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No evidence for the evolution of mating behavior in spider mites due to *Wolbachia*-induced cytoplasmic incompatibility

Leonor R. Rodrigues,[1,2,#] **Flore Zélé,**[1,3,#] **Inês Santos,**[1] **and Sara Magalhães**[1]

[1]*Centre for Ecology, Evolution, and Environmental Changes (cE3c), Faculty of Sciences, University of Lisbon, Lisbon 1749-016, Portugal*

[2]*E-mail: alrodrigues@fc.ul.pt*

[3]*Institute of Evolution Sciences (ISEM), University of Montpellier, CNRS, IRD, EPHE, Montpellier 34095, France*

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Arthropods are often infected with *Wolbachia* inducing cytoplasmic incompatibility (CI), whereby crosses between uninfected females and infected males yield unviable fertilized offspring. Although uninfected females benefit from avoiding mating with *Wolbachia*-infected males, this behavior is not always present in host populations and its evolution may hinge upon various factors. Here, we used spider mites to test whether CI could select for mate preference in uninfected females in absence of kin recognition. We found that uninfected females from several field-derived populations showed no preference for infected or uninfected males, nor evolved a preference after being exposed to CI for 12–15 generations by maintaining uninfected females with both infected and uninfected males (i.e., stable "infection polymorphism"). This suggests that *Wolbachia*-mediated mate choice evolution may require very specific conditions in spider mites. However, after experimental evolution, the copulation duration of *Wolbachia*-infected control males was significantly higher than that of uninfected control males, but not than that of uninfected males from the "infection polymorphism" regime. This result illustrates how gene flow may oppose *Wolbachia*-driven divergence between infected and uninfected hosts in natural populations.

KEY WORDS: **Endosymbiont, mate preference, parasite manipulation, reproductive incompatibility, reproductive isolation, sexual selection.**

Organisms are often exposed to parasites, risking severe fitness costs upon infection. Hosts are thus expected to be under strong selection to avoid being parasitized (Parker et al. 2011; Sarabian et al. 2018). This may be possible via hiding, fleeing from parasites, avoiding infected conspecifics, evading food and habitats where encounters with parasites are likely, or avoiding mating with parasitized conspecifics (Schmid-Hempel 2013; Sarabian et al. 2018; Zélé et al. 2019). Avoidance of infection via mate choice is widespread across different host species (reviewed in Beltran-Bech and Richard 2014), and forms the basis of the Hamilton-Zuk hypothesis, which proposes that individuals choose mates via traits that indicate resistance to parasites (Hamilton and Zuk 1982).

Wolbachia are the most widespread endosymbiotic bacteria found in arthropods (Weinert et al. 2015). Although in some cases, its maintenance and spread in host populations can be attributed to fitness benefits conferred to infected hosts (e.g., increased fecundity, survival, nutritional mutualism, and/or protection against pathogens; Dobson et al. 2004; Barr et al. 2010; Hosokawa et al. 2010; Nikoh et al. 2014; Ross et al. 2019), its success is mostly due to its ability to manipulate the reproduction of its hosts (Werren et al. 2008; Engelstädter and Hurst

[#]Co-first authors.

2009; Kaur et al. 2021). The most common *Wolbachia*-induced reproductive manipulation is cytoplasmic incompatibility (CI), a mechanism that results in the embryonic death of some, or all, fertilized offspring from crosses between uninfected females and infected males (Werren et al. 2008; Shropshire et al. 2020). As all other crosses are compatible, CI promotes the spread of *Wolbachia* by indirectly (i.e., via infected males) increasing the success of infected females relative to that of uninfected females. However, because CI is costly for individuals involved in incompatible crosses, it is expected to exert a strong selective pressure on hosts to evolve strategies that reduce the frequency and/or costs of such matings (Charlat et al. 2003; Engelstädter and Hurst 2009; Sahoo 2016).

Discrimination and avoidance of incompatible mates prior to mating has been proposed as a potential host strategy to avoid CI (Hoffmann et al. 1990; see Sahoo 2016 for a review). Indeed, CI-induced offspring death may select for premating isolation ("reinforcement": Dobzhansky 1937), as demonstrated by theoretical models (Champion de Crespigny et al. 2005; Telschow et al. 2005, 2007). These models predict that selection for the ability to discriminate between *Wolbachia*-infected and uninfected individuals hinges upon populations being exposed to a polymorphic infection state (when both infected and uninfected individuals occur within the same population) for a sufficient amount of time (Champion de Crespigny et al. 2005; Telschow et al. 2005, 2007). Such conditions can be met either when the spread of *Wolbachia* in a host population is sufficiently slow (due to incomplete CI, fecundity costs, and/or imperfect transmission of the symbiont; Champion de Crespigny et al. 2005) or when an uninfected population receives migrants from a *Wolbachia*-infected population (e.g,. in a mainland-island metapopulation; Telschow et al. 2007). Because infection polymorphisms are relatively common in populations of different host species (e.g., Vavre et al. 2002; Baudry et al. 2003; Keller et al. 2004; Hamm et al. 2014), it is likely that mate discrimination evolves frequently. However, empirical studies that experimentally tested mate discrimination in species infected by CI-inducing *Wolbachia* produced variable outcomes (Bi and Wang 2020). Although some studies found no evidence for mate choice (e.g., Hoffmann and Turelli 1988; O'Neill 1991; Wade and Chang 1995; Champion de Crespigny and Wedell 2007; Duron et al. 2011; Arbuthnott et al. 2016; Bagheri et al. 2019), others found that individuals can discriminate between *Wolbachia*-infected and uninfected mates (Vala et al. 2004; Markov et al. 2009), or that *Wolbachia* infection increases levels of mate discrimination between populations (Koukou et al. 2006; Miller et al. 2010).

The evolution of preference for *Wolbachia*-infected or uninfected mates may also depend on the mechanisms underlying mate discrimination. Empirical work suggests that such discrimination may be based on host traits that are altered upon *Wolbachia* infection (e.g., change of host pheromone profiles and/or production; Pontier and Schweisguth 2015; Engl and Kaltenpoth 2018; Fortin et al. 2018; Schneider et al. 2019) or on host traits unrelated to *Wolbachia* infection, such as those associated with kin recognition (e.g., via self-reference phenotype matching; Markov et al. 2009) or local adaptation (e.g., CI-driven mate choice evolution in natural populations of *Drosophila subquinaria*; Jaenike et al. 2006). This is supported by theoretical models predicting that mate discrimination can be selected for when either type of cues is used by hosts (Champion de Crespigny et al. 2005; Telschow et al. 2007). Unfortunately, no experimental study so far has tested whether assortative mating between uninfected and infected hosts evolves under stable polymorphism, and if such evolution can occur based on discrimination of *Wolbachia* infection itself or whether it requires the existence of host traits that indirectly indicate the infection status of potential mates.

