by MD-type incompatibilities only (*Contrasts among these crosses*: χ^2_1=0.04, *p*=0.84; *ca.* 75% decrease compared to compatible crosses: χ^2_1=292.02, *p*<0.0001; and *ca.* 76% decrease when using crosses of category 5: χ^2_8=278.23, p<0.0001; model 1.8; Figure S2c); and (4) *ca.* 9% daughters produced in crosses affected by both FM- and MD-type incompatibilities (*Contrasts among these crosses*: χ^2_3=3.57, *p*=0.31; *ca.* 83% decrease compared to compatible crosses: χ^2_1=606.40, *p*<0.0001).

Both types of incompatibility appeared to have lower consequences on hybrid production when combined than when acting alone. Indeed, we found around 31% decrease in hybrid production due to FM-type incompatibility when comparing groups (3) and (4) (χ^2_1=7.49, *p*=0.03) and close to 65% decrease in hybrid production due to MD-type incompatibility when comparing groups (2) and (4) (χ^2_1=141.97, *p*<0.0001). However, this was only a consequence of their cumulative effects. Indeed, we found a perfect fit between the observed and the predicted proportions of F1 females for crosses affected by both MD- and FM-type incompatibilities, calculated assuming that both affect hybrid production with the same strength when acting either alone or combined (Figure 2c; Goodness-of-fit test: *Gu*♀*xRi1*♂: χ^2_{47}=14.30, *p*=0.58; *Gu*♀*xRi2*♂: χ^2_{47}=8.46, *p*=0.65; *Gi*♀*xRi1*♂: χ^2_{47}=13.90, *p*=0.56; and *Gi*♀*xRi2*♂: χ^2_{48}=7.37, *p*=0.59). Thus, these results show that MD- and FM-type incompatibilities are independent, so that their effects are additive with the former contributing 1.5 to 2 times more than the latter in reducing hybrid production.

F1 offspring fertility and viability of the F2

To estimate the effects of wCI and HI on the fitness of F1 offspring obtained from all aforementioned crosses (except those involving Ru2 and Ri2 populations, *cf.* Methods), we assessed the fertility of virgin F1 females and of F1 males backcrossed to females from their maternal population, and both embryonic and juvenile mortality of the resulting F2 offspring (*i.e.* hybrid breakdown; de Boer 1982b; Sugasawa et al. 2002).

Fertility of F1 females and viability of their offspring (Test 1)

The proportion of fertile F1 females (*i.e.* virgin females that laid at least 1 egg) differed significantly among crosses (χ^2_{15}=214.26, p<0.0001; model 2.1; Figure 3a). Indeed, while the proportion of fertile F1 females resulting from all intra-population crosses was very high (*ca.* 96% on average; *Contrasts among intra-population crosses*; χ^2_7=8.42, p=0.30), more than 99% of the hybrid females resulting from inter-population crosses were unable to lay eggs. Moreover, although 6 hybrid females (over a total of 760), all resulting from crosses between males and females with the same *Wolbachia* infection status (either both infected, or both uninfected), were found to be fertile, they laid very few eggs (average daily oviposition of 0.63 ± 0.15 compared to 6.37 ± 0.09 for females resulting from intra-population crosses; *cf.* Table S3), with no significant difference among inter-population crosses (*Contrasts among inter-population crosses*; χ^2_7=8.59, p=0.28).

None of the eggs laid by the 6 fertile hybrid females hatched (Table S3), which, despite the low number of eggs (15 in total), corresponds to full F2 hybrid breakdown. In contrast, embryonic mortality (mEM_{corr}) of eggs laid by F1 females resulting from intra-population crosses was very low (*ca.* 5%), with only a very small increased mortality (*ca.* 2%) in the brood of F1 females from the green population when infected by *Wolbachia* (*Main cross effect:* χ^2_7=23.33, p=0.001; model 2.3; Figure S3a). Similarly, juvenile mortality (mJM_{corr}) in the offspring (*i.e.* all F2 males) of virgin F1 females resulting from intra-population crosses was very low (below *ca.* 6%), and varied slightly depending on their maternal origin (*Main cross effect:* χ^2_7=18.57, p=0.01; model 2.4; Figure S3b). Indeed, the offspring of infected green F1 females had higher juvenile mortality than the offspring of infected red females (independently of their grandfather; *Contrasts between Gi and Ri1 females*: χ^2_1=12.53, p=0.002), and the offspring of all uninfected F1 females displayed an intermediate mortality (*Contrasts between Gu-Ru1 and Gi females:* χ^2_1=4.28, p=0.17; *Contrasts between Gu-Ru1 and Ri1 females:* χ^2_1=4.49, p=0.17).

