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Leonor R Rodrigues, Flore Zélé, Inês Santos, Sara Magalhães. No evidence for the evolution of mating behavior in spider mites due to Wolbachia -induced cytoplasmic incompatibility. *Evolution - International Journal of Organic Evolution*, 2022, 76 (3), pp.623-635. 10.1111/evo.14429 . hal-03864990

HAL Id: hal-03864990

<https://hal.science/hal-03864990>

Submitted on 22 Nov 2022

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

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10 Running Title:

11 *Wolbachia*, mating behaviour and evolution

12 **Author contributions**

13 Experimental conception and design: LRR, FZ, SM; experimental evolution: FZ, IS;
14 performed the experiment with the field populations: IS; performed the experiment after
15 experimental evolution: LRR; statistical analyses: LRR, FZ; paper writing: LRR, FZ, SM.
16 Funding agencies did not participate in the design or analysis of experiments. All authors
17 have read and approved the final version of the manuscript.

18 **Acknowledgments**

19 We are grateful to Diogo Godinho and João Alpedrinha for useful discussions and comments
20 and all Mite Squad members for unconditional support in the laboratory and elsewhere. This
21 work was funded by an FCT-ANR project (FCT-ANR//BIA- EVF/0013/2012) and an ERC
22 consolidating grant (COMPCON GA 725419) to SM. FZ was funded through FCT Post-Doc
23 fellowship (SFRH/BPD/125020/2016) and LRR through FCT PhD grant
24 (SFRH/BD/87628/2012). This is publication ISEM-2021-XXX of the Institut des Sciences de
25 l'Evolution.

26 **Conflict of interest**

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

28 **Data accessibility**

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/evo.14429](https://doi.org/10.1111/evo.14429).

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29 The data that support the findings of this study is openly available at
30 <https://doi.org/10.6084/m9.figshare.13614608.v1>.

31 **Abstract**

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

47 **Keywords**

48 Endosymbiont; parasite manipulation; reproductive incompatibility; reproductive isolation;
49 sexual selection; mate preference

50 **Introduction**

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

60 *Wolbachia* are the most widespread endosymbiotic bacteria found in arthropods
61 (Weinert et al. 2015). Although, in some cases, its maintenance and spread in host
62 populations can be attributed to fitness benefits conferred to infected hosts (e.g., increased
63 fecundity, survival, nutritional mutualism and/or protection against pathogens; Dobson et al.
64 2004; Barr et al. 2010; Hosokawa et al. 2010; Nikoh et al. 2014; Ross et al. 2019), its success
65 is mostly due to its ability to manipulate the reproduction of its hosts (Werren et al. 2008;
66 Engelstädter and Hurst 2009; Kaur et al. 2021). The most common *Wolbachia*-induced
67 reproductive manipulation is cytoplasmic incompatibility (CI), a mechanism that results in
68 the embryonic death of some, or all, fertilized offspring from crosses between uninfected
69 females and infected males (Werren et al. 2008; Shropshire et al. 2020). As all other crosses
70 are compatible, CI promotes the spread of *Wolbachia* by indirectly (i.e., via infected males)
71 increasing the success of infected females relative to that of uninfected females. However,
72 because CI is costly for individuals involved in incompatible crosses, it is expected to exert a
73 strong selective pressure on hosts to evolve strategies that reduce the frequency and/or costs
74 of such matings (Charlat et al. 2003; Engelstädter and Hurst 2009; Sahoo 2016).

75 Discrimination and avoidance of incompatible mates prior to mating has been
76 proposed as a potential host strategy to avoid CI (Hoffmann et al. 1990; see Sahoo 2016 for a
77 review). Indeed, CI-induced offspring death may select for premating isolation
78 ('reinforcement': Dobzhansky 1937), as demonstrated by different theoretical models
79 (Champion de Crespigny et al. 2005; Telschow et al. 2005, 2007). These models predict that
80 selection for the ability to discriminate between *Wolbachia*-infected and uninfected
81 individuals hinges upon populations being exposed to a polymorphic infection state (when
82 both infected and uninfected individuals occur within the same population) for a sufficient
83 amount of time (Champion de Crespigny et al. 2005; Telschow et al. 2005, 2007). Such
84 conditions can be met either when the spread of *Wolbachia* in a host population is sufficiently
85 slow (due to incomplete CI, fecundity costs and/or imperfect transmission of the symbiont;
86 Champion de Crespigny et al. 2005) or when an uninfected population receives migrants
87 from a *Wolbachia*-infected population (e.g. in a mainland-island metapopulation; Telschow
88 et al. 2007). Because infection polymorphisms are relatively common in populations of
89 different host species (e.g., Vavre et al. 2002; Baudry et al. 2003; Keller et al. 2004; Hamm et
90 al. 2014) it is likely that mate discrimination evolves frequently. However, empirical studies
91 that experimentally tested mate discrimination in species infected by CI-inducing *Wolbachia*
92 produced variable outcomes (Bi and Wang 2020). Whereas some studies found no evidence

93 for mate choice (*e.g.*, Hoffmann and Turelli 1988; O'Neill 1991; Wade and Chang 1995;
94 Champion de Crespigny and Wedell 2007; Duron et al. 2011; Arbuthnott et al. 2016; Bagheri
95 et al. 2019), others found that individuals can discriminate between *Wolbachia*-infected and
96 uninfected mates (Vala et al. 2004; Markov et al. 2009), or that *Wolbachia* infection increases
97 levels of mate discrimination between populations (Koukou et al. 2006; Miller et al. 2010).

