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

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## 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

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

33 The fact that natural populations of many organisms often display variable degrees of
34 reproductive isolation (Scopece et al. 2010; Jennings et al. 2011; Corbett-Detig et al. 2013;
35 Harrison and Larson 2014) offers an excellent opportunity to address the role of wCI in
36 ongoing speciation processes. Still, only few studies have done this, with very contrasting
37 results. Indeed, these studies showed either no wCI in interspecific crosses (Maroja et al.
38 2008; Gazla and Carracedo 2009; Cooper et al. 2017), that wCI caused complete post-mating
39 isolation between species (Bordenstein et al. 2001), or either a complementarity
40 (Shoemaker et al. 1999; Dean and Dobson 2004; see also Gebiola et al. 2016 for CI induced
41 by *Cardinium*), or a synergy (Gotoh et al. 2005) between wCI and host genetic factors in
42 establishing post-mating isolation. However, in the latter scenarios, *i.e.* when both sources
43 of incompatibility jointly reduce gene flow between genetically differentiated host
44 populations and incipient species, whether they have additive or interacting effects, and
45 precise quantification of their relative contribution to post-mating isolation, have not been
46 addressed. This is at odds with the insight it would provide to better understanding the exact
47 contribution of *Wolbachia* to ongoing processes of speciation in arthropods.

48 *Tetranychus* spider mites constitute an excellent system to address the interplay
49 between symbiont-induced and host intrinsic reproductive incompatibilities. Indeed, they
50 are arrhenotokous haplodiploids (*i.e.* males arise from unfertilized eggs and females from
51 fertilized eggs Helle and Bolland 1967), which allows assessing fertilization failure by
52 measuring sex-ratios. Moreover, variable degrees of reproductive isolation have been found
53 both between and within species of this genus (*e.g.* Takafuji and Fujimoto 1985; Navajas et
54 al. 2000; Sato et al. 2015; Clemente et al. 2016; Knegt et al. 2017). This is the case for the
55 well-studied *Tetranychus urticae*, in which two genetically differentiated colour forms have
56 recently diverged (Navajas et al. 1998; Hinomoto et al. 2001; Chen et al. 2014; Matsuda et al.
57 2018). Due to complete reproductive isolation among some populations of the two forms,
58 they were historically described as separate species (*T. urticae* and *T. cinnabarinus*, for the
59 'green' and the 'red' form, respectively; Boudreaux 1956; Van de Bund and Helle 1960; Helle
60 and Van de Bund 1962; Smith 1975). However, subsequent studies reclassified them as semi-
61 species (Goka et al. 1996) or members of the same species (Dupont 1979; Fry 1989; Auger et
62 al. 2013), given that many populations of the two forms are almost completely compatible

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

66 Populations of the two forms of *T. urticae* are also often naturally infected with
67 different *Wolbachia* strains (Gotoh et al. 2003, 2007; Zhang et al. 2016; Zélé et al. 2018a),
68 which induce variable levels of wCI (Perrot-Minnot et al. 2002; Vala et al. 2002; Gotoh et al.
69 2007; Xie et al. 2010; Suh et al. 2015; Zélé et al. 2020b). In spider mites, as in some other
70 haplodiploid species (*e.g.* Breeuwer and Werren 1990; Vavre et al. 2001), diploid zygotes
71 arising from incompatible crosses between an infected male and an uninfected female may
72 survive as haploid males after complete elimination of the paternal set of chromosomes
73 (Male development - MD-type incompatibility; Vala et al. 2000; Perrot-Minnot et al. 2002;
74 Gotoh et al. 2003). In most cases, however, fertilized eggs in incompatible crosses fail to
75 hatch as in diploid species, leading to embryonic mortality of female offspring (Female
76 mortality - FM-CI type incompatibility; Breeuwer 1997; Perrot-Minnot et al. 2002; Vala et al.
77 2002; Gotoh et al. 2003, 2007; Xie et al. 2010; Suh et al. 2015; Bing et al. 2019; Zélé et al.
78 2020b).