Fertility of F1 males and viability of their offspring (Test 2)

The proportion of fertile F1 males (*i.e.* males siring at least one daughter when backcrossed with a female from their maternal population) differed significantly among crosses (χ^2_7=25.58, p<0.001; model 2.5.1, and χ^2_7=15.23, p=0.03; model 2.5.2, for uninfected and infected males, respectively), but this difference was mainly attributable to the maternal

424 populations of the F1 males (hence potentially the females mated with F1 males instead of
425 the males themselves). Indeed, F1 males mated with (and sons of) green females were less
426 fertile than those mated with (and sons of) red females (*ca.* 17.39% and 25.97%, for
427 uninfected and infected males, respectively; *cf.* Figure 3b).

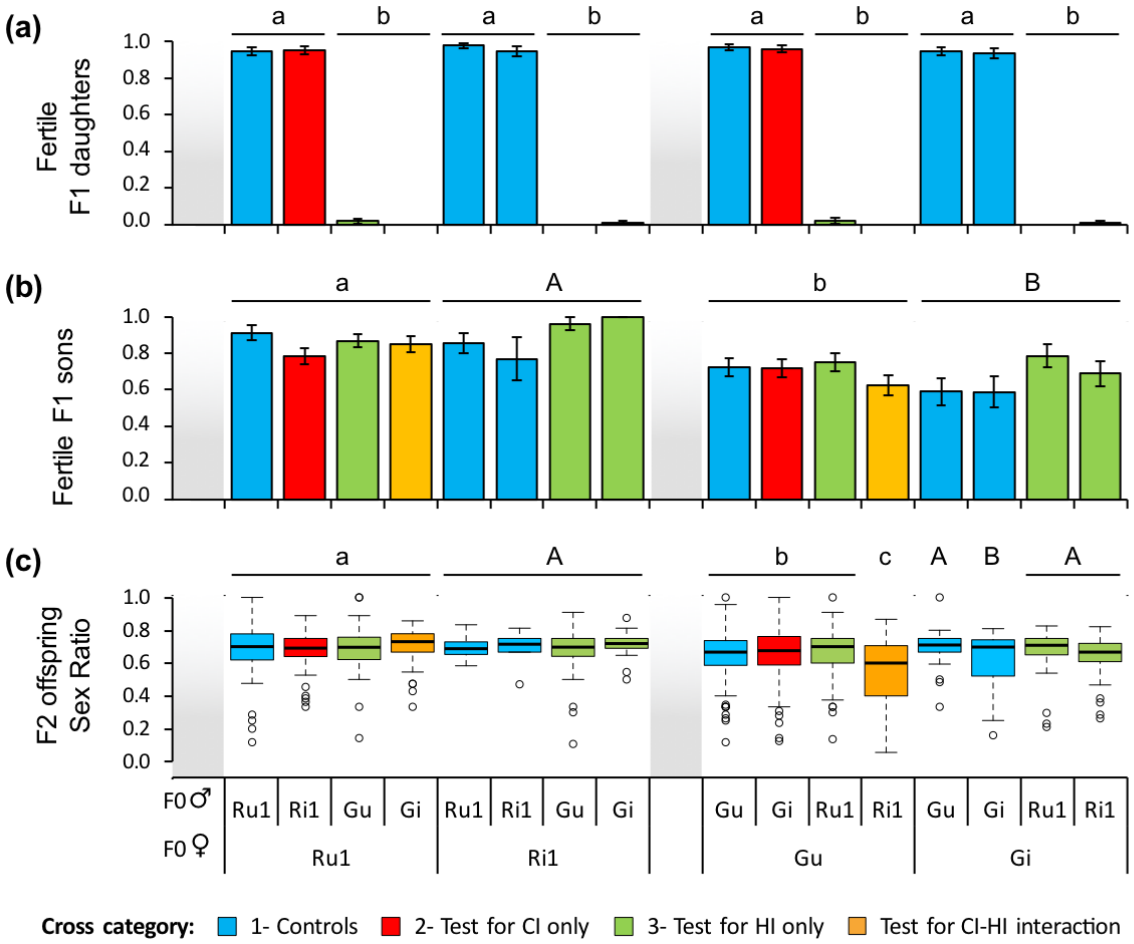

Figure 3. Proportion of fertile F1 female and male offspring resulting from intra- and inter-population crosses using *Wolbachia*-infected and uninfected mites, and sex-ratio of F2 offspring resulting from backcrosses of F1 males. Average proportion (± s.e.) of **(a)** fertile F1 females (*i.e.* proportion of females laying at least 1 egg) and **(b)** fertile F1 males (*i.e.* proportion of males siring at least 1 daughter when mated with a female from the same population as their mother). **(c)** Boxplot of sex ratio (daughters to sons) of F2 offspring sired by F1 males. Mothers are displayed on the bottom level of the x-axis and fathers on the top level. Identical or absent superscripts indicate nonsignificant differences at the 5% level among crosses.

428 The maternal population of fertile F1 males also affected the proportion of daughters
429 they sired, but only when they were uninfected by *Wolbachia* (χ^2_7=42.10, p<0.0001; model
430 2.8.1; Figure 3c). In this case, we found that F1 males mated with (and sons of) red females
431 sired on average more offspring (*ca.* 69%) than F1 males mated with (and sons of) green

females (*ca.* 55% for those mated with red infected males; χ^2_1=32.13, p<0.0001; and *ca.* 65% for those mated with all other males; χ^2_1=8.96, p=0.008). We also found some differences in the sex-ratio of offspring resulting from crosses using infected F1 males (χ^2_7=15.19, p=0.03; model 2.8.2), but this effect was only due to a higher variance (but not median) in the Gi♀xGi♂ control cross, and no difference was found among all other crosses (χ^2_6=9.93, p=0.13; Figure 3c).