98 The evolution of preference for *Wolbachia*-infected or uninfected mates may also
99 depend on the mechanisms underlying mate discrimination. Empirical work suggests that
100 such discrimination may be based on host traits that are altered upon *Wolbachia* infection
101 (*e.g.*, change of host pheromone profiles and/or production; Pontier and Schweisguth 2015;
102 Engl and Kaltenpoth 2018; Fortin et al. 2018; Schneider et al. 2019) or on host traits
103 unrelated to *Wolbachia* infection, such as those associated with kin recognition (*e.g.*, via self-
104 reference phenotype matching; Markov et al. 2009) or local adaptation (*e.g.*, CI-driven mate
105 choice evolution in natural populations of *D. subquinaria*; Jaenike et al. 2006). This is
106 supported by theoretical models predicting that mate discrimination can be selected for when
107 either type of cues is used by hosts (Champion de Crespigny et al. 2005; Telschow et al.
108 2007). Unfortunately, no experimental study so far has tested whether assortative mating
109 between uninfected and infected hosts evolves under stable polymorphism, and if such
110 evolution can occur based on discrimination of *Wolbachia* infection itself or whether it
111 requires the existence of host traits that indirectly indicate the infection status of potential
112 mates.

113 Populations of the two-spotted spider mites *Tetranychus urticae* harbour different CI-
114 inducing *Wolbachia* strains with variable prevalence (from 0 to 100%; *e.g.*, Gotoh et al. 2003,
115 2007; Zhang et al. 2016; Zélé et al. 2018a,b), fitness effects and levels of CI (ranging from
116 costs to benefits on several life history traits, such as longevity and fecundity, and from no CI
117 to complete CI, respectively; *e.g.*, Vala et al. 2002; Gotoh et al. 2007; Xie et al. 2011; Suh et
118 al. 2015; Zélé et al. 2020). In this species, females may mate multiple times, but they use
119 mainly the sperm from their first mating to produce fertilized offspring (*i.e.*, there is first
120 male sperm precedence; Helle 1967; Rodrigues et al. 2020). Unsuccessful matings are thus
121 expected to be highly costly for females, as they risk a reduction in fitness with few chances
122 of recovery upon remating. Evolving the ability to choose males with whom they can
123 successfully reproduce would hence be exceptionally advantageous for such females.
124 Accordingly, female choice is present in this species (Oku 2014). In particular, Vala et al.

125 (2004), showed that *Wolbachia*-uninfected females from a single isofemale line of *T. urticae*
126 prefer uninfected males over infected ones. Here, we tested the generality of this finding by
127 assessing *Wolbachia*-associated mate choice in several field-derived *T. urticae* populations
128 naturally infected by CI-inducing *Wolbachia*. Next, we created outbred populations from
129 these populations and performed experimental evolution to test if pre-copulatory mating
130 behaviour, and avoidance of infected males by uninfected females based on signals
131 associated with *Wolbachia* infection only, could evolve in response to CI.

132 **Materials and Methods**

133 **Spider mite populations and rearing conditions**

134
135 Seven populations belonging to the red form of *T. urticae*, collected on different host plants
136 around Lisbon in late 2013 (Zélé et al. 2018a), were used in this study (Table S1). These
137 populations were established at the University of Lisbon and maintained under standard
138 conditions ($25 \pm 2^\circ\text{C}$, 60% RH, 16/8 h L/D) in the laboratory at high numbers (c.a. 500-1000
139 females) in insect-proof cages containing bean plants (*Phaseolus vulgaris*, Fabaceae, var.
140 *Enana*; Germisem Sementes Lda, Oliveira do Hospital, Portugal). At the time of the
141 experiment, all populations were naturally and fully infected (100% infection frequency in
142 each population) with compatible (or identical) strains of *Wolbachia* that induce variable
143 levels of cytoplasmic incompatibility (ca. 27 to 66% female embryonic mortality; cf. Table
144 S1) and that had variable effects on host fecundity and longevity, ranging from costs to
145 benefits, depending on the host population (Zélé et al. 2020). In absence of *Wolbachia*, these
146 host populations also differed in several life-history traits (fecundity, longevity, juvenile
147 survival and sex-ratio; Zélé et al. 2020), suggesting the existence of genetic variability among
148 populations.

149 **Experimental procedure**

150 ***Mating behaviour in field-derived populations***

151 To measure the mating behaviour of infected and uninfected mites, uninfected sub-
152 populations were created from the *Wolbachia*-infected field-derived populations via
153 antibiotic treatment. Briefly, 30 adult females of each population were placed in petri dishes

154 containing bean leaf fragments on cotton wet with tetracycline solution (0.1 %, w/v) as
155 described in Zélé et al. (2018c). This treatment was applied continuously for three successive
156 generations (Breeuwer 1997), followed by several generations of mass-rearing in an
157 antibiotic-free environment, which limited potential side effects of antibiotic treatment
158 (Ballard and Melvin 2007; Zeh et al. 2012). Before being used in the experiment, a PCR-
159 based diagnostic of the *Wolbachia* infection status was performed on pools of 100 females (as
160 described in Zélé et al. 2018c). As the PCR diagnostic for *Wolbachia* infection gave
161 ambiguous results for two of the tetracycline-treated populations (DF and RF), the
162 experiment was performed with the five remaining populations (AMP, CH, COL, DC, LOU)
163 and their uninfected homologues.

