

1 ***Wolbachia* induce cytoplasmic incompatibility and affect mate**
2 **preference in *Habrobracon hebetor* to increase the chance of its**
3 **transmission to the next generation**

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24 **Abstract**

25 *Wolbachia* are common intracellular bacteria that are generally found in arthropods
26 including a high proportion of insects and also some nematodes. This intracellular
27 symbiont can affect sex ratio with a variety of reproductive anomalies in the host,
28 including cytoplasmic incompatibility (CI) in haplodiploides. In this study, we questioned
29 if the parasitoid wasp, *Habrobracon hebetor* (Hym.: Braconidae), which is one of the
30 most important biological control agents of many lepidopteran larvae, is infected with
31 *Wolbachia*. To test this, DNA was extracted from adult insects and subjected to PCR
32 using specific primers to *Wolbachia* target genes. The results showed high rate of
33 *Wolbachia* infection in this parasitoid wasp. To find out the biological function of
34 *Wolbachia* in *H. hebetor*, we removed this bacterium from the wasps using antibiotic
35 treatment (cured wasps). Results of the crossing experiments revealed that *Wolbachia*
36 induced CI in *H. hebetor* in which cured females crossed with infected males produced
37 only males, while in the progeny of other crosses, both males and females were
38 observed. Also, our result showed that the presence of *Wolbachia* in the females
39 increased fecundity and female offspring of this parasitoid wasp. However, the presence
40 of *Wolbachia* in the males had no significant effect on the fecundity and female
41 production, but might have incurred costs. We also investigated the effect of *Wolbachia*
42 on mate choice and found that *Wolbachia* affects mating behavior of *H. hebetor*.
43 Together, we show that *Wolbachia* induce CI in *H. hebetor* and affect host mating
44 behavior in favor of its transmission. *Wolbachia* utilize these strategies to increase the
45 frequency of infected females in the host population.

46 **Keywords:** Cytoplasmic incompatibility, *Habrobracon hebetor*, mating behaviour,
47 *Wolbachia*,

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50 **1. Introduction**

51 *Wolbachia* are endosymbiotic bacteria that infect more than 52% of arthropod species
52 and also some nematodes. This intracellular symbiont is among the facultative
53 endosymbionts and can be transferred to the next generation by vertical transmission.
54 Utilizing different strategies to expand host range is an exceptional feature of *Wolbachia*;
55 altering sex ratio with a variety of reproductive anomalies in the host (LePage and
56 Bordenstein, 2013), protection from replication of a variety of pathogens (Hedges et al.,
57 2008; Pan et al., 2017; Yixin et al., 2013), alteration in behavior (Miller et al., 2010;
58 Peng et al., 2008; Rohrscheib et al., 2015; Vala et al., 2004) and some metabolic
59 pathway (Brownlie et al., 2009; Hosokawa et al., 2010) are some of *Wolbachia*' effects
60 on the host.

61 *Wolbachia* can alter reproductive system via male-killing (Hurst et al., 1999; Jiggins et
62 al., 1998), feminization in isopods (Rousset et al., 1992), parthenogenesis induction (PI)
63 in haplodiploid species in which unfertilized eggs become females (Stouthamer et al.,
64 1990) and importantly cytoplasmic incompatibility (CI) (Yen and Barr, 1971). CI is
65 commonly expressed when *Wolbachia*-infected males mate with uninfected females
66 (Unidirectional CI) and also occurs in matings between infected individuals harboring
67 different strains of *Wolbachia* (Bidirectional CI) (Hunter et al., 2003; O'Neill and Karr,
68 1990; Perrot-Minnot et al., 1996b). In unidirectional CI crosses, paternal chromatin fails

69 to condense properly in the first cell cycle and line on the metaphase plate during the
70 first mitosis. Such a cross results in the death of the embryo (Beckmann et al., 2017).
71 This strategy can be used in the mass production and release of incompatible male
72 insects to control wild populations of disease vectors such as the mosquito *Culex*
73 *pipiens* (Laven, 1967) and of agricultural pests such as *Liriomyza trifolii* (Tagami et al.,
74 2006).

75 It has been reported that *Wolbachia* may have positive effects on the fitness of host. For
76 example, it was shown that *Wolbachia* increased fecundity in the parasitoid wasp
77 *Trichogramma bourarachae* (Vavre et al., 1999). It has also been reported that
78 *Wolbachia* infection positively affects the life history and reproductive traits of
79 *Callosobruchus chinensis* males and females (Okayama et al., 2016). Given that
80 parasitoid wasps play an important role in the biological control of insect pests, the
81 presence of *Wolbachia* can affect their function. Previous studies have reported the
82 effect of *Wolbachia* on reproduction of some parasitoid wasps; for example, PI-
83 *Wolbachia* has been reported in *Trichogramma* sp. (Trichogrammatidae) (Stouthamer
84 and Werren, 1993) and *Encarsia* sp. (Aphelinidae) (Stouthamer and Mak, 2002; Wang
85 et al., 2017; Zchori-Fein et al., 1992). Also, in the parasitoid wasp *Asobara tabida*
86 (Braconidae), it has been shown that two strains of *Wolbachia* (i.e. *wAtab1* and *wAtab2*)
87 induce CI, but *wAtab3* strain is required for the host oogenesis (Dedeine et al., 2005).
88 CI-*Wolbachia* has been reported from other wasps such as *Aphytis melinus*
89 (Aphelinidae) (Vasquez et al., 2011) and *Nasonia vitripennis* (Pteromalidae) (Breeuwer
90 and Werren, 1993).