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

## Methods

86 **Spider mite populations**

87 Three different populations of spider mites, all collected in Portugal and naturally infected
88 with *Wolbachia*, were used in this study. Two populations, 'Ri1' and 'Ri2', belong to the red
89 form of *T. urticae* and share the same *ITS2* rDNA and *COI* mtDNA sequences. The third
90 population, 'Gi', belongs to the green form of *T. urticae* and differs from the former two
91 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<br>Ru2 x Ru2 and Ri2 x Ri2<br>Gu x Gu and Gi x Gi |
| 2 – Test for wCI only | intra-population crosses using uninfected ♀ and infected ♂ | Ru1 x Ri1<br>Ru2 x Ri2<br>Gu x Gi |
| 3 – Test for HI only (without *Wolbachia*) | inter-population crosses using uninfected ♀ and uninfected ♂ | Ru1 x Ru2 or Gu<br>Ru2 x Ru1 or Gu<br>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<br>Ru2 or Ri2 x Ri1 or Gi<br>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<br>Ri2 x Ru1 or Gu<br>Gi x Ru1 or Ru2<br>(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).

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

172 $$JM_{corr} = \frac{JM_{obs} - CCJM}{1 - CCJM}$$

173 where $JM_{obs}$ = number of dead juveniles/total number of eggs, and $CCJM$ (calculated as
174 $JM_{obs}$) is the mean juvenile mortality observed in control crosses.

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

178 $$FP = \frac{number\ of\ F1\ females}{total\ number\ of\ eggs}$$

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

183 $$FP_{pred} = \frac{FP_{md} \times FP_{fm}}{FP_{comp}}$$

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

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

194 $$\chi^2_{df} = \sum_j \frac{(FP_{obs_j} - FP_{pred})^2}{FP_{pred}}$$

195 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

228 was counted 5 days later (day 9), and the numbers of dead juveniles and adult males were
229 counted 12 days later (day 16; as mothers were virgin, they could only produce sons).
230 As F1 female fertility corresponds to their ability to lay at least one egg, and/or a
231 normal number of eggs (Navajas et al. 2000), we estimated both the proportion of
232 ovipositing females and the daily oviposition of these females, taking into account their daily
233 mortality (*i.e.* total number of eggs laid by each female/total number of days each female
234 was alive). Hybrid breakdown was assessed as male embryonic and juvenile mortality
235 accounting for variation in background mortality (*i.e.* not related to hybrid breakdown). The
236 corresponding $mEM_{corr}$ and $mJM_{corr}$ indexes were calculated as follows:

237 $$mEM_{corr} = \frac{mEM_{obs} - CCmEM}{1 - CCmEM}$$

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

240 $$mJM_{corr} = \frac{mJM_{obs} - CCmJM}{1 - CCmJM}$$

241 where $mJM_{obs}$ = number of dead juveniles/total number of eggs, and $CCmJM$ (calculated as
242 $mJM_{obs}$) is the mean juvenile mortality observed in control crosses (*i.e.* category 1).
243
244 *Test 2: F1 male fertility and F2 viability*
245 As in haplodiploids sons are hemiclones of their mothers, they inherit a single maternal
246 chromosome. Thus, in absence of reproductive anomalies they should be fully compatible
247 with females from their maternal population, whereas the expression of an incompatibility
248 may indicate that these males are aneuploid. To test this, adult F1 males were collected
249 from each cross performed in panmixia and placed on 9 $cm^2$ bean leaf fragments 2 days prior
250 to the beginning of experiment (day -2) to avoid sperm depletion. On the first experimental
251 day (day 0), each male was installed with one virgin female (obtained from age cohorts
252 created as in Experiment 1) from the same population as its mother on a 2.5 $cm^2$ bean leaf
253 disc. Four days were given for males to mate and for the females to lay eggs before both
254 were discarded (day 4). The number of unhatched eggs was counted 5 days later (day 9), and
255 the numbers of dead juveniles, adult males and adult females were counted 12 days later
256 (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

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

## Results

298 **F1 offspring production and viability**

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

311

312 *Overproduction of males (MD-type incompatibility)*

313 Overall, we found an overproduction of males (*i.e.* higher values of the $MD_{corr}$ index; *cf.*
314 *Methods*) in all inter-population crosses involving females from the green population (*ca.*
315 46.4 to 64.3%) relative to all other crosses (*ca.* 5.6 to 21.5%; *Main cross effect*: $\chi^2_{26}$=460.70,