Finally, both embryonic mortality (fEM_{corr}) and juvenile mortality (fJM_{corr}) varied among the offspring of F1 uninfected males (*Main cross effect on* fEM_{corr}*:* χ^2_7=26.31, p<0.001; model 2.6.1, and *on* fJM_{corr}*:* χ^2_7=22.64, p=0.002; model 2.7.1). Indeed, uninfected F1 males mated with (and sons of) green females sired offspring with a higher embryonic mortality (*ca.* 7% on average) than those mated with, or sons of, red females (*ca.* 4% in average). However, the variations observed in juvenile mortality cannot necessarily be explained by the maternal populations of F1 uninfected males (or the females they mated with), nor by *Wolbachia* infection in the mates of their mother (see Figure S4b). In contrast, no differences were detected among the offspring of infected F1 males (*Main cross effect on* fEM_{corr}*:* χ^2_7=5.58, p=0.59; model 2.6.2, and *on* fJM_{corr}*:* χ^2_7=11.68, p=0.11; model 2.7.2).

Discussion

Using three populations of the two genetically differentiated colour forms of *T. urticae*, each naturally infected or cured from *Wolbachia*, we assessed the relative contribution of *Wolbachia*-induced CI (wCI) and of host-associated incompatibilities (HI) to post-mating isolation. Our results revealed that both sources of incompatibility jointly reduced the production of F1 hybrid females in the same direction, albeit through different and independent mechanisms, and with HI contributing up to 2 times more than wCI. Additionally, we found a *Wolbachia*-independent near-complete F1 hybrid female sterility and F2 hybrid breakdown in all reciprocal crosses between the green and the red populations.

Expression of host-associated incompatibilities

Crosses performed among uninfected host populations in absence of *Wolbachia* infection confirmed that the two populations belonging to the red form of *T. urticae* were mutually

461 compatible (Zélé et al. 2020b), but were fully isolated from the green-form population. We
462 found three different types of post-mating reproductive barriers between these populations:
463 (1) a sharp and unidirectional reduction in F1 hybrid (female) production, concurrent with an
464 increased production of F1 males (*i.e.* MD-type incompatibility between females from the
465 green population and males from the red populations); (2) near-complete F1 hybrid sterility
466 (> 99%) in all reciprocal crosses between the red and the green population; and (3) full F2
467 hybrid breakdown, as none of the few eggs produced by virgin hybrid females hatched (*i.e.*
468 corresponding to F1 hybrid males, as spider mites are haplodiploid).

