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# ***Wolbachia* increases susceptibility to *Plasmodium* infection in a natural system**

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## **Abstract**

Current views about the impact of *Wolbachia* on *Plasmodium* infections are almost entirely based on data regarding artificially transfected mosquitoes. This work has shown that *Wolbachia* reduces the intensity of *Plasmodium* infections in mosquitoes, raising the exciting possibility of using *Wolbachia* to control or limit the spread of malaria. Whether natural *Wolbachia* infections have the same parasite-inhibiting properties is not yet clear. *Wolbachia*-mosquito combinations with a long evolutionary history are, however, key for understanding what may happen with *Wolbachia*-transfected mosquitoes after several generations of coevolution. We investigate this issue using an entirely natural mosquito-*Wolbachia*-*Plasmodium* combination. In contrast to most previous studies, which have been centered on the quantification of the midgut stages of *Plasmodium*, we obtain a measurement of parasitaemia that relates directly to transmission by following infections to the salivary gland stages. We show that *Wolbachia* increases the susceptibility of *Culex pipiens* mosquitoes to *Plasmodium relictum*, significantly increasing the prevalence of salivary gland stage infections. This effect is independent of the density of *Wolbachia* in the mosquito. These results suggest that naturally *Wolbachia*-infected mosquitoes may, in fact, be better vectors of malaria than *Wolbachia*-free ones.

**Key words:** symbiont-mediated protection, vectorial competence, infection prevalence, infection intensity, oocysts, sporozoites.

## 1 **Introduction**

2 Individual hosts are often simultaneously infected with more than one parasite species. Co-  
3 infections can impact both host fitness and parasite transmissibility, and can therefore have  
4 important evolutionary and epidemiological consequences [1, 2]. Within a host, parasites may  
5 interact in different ways. They may suppress each other because they are in competition for  
6 a resource in limited supply, such as a particular nutrient or tissue, or because they stimulate  
7 the same branch of the immune system [3]. In the most extreme cases, parasites can excrete  
8 molecules that directly inhibit the growth of competitors [4]. Host sharing may also, however,  
9 facilitate parasite development, most notably when one of the parasites immunosuppresses the  
10 host [2]. Co-infections have been intensely investigated in the biomedical literature, as  
11 several important human infections are known to be complicated by the arrival of secondary  
12 or opportunistic pathogens [3]. More recently, however, a great deal of attention has been  
13 drawn to the impact of co-infections on vector-transmitted diseases with the realization that,  
14 in the field, arthropod vectors are also often infected by multiple parasites [5-7].

15         A few years ago, two seminal papers showed that *Wolbachia*, a maternally transmitted  
16 bacterial endosymbiont of arthropods, protects *Drosophila* flies from several viral infections  
17 [8, 9]. This stimulated a great deal of research into *Wolbachia*-mediated parasite interference  
18 in other insect systems (see Supplementary Materials, Table S1), and raised the exciting  
19 possibility of using *Wolbachia* to control or limit the spread of mosquito-transmitted diseases  
20 such as dengue and malaria. Interestingly, although neither *Aedes aegypti* (vector of the  
21 dengue virus) nor *Anopheles gambiae* or *An. stephensi* (vectors of *Plasmodium falciparum*)  
22 are naturally infected by *Wolbachia*, they can be successfully transfected in the laboratory  
23 using bacteria isolated from other insect species [10-12] although not always stably (in *An*  
24 *gambiae* the infections are somatic and do not transmit vertically to the offspring [13, 14]).  
25 As a consequence, in the last few years, a large number of studies have been conducted using  
26 transfected mosquitoes. These studies have largely confirmed the results obtained in naturally  
27 infected *Drosophila*: transfected *Wolbachia* exhibit considerable pathogen-interference  
28 properties against a wide range of parasite taxa (e.g. [12, 13, 15-17]; see also Table S1). In  
29 contrast, studies of natural *Wolbachia* infections in mosquitoes have been much less  
30 conclusive; some studies have shown no effect of *Wolbachia* on pathogen development [17-  
31 19] while others have shown that *Wolbachia* facilitates [20] or blocks [21] pathogen  
32 replication (see Table S1 for a summary). This raises the question of whether the *Wolbachia*-  
33 mediated parasite protection observed in recently transfected mosquitoes can be maintained

34 across generations. *Wolbachia*-mosquito combinations with a long evolutionary history may  
35 be key for understanding what will happen with *Wolbachia* transfected mosquitoes several  
36 generations down the line if, as has been shown in other systems [22, 23], the novel  
37 *Wolbachia*-mosquito interactions evolve rapidly.