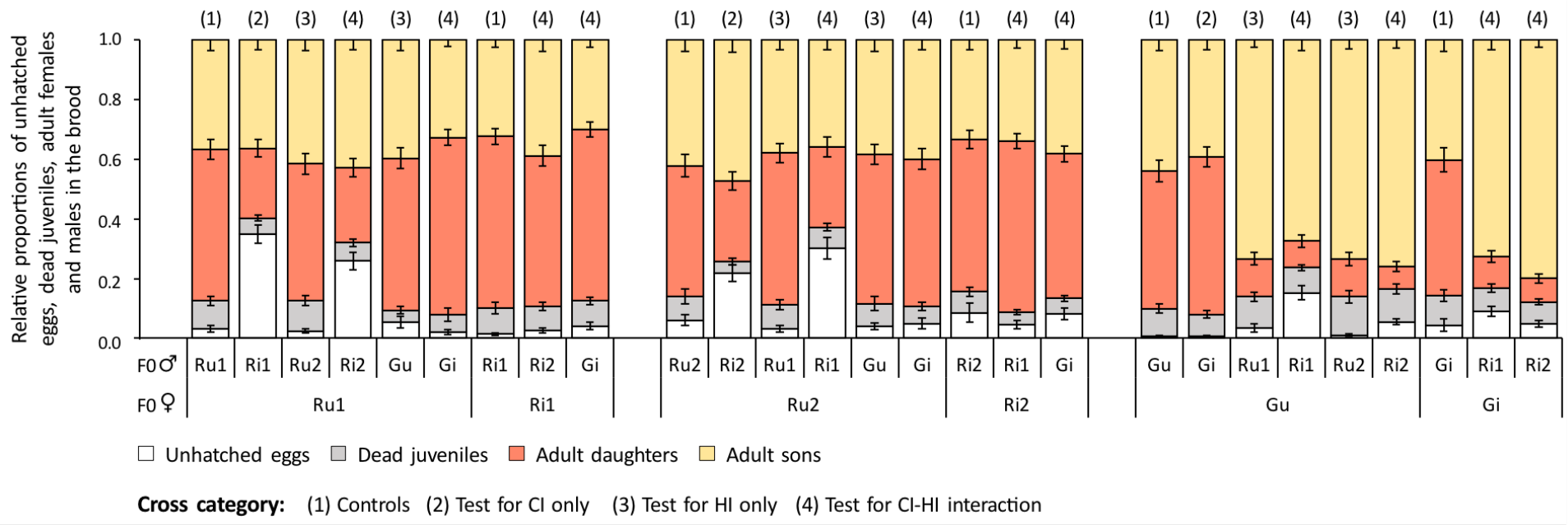


**Figure 1. Summary of the development of *T. urticae* eggs resulting from intra- and inter-population crosses between *Wolbachia*-infected and -uninfected mites.** Bar plots represent mean ± s.e. relative proportions of unhatched eggs (i.e. embryonic mortality), dead juveniles (i.e. juvenile mortality), 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*♀: $\chi^2_5$=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*: $\chi^2_{20}$=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*: $\chi^2_8$=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*: $\chi^2_{26}$=506.20, *p*<0.0001; model 1.3; Figure 2b). In addition, there were no
347 significant differences among these crosses ($\chi^2_7$=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* $FM_{corr}$: $\chi^2_{16}$=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 $\chi^2_{26}$=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