91 *Habrobracon hebetor* (Hym.: Braconidae) is one of the most important biological control
92 agents of many lepidopteran larvae, mainly pyralid moths infesting stored products
93 (Ghimire, 2008). In previous studies, the presence of *Wolbachia* in *H. hebetor* has been
94 reported (Kageyama et al., 2010); however, the effect of *Wolbachia* on this parasitoid
95 wasp has not been investigated yet. Therefore, in this study we questioned whether
96 *Wolbachia* affects the reproduction of *H. hebetor*. Our results indicated the presence of
97 *Wolbachia* in the population of *H. hebetor* collected from Karaj, Iran. We show that
98 *Wolbachia* induce CI in *H. hebetor* and affect the wasp mating behavior in favor of
99 *Wolbachia* transmission.

100

101 **2. Materials and Methods**

102 2.1. Insects

103 The original population of the *H. hebetor* was collected from Karaj, Iran. The wasps
104 were reared under the laboratory condition at 25 ± 5 °C, $60 \pm 5\%$ RH and a photoperiod
105 of 16:8 h and fed daily with diluted raw honey (90% honey and 10% water). The female
106 wasps were presented with fifth instar mill moth (*Ephestia kuehniella*) larvae for
107 oviposition.

108

109 2.2. *Wolbachia* detection

110 Total DNA was extracted from single individuals of *H. hebetor* (n=100) using the
111 previously described procedure (O'Neill et al., 1992). In order to detect *Wolbachia*, the
112 DNA samples were subjected to PCR by using the specific primers targeting *Wolbachia*
113 *wsp* gene (Narita et al., 2007). The PCR reactions were conducted under a temperature

114 profile of 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 sec, 56 °C for 30 sec
115 and 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR products were
116 subjected to agarose (1%) gel electrophoresis. Moreover, to compare *Wolbachia*
117 density, DNA was extracted from each developmental stage of the wasp including the
118 egg, larva (1st, 2d, 3th and 4th instar), pupa (3-, 5- and 7-days old), and adult (1-, 15-,
119 20-days-old males; and 1-, 15-, 20-, 25-, 30-days-old females) as described above.
120 Concentrations of the DNA samples were measured using a Epoch instrument (BioTek),
121 and 10 ng from each genomic DNA sample was used for quantitative PCR (qPCR)
122 using SYBR Green Mix without ROX (Ampliqon) with a Mic real time PCR (BMS) under
123 the following conditions: 95 °C for 15 min, followed by 45 cycles of 95 °C for 10 sec, 30
124 sec at the annealing temperature, and 72 °C for 30 sec, followed by the melting curve
125 (72–95 °C). Specific primers targeting *Wolbachia ftsZ* gene (Kruse et al., 2017) and the
126 insect *18s rRNA* gene as a reference gene (Karamipour et al., 2016) were used (Table
127 1). Reactions from three biological replicates were repeated three times.

128

129 2.3. Antibiotic treatments

130 To find out the biological function of *Wolbachia* in *H. hebetor*, four isolines (i.e. two
131 *Wolbachia*-infected (W^+) and two uninfected or cured (W^-)) were generated using
132 tetracycline treatment (0.2%, w/v with diluted raw honey) for seven days. Thereafter, the
133 wasps were allowed to lay eggs on the host, mill moth larvae. Tetracycline treatment
134 was continued for three generations (seven days treatment of young adults per
135 generation), and the number of 10 wasps were then randomly selected to test for
136 *Wolbachia* infection using qPCR as described above. After successful removal of

137 *Wolbachia* from the wasps, they were reared for five generations. The resultant adult
138 wasps from the 8th generation were considered as the uninfected isolines. The infected
139 isolines were also treated the same; however the antibiotic was not used. The adult
140 wasps from these isolines were used for the cross-mating experiments.

141

142 2.4. Crossing experiments and assessing the role of *Wolbachia* in *H. hebetor*

143 For the crossing experiments, we collected 100 pupae from the infected and uninfected
144 isolines and allowed them to emerge. Then, 20 pairs of unmated individuals (virgin)
145 from these isolines were used for the crossing tests as follow: infected female—infected
146 male, infected female—uninfected male, uninfected female—infected male and
147 uninfected female—uninfected male. We counted the number of the resultant eggs, rate
148 of hatching eggs, numbers of larvae, pupae and offspring during three days after
149 crossing. We also estimated the sex ratio of the emerged wasps.

150

151 2.5. Effect of *Wolbachia* on *H. hebetor* mate choice

152 In order to investigate the effect of *Wolbachia* on mate choice, we placed virgin infected
153 or uninfected females individually in 5-cm-diameter cups with two virgin males, one
154 infected and one uninfected (i.e. a virgin infected female and two virgin males; a virgin
155 uninfected female and two virgin males). To differentiate between the males, either
156 infected or uninfected males (equally) were marked with a point on the end of their
157 wings using a black pen. Forty replicates of each combination were conducted and the
158 rates of successful mating were counted for 15 min. In successful mating, a female
159 accepts a male copulation attempt by moving her ovipositor laterally and then remaining

160 immobile. But a female prevents successful mating by walking, knocking the male with
161 the hind legs, or bending down the abdomen.

162

163 2.6. Data analysis

164 *Wolbachia* densities in different developmental stages of *H. hebetor* were compared
165 using ANOVA followed by Tukey's multiple comparisons test. *Wolbachia* density in the
166 infected (W^+) and the uninfected (W^-) parasitoid wasps were compared using unpaired
167 *t*-test. Comparisons of the mean number of eggs per female wasp within three days in
168 different crosses were analyzed with Two-way ANOVA followed by Tukey's (HSD) test.
169 The mean number of eggs, the rate of hatching eggs, and the percentage of pupae
170 formation, the percentage of progeny emergence and the sex-ratio of progeny
171 emergence per female in different crosses were compared using Mann-Whitney *U*-test.
172 The percentage of mating was analyzed using Kruskal-Wallis test. Statistical analyses
173 were performed using the software SPSS Statistics 17.0 and the graphs were created
174 by using Graph Pad Prism.