Populations of the two-spotted spider mites *Tetranychus urticae* harbor different CI-inducing *Wolbachia* strains with variable prevalence (from 0% to 100%; e.g., Gotoh et al. 2003, Gotoh et al. 2007; Zhang et al. 2016; Zélé et al. 2018a,b), fitness effects and levels of CI (ranging from costs to benefits on several life history traits, such as longevity and fecundity, and from no CI to complete CI, respectively; e.g., Vala et al. 2002; Gotoh et al. 2007; Xie et al. 2011; Suh et al. 2015; Zélé et al. 2020). In this species, females may mate multiple times, but they use mainly the sperm from their first mating to produce fertilized offspring (i.e., there is first male sperm precedence; Helle 1967; Rodrigues et al. 2020). Unsuccessful matings are thus expected to be highly costly for females, as they risk a reduction in fitness with few chances of recovery upon remating. Evolving the ability to choose males with whom they can successfully reproduce would hence be exceptionally advantageous for such females. Accordingly, female choice is present in this species (Oku 2014). In particular, Vala et al. (2004) showed that *Wolbachia*-uninfected females from a single isofemale line of *T. urticae* prefer uninfected males over infected ones. Here, we tested the generality of this finding by assessing *Wolbachia*-associated mate choice in several field-derived *T. urticae* populations naturally infected by CI-inducing *Wolbachia*. Next, we created outbred populations from these populations and performed experimental evolution to test if pre-copulatory mating behavior, and avoidance of infected males by uninfected females, based on signals associated with *Wolbachia* infection only, could evolve in response to CI.

Materials and Methods

SPIDER MITE POPULATIONS AND REARING CONDITIONS

Seven populations belonging to the red form of *T. urticae*, collected on different host plants around Lisbon in late 2013

(Zélé et al. 2018a), were used in this study (Table S1). These populations were established at the University of Lisbon and maintained under standard conditions (25 ± 2°C, 60% RH, 16/8 h L/D) at the laboratory in high numbers (about 500–1000 females) inside insect-proof cages containing bean plants (*Phaseolus vulgaris*, Fabaceae, var. *Enana*; Germisem Sementes Lda, Oliveira do Hospital, Portugal). At the time of the experiment, all populations were naturally and fully infected (100% infection frequency in each population) with compatible (or identical) strains of *Wolbachia* that induce variable levels of CI (about 27-66% female embryonic mortality; cf. Table S1) and that had variable effects on host fecundity and longevity, ranging from costs to benefits, depending on the host population (Zélé et al. 2020). In absence of *Wolbachia*, these host populations also differed in several life-history traits (fecundity, longevity, juvenile survival, and sex ratio; Zélé et al. 2020), suggesting the existence of genetic variability among populations.

EXPERIMENTAL PROCEDURE

Mating behavior in field-derived populations

To measure the mating behavior of infected and uninfected mites, uninfected subpopulations were created from the *Wolbachia*-infected field-derived populations via antibiotic treatment. Briefly, 30 adult females of each population were placed in petri dishes containing bean leaf fragments on cotton wet with tetracycline solution (0.1 %, w/v) as described in Zélé et al. (2018c). This treatment was applied continuously for three successive generations (Breeuwer 1997), followed by several generations of mass-rearing in an antibiotic-free environment, which limited potential side effects of antibiotic treatment (Ballard and Melvin 2007; Zeh et al. 2012). Before being used in the experiment, a PCR-based diagnostic of the *Wolbachia* infection status was performed on pools of 100 females (as described in Zélé et al. 2018c). As the PCR diagnostic for *Wolbachia* infection gave ambiguous results for two of the tetracycline-treated populations (DF and RF), the experiment was performed with the five remaining populations (AMP, CH, COL, DC, and LOU) and their uninfected homologues.

One day prior to the experiment, *Wolbachia*-infected and uninfected adult males and *Wolbachia*-uninfected quiescent females (i.e., in the last moulting stage) were isolated from their base populations onto 8 cm^2 leaf squares placed on water-saturated cotton. The next day, quiescent females became virgin adults, roughly of the same age, whereas adult males had been isolated for about 24 hours, which guarantees increased eagerness to mate (Krainacker and Carey 1990). Before the test, males of each population were painted with one of two distinct colors of water-based paint using a fine brush. Colors were randomized across treatments (i.e., infected males were painted in yellow and uninfected males in white for half of the mating trials, and the reverse for the other half). Preference tests were done on 0.5 cm^2 leaf discs (hereafter called "arenas"). Two males, from the same population but with a different infection status, were placed on each arena. The test started as soon as a *Wolbachia*-uninfected virgin female from the same population was added to the arena. Each preference test lasted 30 minutes and the time until the beginning of mating ("latency to copulation") and its duration ("copulation duration") were measured using a stopwatch (www.online-stopwatch.com). Simultaneously, the color of the male that first copulated with the female was registered, and later assigned to a male type. To ensure observer blindness, the correspondence between male type and color was only determined after observations. Trials where no mating occurred during 30 min were excluded from the statistical analysis. In total, 32-38 preference tests per population were performed (cf. Table S2).

Establishment of base populations for experimental evolution

To start experimental evolution with high genetic diversity, we created uninfected and infected base populations by mixing equal subsets of antibiotic-treated and untreated field-derived populations, respectively (Fig. 1). To ensure that *Wolbachia*-infected and uninfected population subsets were obtained from the same founding females from each field-derived population, new tetracycline treatments were employed within the procedure used to create these base populations. To this aim, four groups of 25 adult females were randomly sampled from each of the seven field-derived populations infected with *Wolbachia* and each group was allowed to oviposit on a patch for 3 days. Fifty adult offspring females obtained from each of these four patches (G_{-6} before experimental evolution) were then divided into two new patches: one treated with antibiotics for three generations to remove *Wolbachia* infection (as described above), and the other maintained in the same conditions in absence of antibiotics.