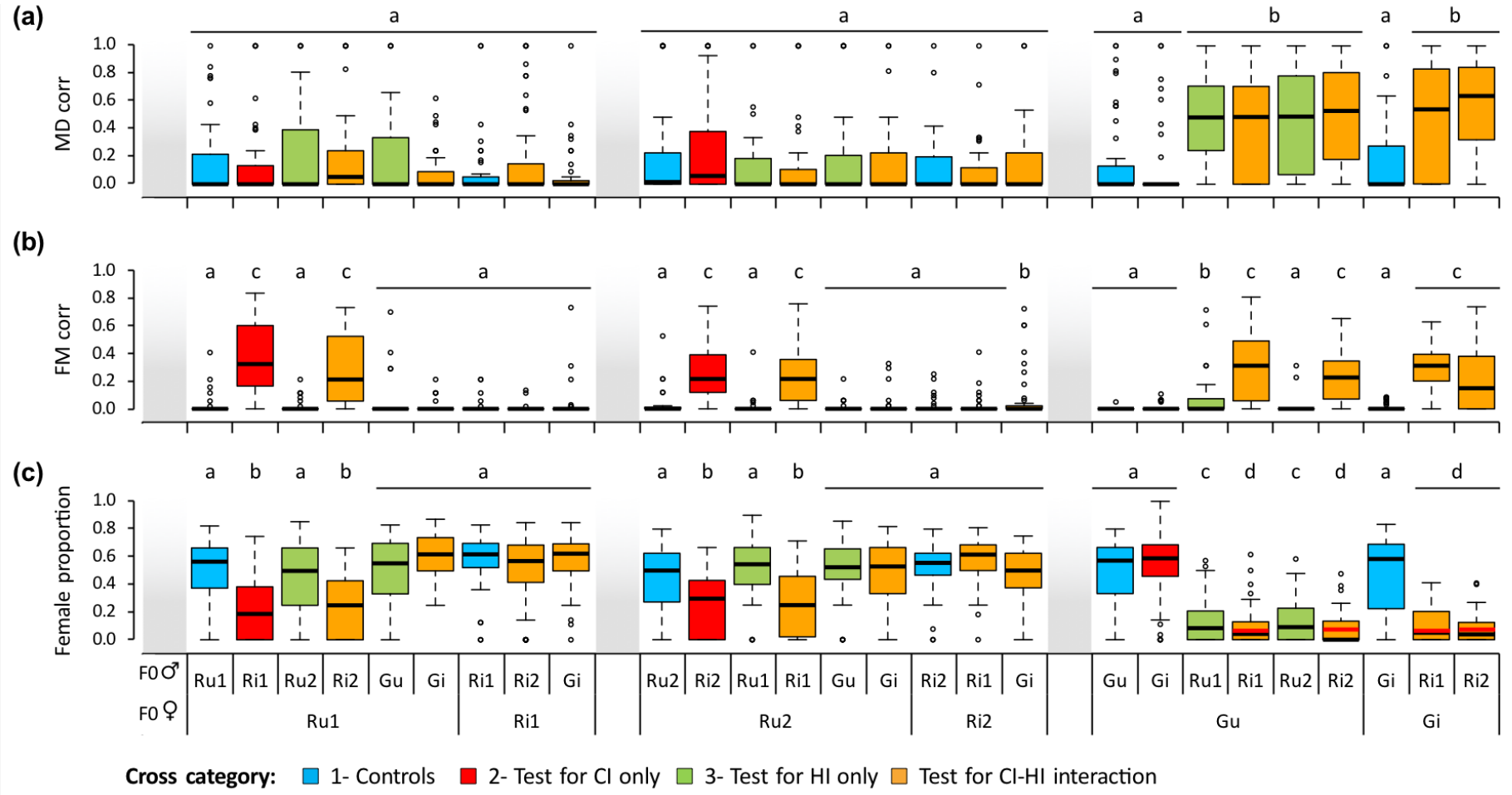


**Figure 2. Overproduction of males, female embryonic mortality, and resulting hybrid production in intra- and inter-population crosses using *Wolbachia*-infected and uninfected mites. (a)** Boxplot of the proportion of males produced in all crosses relative to that in control crosses ($MD_{corr}$). **(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.