175

176 3. Results

177 3.1. *Wolbachia* are persistently present during development of *H. hebetor*

178 To detect and estimate *Wolbachia* infection rate, DNA was extracted from 100
179 individuals (both sexes) of *H. hebetor*. The results showed that all the tested individuals
180 were infected with *Wolbachia* (Fig. 1A). Also, to estimate the relative density of
181 *Wolbachia* in different developmental stages of *H. hebetor*, DNA samples of each
182 developmental stage (i.e. egg, larva, pupa, and adult) were screened by qPCR. We

183 found that *Wolbachia* was present in all the developmental stages of the parasitoid
184 wasp and its density in the eggs and adults were higher than that of the other stages
185 (Fig. 1B; $F_{9, 20} = 70.86$, $P < 0.0001$). The density of *Wolbachia* in different larval and
186 pupal stages showed no significant difference. Also, the result showed that *Wolbachia*
187 density increased along with the increase in the age of females ($F_{4, 10} = 556.3$, $P <$
188 0.0001), while *Wolbachia* density did not differ significantly among 1-day, 15-day and
189 20-day-old males (Fig. 1C).

190

191 3.2. *Wolbachia* induce cytoplasmic incompatibility in *H. hebetor*

192 To investigate the effect of *Wolbachia* infection on the reproduction and fitness of *H.*
193 *hebetor*, we created uninfected *H. hebetor* isolines by treating the wasps with
194 tetracycline. Following confirmation of removal of *Wolbachia* from tetracycline-treated
195 wasps (Fig. 2A), we tested if the antibiotic treatments affected mitochondria density
196 (Ballard and Melvin, 2007) and microbiota population (Karamipour et al., 2016) in the
197 treated wasps and found no significant differences with the control wasps (Fig. S1).
198 Then, we created four different crosses and analyzed different parameters associated
199 with reproduction of the wasp. The results of the crosses showed that all the crosses
200 produced both males and females progeny, except when infected males were crossed
201 with the uninfected females which resulted in only male progeny (Fig. 2B). It is worth
202 mentioning that in hymenopterans, sex determination is based on haplodiploidy system.
203 In other words, males are haploid and females are diploid. Therefore, CI only appears in
204 the female progeny (Breeuwer and Werren, 1993; Perrot-Minnot et al., 1996a). Our
205 results also suggest that *Wolbachia* induced CI in the parasitoid wasp, *H. hebetor*.

206 We also compared the number of resultant eggs of the crosses during three days
207 oviposition. The mean number of eggs within three days per female in infected female-
208 infected male and infected female-uninfected male crosses was significantly higher than
209 those of the other two crosses (Fig. 3A). In addition, the rate of egg hatching in
210 uninfected female-infected male cross was significantly lower than that of other crosses
211 (Fig. 3B). The rates of pupa formation were not significantly different in different crosses
212 (Fig. 3C), while the progeny emergence rate of infected female-uninfected male was
213 significantly higher than that of other crosses (Fig. 3D). Also, the results showed that the
214 presence of *Wolbachia* in both male and female (i.e. infected female—infected male
215 cross) significantly increase the progeny emergence rate compared with the absence of
216 *Wolbachia* (cured female-cured male cross) (Fig. 3D).

217 Moreover, we compared the mean number of eggs per day in each cross. On the first
218 day, the mean number of eggs in infected female-infected male cross was significantly
219 higher than that of the other crosses. On the second day, there was no significant
220 difference among the crosses in term of the mean number of eggs. On the third day,
221 however, the mean number of eggs in infected female-uninfected male cross was
222 significantly higher than that of others (Fig. 4, Table 2). Notably, the highest numbers of
223 progeny females were observed in infected female—infected male and infected
224 female—uninfected male crosses (Fig. 2B).

225

226 3.3. *Wolbachia* affect mate choice in *H. hebetor*

227 In order to investigate the effect of *Wolbachia* on mate choice, we designed a choice
228 mating test. In this test, two infected and uninfected males were exposed to either an

229 infected or an uninfected female. The results showed that the infected females mated at
230 a higher rate with infected males compared to the uninfected males (K-W test, $\chi^2= 5.00$,
231 $P = 0.03$). However, there was no difference between mate choices of uninfected
232 females (K-W test, $\chi^2= 0.8$, $P = 0.3$) (Fig. 5).

233

234 **4. Discussion**

235 *Wolbachia* have evolved several mechanisms of host reproductive manipulations to
236 increase their worldwide prevalence, including feminization, parthenogenesis, male-
237 killing, and cytoplasmic incompatibility (CI). These phenotypes, except CI, serve to
238 increase the frequency of *Wolbachia*-infected females, the transmitting sex of the
239 bacteria, in a host population as males are an evolutionary dead end for *Wolbachia*
240 (Hurst and Frost, 2015; Serbus et al., 2008; Werren et al., 2008). The presence
241 *Wolbachia* has been reported in many insects. Given that *Wolbachia* manipulate
242 reproductive system of their insect hosts, they have good potential in biological control,
243 either to control insect pests or to increase the population of biological control agents
244 and improve their functions.