At the end of the treatment (G_{-3}), 25 adult females from each of the four tetracycline-treated (or nontreated) patches belonging to the same population were merged into a single plastic box ($14 \times 14 \times 20$ cm^3) containing two bean plants whose stem was imbibed in wet cotton. At the following generation (G_{-2}), 200 adult offspring females were randomly collected from each box and transferred into a new box to build the next generation (G_{-1}). At G_{-1}, pools of 100 females, randomly sampled from each population, were checked by PCR as before, to confirm the *Wolbachia* infection status prior to the creation of the base populations. As a weak ambiguous signal for *Wolbachia* infection appeared for the tetracycline-treated subset obtained from the field-derived population LOU, we opted for excluding this population from the base populations. The six remaining *Wolbachia*-infected population subsets and their six uninfected homologues were then used to create five *Wolbachia*-infected and five uninfected base

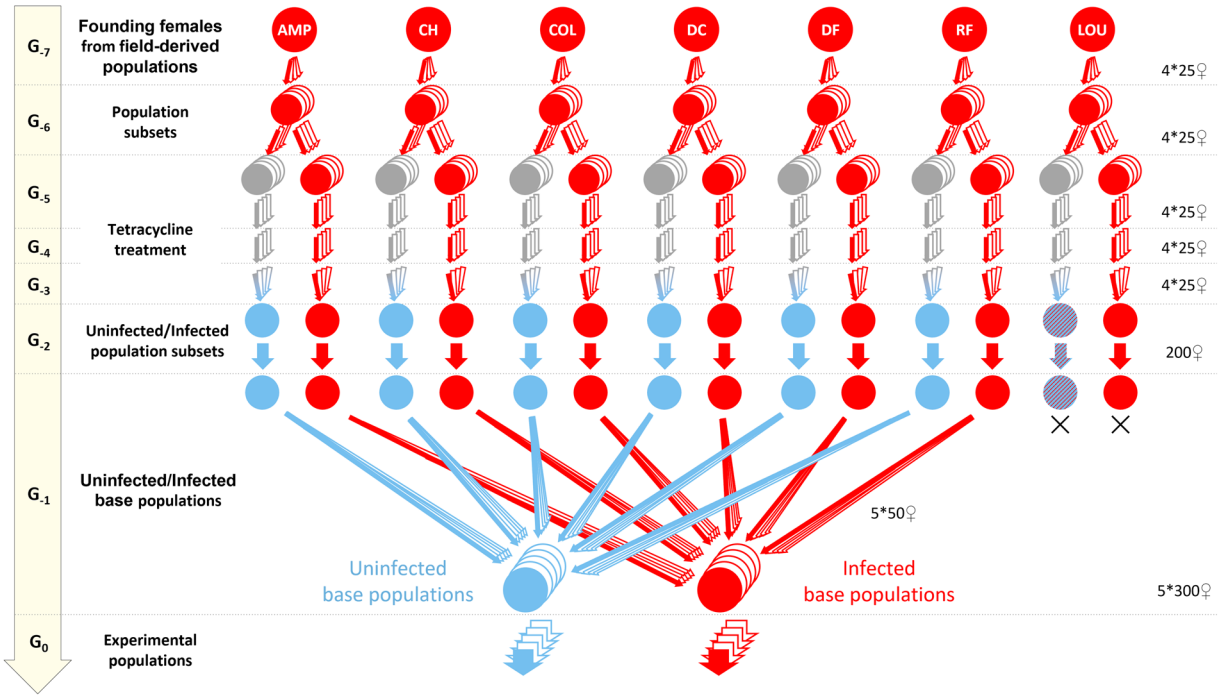

Figure 1. Creation of *Wolbachia*-infected and uninfected base populations. Two subsets established from the same founding individuals (n = 100 females that were distributed among four leaf patches with 25 females each at G_{-7}) of each field-derived population were created at G_{-6}. One of the subsets was treated with tetracycline hydrochloride during three generations (G_{-6} to G_{-3}), followed by three generations without antibiotics (G_{-3} to G_0) before the establishment of populations for experimental evolution at G_0. At G_{-2}, 25 adult females from each of the four leaf patches of a given subset were merged. At G_{-1}, the infection status of each population subset was confirmed by PCR, then five uninfected and five infected base populations were created independently, by mixing five times 50 females from each of the treated and untreated field-derived population subsets, respectively. All population subsets were used, except those derived from the population LOU due to an unclear *Wolbachia* infection status, hence a total of 250 females were added to each base population. Each arrow corresponds to the transfer of adult mated females to found a new generation. The generations before starting the experimental evolution (from G_{-7} to G_0) and each step of the procedure are provided on the left side of the figure and the numbers of females transferred per population and/or treatment at each generation are provided on the right side. Red fill: *Wolbachia*-infected mites; gray fill: mites being treated with tetracycline; blue fill: uninfected mites; blue/red dashed fill: ambiguous PCR results for *Wolbachia* infection.

populations, respectively. Each base population was created by mixing 50 females from each of the six population subsets (infected or not), totaling 300 females. As all field-derived populations were fully compatible (Zélé et al. 2020), they were all expected to be represented and to contribute to the high genetic variability of the merged populations (Godinho et al. 2020). The experimental evolution started from these base populations at the next generation (G_0), hence three generations after the end of the tetracycline treatment.

Experimental evolution

Three experimental selection regimes were created, each with five independent population replicates, initially corresponding to the five base populations (Fig. 2): (a) an infected control (iC) regime composed of *Wolbachia*-infected individuals only, (b) an uninfected control (uC) regime composed of *Wolbachia*-uninfected individuals only, and (c) an uninfected mixed (uM) regime composed of *Wolbachia*-uninfected females and an even proportion of *Wolbachia*-infected and uninfected males (i.e., simulating a stable infection polymorphism in a scenario akin the mainland-island model of Telschow et al. 2007, but preventing *Wolbachia* invasion as females are never infected). In the latter regime, uninfected females were exposed to a 50/50 ratio of infected/uninfected males for three main reasons. First, although a higher frequency of infected males should lead to stronger selection for mate discrimination in uninfected females (Sahoo 2016), it would also increase gene flow from the infected populations into the uninfected populations (because CI is incomplete in this system), thereby hampering the effect of natural selection (Liou and Price 1994; Telschow et al. 2007). Second, a 50/50 ratio gives an equal opportunity to infected and uninfected males to access females, thereby avoiding a bias in encounter rate. Finally, this ratio is close to the average infection prevalence (about 61%) previously found in natural populations of *T. urticae* in South-Western