361 produced in compatible crosses (*i.e.* unaffected by both incompatibilities; *Contrasts among*
362 *these crosses*: $\chi^2_{16}$=21.22, *p*=0.17); (2) *ca.* 26% daughters produced in crosses affected by
363 FM-type incompatibilities only (*Contrasts among these crosses*: $\chi^2_3$=2.98, *p*=0.40; *ca.* 50%
364 decrease compared to compatible crosses: $\chi^2_1$=187.67, *p*<0.0001); (3) *ca.* 13% daughters
365 produced in crosses affected by MD-type incompatibilities only (*Contrasts among these*
366 *crosses*: $\chi^2_1$=0.04, *p*=0.84; *ca.* 75% decrease compared to compatible crosses: $\chi^2_1$=292.02,
367 *p*<0.0001; and *ca.* 76% decrease when using crosses of category 5: $\chi^2_8$=278.23, p<0.0001;
368 model 1.8; Figure S2c); and (4) *ca.* 9% daughters produced in crosses affected by both FM-
369 and MD-type incompatibilities (*Contrasts among these crosses*: $\chi^2_3$=3.57, *p*=0.31; *ca.* 83%
370 decrease compared to compatible crosses: $\chi^2_1$=606.40, *p*<0.0001).
371 Both types of incompatibility appeared to have lower consequences on hybrid
372 production when combined than when acting alone. Indeed, we found around 31% decrease
373 in hybrid production due to FM-type incompatibility when comparing groups (3) and (4)
374 ($\chi^2_1$=7.49, *p*=0.03) and close to 65% decrease in hybrid production due to MD-type
375 incompatibility when comparing groups (2) and (4) ($\chi^2_1$=141.97, *p*<0.0001). However, this
376 was only a consequence of their cumulative effects. Indeed, we found a perfect fit between
377 the observed and the predicted proportions of F1 females for crosses affected by both MD-
378 and FM-type incompatibilities, calculated assuming that both affect hybrid production with
379 the same strength when acting either alone or combined (Figure 2c; Goodness-of-fit test:
380 *Gu*♀*xRi1*♂: $\chi^2_{47}$=14.30, *p*=0.58; *Gu*♀*xRi2*♂: $\chi^2_{47}$=8.46, *p*=0.65; *Gi*♀*xRi1*♂: $\chi^2_{47}$=13.90, *p*=0.56;
381 and *Gi*♀*xRi2*♂: $\chi^2_{48}$=7.37, *p*=0.59). Thus, these results show that MD- and FM-type
382 incompatibilities are independent, so that their effects are additive with the former
383 contributing 1.5 to 2 times more than the latter in reducing hybrid production.
384
385 **F1 offspring fertility and viability of the F2**
386 To estimate the effects of wCI and HI on the fitness of F1 offspring obtained from all
387 aforementioned crosses (except those involving Ru2 and Ri2 populations, *cf.* Methods), we
388 assessed the fertility of virgin F1 females and of F1 males backcrossed to females from their
389 maternal population, and both embryonic and juvenile mortality of the resulting F2 offspring
390 (*i.e.* hybrid breakdown; de Boer 1982b; Sugasawa et al. 2002).
391

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

393 The proportion of fertile F1 females (*i.e.* virgin females that laid at least 1 egg) differed
394 significantly among crosses ($\chi^2_{15}$=214.26, $p$<0.0001; model 2.1; Figure 3a). Indeed, while the
395 proportion of fertile F1 females resulting from all intra-population crosses was very high (*ca.*
396 96% on average; *Contrasts among intra-population crosses*; $\chi^2_7$=8.42, $p$=0.30), more than
397 99% of the hybrid females resulting from inter-population crosses were unable to lay eggs.
398 Moreover, although 6 hybrid females (over a total of 760), all resulting from crosses
399 between males and females with the same *Wolbachia* infection status (either both infected,
400 or both uninfected), were found to be fertile, they laid very few eggs (average daily
401 oviposition of 0.63 ± 0.15 compared to 6.37 ± 0.09 for females resulting from intra-
402 population crosses; *cf.* Table S3), with no significant difference among inter-population
403 crosses (*Contrasts among inter-population crosses*; $\chi^2_7$=8.59, $p$=0.28).

404 None of the eggs laid by the 6 fertile hybrid females hatched (Table S3), which,
405 despite the low number of eggs (15 in total), corresponds to full F2 hybrid breakdown. In
406 contrast, embryonic mortality ($mEM_{corr}$) of eggs laid by F1 females resulting from intra-
407 population crosses was very low (*ca.* 5%), with only a very small increased mortality (*ca.* 2%)
408 in the brood of F1 females from the green population when infected by *Wolbachia* (*Main*
409 *cross effect:* $\chi^2_7$=23.33, $p$=0.001; model 2.3; Figure S3a). Similarly, juvenile mortality ($mJM_{corr}$)
410 in the offspring (*i.e.* all F2 males) of virgin F1 females resulting from intra-population crosses
411 was very low (below *ca.* 6%), and varied slightly depending on their maternal origin (*Main*
412 *cross effect:* $\chi^2_7$=18.57, $p$=0.01; model 2.4; Figure S3b). Indeed, the offspring of infected
413 green F1 females had higher juvenile mortality than the offspring of infected red females
414 (independently of their grandfather; *Contrasts between Gi and Ri1 females*: $\chi^2_1$=12.53,
415 $p$=0.002), and the offspring of all uninfected F1 females displayed an intermediate mortality
416 (*Contrasts between Gu-Ru1 and Gi females:* $\chi^2_1$=4.28, $p$=0.17; *Contrasts between Gu-Ru1 and*
417 *Ri1 females:* $\chi^2_1$=4.49, $p$=0.17).