245 Here, we investigated the effect of *Wolbachia* on the reproductive biology of the
246 parasitoid wasp *H. hebetor* which is one of the important biological control agents of
247 many lepidopteran larvae. We detected *Wolbachia* from all the field-collected individuals
248 of *H. hebetor* and the prevalence rate of infection was 100%. High rate of *Wolbachia*
249 infection in natural population of *H. hebetor* could be explained by fitness benefit that
250 *Wolbachia* confers to the wasp. Also, we estimated relative density of *Wolbachia* in
251 different developmental stages of *H. hebetor* and found that *Wolbachia* densities in the

252 eggs and adult females were higher than other stages of the parasitoid wasps. These
253 results indicate efficient vertical transmission of *Wolbachia* in this parasitoid wasp.
254 Developmental-specific densities of *Wolbachia* have also been reported in other insects
255 such as *Tribolium confusum* (Ming et al., 2015) and *Brontispa longissima* (Ali et al.,
256 2018) with higher levels in eggs and adults than other stages. *Wolbachia* density in
257 *Diaphorina citri* (Dossi et al., 2014) and *Drosophila melanogaster* (Goto et al., 2006)
258 was shown to increase as development proceeds. Considering our results and those
259 reported from other studies, it can be hypothesized that *Wolbachia* may affect or be
260 regulated throughout the host development. Moreover, high rate of *Wolbachia*
261 transmission might be achieved by investing high population of the bacteria within the
262 host egg.

263 In addition, we investigated the effect of ageing on the *Wolbachia* density in the adult
264 males and females. The result showed that *Wolbachia* density increased in aged
265 females that is in congruence to the study that showed *Wolbachia* density increase with
266 aging in *Laodelphax striatellus* females (Noda et al., 2001). We found no change in
267 *Wolbachia* density over time in the males. Similar to our results, it has been shown that
268 *Wolbachia* density in the males of *Tribolium confusum* remains unchanged over time
269 (Ming et al., 2015). *Wolbachia* are positively associated with CI strength (Bourtzis et al.,
270 1996; LePage and Bordenstein, 2013; Sinkins et al., 1995). Reduction in *Wolbachia*
271 density during aging in males has been suggested to be associated with reduction in the
272 ability to cause CI. This phenomenon has been reported in *Sogatella furcifera* males
273 (Noda et al., 2001) and *Aedes albopictus* males infected with *wAlbA* strain of
274 *Wolbachia* (Tortosa et al., 2010). Therefore, based on our results, it could be

275 hypothesized that *Wolbachia*-induced CI increase over time in the female wasps, while
276 it remains almost constant in the males. Males suffer from fertility reduction in CI
277 crosses, therefore by removing/reducing the infection during development they may
278 suppress the expression of CI (Tortosa et al., 2010). In the present study, the density of
279 *Wolbachia* in 1-day, 15-day and 20-day-old males was almost the same, and it can be
280 concluded that males prevent *Wolbachia* growth to suppress CI. However, density of
281 *Wolbachia* increases in females with aging, with 30-day-olds having the highest
282 bacterial density.

283 Given the high rate of *Wolbachia* infection and their presence during the wasps'
284 development, we next examined the functional role of *Wolbachia* in this insect. To
285 assess the effect of *Wolbachia* infection on the reproduction of *H. hebetor*, we designed
286 four different crosses and found that the infected males crossed with uninfected females
287 resulted in only male progeny (i.e. incompatible cross). The rates of eggs hatching,
288 pupa formation and adult emergence of this cross were also lower than that of others.
289 Two types of CI have been suggested in haplodiploids: female mortality or conversion to
290 males (Bordenstein et al., 2003). Given low hatching rate and all male progeny in the
291 incompatible cross of our experiments, the mortality type of CI is suggested for *H.*
292 *hebetor*. Female mortality has been suggested as the normal and most common type of
293 CI in haplodiploid insects including parasitoid wasps such as *Nasonia* (Perrot-Minnot et
294 al., 1996a), *Leptopilina* (Vavre et al., 2000) and *Trichopria* (Vavre et al., 2002), whereas
295 female conversion to males is an exception (Bordenstein et al., 2003).

296 Our data showed that the presence of *Wolbachia* in the female wasps improved their
297 fecundity. The number of eggs laid by the infected female wasps was significantly

298 higher in the compatible crosses (i.e. infected female-infected male cross and infected
299 female-uninfected male cross) compared with the others. Consistent to these results, it
300 has been reported that *Wolbachia*-infection improves fecundity in *Aedes albopictus*
301 (Dobson et al., 2004), *Trichogramma* (Vavre et al., 1999), *D. melanogaster* (Fry et al.,
302 2004) and *Tetranychus urticae* (Zhao et al., 2013). Higher fecundity of the infected
303 females would increase infected individuals thereby accelerate *Wolbachia* distribution
304 within the host population.

305 Moreover, our data showed that the compatible crosses transmitting *Wolbachia* to the
306 next generation had higher reproductive fitness compared to the others. The rates of
307 egg hatching and progeny emergence in the infected female-uninfected male cross
308 were significantly higher than those in infected female-infected male cross. In addition,
309 the number of resultant females from infected female-uninfected male cross was similar
310 to those produced from infected female—infected male cross. These data suggest that
311 the presence of *Wolbachia* in the males have no significant effect on fecundity, fertility
312 and number of female progeny when female is also infected with the same *Wolbachia*.
313 Therefore, in the compatible crosses transmitting *Wolbachia*, female infection matters
314 regardless of male infection.