164 One day prior to the experiment, *Wolbachia*-infected and -uninfected adult males and
165 *Wolbachia*-uninfected quiescent females were isolated from their base populations onto 8
166 cm² leaf squares placed on water-saturated cotton. The next day, quiescent females became
167 virgin adults, roughly of the same age, while adult males had been isolated for *ca.* 24 hours,
168 which guarantees increased eagerness to mate (Krainacker and Carey 1990). Before the test,
169 males of each population were painted with one of two distinct colours of water-based paint
170 using a fine brush. Colours were randomized across treatments (*i.e.*, infected males were
171 painted in yellow and uninfected males in white for half of the mating trials, and the reverse
172 for the other half). Preference tests were done on 0.5 cm² leaf discs (hereafter called
173 “arenas”). Two males, from the same population but with a different infection status, were
174 placed on each arena. The test started as soon as a *Wolbachia*-uninfected virgin female from
175 the same population was added to the arena. Each preference test lasted for thirty minutes and
176 the time until the beginning of mating (‘latency to copulation’) and its duration (‘copulation
177 duration’) were measured using a stopwatch (www.online-stopwatch.com). Simultaneously,
178 the colour of the male that first copulated with the female was registered, and later assigned
179 to a male type. To ensure observer blindness, the correspondence between male type and
180 colour was only determined after observations. Trials where no mating occurred for 30
181 minutes were excluded from the statistical analysis. In total, 32 to 38 preference tests per
182 population were performed (*cf.* Table S2).

183 ***Establishment of base populations for experimental evolution***

184 To start experimental evolution with high genetic diversity, we created uninfected and
185 infected base populations by mixing equal subsets of the antibiotic-treated and untreated

186 field-derived populations, respectively (Fig. 1). To ensure that *Wolbachia*-infected and
187 uninfected population subsets were obtained from the same founding females from each
188 field-derived population, new tetracycline treatments were employed within the procedure
189 used to create these base populations. To this aim, four groups of 25 adult females were
190 randomly sampled from each of the seven field-derived population infected with *Wolbachia*
191 and each group was allowed to oviposit on a patch for three days. 50 adult offspring females
192 obtained from each of these four patches (G-6 before experimental evolution) were then
193 divided into two new patches: one treated with antibiotics for three generations to remove
194 *Wolbachia* infection (as described above), and the other maintained in the same conditions in
195 absence of antibiotics.

196 At the end of the treatment (G₃), 25 adult females from each of the 4 tetracycline-
197 treated (or non-treated) patches belonging to the same population were merged into a single
198 plastic box (14x14x20cm) containing two bean plants whose stem was imbibed in wet cotton.
199 At the following generation (G₂), 200 adult offspring females were randomly collected from
200 each box and transferred into a new box to build the next generation (G₁). At G₁, pools of
201 100 females, randomly sampled from each population, were checked by PCR as before, to
202 confirm the *Wolbachia* infection status prior to the creation of the base populations. As a
203 weak ambiguous signal for *Wolbachia* infection appeared for one of the tetracycline-treated
204 subsets obtained from the field-derived population LOU, we opted for excluding this
205 population from the base populations. The 6 remaining *Wolbachia*-infected population
206 subsets and their 6 uninfected homologues were then used to create 5 *Wolbachia*-infected and
207 5 uninfected base populations, respectively. Each base population was created by mixing 50
208 females from each of the 6 population subsets (infected or not), totalling 300 females. As all
209 field-derived populations were fully compatible (Zélé et al. 2020), they were all expected to
210 be represented and to contribute to the high genetic variability of the merged populations
211 (Godinho et al. 2020). The experimental evolution started from these base populations at the
212 next generation (G₀), 3 generations after the end of the tetracycline treatment.

214 ***Experimental Evolution***

215 Three experimental selection regimes were created, each with five independent population
216 replicates, initially corresponding to the 5 base populations (Fig. 2): (a) an infected control
217 (iC) regime composed of *Wolbachia*-infected individuals only, (b) an uninfected control (uC)

218 regime composed of *Wolbachia*-uninfected individuals only, and (c) an uninfected mixed
219 (uM) regime composed of *Wolbachia*-uninfected females and an even proportion of
220 *Wolbachia*-infected and -uninfected males (*i.e.*, simulating a stable infection polymorphism
221 in a scenario akin the mainland-island model of Telschow et al. (2007), but preventing
222 *Wolbachia* invasion as females are never infected). In the latter regime, uninfected females
223 were exposed to a 50/50 ratio of infected/uninfected males for three main reasons. First,
224 although a higher frequency of infected males should lead to stronger selection for mate
225 discrimination in uninfected females (Sahoo 2016), it also increases gene flow from the
226 infected populations into the uninfected populations (because CI is incomplete in this
227 system), thereby hampering the effect of natural selection (Liou and Price 1994; Telschow et
228 al. 2007). Second, a 50/50 ratio gives an equal opportunity to infected and uninfected males
229 to access females, thereby avoiding a bias in encounter rate. Finally, this ratio is close to the
230 average infection prevalence (ca. 61%) previously found in natural populations of *T. urticae*
231 in South-Western Europe (although substantial variation was found among populations: from
232 ca. 15% to 100%; Zélé et al. 2018a).