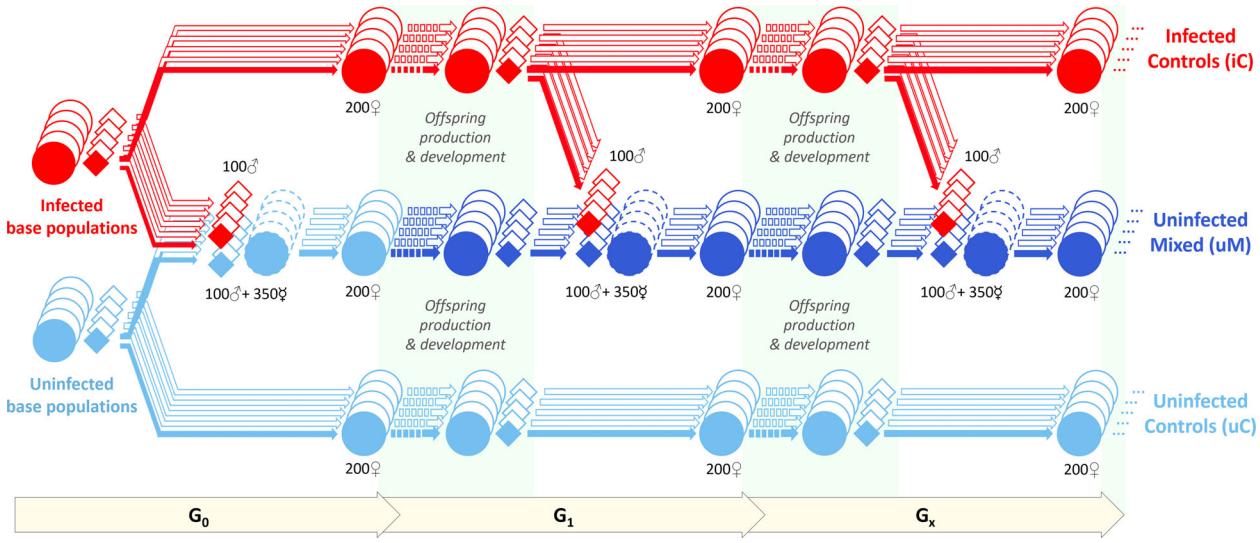

Figure 2. **Procedure used for experimental evolution of spider mites exposed or not to *Wolbachia*-induced cytoplasmic incompatibility. In the uninfected mixed regime, the infection status of males mating with uninfected females was controlled at each generation (50:50 ratio), whereas in the two control regimes matings occurred before transferring adult females into the next generation's box. The entire procedure was repeated in five independent replicates. White background and solid arrows: experimental transfers; green-shaded background and dashed arrows: offspring production and development. Circles: females; lozenge: males; solid-lined symbols: mated females (♀) and males (♂); dashed-lined symbols: virgin females (☿); red fill: *Wolbachia*-infected control (iC); dark blue fill: *Wolbachia*-uninfected females mixed with infected and uninfected males (uM); light blue fill: uninfected control (uC).**

Europe (although substantial variation was found among populations: from about 15 to 100%; Zélé et al. 2018a).

Each population of experimental evolution was initiated by placing 200 females from one of the base populations at 23.5°C in an experimental box ($14 \times 14 \times 20$ cm^3) containing two bean plants (17 days old), whose stem was imbibed in wet cotton. A fresh bean plant was added to each experimental box after 7 days, to avoid resource depletion. The eggs laid by the females in the experimental boxes hatched and reached adulthood within about 11 days (±2 days; Boudreaux 1963; Helle and Sabelis 1985). A new experimental box was created 2 days later to ensure that matings occurred between males and females of roughly the same age prior to female transfer. A discrete generation time of 14 days was thus used for all selection regimes. For the two control regimes, 200 young mated daughters were randomly picked from the old plants and transferred onto two fresh bean plants in a new experimental box at each generation. For the uninfected mixed regime, 350 young quiescent females, 100 males from the uninfected mixed regime and 100 males from the infected control regime were randomly picked from the previous experimental boxes at day 12 of each generation and mixed into a petri dish containing a bean leaf placed on water-saturated cotton. Females emerged as adult virgins and could mate for 2 days, after which 200 of them were transferred to fresh plants in a new box to build the next generation. Despite considerable care, one replicate of the uninfected mixed regime was contaminated by *Wolbachia*-infected females at generation 13 of experimental evolution. This replicate was thus excluded from the entire experiment, along with its respective controls.

Mating behavior after experimental evolution

After 12-15 generations of experimental evolution, *Wolbachia*-uninfected females belonging to the control or mixed regimes were given the choice between different combinations of males (called "treatment" hereafter): (a) males from the uninfected control and from the uninfected mixed regime, (b) males from the infected control and from the uninfected mixed regime, and (c) males from the infected control and from the uninfected control regimes. Our main prediction was that mate preference would evolve in the uninfected mixed regime only. To ensure that preference, if present, would be due to self-referent phenotype matching based solely on *Wolbachia*-induced cues (i.e., excluding kin recognition or familiarity), females and males of each preference test belonged to different replicates: females from the replicates 1, 2, 3, and 4 were presented to males from the replicates 2, 3, 4, and 1, respectively. The protocol followed here was similar to that of the experiment with the field-derived populations except for two minor differences. First, males, like females, were isolated 1 day prior to the experiment as quiescent from a subset of their base populations to ensure that all individuals were virgin and roughly of the same age. Second, trials where no mating occurred for 30 min were included in the final analysis, to test

whether mating propensity (i.e., whether individuals mated during the time of observation) evolved, as uninfected females could become less receptive to matings involving *Wolbachia*-infected males. A total of 25-39 females were tested per experimental evolution replicate and per treatment (for a total of 117-128 females per treatment; cf. Table S3).

STATISTICAL ANALYSES

All analyses were carried out using R (version 3.6.3; R Core Team 2021). The raw datasets used and the corresponding R scripts are available online (Rodrigues et al. 2021). The different statistical models built to analyze the data are described in Table S4. The general procedure was as follows: Mate choice and mating propensity were computed as binary response variables and analyzed using generalized liner mixed-effect models (*glmer* function of the *lme4* package; Bates et al. 2015) with a binomial error distribution. For the analyses of mate choice, the intercept of the models was forced to zero, which gives the estimate of the fixed factor as the difference to a probability of 0.5 in a model with categorical factors and a binomial distribution (Crawley 2007). Latency to copulation and copulation duration were analyzed using a cox proportional hazard mixed-effect model (*coxme* function of the *coxme* package), a nonparametric method to analyze time-to-event data (Crawley 2007).