315 Considering the reproductive fitness that *Wolbachia* impose on *H. hebetor*, we
316 questioned whether that affects mate choice of the female wasp. Our data showed that
317 infected females chose infected males more frequently than uninfected males.
318 However, there was no difference in male mate choice of the uninfected females. These
319 data suggest that *Wolbachia* infection may modify the mate choice of this wasp. In
320 congruence to these results, it has been reported that in the two-spotted mite,

321 *Tetranychus urticae*, *Wolbachia*-infected females mostly choose infected males to
322 decrease the chance of CI occurring (Vala et al., 2004). Also, it has been shown that
323 *Wolbachia* affect reproduction preference and reproduction isolation among different
324 populations of *D. melanogaster*. This observation may strengthen the hypothesis that
325 infected females are more attractive to males than their uninfected counterparts. This
326 active bias could be due to differences in pheromone compounds of infected and
327 uninfected females that attracts the infected males more than the uninfected males.

328

329 **5. Conclusion**

330 In this study we showed that *Wolbachia* were persistently present during development
331 of the parasitoid wasp, *H. hebetor* highlighting efficient vertical transmission of
332 *Wolbachia*. Different densities of *Wolbachia* during development of *H. hebetor* may be
333 due to specific regulatory mechanisms (such as metamorphosis) that allow it to
334 populate the host at certain stages. By elimination of *Wolbachia* from *H. hebetor* and
335 conducting crossing experiments, we confirmed that *Wolbachia* induced CI in this wasp
336 is most probably through killing female embryos in incompatible crosses. Moreover, we
337 reported that *Wolbachia* affects mate choice and fecundity of *H. hebetor* (in compatible
338 crosses); thereby incur direct fitness to the host that transmits it to the next generation.
339 This strategy facilitates *Wolbachia* spread through populations of the host insects.

340

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346

347 **Authors' contributions**

348 M.M. and Z.B. conceived and designed the experiments. Z.B. performed the
349 experiments. Z.B. and M.M. and A.A.T. analyzed the data and prepared the figures and
350 tables. Z.B., S.A. and M.M. wrote the manuscript. All of the authors reviewed the
351 manuscript.

352

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514 **Figure legends**

515 **Fig. 1. Detection of *Wolbachia* and their density fluctuations during development**

516 **of *H. hebetor*.** A: A representative diagnostic PCR of *Wolbachia* in adults of *H. hebetor*

517 collected from Karaj using *wsp* primers. C⁻, negative control lacking DNA sample. B:

518 Quantitative PCR (qPCR) detection of *ftsZ* gene of *Wolbachia* during development of *H.*

519 *hebetor*. C: Effect of aging on *Wolbachia* density in female and male adult of *H. hebetor*.

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521 **Fig. 2. Confirmation of *Wolbachia* removal and the effect of *Wolbachia* on progeny**

522 **sex ratio.** A: Antibiotic treatment resulted in *Wolbachia* elimination in *H. hebetor*. qPCR

523 analysis of *Wolbachia* density in the *Wolbachia* infected (*W*⁺) and the *Wolbachia* cured

524 (*W*⁻) parasitoid wasps. *** P<0.001. B: Sex ratio of the offspring resulted from different

525 crosses during three days oviposition. Each data point represents a total number of
526 female offspring produced per female during three days. Female (F), Male (M),
527 *Wolbachia*-infected (W^+) and *Wolbachia*-cured (W^-). Mann-Whitney *U* test, *** signifies
528 $P < 0.001$.

529

530 **Fig. 3. Comparison of biological parameters of *H. hebetor* in different crosses.** A:
531 Comparison of the mean number of eggs per female. B: The rate of hatching. C: The
532 percentage of pupae formation. D: The percentage progeny emergence in different
533 crosses. Female (F), Male (M), *Wolbachia*-infected (W^+) and *Wolbachia*-cured (W^-).
534 Each data point represents a sum of data obtained per female during three days.
535 Mann-Whitney *U* test, *signifies $P < 0.05$, **signifies $P < 0.01$, *** signifies $P < 0.001$.

536

537 **Fig. 4. Comparison of the mean number of eggs per female in three days in**
538 **different crosses.** Each data point represents a number of eggs per female. Means
539 were compared by Tukey's (HSD) test. Day (D), *Wolbachia* infected (W^+) and *Wolbachia*
540 cured (W^-). *signifies $P < 0.05$, **signifies $P < 0.01$, *** signifies $P < 0.001$, **** signifies
541 $P < 0.0001$

542

543 **Fig. 5. Effect of *Wolbachia* on female mate choice.** Female (F), Male (M), *Wolbachia*
544 infected (W^+) and *Wolbachia*-cured (W^-). Kruskal-Wallis (K-W) test, * signifies $P < 0.05$.

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Table 1. Primers used in this study.

Target gene	Primer	Primer sequence (5' -3')	Fwd./Rev	Annealing temp. (°C)	Ref.
<i>wsp</i>	wsp81F	TGGTCCAATAAGTGATGAAGAAAC	Fwd.	56	(Narita et al., 2007)
	wsp691R	AAAAATTAACGCTACTCCA	Rev.		
18s rRNA	penta-18srRNA-F	CCTGCGGCTTAATTTGACTC	Fwd.	57	(Karamipour et al., 2016)
	penta-18srRNA-R	AACTAAGAACGGCCATGCAC	Rev.		
<i>FtsZ</i>	<i>ftsZ</i> -F(RT)	AGCAGCCAGAGAAGCAAGAG	Fwd.	57	(Kruse et al., 2017)
	<i>ftsZ</i> -R(RT)	TACGTCGCACACCTTCAAAA	Rev.		

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Table 2. Analysis of Variance (ANOVA). The effect of day on number of eggs in different crosses in *H. hebetor*.