233 Each population of experimental evolution was initiated by placing 200 females from
234 one of the base populations at 23.5°C in an experimental box (14x14x20cm) containing two
235 bean plants (17 days old), whose stem was imbibed in wet cotton. A fresh bean plant was
236 added to each experimental box after 7 days, to avoid resource depletion. The eggs laid by the
237 females in the experimental boxes hatched and reached adulthood within c.a. 11 days (± 2
238 days; Boudreaux 1963; Helle and Sabelis 1985). A new experimental box was created 2 days
239 later to ensure that matings occurred between males and females of roughly the same age
240 prior to female transfer. A discrete generation time of 14 days was thus used for all selection
241 regimes. For the two control regimes, 200 young mated daughters were randomly picked
242 from the old plants and transferred onto 2 fresh bean plants in a new experimental box at each
243 generation (*i.e.*, every 14 days). For the uninfected mixed regime, 350 young quiescent
244 females and 100 males from the uninfected mixed regime and 100 males from the infected
245 control regime, were randomly picked from the previous experimental boxes at day 12 of
246 each generation and mixed into a petri dish containing a bean leaf placed on water-saturated
247 cotton. Females emerged as adult virgins and could mate for two days, after which 200 of
248 them were transferred to fresh plants in a new box to build the next generation. Despite
249 considerable care, one replicate of the uninfected mixed regime was contaminated by

250 *Wolbachia*-infected females at generation 13 of experimental evolution. This replicate was
251 thus excluded from the entire experiment, along with its respective controls.

252 ***Mating behaviour after experimental evolution***

253 After 12 to 15 generations of experimental evolution *Wolbachia*-uninfected females
254 belonging to the control or mixed regimes were given the choice between different
255 combinations of males (called 'treatment' hereafter): (a) males from the uninfected control
256 and from the uninfected mixed regime, (b) males from the infected control and from the
257 uninfected mixed regime, and (c) males from the infected control and from the uninfected
258 control regimes. Our main prediction was that mate preference would evolve only in the
259 uninfected mixed regime. To ensure that preference, if present, would be due to self-referent
260 phenotype matching based on *Wolbachia*-induced cues (*i.e.*, excluding kin recognition or
261 familiarity), females and males of each preference test belonged to different replicates:
262 females from the replicates 1, 2, 3 and 4 were presented to males from the replicates 2, 3, 4
263 and 1, respectively. The protocol followed here was similar to that of the first experiment
264 except for two minor differences. First, males, like females, were isolated one day prior to the
265 experiment as quiescent from a subset of their base populations to ensure that all individuals
266 were virgin and roughly of the same age. Second, trials where no mating occurred for 30
267 minutes were included in the final analysis, to test whether mating propensity (*i.e.*, whether
268 individuals mated during the time of the observations) evolved, as uninfected females could
269 become less receptive to matings involving *Wolbachia*-infected males. 25 to 39 females were
270 tested per experimental evolution replicate and per treatment (for a total of 117 to 128
271 females per treatment; *cf.* Table S3).

272 **Statistical Analyses**

273 All analyses were carried out using R (v. 3.6.3; R Core Team 2021). The raw datasets used,
274 and the corresponding R scripts are available online (Rodrigues et al. 2021). The different
275 statistical models built to analyse the data are described in the Supporting Information Table
276 S4. The general procedure was as follows: Mate choice and mating propensity were
277 computed as binary response variables and analysed using generalized liner mixed-effect
278 models (*glmer* function of the *lme4* package; Bates et al. 2015) with a binomial error
279 distribution. For the analyses of mate choice, the intercept of the models was forced to zero,
280 which gives the estimate of the fixed factor as the difference to a probability of 0.5 in a model

281 with categorical factors and a binomial distribution (Crawley 2007). Latency to copulation
282 and copulation duration were analysed using a cox proportional hazard mixed-effect model
283 (*coxme* function of the *coxme* package), a non-parametric method to analyse time-to-event
284 data (Crawley 2007).

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

303 For the analyses of mating behaviour after experimental evolution, the selection
304 regime of the female and of the two males introduced in the arena (for mating propensity and
305 mate choice), or of the chosen male (for latency to copulation and copulation duration) were
306 fit as fixed explanatory variables. The date of observation, the experimental evolution
307 replicate, the colour of the chosen male (for the analysis of mate choice, latency to copulation
308 and copulation duration), and the selection regime of the chosen male (for the analysis of
309 latency to copulation and copulation duration) were fit as random explanatory variables (see
310 Table S4). Maximal models, including all higher-order interactions, were simplified by
311 sequentially eliminating non-significant terms and interactions to establish a minimal model,
312 and the significance of the explanatory variables was established using chi-squared tests
313 (Crawley 2007). When factors with more than two levels were significant, differences among

314 factor levels were subsequently analysed using multiple comparisons (function *pairs* of the
315 package *emmeans*; Lenth et al. 2018) with false discovery rate (FDR) correction (Benjamini
316 and Hochberg 1995).