418

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

420 The proportion of fertile F1 males (*i.e.* males siring at least one daughter when backcrossed
421 with a female from their maternal population) differed significantly among crosses
422 ($\chi^2_7$=25.58, $p$<0.001; model 2.5.1, and $\chi^2_7$=15.23, $p$=0.03; model 2.5.2, for uninfected and
423 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).

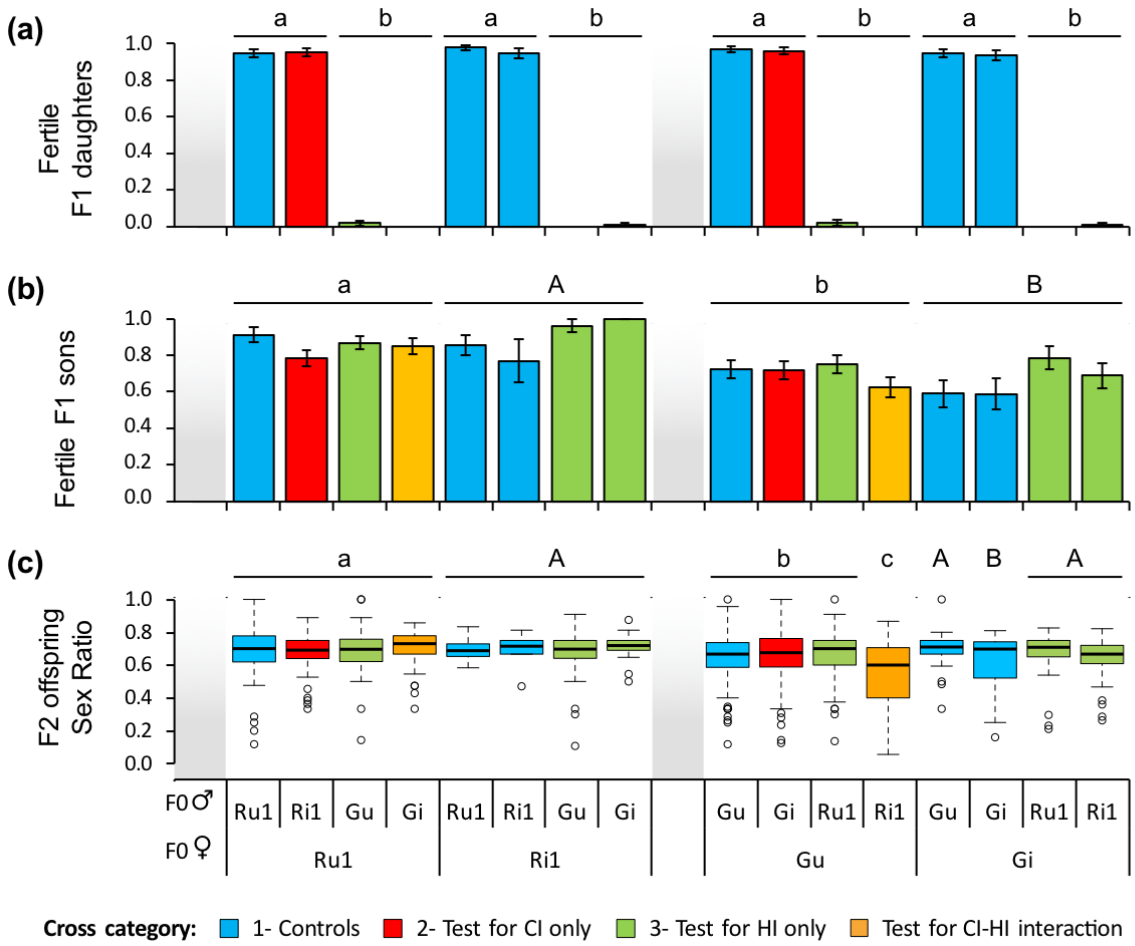


**Figure 3. Proportion of fertile F1 female and male offspring resulting from intra- and inter-population crosses using *Wolbachia*-infected and uninfected mites, and sex-ratio of F2 offspring resulting from backcrosses of F1 males.** Average proportion (± s.e.) of **(a)** fertile F1 females (*i.e.* 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* ($\chi^2_7$=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

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

438 Finally, both embryonic mortality ($fEM_{corr}$) and juvenile mortality ($fJM_{corr}$) varied
439 among the offspring of F1 uninfected males (*Main cross effect on* $fEM_{corr}$*:* $\chi^2_7$=26.31,
440 *p*<0.001; model 2.6.1, and *on* $fJM_{corr}$*:* $\chi^2_7$=22.64, *p*=0.002; model 2.7.1). Indeed, uninfected F1
441 males mated with (and sons of) green females sired offspring with a higher embryonic
442 mortality (*ca.* 7% on average) than those mated with, or sons of, red females (*ca.* 4% in
443 average). However, the variations observed in juvenile mortality cannot necessarily be
444 explained by the maternal populations of F1 uninfected males (or the females they mated
445 with), nor by *Wolbachia* infection in the mates of their mother (see Figure S4b). In contrast,
446 no differences were detected among the offspring of infected F1 males (*Main cross effect on*
447 $fEM_{corr}$*:* $\chi^2_7$=5.58, *p*=0.59; model 2.6.2, and *on* $fJM_{corr}$*:* $\chi^2_7$=11.68, *p*=0.11; model 2.7.2).