	P	Df	F
Day	****	2	34.50
Cross	****	3	9.02
Day × Cross	****	6	7.01

**** signifies $P < 0.0001$.

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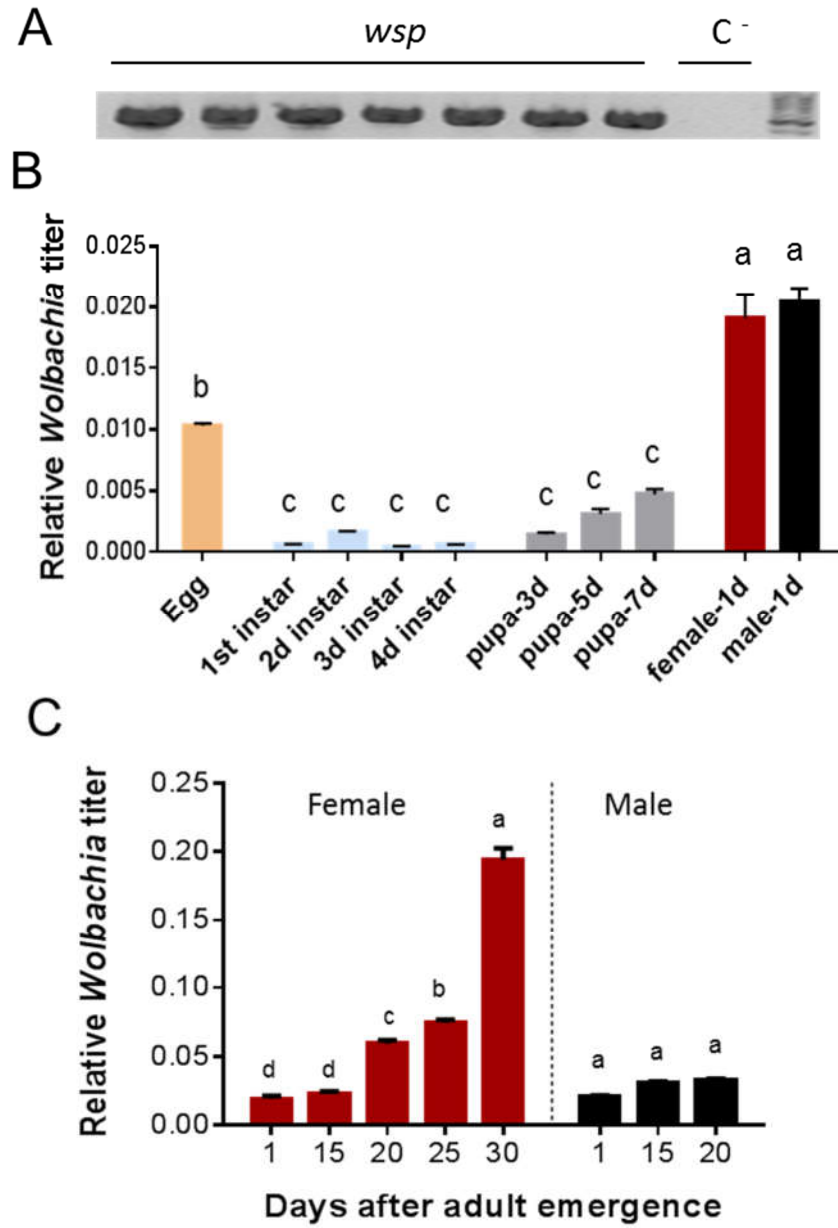
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Fig. 1



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570 *Fig. 2*

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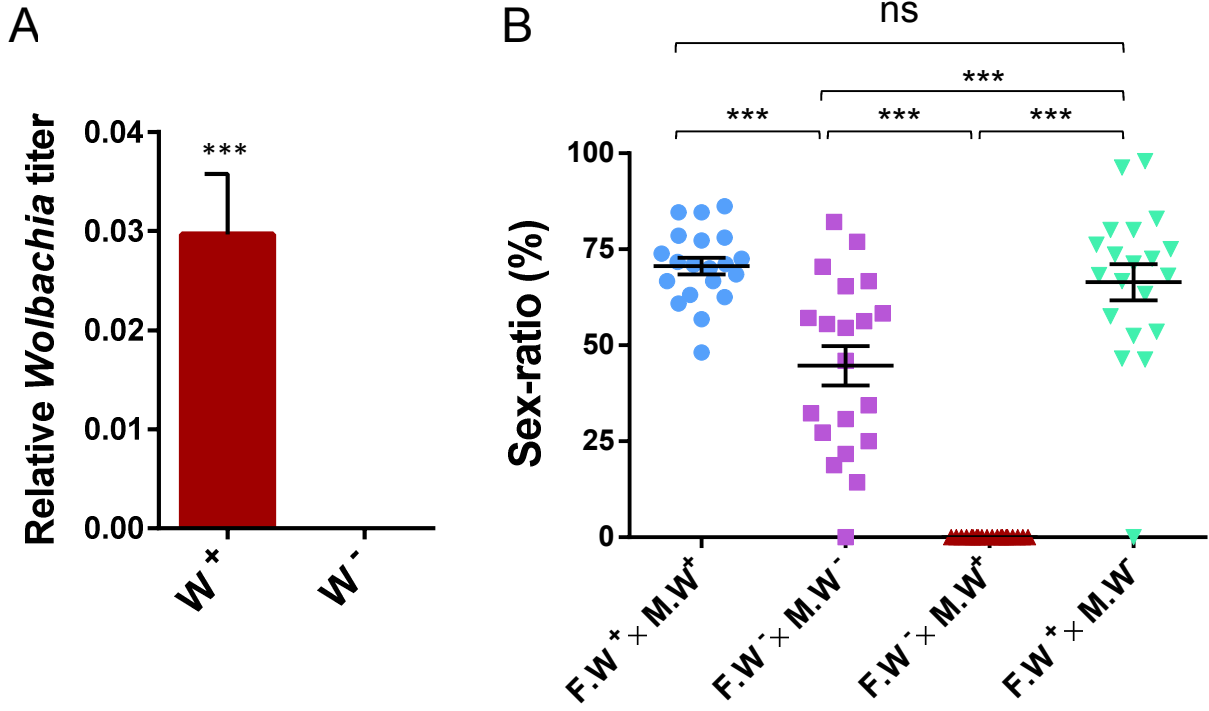
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Fig. 3