317

318 **Results**

319 **Mating behaviour in field-derived populations**

320 Uninfected females showed no significant preference for uninfected or infected males in any
321 of the populations tested (Fig. 3a; see Table 2 for statistical results). Although latency to
322 copulation and copulation duration did not differ significantly between crosses involving
323 infected or uninfected males in most populations tested (Fig. 3b and 3c, respectively; Table
324 2), *Wolbachia*-infected males had shorter latency to copulation than uninfected males in the
325 population CH ($Z=2.04$, $P=0.04$) and longer copulation duration than uninfected males in the
326 population COL ($Z=-2.91$, $P=0.004$).

327 **Mating behaviour after experimental evolution**

328 The selection regime of the females and of the two males introduced in the arena, as well as
329 the interaction between these two factors, did not significantly affect mating propensity (Fig.
330 4a) nor mate choice (Fig. 4b). Furthermore, no effect of the selection regime of the female, of
331 the selection regime of the chosen male, nor of their interaction was found for latency to
332 copulation (Fig. 4c). In contrast, copulation duration was significantly affected by the
333 selection regime of the chosen male ($X^2_2=9.17$, $P=0.01$), but not by the selection regime of
334 the female ($X^2_1=0.16$, $P=0.69$), nor by the interaction between these factors ($X^2_2=2.73$,
335 $P=0.26$; Fig. 4d). Indeed, females from both selection regimes engaged in longer copulations
336 with *Wolbachia*-infected males than with uninfected males from the control regime (infected
337 control vs uninfected control: $Z=-2.99$, $P=0.008$), while no difference was found when
338 comparing the other types of males (uninfected control vs uninfected mixed: $Z=1.94$, $P=0.08$;
339 and infected control vs uninfected mixed: $Z=-1.17$, $P=0.24$).

340

341 **Discussion**

342 We studied the mating behaviour of *Wolbachia*-uninfected females prior and after 12 to 15
343 generations of selection in the presence (*i.e.*, stable *Wolbachia* infection polymorphism in

344 males; ‘mixed regime’) or absence (‘uninfected control’) of CI. When comparing matings
345 with infected and uninfected males before experimental evolution, we found a shorter latency
346 to copulation in one field population and a longer copulation duration in another. After
347 experimental evolution, these differences were not recapitulated when using uninfected males
348 from the mixed regime, but copulation duration was longer in infected males than in
349 uninfected males from the control regimes. Finally, mate choice was not observed in any of
350 the field-derived populations, nor did it evolve in uninfected mites exposed to CI
351 (polymorphic infection).

352

353 **Wolbachia effect on male mating behaviour before and after experimental evolution**

354 Behavioural advantages conferred to infected males by *Wolbachia*, such as increased
355 competitiveness and mating rate, has been previously shown in *Drosophila* (Champion de
356 Crespigny and Wedell 2006; Panteleev et al. 2007). In line with this, the shorter mating
357 latency observed for infected males relative to uninfected males in the population CH,
358 together with the tendency for these males to mate with more females than uninfected males
359 in the mate choice test (ca. 64 %), may indicate that *Wolbachia* increased male
360 competitiveness in this population, as found in other systems (Hosken et al. 2008; Katsuki et
361 al. 2016). However, this tendency was not observed in the other populations studied here, and
362 a previous study in *T. urticae* did not find an effect of *Wolbachia* on male competitiveness
363 (Zhao et al. 2013). Moreover, no effect of *Wolbachia* on male latency to copulation or mating
364 propensity was found after experimental evolution.

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

375 males, as found in several species (Simmons 2001). In spider mites, interrupted matings lead
376 to some second male paternity, suggesting that longer matings ensure higher paternity share
377 of the first males (Potter and Wrensch 1978; Satoh et al. 2001). This should also be beneficial
378 for *Wolbachia*, as it is expected to reduce the chances that uninfected females mated with
379 infected males produce offspring from subsequent matings. Finally, *Wolbachia*-infected
380 males may be compensating for a *Wolbachia*-induced decrease in sperm quality or quantity,
381 which is known to occur in several hosts (Snook et al. 2000; Champion de Crespigny et al.
382 2006; Engelstädter and Hurst 2009; Lewis et al. 2011; Awrahman et al. 2014). Such host
383 behavioural and/or reproductive compensations for several types of costs induced by
384 *Wolbachia* have been suggested in several systems, including spider mites (e.g., Vala et al.
385 2003; Champion de Crespigny et al. 2006; Koop et al. 2009).

386 Longer copulation duration was also found after experimental evolution for
387 *Wolbachia*-infected control males when compared with uninfected control males, which
388 suggests that this trait, initially present only in the population COL, was selected during
389 experimental evolution in the infected control regime. Hence, this trait may be beneficial for
390 infected individuals even in absence of CI. This also suggests that *Wolbachia* infection could
391 increase trait divergence between uninfected allopatric populations (here between uninfected
392 control and mixed regimes) on a longer term, as observed in natural populations of *D.*
393 *subquinaria* (Jaenike et al. 2006). In contrast, mating duration did not differ between
394 uninfected males from the mixed-infection regime and infected males from the control
395 regime. Two non-exclusive possibilities may explain this: (i) copulation duration may have
396 increased in uninfected males from the mixed-infection regime in response to competition
397 with infected males; (ii) continuous unidirectional introgression from the infected control
398 regime into the uninfected mixed regime (as CI is incomplete in these populations; Zélé et al.
399 2020) may have equalized this trait among the two types of males. If this is the case, it seems
400 that even limited gene flow may prevent divergence between populations in sympatry,
401 decreasing the advantage conferred by *Wolbachia* to infected hosts relative to uninfected ones
402 (Liou and Price 1994). Regardless of the evolutionary mechanisms underlying the differences
403 in copulation duration observed across selection regimes, the benefits of a longer copulation
404 duration remain elusive, and more studies are necessary to unveil the function of this
405 behaviour in this context.