38 Here we investigate whether a natural *Wolbachia* infection interferes or facilitates  
39 *Plasmodium* development in mosquitoes. Previous work on the outcome of *Plasmodium*-  
40 *Wolbachia* coinfections has been carried out using transfected *Wolbachia* and/or mosquito-  
41 *Plasmodium* combinations that work well in the laboratory but do not exist in nature (Table  
42 S1). The results obtained range from an increase [14, 19] to a decrease [12-15] in  
43 *Plasmodium* parasitaemia in the presence of *Wolbachia*, depending on the particular  
44 *Wolbachia*-mosquito-*Plasmodium* combination used. Results from artificial mosquito-  
45 *Plasmodium* combinations are particularly difficult to interpret because there is growing  
46 evidence that they do not behave in the same way as natural combinations [24, 25]. One  
47 intriguing example from the *Wolbachia* literature is that of the human malaria vector, *An.*  
48 *gambiae*, transfected with the wAlbB strain of *Wolbachia*. This strain of *Wolbachia* decreases  
49 parasitaemia when mosquitoes are infected with a human (*Plasmodium falciparum*) malaria  
50 parasite [13], but has the opposite effect when mosquitoes are infected with a rodent (*P.*  
51 *berghei*) malaria parasite [14]. The reasons for these contrasting results are not yet known,  
52 but one possibility is that the disparity may be immune-mediated, as the natural (*P.*  
53 *falciparum*) and unnatural (*P. berghei*) parasites are controlled by different immune pathways  
54 in *An gambiae* mosquitoes [25].

55 We used an entirely natural system, consisting of the avian malaria parasite *P.*  
56 *relictum*, its natural vector, the mosquito *Cx pipiens*, and its native (wPip) *Wolbachia* strain.  
57 The aim was to establish whether the infection with *Wolbachia* decreases the prevalence  
58 and/or intensity of *Plasmodium* infection. In contrast to most previous studies which have  
59 been exclusively centered on the quantification of oocysts in the midgut of mosquitoes 7 days  
60 after the infection (but see [12]), we aimed to obtain a measurement of parasitaemia that  
61 would relate more directly to transmission by following the infections all the way to day 14,  
62 when the sporozoites have infected the salivary glands of the female. Indeed the  
63 epidemiological significance of having more or less oocysts in the gut remains to be  
64 demonstrated: a single oocyst produces thousands of sporozoites, but as few as ten of these  
65 sporozoites suffice to initiate a new infection in a host [26]. Thus, despite earlier studies  
66 showing a difference in *Plasmodium* oocystaemia in *Wolbachia*-infected mosquitoes, the

67 question of whether natural *Wolbachia* infections can interfere with *Plasmodium* transmission  
68 in mosquitoes has not been entirely resolved.

69

70

## 71 **Material and methods**

72

### 73 ***Mosquito lines***

74 We used two isogenic lines of *Cx. pipiens quinquefasciatus* that share the same nuclear  
75 genome but differ in their *Wolbachia* infection. The first line ( $w^{SL}$ ) is naturally infected by  
76 the *Wolbachia* wPip(SI) strain. The second line ( $w^{(-)}$ ) was generated by antibiotic treatment of  
77  $w^{SL}$  larvae to eliminate the *Wolbachia* infection (see [27] for details of the lines). The  $w^{(-)}$   
78 was reared for ca. 30 generations before the experiment to eliminate side effects of the  
79 tetracycline. Both lines,  $w^{SL}$  and  $w^{(-)}$  were reared throughout under identical conditions.  
80 Newly hatched (L1) larvae from these two different lines were placed in plastic trays (34 x 23  
81 x 7 cm) filled with 1L of water at a constant density of 300 larvae per tray ( $n = 10$  trays per  
82 line). The experiment took place under standard temperature ( $24 \pm 2^\circ\text{C}$ ), humidity ( $65 \pm 5\%$ )  
83 and photoperiod (12L:12D) conditions. Larvae were fed *ad libitum* on brewer's yeast on the  
84 first day, and thereafter on ground Tetramin<sup>®</sup> fish flakes. On day 7 post hatching, each plastic  
85 tray was individually placed inside an “emergence cage” (40 x 28 x 31 cm) and emerged  
86 adults were allowed to feed *ad libitum* on a 10% glucose water solution.

87

### 88 ***Plasmodium strain and bird infections***

89 We used a lineage of *P. relictum* known as SGS1. It is the most prevalent avian malaria  
90 lineage in Europe, both in wild Passeriformes birds and in *Cx. pipiens* mosquitoes (MalAvi  
91 database ; see [28]). The strain used in the experiment was isolated from wild sparrows and  
92 has been since maintained in our animal house by carrying out regular passages between our  
93 stock canaries every ca. 3 weeks [29]. Experimental canaries ( $n = 6$ ) were haphazardly  
94 allocated to one of two treatments: half of them were experimentally infected with our SGS1  
95 *Plasmodium* lineage (“infected cages”), the other half were left as uninfected controls  
96 (“control cages”). Experimental infections took place by intraperitoneal injection of ca. 50-  
97 100  $\mu\text{l}$  of blood from our infected canary stock, and mosquito blood feeding took place 10  
98 days after the infection, to coincide with the acute phase of the parasitaemia [29].