469 MD-type incompatibilities have already been reported, both between populations
470 from the two colour forms of *T. urticae* (Murtaugh and Wrensch 1978; Sugasawa et al. 2002;
471 Lu et al. 2017), as well as among populations of the same colour form (Navajas et al. 2000;
472 Perrot-Minnot et al. 2004). In haplodiploids, this type of incompatibility can result from
473 either fertilization failure or haploidization of fertilized F1 eggs, resulting in an excess of F1
474 males. Partial haploidization of fertilized eggs is unlikely here, as males surviving such defect
475 would be aneuploid, and thus, should produce fewer daughters, an outcome we did not find
476 when testing F1 males. Moreover, there is yet no evidence that aneuploid embryos can
477 actually be viable in spider mites. Conversely, complete haploidization of fertilized eggs is a
478 plausible explanation, as *Wolbachia* can cause MD-type incompatibility in *T. urticae* (Vala et
479 al. 2000; Perrot-Minnot et al. 2002; Gotoh et al. 2003), and this outcome was shown to
480 result from paternal genome elimination following fertilization in haplodiploids (*i.e.* it
481 restores haploidy and thus leads to the production of viable males; Breeuwer and Werren
482 1990; Tram et al. 2006). However, because, in spider mites, males come from unfertilized
483 eggs (*i.e.* arrhenotoky; Helle and Bolland 1967) and not from the elimination of the paternal
484 genome in fertilized eggs (*i.e.* pseudo-arrhenotoky; Nelson-Rees et al. 1980; Sabelis and
485 Nagelkerke 1988), fertilization failure due to sperm-egg incompatibility is another possible
486 explanation for this type of incompatibility between populations (Takafuji and Fujimoto
487 1985; Perrot-Minnot et al. 2004). Nevertheless, only direct observations of the fertilization
488 process and early embryogenesis of the offspring in these crosses would provide a test to
489 this hypothesis.

490 Irrespective of the underlying cytological mechanisms, we found both asymmetric
491 (MD-type) and symmetric (F1 hybrid sterility and F2 hybrid breakdown) patterns of
492 reproductive incompatibilities between spider mite populations. In general, asymmetric

493 incompatibilities have been tied to "Bateson-Dobzhansky-Muller incompatibilities" (BDMIs –
494 negative epistatic interactions between alleles from independently evolving lineages)
495 between autosomal loci and uniparentally inherited factors (*e.g.* maternal transcripts;
496 Sawamura 1996; Turelli and Orr 2000; or cytoplasmic elements such as mitochondrial genes;
497 Burton and Barreto 2012). In contrast, symmetrical patterns of incompatibilities are
498 generally associated to BDMIs between nuclear genes inherited from both parents (Turelli
499 and Moyle 2007). This suggests that MD-type incompatibilities are caused by cytonuclear
500 interactions, whereas hybrid sterility and hybrid breakdown are mainly due to
501 incompatibilities between nuclear genes. This is in line with some evidence from previous
502 work using spider mites, albeit several of these studies also highlight a role for cytonuclear
503 interactions in hybrid sterility and hybrid breakdown (Overmeer and van Zon 1976; de Boer
504 1982b; Fry 1989; Sugasawa et al. 2002; Perrot-Minnot et al. 2004).
505
506 **Expression of *Wolbachia*-induced CI within and among populations**
507 Crosses between *Wolbachia*-infected males and uninfected females within and among
508 populations showed that the *Wolbachia* strains infecting the two populations of the red
509 form of *T. urticae* induced imperfect FM-type incompatibility (*ca.* 22 to 43% female
510 embryonic mortality) and were mutually compatible (as found by Zélé et al; 2020b). Here,
511 we further showed that wCI had the same penetrance within and among host populations,
512 including the population from the green form. Conversely, the strain infecting the
513 population of the green form of *T. urticae* did not induce CI within or between populations,
514 neither of the FM-type nor of the MD-type. Moreover, this strain did not rescue the CI
515 induced by the strain infecting the red form populations, in contrast to some other non CI-
516 inducing *Wolbachia* strains in *T. urticae* (Vala et al. 2002). Thus, taken together, these results
517 show a unidirectional pattern of wCI, which reduces hybrid production between the
518 *Wolbachia*-infected red-form populations and the green-form population, regardless of
519 *Wolbachia* infection in the latter. Finally, we found no evidence for hybrid breakdown (*i.e.*
520 increased mortality of F2 offspring produced by F1 females escaping wCI) induced by any of
521 the *Wolbachia* strains, suggesting that such effect is not a common feature in spider mites,
522 or that it is restricted to strains inducing MD-type incompatibilities (Vala et al. 2000).
523

The combined effects of incompatibility types for hybrid production and gene flow