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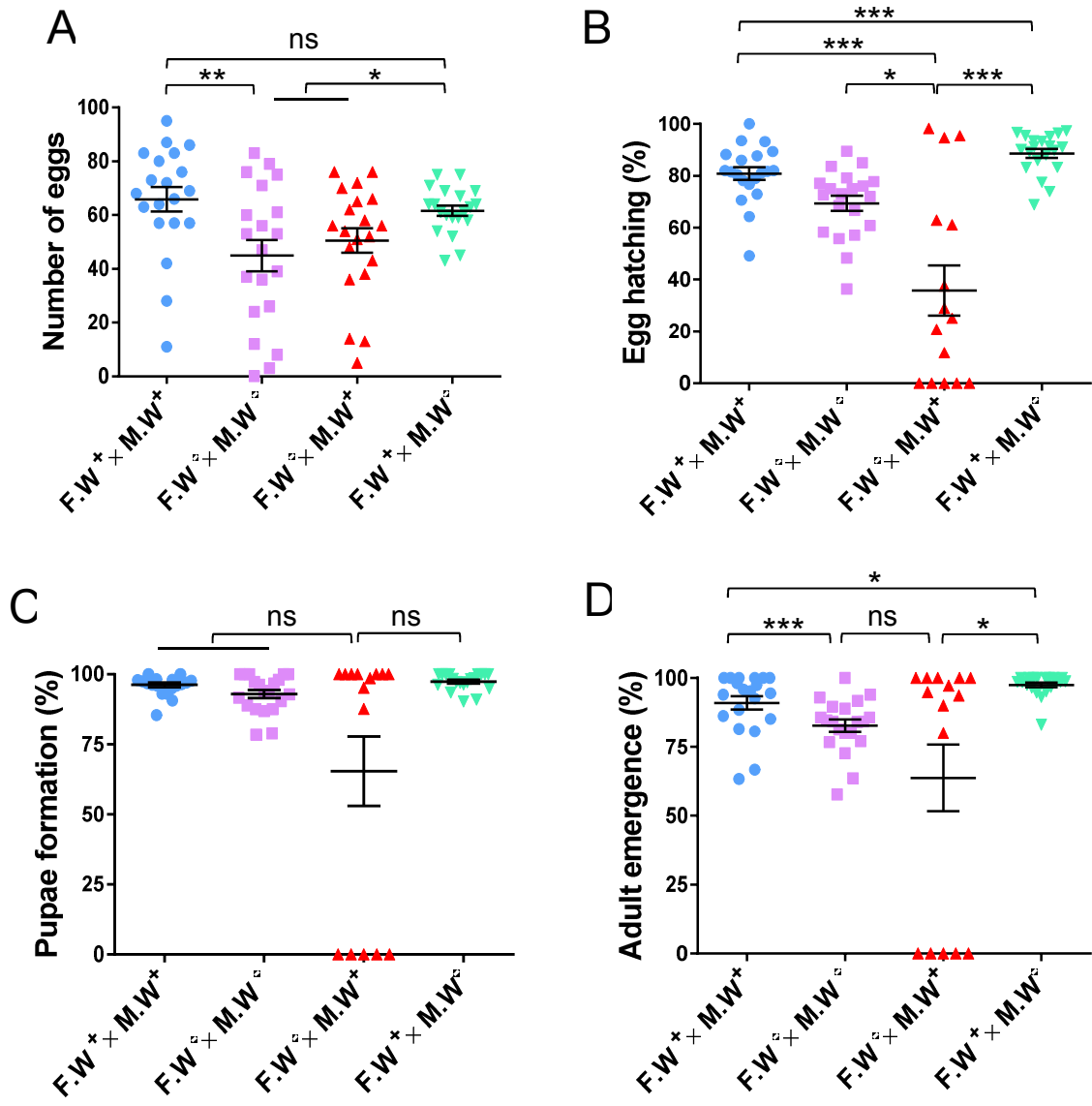
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607 *Fig. 4*