99

## 100 ***Mosquito experimental infections and dissections***

101 To estimate *Plasmodium* burden and *Wolbachia* density simultaneously, groups of 90 adult  
102 *Cx. pipiens* females (8-10 days old) from each line ( $w^{SL}$  and  $w^{(-)}$ ) were haphazardly chosen  
103 from the different emergence cages and placed together to feed overnight inside an  
104 experimental cage (n=3 infected cages, n=3 control cages). After the blood meal, the birds  
105 were taken out and all the cages were supplied with *ad libitum* glucose water until the end of  
106 the experiment. Mosquitoes that had not taken a blood meal (less than 8%) were removed  
107 from the cages. To simplify the identification of the strains, three days before the blood meal  
108 the mosquitoes were marked using a small amount (1 $\mu$ g/female) of either pink or blue  
109 fluorescent powder (RadGlo<sup>®</sup> JST) applied as a dust storm. Preliminary trials have shown  
110 that at this concentration the dust has no effect on mosquito survival or parasite burden [27].  
111 The two colours were used in rotation to mark the two strains so that the strain-colour code  
112 was switched from cage to cage.

113 To count oocysts in the mosquito gut, 20 blood-fed females of each line were  
114 haphazardly chosen from each cage 7-8 days post blood meal (dpbm), and dissected under a  
115 binocular microscope in 100 $\mu$ l of 0.01M phosphate-buffered saline (PBS). One wing was also  
116 extracted and measured along its longest axis as an estimate of female size. The dissected  
117 midguts were stained with a 5% mercurochrome solution to assess infection rate (oocysts  
118 present/absent) and oocyst burden (number of oocysts) under a phase contrast microscope.  
119 The dissected abdomens (minus the midguts) were individually frozen at -20°C for the  
120 subsequent *Wolbachia* quantification. A similar procedure was carried out at day 14 pbm,  
121 when the sporozoites have migrated to the salivary glands. At this time, 40 blood-fed females  
122 from each mosquito line were haphazardly sampled from each of the cages. Females were  
123 first dissected to get rid of the midgut (at this stage, all oocysts in the midgut are expected to  
124 have burst), and then the mosquito was severed to separate the thorax (containing the salivary  
125 glands) and the abdomen, both of which were individually frozen at -20°C for the subsequent  
126 quantification of *Plasmodium* and *Wolbachia* infections, respectively.

127

## 128 ***Wolbachia and Plasmodium sporozoite quantification***

129 Real-time quantitative PCR was used to estimate the relative density of *Wolbachia*  
130 (abdomen) and *Plasmodium* sporozoites (thorax) in each mosquito. We carried out two PCRs  
131 on each of the body segments: one was specific for the *Culex ace-2* locus [30], and the other  
132 was either specific for the *Wolbachia wsp* locus [31] or for the mtDNA *cytb* gene of  
133 *Plasmodium*. For the latter we used the primers CytSPO7F (5'-



134 AGTTTCATGGATATGTGGTGGGA-3') and CytSPO10R (5'-  
135 AAAGATTTGGATAGAAGGGTATTT-3'). For each of the genes under study, the 5 $\mu$ L  
136 reaction mixture contained 1 $\mu$ l of template DNA (thorax at 5ng/ $\mu$ l and abdomens at 10ng/ $\mu$ l),  
137 2.5 $\mu$ l of 2X LightCycler DNA Master SYBR Green I (Roche Applied Science), 0.25 $\mu$ l of  
138 primers at 10 $\mu$ M, and 1 $\mu$ l of RNase-Free Water (QIAGEN). Amplification conditions were  
139 as follows: 8 min at 95°C, followed by 45 cycles of 95°C for 10 s, 58°C for 20 s, 65°C for 20  
140 s. Standard curves were plotted using dilutions of a pBluescriptKS vector containing one  
141 copy of each of the *ace-2*, *wsp* and *cytb* gene fragments. Each abdomen (or thorax) DNA  
142 template was analysed in triplicate for *ace-2* and *wsp* (or *cytb*) quantification. Assuming that  
143 each gene is present in a single copy per haploid genome, the ratio between the *wsp* (or *cytb*)  
144 and *ace-2* provides the number of *Wolbachia* (or *Plasmodium*) genomes relative to the *Culex*  
145 genomes.