406

407 **Uninfected females did not evolve avoidance of *Wolbachia*-infected males**

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

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

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

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

457 Fourth, mate discrimination in favour of uninfected individuals may have not been
458 observed due to a trade-off between traits. For instance, female preference could be
459 counterbalanced by different male competitive abilities (Oku et al. 2014). Moreover, if
460 compatible males are of lower quality, females could be trading off male compatibility and
461 quality when choosing (Colegrave et al. 2002; Neff and Pitcher 2005). That could be the case,
462 for example, in *D. melanogaster*, where uninfected females have extended life span after
463 mating with *Wolbachia*-infected males compared to those mated with uninfected males (He et
464 al. 2018). In line with this, *T. urticae* females from two field-collected populations (DC and
465 RF) had increased survival when mated with infected males (although the opposite was found
466 in the populations COL and LOU; Zélé et al. 2020). Another possibility is that the selective
467 pressure applied here may also have led to the evolution of another trait that renders pre-
468 copulatory strategies unnecessary. For instance, uninfected mites may have evolved
469 disruption of their pattern of sperm precedence, allowing sperm choice or improved sperm
470 competitive ability to avoid incompatible matings, as seen in other species (Price and Wedell
471 2008; Wedell 2013).

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

498 499 **Conclusions**

500 Our results show that *Wolbachia*-induced CI did not select for assortative mating under our
501 experimental conditions, despite the clear benefit that avoidance of infected males would
502 confer to uninfected females. We proposed a wide range of hypotheses to explain why such
503 behaviour did not evolve, which will hopefully provide guidance for future studies addressing

504 this issue. In particular, this result supports the hypothesis that *Wolbachia* driven host
505 speciation may not only require a strong selection pressure due to CI under stable infection
506 polymorphism, but also hinge upon other specific conditions, such as local adaptation in
507 structured populations and kin recognition (Telschow et al. 2007). Nevertheless, our study
508 shows that, within only 15 generations, *Wolbachia* can drive the evolution of other mating
509 behaviours in a fully infected population, as well as in uninfected hosts in a polymorphic
510 population. This suggests that *Wolbachia* has the potential to affect the evolution of host
511 reproductive strategies more broadly and could accelerate divergence between allopatric
512 populations.