For the analyses of mating behavior in the field-derived populations, population identity was fit as a fixed explanatory variable, whereas the date of observation and the color of the chosen male were fit as random explanatory variables (Table S4). In the analysis of latency to copulation and copulation duration, the *Wolbachia* infection status of the chosen male and its interaction with population identity were also added as fixed explanatory variables (Table S4). Because each population was tested in a different period, depending on spider mite availability and owing to excessive workload, any significant effect of "population" cannot be unambiguously attributed to differences among populations (i.e., due to possible confounding effects of abiotic conditions and/or plant quality despite controlled conditions for plant growing). Therefore, differences among populations were not tested. Instead, post hoc comparisons between estimated regression coefficients, obtained from the maximal models, were performed using the package *emmeans* (Lenth et al. 2018) to determine, for each population, the difference to random mating (i.e., the difference between the estimate and the zero-intercept using the function *test*) and the effect of *Wolbachia* infection in the chosen male on latency to copulation and copulation duration (i.e., the difference between estimates obtained for infected and uninfected males within each population using the function *pairs*). As the data were used only once in each of these analyses, no *P*-value adjustment was performed.

For the analyses of mating behavior after experimental evolution, the selection regimes of the female and of the two males introduced in the arena (for mating propensity and mate choice) or of the chosen male (for latency to copulation and copulation duration) were fit as fixed explanatory variables. The date of observation, the experimental evolution replicate, the color of the chosen male (for the analysis of mate choice, latency to copulation, and copulation duration), and the selection regime of the two males introduced in the arena (for the analysis of latency to copulation and copulation duration) were fit as random explanatory variables (see Table S4). Maximal models, including all higher order interactions, were simplified by sequentially eliminating nonsignificant terms and interactions to establish a minimal model, and the significance of the explanatory variables was established using χ^2-tests (Crawley 2007). When factors with more than two levels were significant, differences among factor levels were subsequently analyzed using multiple comparisons (function *pairs* of the package *emmeans*; Lenth et al. 2018) with false discovery rate (FDR) correction (Benjamini and Hochberg 1995).

Results

MATING BEHAVIOR IN FIELD-DERIVED POPULATIONS

Uninfected females showed no significant preference for uninfected or infected males in any of the populations tested (Fig. 3a; see Table 1 for statistical results). Although latency to copulation and copulation duration did not differ significantly between crosses involving infected or uninfected males in most populations tested (Figs. 3b and 3c, respectively; Table 1), *Wolbachia*-infected males had shorter latency to copulation than uninfected males in the population CH ($Z = 2.04$, $P = 0.04$) and longer copulation duration than uninfected males in the population COL ($Z = -2.91$, $P = 0.004$).

MATING BEHAVIOR AFTER EXPERIMENTAL EVOLUTION

The selection regime of the females and of the two males introduced in the arena, as well as the interaction between these two factors, did not significantly affect mating propensity (Fig. 4a; see Table 2 for statistical results) or mate choice (Fig. 4b; Table 2). Furthermore, no effect of the selection regime of the female, of the selection regime of the chosen male, or of their interaction was found for latency to copulation (Fig. 4c; Table 2). In contrast, copulation duration was significantly affected by the selection regime of the chosen male ($\chi^2_2 = 9.17$, $P = 0.01$), but not by the selection regime of the female ($\chi^2_1 = 0.16$, $P = 0.69$), nor by the interaction between these factors ($\chi^2_2 = 2.73$, $P = 0.26$; Fig. 4d; Table 2). Indeed, females from both selection

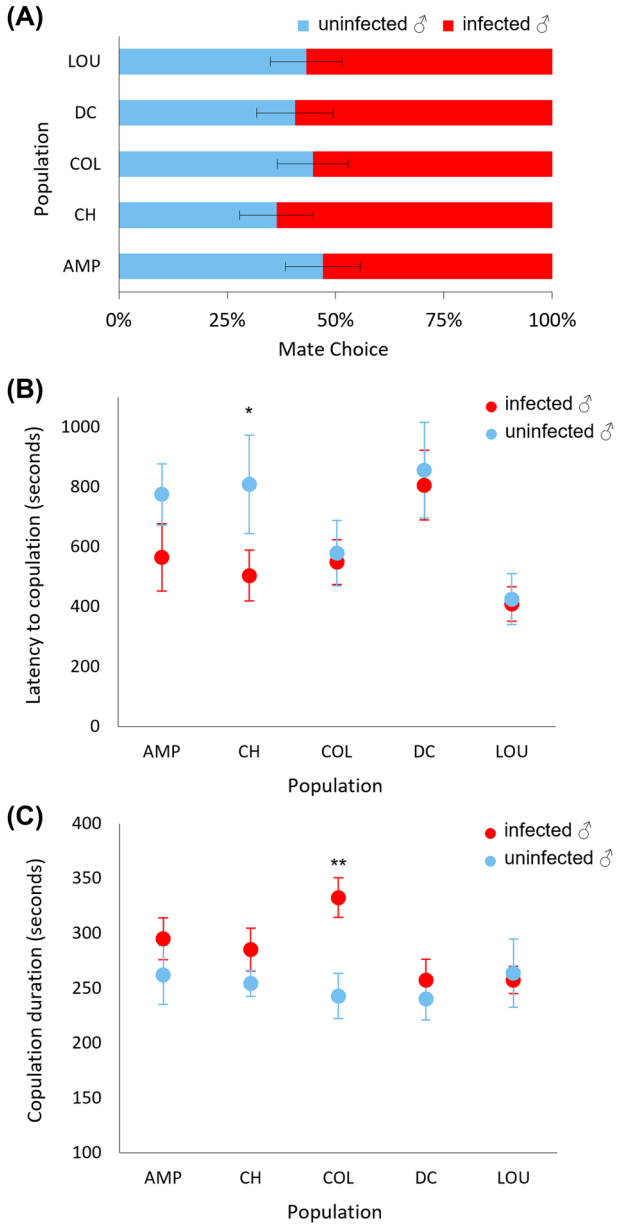

Figure 3. **Mating behavior of *Wolbachia*-uninfected females exposed to *Wolbachia*-infected and uninfected males in five field-derived populations of *T. urticae*. (A) Mate choice: bars represent mean (± SE) proportion of *Wolbachia*-infected males (red bars) or uninfected males (light blue bars) chosen by *Wolbachia*-uninfected females. (B) Latency to copulation and (C) copulation duration: circles represent mean (± SE) time (in seconds) for *Wolbachia*-infected males (red circles) or uninfected males (light blue circles).**

regimes engaged in longer copulations with *Wolbachia*-infected males than with uninfected males from the control regime (infected control vs. uninfected control: $Z = -2.99$, $P = 0.008$), whereas no difference was found when comparing the other types of males (uninfected control vs. uninfected mixed: $Z = 1.94$, $P = 0.08$; and infected control vs. uninfected mixed: $Z = -1.17$, $P = 0.24$).