## Discussion

448 Using three populations of the two genetically differentiated colour forms of *T. urticae*, each
449 naturally infected or cured from *Wolbachia*, we assessed the relative contribution of
450 *Wolbachia*-induced CI (wCI) and of host-associated incompatibilities (HI) to post-mating
451 isolation. Our results revealed that both sources of incompatibility jointly reduced the
452 production of F1 hybrid females in the same direction, albeit through different and
453 independent mechanisms, and with HI contributing up to 2 times more than wCI.
454 Additionally, we found a *Wolbachia*-independent near-complete F1 hybrid female sterility
455 and F2 hybrid breakdown in all reciprocal crosses between the green and the red
456 populations.
457

458 **Expression of host-associated incompatibilities**

459 Crosses performed among uninfected host populations in absence of *Wolbachia* infection
460 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

incompatibilities have been tied to "Bateson-Dobzhansky-Muller incompatibilities" (BDMIs – negative epistatic interactions between alleles from independently evolving lineages) between autosomal loci and uniparentally inherited factors (*e.g.* maternal transcripts; Sawamura 1996; Turelli and Orr 2000; or cytoplasmic elements such as mitochondrial genes; Burton and Barreto 2012). In contrast, symmetrical patterns of incompatibilities are generally associated to BDMIs between nuclear genes inherited from both parents (Turelli and Moyle 2007). This suggests that MD-type incompatibilities are caused by cytonuclear interactions, whereas hybrid sterility and hybrid breakdown are mainly due to incompatibilities between nuclear genes. This is in line with some evidence from previous work using spider mites, albeit several of these studies also highlight a role for cytonuclear interactions in hybrid sterility and hybrid breakdown (Overmeer and van Zon 1976; de Boer 1982b; Fry 1989; Sugasawa et al. 2002; Perrot-Minnot et al. 2004).