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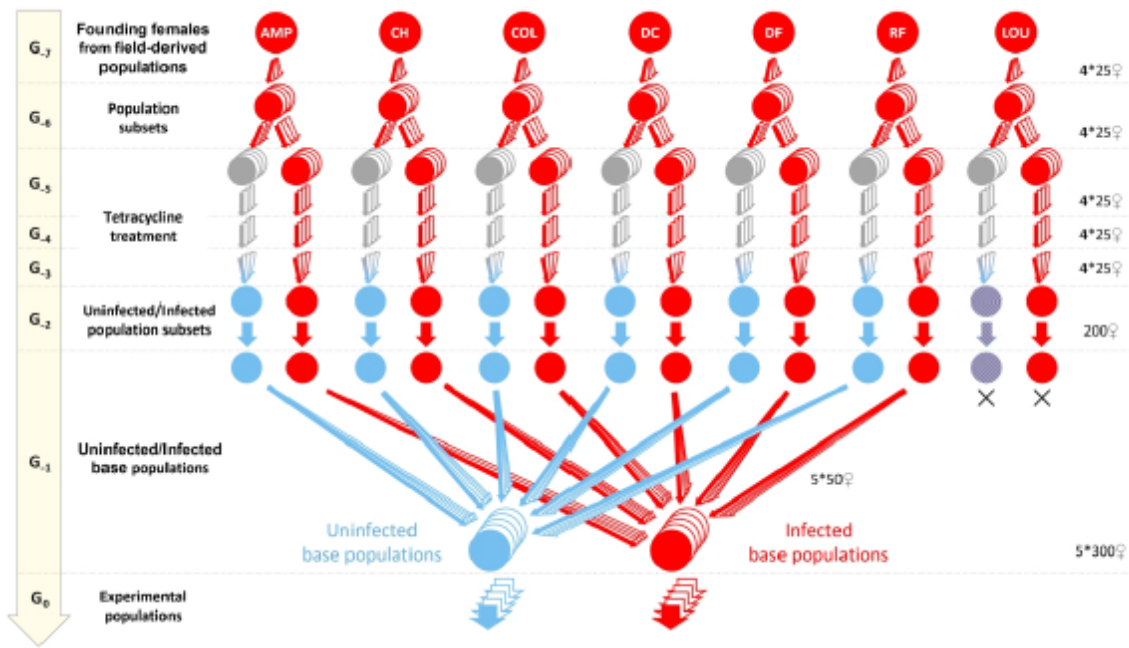
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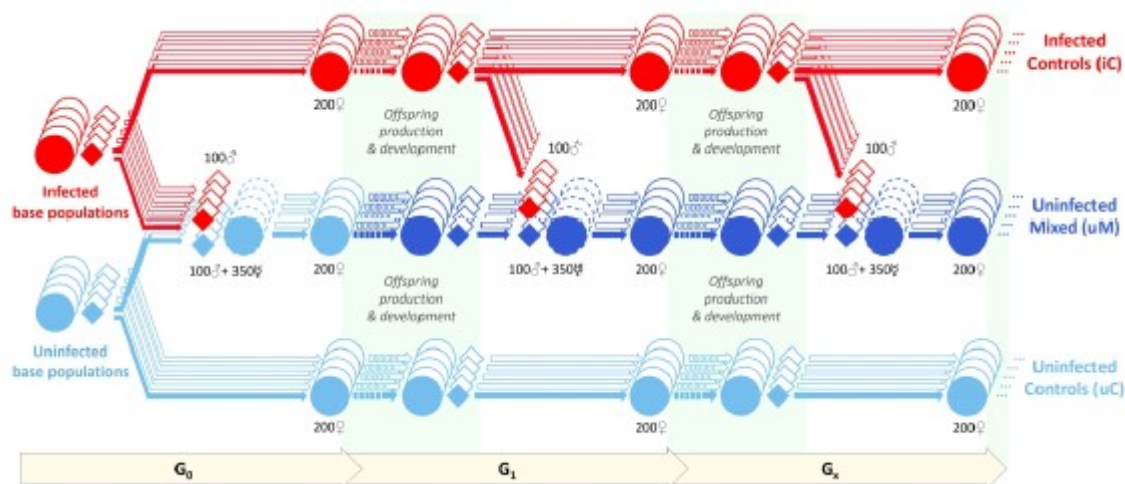
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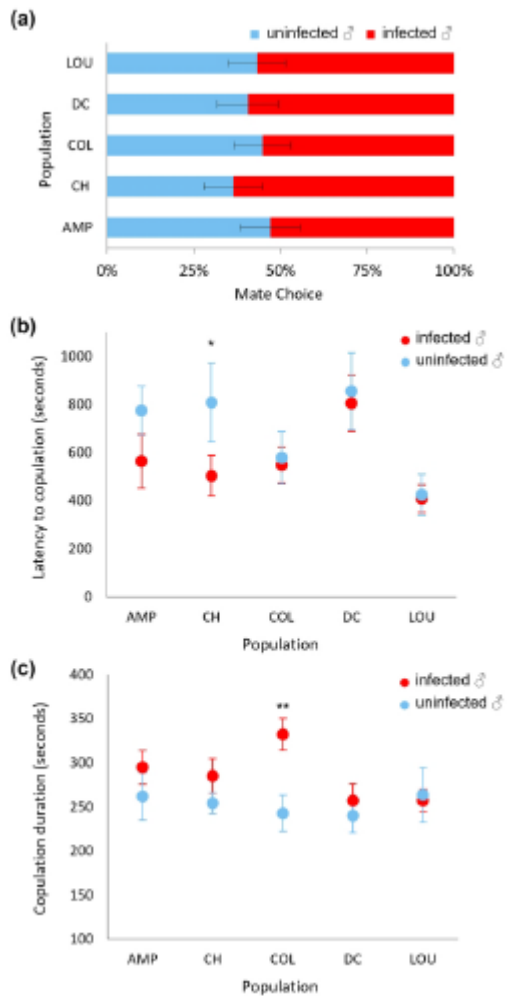
746
 747 **Figure 1. Creation of *Wolbachia*-infected and -uninfected base populations.** Two subsets
 748 established from the same founding individuals ($n=100$ females, distributed among 4 leaf patches with
 749 25 females each at G_{-7}) of each field-derived population were created at G_{-6} . One of the subsets was
 750 treated with tetracycline hydrochloride during three generations (G_{-6} to G_{-3}), followed by three
 751 generations without antibiotics (G_{-3} to G_0) before the establishment of populations for experimental
 752 evolution at G_0 . At G_{-2} the 4 leaf patches of each subset were merged. At G_{-1} , the infection status of
 753 each population subset was confirmed by PCR, then 5 uninfected and 5 infected base populations
 754 were created by mixing 5 times independently 50 females from each of the treated and untreated field-
 755 derived population subsets, respectively. All population subsets were used, except those derived from
 756 the population LOU due to uncertain *Wolbachia* infection status, hence a total of 250 females were
 757 added to each base population. Each arrow corresponds to the transfer of adult mated females to found
 758 a new generation. Generations before starting the experimental evolution (from G_{-7} to G_0) and each
 759 step of the procedure are provided on the left side of the figure and the numbers of females transferred
 760 per population and/or treatment at each generation are provided on the right side of the figure. Red

761 fill: *Wolbachia*-infected mites; Grey fill: mites being treated with tetracycline; Blue fill: uninfected
 762 mites; Blue/red dashed fill: ambiguous PCR results for *Wolbachia* infection.