Table 1. **Effect size for *Wolbachia* infection in males on mating behavior in the field-derived populations. For each response variable, regression coefficients (β), standard errors (SE), *z* ratios, and *P*-values (Pr(>|z|)) associated to the difference with a 50-50 choice between *Wolbachia*-infected and uninfected males (i.e., intercept forced at zero for mate choice), or to the effect of *Wolbachia* infection in males on latency to copulation and copulation duration, were obtained from *Z*-tests performed on the models described in Table S4. Significant effects are displayed in bold.**

Response variable	Population	β	SE (β)	*z* ratio	Pr(>|*z*|)
Mate choice	AMP	−0.228	0.514	−0.443	0.658
	CH	−0.482	0.496	−0.971	0.331
	COL	−0.229	0.441	−0.521	0.603
	DC	−0.350	0.480	−0.728	0.466
	LOU	−0.300	0.447	−0.672	0.501
Latency to copulation	AMP	0.386	0.344	1.120	0.263
	CH	**0.775**	**0.380**	**2.040**	**0.041**
	COL	0.118	0.328	0.360	0.719
	DC	0.210	0.365	0.575	0.566
	LOU	0.091	0.333	0.273	0.785
Copulation duration	AMP	−0.195	0.346	−0.564	0.573
	CH	−0.636	0.367	−1.735	0.083
	COL	**−0.962**	**0.330**	**−2.912**	**0.004**
	DC	−0.346	0.362	−0.956	0.339
	LOU	0.263	0.344	0.765	0.445

Discussion

We studied the mating behavior of *Wolbachia*-uninfected females prior and after 12-15 generations of selection in the presence (i.e., stable *Wolbachia* infection polymorphism in males; "mixed regime") or absence ("uninfected control") of CI. When comparing matings with infected and uninfected males before experimental evolution, we found a shorter latency to copulation in one field population and a longer copulation duration in another. After experimental evolution, these differences were not recapitulated when using uninfected males from the mixed regime, but copulation duration was longer in infected males than in uninfected males from the control regimes. Finally, mate choice was not observed in any of the field-derived populations, nor did it evolve in uninfected mites exposed to CI (polymorphic infection).

Wolbachia EFFECT ON MALE MATING BEHAVIOR BEFORE AND AFTER EXPERIMENTAL EVOLUTION

Behavioral advantages conferred to infected males by *Wolbachia*, such as increased competitiveness and mating rate, have been previously shown in *Drosophila* (Champion de Crespigny et al. 2006; Panteleev et al. 2007). In line with this, the shorter mating

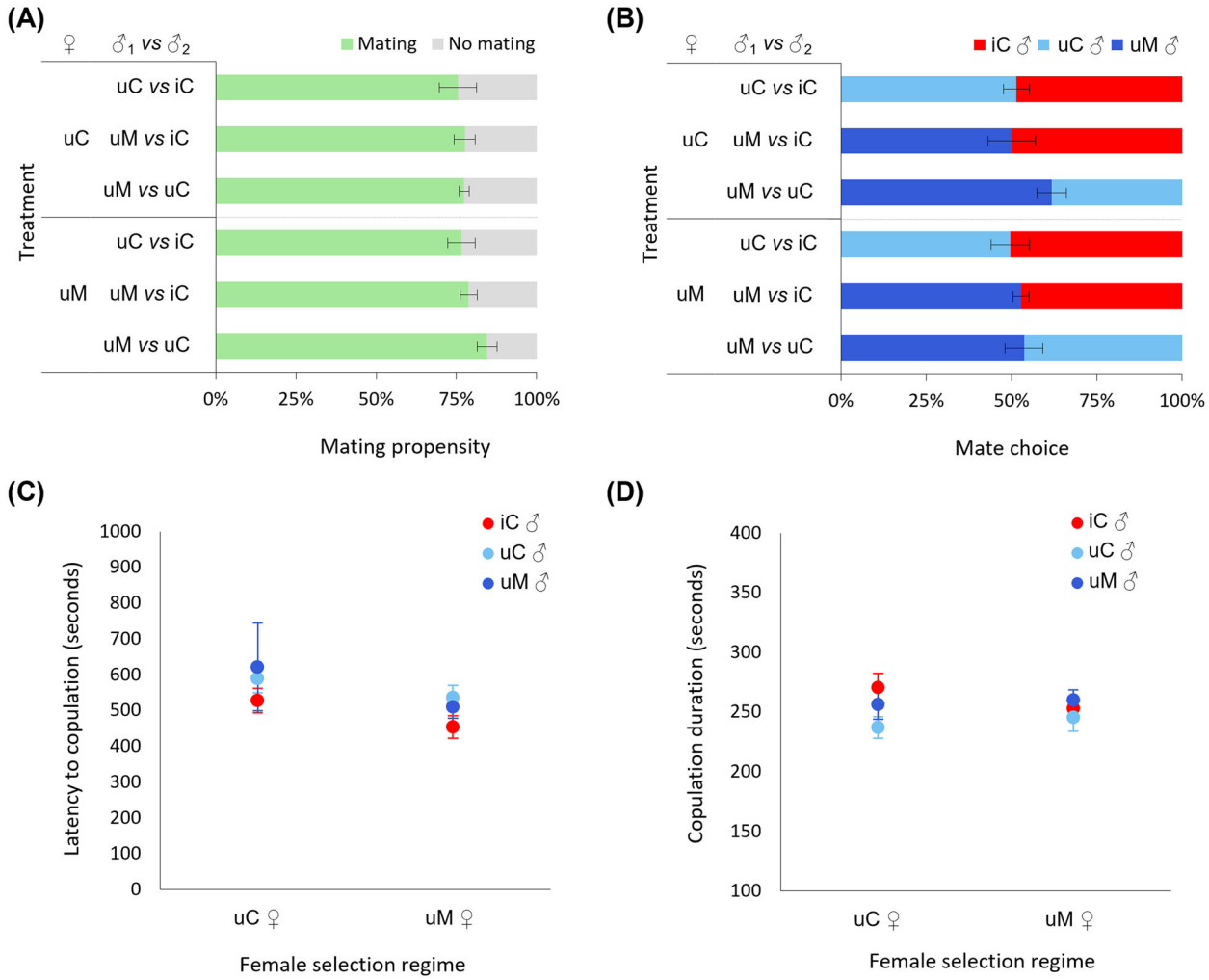

Figure 4. **Mating behavior of *Wolbachia*-uninfected females exposed to males from different experimental evolution regimes. *Wolbachia*-uninfected females from the control and the mixed regimes (uC and uM, respectively) were given the choice between two males from the three different regimes: uninfected control (uC), *Wolbachia*-infected control (iC), or uninfected mixed (uM). (A) Mating propensity: bars represent mean (± SE) percentage of trials where mating occurred (green bars) or not (gray bars) within the time of the observation. (B) Mate choice: bars represent mean (± SE) proportion of females choosing males from the uninfected control (uC: light blue bars), infected control (iC: red bars), or uninfected mixed (uM: dark blue bars) regimes. (C) Latency to copulation and (D) copulation duration: Circles represent mean (± SE) time (in seconds) for males from the uninfected control (uC: light blue circles), infected control (iC: red circles), or uninfected mixed (uM: dark blue circles) regimes.**