In some systems, wCI may play a greater role than HI in reducing gene flow between hosts. For instance, complete post-mating isolation due to bidirectional wCI has been found in interspecific crosses between the mosquitoes *Aedes polyniensis* and *Ae. riversi* (Dean and Dobson 2004), and between the parasitoid wasps *Nasonia giraulti* and *N. vitripennis* (Breeuwer and Werren 1990, 1995), while only partial isolation was found in interspecific crosses upon *Wolbachia* removal (asymmetrical hybrid production and F2 hybrid breakdown, respectively). In other systems, however, CI induced by symbionts and host intrinsic factors can complement each other when acting in opposite directions, as found between *Encarsia gennaroi* and *Cardinium*-infected *E. suzannae* (Gebiola et al. 2016), or can act synergistically to reduce gene flow in the same direction. This was found between some populations of the spider mite *Panonychus mori*, where wCI mainly results in haploidization of fertilized eggs and can increase existing MD-type incompatibilities between populations (Gotoh et al. 2005). However, the relative contribution of wCI and HI to post-mating isolation was not quantified in such cases, nor whether they have additive or interacting effects.

In our system, we found that HI and wCI act synergistically to prevent the production of F1 hybrid offspring in crosses between green females and red males. Moreover, we showed that they act independently and additively, with HI contributing 1.5 to 2 times more than wCI to the reduction in hybrid production. However, because all hybrids were either sterile or produced unviable eggs, *Wolbachia* did not affect gene flow between the red and green form populations studied here. Nonetheless, these results suggest that wCI may have an important role in restricting gene flow between populations of *T. urticae* that are only partially isolated, *i.e.* when hybrids are fertile and hybrid breakdown incomplete (Dupont 1979; Sugasawa et al. 2002). In particular, the effects of wCI may be considerable when MD-type incompatibilities between hosts are weaker (Murtaugh and Wrensch 1978; Navajas et al. 2000; Sugasawa et al. 2002), and when the two types of incompatibilities act in opposite directions (Gebiola et al. 2016).

The role of *Wolbachia* on ongoing processes of speciation in spider mites

Partial to complete reproductive incompatibility between populations of different origin is a common phenomenon in many spider mite species (*e.g.* Van de Bund and Helle 1960; Navajas et al. 2001; Sato et al. 2015; Knegt et al. 2017), including *T. urticae* (*e.g.* Fry 1989;

Navajas et al. 2000; Sugasawa et al. 2002; Perrot-Minnot et al. 2004). This suggests that incompatibilities can evolve very quickly in spider mites, for instance due to local adaptation (*e.g.* host-plant adaptation; Sousa et al. 2019). Possibly, such incompatibilities have evolved independently of *Wolbachia*, which could have been acquired later on by horizontal transfer. Such scenario is supported by the absence of an association between mtDNA haplotypes and *Wolbachia* infection in *T. urticae* (Yu et al. 2011; Zélé et al. 2018a). However, this does not rule out the possibility that transient infections can play a role at early stages of host divergence. Moreover, the fact that wCI and HI affected hybrid production in the same cross direction is compatible with *Wolbachia* playing a role in early stages of population divergence. Thus, a potential evolutionary scenario could be that wCI, by reducing the introgression of nuclear genes from the red populations into the cytoplasm of the green population, could have initiated the divergence of coadapted cytonuclear complexes between these populations, thereby further increasing post-mating barriers to gene flow and subsequent genetic divergence until complete post-mating isolation (Hill 2015), as in the *Nasonia* wasp complex (Breeuwer and Werren 1990, 1995; Bordenstein et al. 2001; Niehuis et al. 2008). Although we found only partial unidirectional wCI between our populations, while complete bidirectional wCI is involved in the evolution of reproductive isolation in *Nasonia*, several studies have shown that unidirectional wCI causes gene flow reduction between host populations (reviewed by Engelstädter and Telschow; 2009), and that divergence between lineages can occur in the face of ongoing gene flow (Pinho and Hey 2010; Nosil 2012; Muirhead and Presgraves 2016). Nevertheless, studies using population pairs with variable degrees of post-mating isolation are needed to better understand the role played by *Wolbachia* in the speciation processes currently ongoing in spider mites.