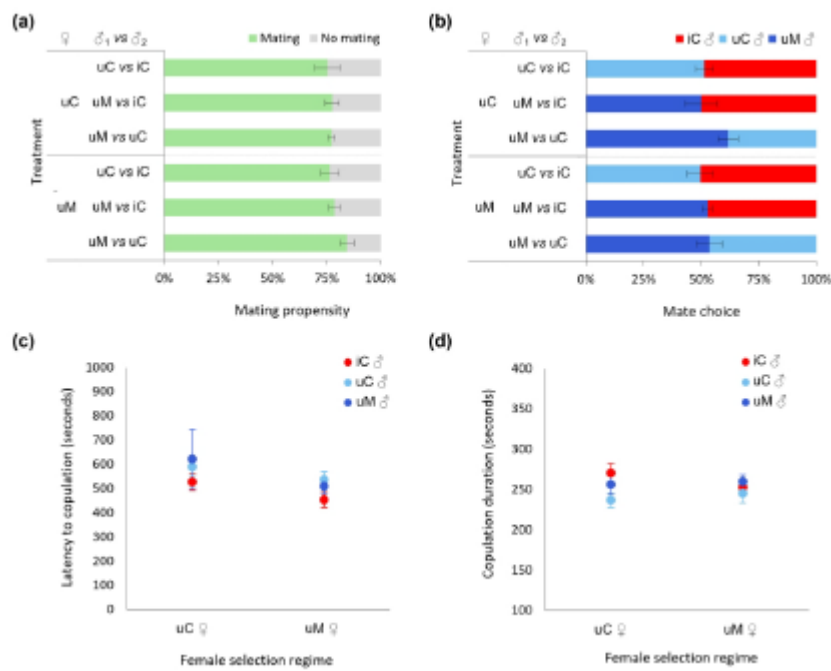


763

764 **Figure 2. Procedure used for experimental evolution of spider mites exposed or not to**
 765 ***Wolbachia*-induced cytoplasmic incompatibility.** In the uninfected mixed regime, the infection
 766 status of males mating with uninfected females was controlled at each generation (1:1 ratio), while in
 767 the two control regimes matings occurred before transferring adult females into the next generation's
 768 box. The entire procedure was repeated in 5 independent replicates. White background and solid
 769 arrows: experimental transfers; Green shaded background and dashed arrows: offspring production
 770 and development. Circles: females; orange: males; Solid-lined symbols: mated females (♀) and
 771 males (♂); Dashed-lined symbols: virgin females (♀); Red fill: *Wolbachia*-infected control (iC); Blue
 772 fill: *Wolbachia*-uninfected females mixed with infected and uninfected males (uM); Light blue
 773 fill: uninfected control (uC).



774
775 **Figure 3. Mating behaviour of *Wolbachia*-uninfected females exposed to *Wolbachia*-infected and**
776 **-uninfected males in five field-derived populations of *T. urticae*.** (a) Mate choice: Bars represent
777 mean (\pm s.e.) proportion of *Wolbachia*-infected males (red bars) or uninfected males (light blue bars)
778 chosen by *Wolbachia*-uninfected females. (b) Latency to copulation and (c) Copulation duration:
779 Circles represent mean (\pm s.e.) time (in seconds) for *Wolbachia*-infected males (red circles) or
780 uninfected males (light blue circles).



781
782 **Figure 4. Mating behaviour of *Wolbachia*-uninfected females exposed to males from different**
783 **experimental evolution regimes.** *Wolbachia*-uninfected females from the control and the mixed
784 regimes (uC and uM, respectively) were given the choice between two males from the three different
785 regimes: uninfected control (uC), *Wolbachia*-infected control (iC), or uninfected mixed (uM). (a)
786 Mating propensity: bars represent mean (\pm s.e.) percentage of trials where mating occurred (green
787 bars) or not (grey bars) within the time of the observation. (b) Mate choice: bars represent mean (\pm
788 s.e.) proportion of females choosing males from the uninfected control (uC: light blue bars), infected
789 control (iC: red bars), or uninfected mixed (uM: dark blue bars) regimes. (c) Latency to copulation
790 and (d) Copulation duration: Circles represent mean (\pm s.e.) time (in seconds) for males from the
791 uninfected control (uC: light blue circles), infected control (iC: red circles), or uninfected mixed (uM:
792 dark blue circles) regimes.

800 **Table 1. Effect size for *Wolbachia* infection in males on mating behaviour in the field-**
 801 **derived populations.** For each response variable, regression coefficients (β), standard errors
 802 (SE), z ratios and P-values associated to the difference with a 50-50 choice between
 803 *Wolbachia*-infected and uninfected males (*i.e.*, intercept forced at zero for mate choice), or to
 804 the effect of *Wolbachia* infection in males on latency to copulation and copulation duration,
 805 were obtained from Z-tests performed on the models described in Table S4. Significant
 806 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

808
 809
 810 **Table 2. Statistical results for the effect of female and male selection regimes, and their**
 811 **interaction, on mating behaviour in the evolved populations.** For each response variable,
 812 Chi-square (χ^2) and P-values were obtained before deletion of the explanatory variable from
 813 the minimal models (cf. Table S4). Significant effects are displayed in bold. females:
 814 selection regime of the female; males: selection regime of the two males introduced in the
 815 arena with each tested female; chosen: selection regime of the male with which the female
 816 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
	Females* chosen	2	0.14	0.93
Latency to copulation	Females	1	1.84	0.18
	Chosen	2	4.48	0.11
	Females* chosen	2	2.73	0.26
Copulation duration	Females	1	0.16	0.69
	Chosen	2	9.17	0.01