latency observed for infected males relative to uninfected males in the population CH, together with the tendency for these males to mate with more females than uninfected males in the mate choice test (about 64 %), may indicate that *Wolbachia* increased male competitiveness in this population, as found in other systems (Hosken et al. 2008; Katsuki et al. 2016). However, this tendency was not observed in the other populations studied here, and a previous study in *T. urticae* did not find an effect of *Wolbachia* on male competitiveness (Zhao et al. 2013). Moreover, no effect of *Wolbachia* on male latency to copulation or mating propensity was found after experimental evolution.

The increase in copulation duration induced by *Wolbachia* in the COL population may be advantageous to infected males (and to *Wolbachia*), as longer copulations may entail more offspring production (Simmons 2001). Such an advantage was found in *Wolbachia*-infected males of the flour beetle *Tribolium confusum* (Wade and Chang 1995). However, this seems unlikely here, as in *T. urticae* no correlation was observed between copulation duration and the number of fertilized eggs (i.e., female offspring, given that this species is arrhenotokous) in the absence of *Wolbachia* (Satoh et al. 2001). Accordingly, no differences in the number of fertilized eggs were found among crosses involving infected and uninfected males from the COL population (Zélé et al. 2020). Alternatively, prolonged copulations may serve as a mechanism of paternity assurance (i.e., postcopulatory guarding) for infected males, as found in several species (Simmons

Table 2. Statistical results for the effect of female and male selection regimes, and their interaction, on mating behavior in the evolved populations. For each response variable, Chi-square (χ^2) and *P*-values were obtained before deletion of the explanatory variable from the minimal models (cf. Table S4). Significant effects are displayed in bold. Females: selection regime of the female; males: selection regime of the two males introduced in the arena with each tested female; chosen: selection regime of the male with which the female mated.

Variable of interest	Explanatory variable	Df	χ^2	*P*-value
Mating propensity	Females × males	2	1.03	0.60
	Females	1	0.74	0.39
	Males	2	2.09	0.35
Mate choice	Females × males	2	1.07	0.58
	Females	2	2.24	0.33
	Males	3	3.93	0.27
Latency to copulation	Females × chosen	2	0.14	0.93
	Females	1	1.84	0.18
	Chosen	2	4.48	0.11
Copulation duration	Females × chosen	2	2.73	0.26
	Females	1	0.16	0.69
	Chosen	**2**	**9.17**	**0.01**

2001). In spider mites, interrupted matings lead to some second male paternity, suggesting that longer matings ensure higher paternity share of the first males (Potter and Wrensch 1978; Satoh et al. 2001). This should also be beneficial for *Wolbachia*, as it is expected to reduce the chances that uninfected females mated with infected males produce offspring from subsequent matings. Finally, *Wolbachia*-infected males may be compensating for a *Wolbachia*-induced decrease in sperm quality or quantity, which is known to occur in several hosts (Snook et al. 2000; Engelstädter and Hurst 2009; Lewis et al. 2011; Awrahman et al. 2014). Such host behavioral and/or reproductive compensations for several types of costs induced by *Wolbachia* have been suggested in several systems, including spider mites (e.g., Vala et al. 2003; Champion de Crespigny et al. 2006; Koop et al. 2009).

Longer copulation duration was also found after experimental evolution for *Wolbachia*-infected control males when compared with uninfected control males, which suggests that this trait, initially present only in the population COL, was selected during experimental evolution in the infected control regime. Hence, this trait may be beneficial for infected individuals even in absence of CI. This also suggests that *Wolbachia* infection could increase trait divergence between uninfected allopatric populations (here between uninfected control and mixed regimes) on a longer term, as observed in natural populations of *D. subquinaria* (Jaenike et al. 2006). However, we found that mating duration did not differ between uninfected males from the mixed-infection regime and infected males from the control regime. Two nonexclusive possibilities may explain this: (i) copulation duration may have increased in uninfected males from the mixed-infection regime (as compared to uninfected males from the control regime) in response to competition with infected males; (ii) continuous unidirectional introgression from the infected control regime into the uninfected mixed regime (as CI is incomplete in these populations; Zélé et al. 2020) may have equalized this trait among the two types of males. If this is the case, it seems that even limited gene flow may prevent divergence between populations in sympatry, decreasing the advantage conferred by *Wolbachia* to infected hosts relative to uninfected ones (Liou and Price 1994). Regardless of the evolutionary mechanisms underlying the differences in copulation duration observed across selection regimes, the benefits of a longer copulation duration remain elusive, and more studies are necessary to unveil the function of this behavior in this context.

UNINFECTED FEMALES DID NOT EVOLVE AVOIDANCE OF *Wolbachia*-INFECTED MALES

In contrast to copulation duration, we found no evidence for a preference between *Wolbachia*-infected and uninfected males across all field-derived populations. This suggests that the results obtained by Vala et al. (2004) are not representative of the reproductive behavior of this species. Moreover, uninfected females did not evolve avoidance of males infected with CI-inducing *Wolbachia* when they were given an equal opportunity to mate with either infected or uninfected males for 12-15 generations. Below we discuss several, nonexclusive, explanations for these results.

First, the field populations tested may lack sufficient genetic variation for choice to evolve in response to CI. It is unlikely that these populations were deprived of genetic variation because (a) spider mite populations are often found in very high numbers in the field, (b) these populations were founded in the lab using a moderate/large number of individuals (from 65 to 400; Zélé et al. 2020), and (c) we found variation for several other traits in these populations (Zélé et al. 2020). Still, it is possible that the specific trait(s) underlying mate choice lack genetic variation in these populations. In line with this, the combination of these five populations may also have harbored insufficient variation for a response to evolve. However, most experimental evolution studies that observed a response to selection, including in this system, used populations with fewer individuals and from one initial field population (Kawecki et al. 2012; Sousa et al. 2019). Therefore, if the lack of evolution of premating isolation is due to insufficient genetic variation for choice in our base populations, we expect this trait to be rare. To confirm this, more studies addressing this issue in several natural populations and in different systems are needed.