**Expression of *Wolbachia*-induced CI within and among populations**

Crosses between *Wolbachia*-infected males and uninfected females within and among populations showed that the *Wolbachia* strains infecting the two populations of the red form of *T. urticae* induced imperfect FM-type incompatibility (*ca.* 22 to 43% female embryonic mortality) and were mutually compatible (as found by Zélé et al; 2020b). Here, we further showed that wCI had the same penetrance within and among host populations, including the population from the green form. Conversely, the strain infecting the population of the green form of *T. urticae* did not induce CI within or between populations, neither of the FM-type nor of the MD-type. Moreover, this strain did not rescue the CI induced by the strain infecting the red form populations, in contrast to some other non CI-inducing *Wolbachia* strains in *T. urticae* (Vala et al. 2002). Thus, taken together, these results show a unidirectional pattern of wCI, which reduces hybrid production between the *Wolbachia*-infected red-form populations and the green-form population, regardless of *Wolbachia* infection in the latter. Finally, we found no evidence for hybrid breakdown (*i.e.* increased mortality of F2 offspring produced by F1 females escaping wCI) induced by any of the *Wolbachia* strains, suggesting that such effect is not a common feature in spider mites, or that it is restricted to strains inducing MD-type incompatibilities (Vala et al. 2000).

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

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

539 In our system, we found that HI and wCI act synergistically to prevent the production
540 of F1 hybrid offspring in crosses between green females and red males. Moreover, we
541 showed that they act independently and additively, with HI contributing 1.5 to 2 times more
542 than wCI to the reduction in hybrid production. However, because all hybrids were either
543 sterile or produced unviable eggs, *Wolbachia* did not affect gene flow between the red and
544 green form populations studied here. Nonetheless, these results suggest that wCI may have
545 an important role in restricting gene flow between populations of *T. urticae* that are only
546 partially isolated, *i.e.* when hybrids are fertile and hybrid breakdown incomplete (Dupont
547 1979; Sugasawa et al. 2002). In particular, the effects of wCI may be considerable when MD-
548 type incompatibilities between hosts are weaker (Murtaugh and Wrensch 1978; Navajas et
549 al. 2000; Sugasawa et al. 2002), and when the two types of incompatibilities act in opposite
550 directions (Gebiola et al. 2016).

551

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

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

556 Navajas et al. 2000; Sugasawa et al. 2002; Perrot-Minnot et al. 2004). This suggests that
557 incompatibilities can evolve very quickly in spider mites, for instance due to local adaptation
558 (*e.g.* host-plant adaptation; Sousa et al. 2019). Possibly, such incompatibilities have evolved
559 independently of *Wolbachia*, which could have been acquired later on by horizontal transfer.
560 Such scenario is supported by the absence of an association between mtDNA haplotypes and
561 *Wolbachia* infection in *T. urticae* (Yu et al. 2011; Zélé et al. 2018a). However, this does not
562 rule out the possibility that transient infections can play a role at early stages of host
563 divergence. Moreover, the fact that wCI and HI affected hybrid production in the same cross
564 direction is compatible with *Wolbachia* playing a role in early stages of population
565 divergence. Thus, a potential evolutionary scenario could be that wCI, by reducing the
566 introgression of nuclear genes from the red populations into the cytoplasm of the green
567 population, could have initiated the divergence of coadapted cytonuclear complexes
568 between these populations, thereby further increasing post-mating barriers to gene flow
569 and subsequent genetic divergence until complete post-mating isolation (Hill 2015), as in the
570 *Nasonia* wasp complex (Breeuwer and Werren 1990, 1995; Bordenstein et al. 2001; Niehuis
571 et al. 2008). Although we found only partial unidirectional wCI between our populations,
572 while complete bidirectional wCI is involved in the evolution of reproductive isolation in
573 *Nasonia*, several studies have shown that unidirectional wCI causes gene flow reduction
574 between host populations (reviewed by Engelstädter and Telschow; 2009), and that
575 divergence between lineages can occur in the face of ongoing gene flow (Pinho and Hey
576 2010; Nosil 2012; Muirhead and Presgraves 2016). Nevertheless, studies using population
577 pairs with variable degrees of post-mating isolation are needed to better understand the
578 role played by *Wolbachia* in the speciation processes currently ongoing in spider mites.
579

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

581 Our results show strong reproductive interference (see Gröning and Hochkirch 2008;
582 Burdfield-Steel and Shuker 2011) between the populations from the two forms of *T. urticae*,
583 which may potentially impact their dynamics by favouring the green form population.
584 Indeed, green females mated with red males produce less (sterile) hybrid daughters but
585 more (fertile) sons than red females mated with green males, and this overproduction of
586 sons may have important consequences for population persistence. Indeed, despite our
587 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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