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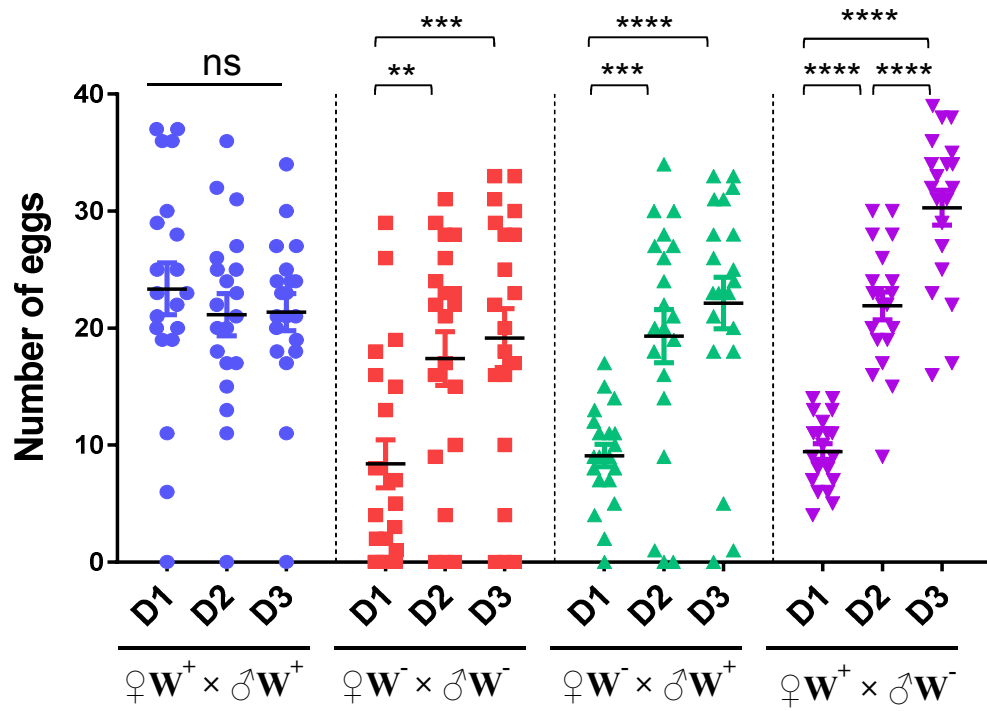
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627 *Fig. 5*

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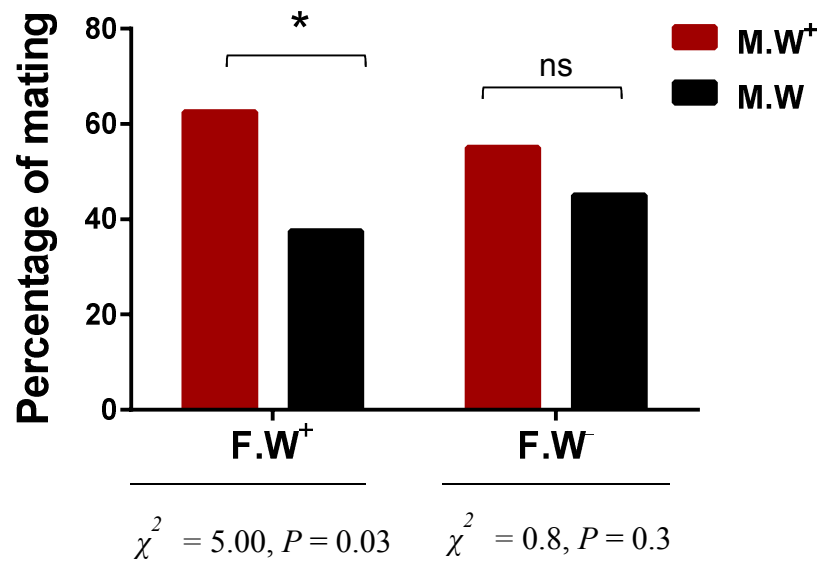
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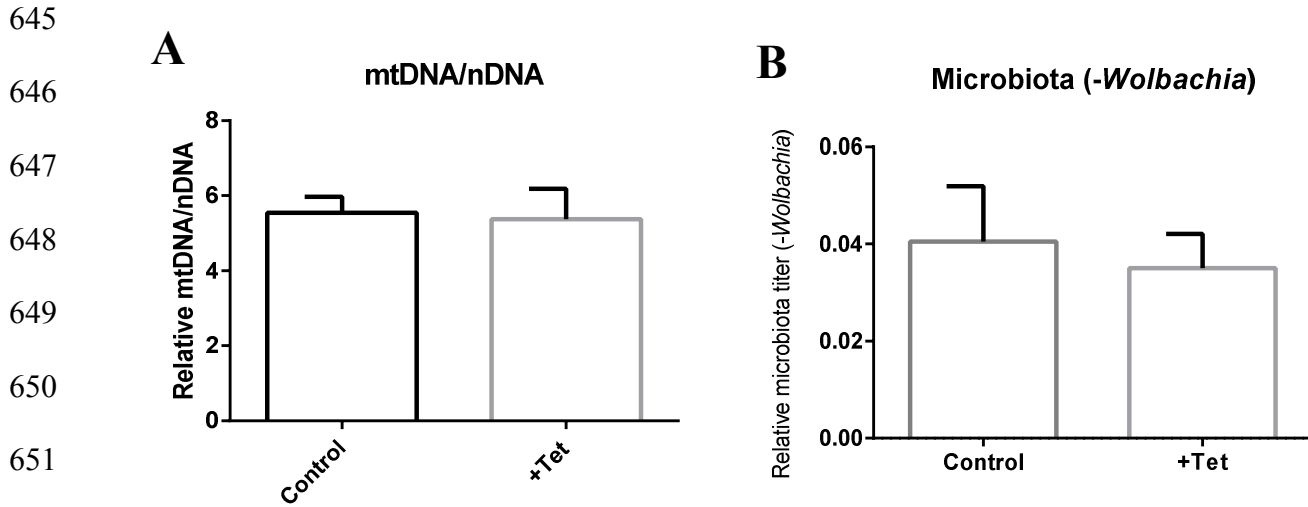
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Fig. S1



655 Fig. S1. Effect of tetracycline treatment on (A) mitochondria density and (B)
656 microbiota population of *H. hebetor* (8th generation after treatment). There was no
657 significant differences between the control and treated wasps.

658