Ecological implications of host-associated and *Wolbachia*-induced incompatibilities

Our results show strong reproductive interference (see Gröning and Hochkirch 2008; Burdfield-Steel and Shuker 2011) between the populations from the two forms of *T. urticae*, which may potentially impact their dynamics by favouring the green form population. Indeed, green females mated with red males produce less (sterile) hybrid daughters but more (fertile) sons than red females mated with green males, and this overproduction of sons may have important consequences for population persistence. Indeed, despite our finding that F1 green males had a slightly lower fitness than F1 red males (*i.e.* lower fertility

588 and higher embryonic mortality of their daughters), their overproduction should allow green
589 females to transmit more genes (thereby mitigating the costs of heterospecific matings;
590 Feldhaar et al. 2008). Moreover, it should also increase, at the next generation, the
591 probability of conspecific matings (*e.g.* in *Callosobruchus* beetles; Kyogoku and Nishida 2012)
592 for green females, and of heterospecific matings for red females, which may again favour
593 the green population.

594 *Wolbachia* may also affect the balance of the interactions between these
595 populations, both due to the direct effects of infection on host fitness (*i.e. Wolbachia* slightly
596 increases the embryonic and juvenile mortality of F2 sons of green, but not red, F1 females),
597 but also due to wCI. Indeed, although wCI leads to embryonic mortality of hybrid daughters
598 of green females, all these daughters are sterile. Conversely, wCI leads to embryonic
599 mortality of fertile daughters of red females, which may further disadvantage red females in
600 populations that are polymorphic for *Wolbachia* infection (as often found in spider mites;
601 Breeuwer and Jacobs 1996; Zhang et al. 2013; Zélé et al. 2018a). Note, however, that the
602 effect of wCI between partially isolated populations of the two forms (*e.g.* de Boer 1982b;
603 Sugasawa et al. 2002) may lead to different scenarios, as it could also affect fertile hybrid
604 daughters produced by green females.

605 Such ecological scenarios are likely to occur in natural populations of *T. urticae*, as
606 incompatible populations (both of the same and of different colour forms) often co-occur in
607 the field (Helle and Pieterse 1965; Lu et al. 2017), and the populations used in this study
608 were collected in the same geographical area (*cf.* Box S1). However, these scenarios will also
609 depend on the strength and the symmetry of pre-mating and post-mating prezygotic
610 reproductive barriers between populations (Sato et al. 2015, 2018; Gebiola et al. 2017;
611 Clemente et al. 2018). Indeed, although one study reported no assortative mating between
612 the colour forms of *T. urticae* (Murtaugh and Wrensch 1978), this may vary between
613 populations, as found between *T. urticae* and *T. evansi* (Sato et al. 2014; Clemente et al.
614 2016). In line with this, contrasting results were also found concerning the effect of
615 *Wolbachia* on spider mite mating behaviour (Vala et al. 2004; Rodrigues et al. 2018). Thus, to
616 understand the implications of reproductive interference in this system, future studies
617 should focus on prezygotic isolation between *T. urticae* populations, infected or not by
618 *Wolbachia*.
619

Conclusions

Our results show that host-associated and *Wolbachia*-induced incompatibilities in this system lead to different outcomes and that both contribute to counter hybridization between populations of the two *T. urticae* colour forms. Furthermore, these two types of incompatibility have additive effects in the same direction of crosses, which hints at a possible role of *Wolbachia*-induced incompatibilities in host population divergence and subsequent evolution of intrinsic reproductive barriers. Although the level of divergence between the populations studied here narrows our understanding of the contribution by *Wolbachia* in this system (because they are either not or fully isolated), our results suggest that this reproductive manipulator may have a considerable effect between partially isolated populations and, thus, could play an important role in the processes of speciation currently ongoing in spider mites. Finally, our results raise important questions about the ecological consequences of *Wolbachia*-driven reproductive interference in arthropods, and call for further studies to understand its impact on the dynamics and distribution of natural populations from the same species, but also from closely-related species.

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Authors' contributions

Experimental conception and design of the first experiment: FZ with discussions with SM. Experimental conception and design of the second experiment: MC, FZ, SM, ES; Acquisition of data, statistical analyses, and writing of the first version of the manuscript: MC, FZ. Subsequent versions were written with input from SM and ES. All authors have approved the final version for publication.

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Abbreviations

CI: cytoplasmic incompatibility; wCI: *Wolbachia*-induced cytoplasmic incompatibility; HI: Host-associated incompatibility; EM: Embryonic mortality; FM: Female mortality; MD: Male development; JM: Juvenile mortality; FP: Female proportion over total number of eggs laid; SR: Sex ratio (here ratio of females to males in the offspring).

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