Second, the ability to discriminate might have been present originally in the field-derived populations but lost in the

laboratory before the populations were tested, 10-17 generations after being collected. Indeed, infection rapidly reached fixation in the laboratory (Zélé et al. 2020), ruling out the benefit of choice. If choice is costly, we expect it to be rapidly lost, hence not detected when the field-derived and experimentally evolved populations were tested. However, the *T. urticae* isofemale line in which mate choice was observed (Vala et al. 2004) was created from a fully infected population formerly maintained in the laboratory for at least 2 years (Vala et al. 2000), which suggests that female choice was not costly. Still, reducing the lag between collection of field populations and laboratory testing might increase the likelihood of observing mate discrimination.

Third, alleles associated to the ability to choose may have been present at a low frequency in the field-derived populations, but have not increased in frequency during experimental evolution. Indeed, the selection pressure might not have lasted long enough for the preference trait to spread in the populations. A mathematical model by Champion de Crespigny et al. (2005) showed that the frequency of a costless dominant preference allele initially at 1% in the population increases only after about 60 generations. However, in this model, the conditions favoring the spread of the preference allele (i.e., 30-90% *Wolbachia* infection frequencies) are present for less than 15 generations, during which the frequency of the preference allele increased by about 17%. Given that in our study intermediate infection frequencies were always present, this suggests that the number of generations elapsed was sufficient to observe a response. Still, our conditions may not match those underlying the model of Champion de Crespigny et al. (2005). Indeed, spider mites are haplodiploid, thus females involved in incompatible crosses pass on their genes via haploid sons. Moreover, this prediction from the model is based on a higher CI level than that of our study. Finally, we are unaware of the genetic basis of choice in our system, if any. Regardless, if 15 generations of selection (i.e., 7.5 months in mites) are not sufficient for mate choice evolution in this system, such choice is unlikely to be selected for in natural populations, not the least because of the intrinsic seasonality of the mite-plant system.

Fourth, mate discrimination in favor of uninfected individuals may have not been observed due to a trade-off between traits. For instance, female preference could be counterbalanced by different male competitive abilities (Oku 2014). Moreover, if compatible males are of lower quality, females could be trading off male compatibility and quality when choosing (Colegrave et al. 2002; Neff and Pitcher 2005). This could be the case, for example, in *Drosophila melanogaster*, where uninfected females have extended life span after mating with *Wolbachia*-infected males compared to those mated with uninfected males (He et al. 2018). In line with this, *T. urticae* females from two field-collected populations (DC and RF) had increased survival when mated with infected males (although the opposite was found in the populations COL and LOU; Zélé et al. 2020). Another possibility is that the selective pressure applied here may also have led to the evolution of another trait that renders precopulatory strategies unnecessary. For instance, uninfected mites may have evolved disruption of their pattern of sperm precedence, allowing sperm choice or improved sperm competitive ability to avoid incompatible matings, as seen in other species (Price and Wedell 2008; Wedell 2013).

Finally, the cues allowing the discrimination between *Wolbachia*-infected and uninfected males may be absent or unperceivable by females in our populations. Indeed, even though microbial infections (including by *Wolbachia*) have been shown to alter molecular cues used for mate recognition in diverse arthropod hosts (Beltran-Bech and Richard 2014; Richard 2017; Engl and Kaltenpoth 2018; Fortin et al. 2018; Schneider et al. 2019; Bi and Wang 2020), many symbionts (including parasites) are capable of remaining undetected (Schmid-Hempel 2013). Therefore, it has been proposed that discrimination between infected and uninfected individuals could instead occur via traits of locally adapted males that females evolved to use as a preference cue (Telschow et al. 2007; Engelstädter and Telschow 2009). For example, due to CI between *Wolbachia*-uninfected *D. subquinaria* and *Wolbachia*-infected *Drosophila recens*, *D. subquinaria* females from field populations sympatric with *D. recens* evolved avoidance of *D. recens* males (Jaenike et al. 2006). However, these females also discriminate against allopatric (uninfected) conspecific males, whereas females from populations allopatric with *D. recens* show no discrimination against any conspecific males. Another possibility is that discrimination occurs via kin recognition, if uninfected females evolve the ability to discriminate against infected males via discrimination against unrelated (or unfamiliar) males (Beltran-Bech and Richard 2014). Mate discrimination based on relatedness, familiarity, or the local environment (e.g., the host plant) could occur in *T. urticae*, as it has been shown in absence of *Wolbachia* infection (Tien et al. 2011; Yoshioka and Yano 2014). In the context of *Wolbachia* infection, experimental studies, including on spider mites, show that relatedness alone, or cues associated to locally adapted traits, cannot explain the effect of *Wolbachia* on mate discrimination (Vala et al. 2004; Koukou et al. 2006; Miller et al. 2010), but they do not exclude a potential familiarity effect, as males and females from the same infection status likely developed together. Our experimental procedure was specifically designed to test for a direct effect of *Wolbachia* infection, not allowing for such indirect cues to affect preference.

Conclusions

Our results show that *Wolbachia*-induced CI did not select for assortative mating under our experimental conditions, despite the

clear benefit that avoidance of infected males would confer to uninfected females. We proposed a wide range of hypotheses to explain why such behavior did not evolve, which will hopefully provide guidance for future studies addressing this issue. In particular, this result supports the hypothesis that *Wolbachia*-driven host speciation may not only require a strong selection pressure due to CI under stable infection polymorphism, but also hinge upon other specific conditions, such as local adaptation in structured populations and kin recognition (Telschow et al. 2007). Nevertheless, our study shows that, within only 15 generations, *Wolbachia* can drive the evolution of other mating behaviors in a fully infected population, as well as in uninfected hosts in a polymorphic population. This suggests that *Wolbachia* has the potential to affect the evolution of host reproductive strategies more broadly and could accelerate divergence between allopatric populations.

AUTHOR CONTRIBUTIONS

LRR, FZ, and SM conceptualized and designed the experiments. FZ and IS performed experimental evolution. IS performed the experiments with the field populations. LRR performed the experiments after experimental evolution. LRR and FZ performed statistical analyses. LRR, FZ, and SM wrote this article. Funding agencies did not participate in the design or analysis of experiments. All authors have read and approved the final version of the manuscript.

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DATA ARCHIVING

The data that support the findings of this study are openly available at https://doi.org/10.6084/m9.figshare.13614608.v1.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Description of the field-derived spider mite populations.
Table S2. Summary of the results for mating behavior in the field-derived populations.
Table S3. Summary of the results for mating behavior in the evolved populations.
Table S4. Description of the statistical models used in the analyses of field-derived and evolved populations.