146

#### 147 **Statistical analysis**

148 Analyses were carried out using the R statistical package (v2.12.0). The different statistical  
149 models built to analyse the data are described in the electronic supplementary material (Table  
150 S2). The general procedure for building the statistical models was as follows: mosquito lines  
151 ( $w^{SL}$ , and  $w^{(-)}$ ), dissection day (7-8 days pbm) and mosquito wing size were fitted as fixed  
152 explanatory variables, whereas bird and qPCR plate were fitted as random explanatory  
153 variables. *Plasmodium* infection prevalence (proportion of mosquitoes containing at least one  
154 parasite; models 1 to 5, Table S2) was analyzed using generalized linear mixed models with a  
155 binomial error distribution (*lmer*, *lme4* package). *Plasmodium* infection intensity (oocyst and  
156 sporozoite loads) was analyzed by including only individuals that became infected. As found  
157 in other systems [32], oocyst count data were greatly overdispersed. One way of handling this  
158 overdispersion is by using negative binomial pseudo distributions [32]. However, to our  
159 knowledge, it is not currently possible to account for negative binomial distributions within a  
160 mixed model *lmer* procedure. For this reason we used instead a *glm* model with a negative  
161 binomial error distribution (*glm.nb*, MASS package; models 6 and 8, Table S2) and we fitted  
162 bird and qPCR plate as fixed factors, next to our variables of interest (i.e. mosquito strain,  
163 dissection day, mosquito wing size). Using fixed rather than mixed models results in some  
164 loss of statistical power, but the results are likely to be conservative [33]. Sporozoite load  
165 data were analysed using a *glm* model with a quasi error distribution and a log link with a  
166 variance equal to  $\mu^2$  to correct for overdispersion (models 7 and 9). *Wolbachia* density was  
167 Box-Cox transformed [34] (models 10 and 11) and subsequently analyzed using linear

168 mixed-effect models (lme, nlme package). Differences in wing size between the lines were  
169 analysed using an ANOVA (aov). Maximal models, including all higher-order interactions,  
170 were simplified by sequentially eliminating non-significant terms and interactions to establish  
171 a minimal model [34]. The significance of the explanatory variables was established using a  
172 likelihood ratio test (LRT), which is approximately distributed as a  $\chi^2$  distribution [33]. The  
173 significant  $\chi^2$  values given in the text are for the minimal model [34]. Full dataset has been  
174 deposited in the Dryad Digital Repository (doi.org/10.5061/dryad.m3752).

175

### 176 ***Ethical statement***

177 Animal experiments were carried out in strict accordance with the “National Charter on the  
178 Ethics of Animal Experimentation” of the French Government, and all efforts were made to  
179 minimize suffering. Experiments were approved by the Ethical Committee for Animal  
180 Experimentation established by the authors’ institution (CNRS) under the auspices of the  
181 French Ministry of Education and Research (permit number CEEA- LR-1051).

182

183

### 184 **Results**

185

186 During the blood meal, one infected canary died for an unknown reason, so this replicate was  
187 eliminated from all subsequent analyses. The percentages of mosquitoes that did not blood  
188 feed, or died before the dissections are detailed in the electronic supplementary material  
189 (Table S3). In the end, a total of 77  $w^{SL}$  and 79  $w^{(-)}$  mosquitoes and 81  $w^{SL}$  and 83  $w^{(-)}$   
190 mosquitoes were dissected at the oocyst (day 7-8 pbm) and sporozoite (day 14 pbm) stages  
191 respectively. Overall,  $w^{(-)}$  females were smaller than  $w^{SL}$  ones (mean  $\pm$  se,  $w^{(-)}$   $3.52 \pm 0.01$   
192 mm,  $w^{SL}$   $3.62 \pm 0.01$  mm,  $\chi_1^2 = 8347$ ,  $p < 0.0001$ ).

193

194 We first analysed whether *Wolbachia* influences *Plasmodium* prevalence. Our results  
195 show that the probability of becoming infected with *P. relictum* is significantly higher when  
196 *Wolbachia* is present ( $w^{SL}$ ). This effect is consistent across the oocyst (probability of  
197 infection in  $w^{SL}$  is on average  $15.9 \pm 7.1$  % higher than in  $w^{(-)}$ ,  $\chi_1^2 = 5.42$ ,  $p = 0.02$ , model 1.)  
198 and the sporozoite ( $20.6 \pm 7.7$  % higher,  $\chi_1^2 = 10.74$ ,  $p = 0.001$ , model 2) stages (Figure 1).

199

200 The combined analysis of the two measurement times revealed a mean ( $\pm$  standard error)  
decrease of  $26.2 (\pm 5.3)$  % in the *Plasmodium* prevalence between 7-8 and 14 dpbm  
(*Plasmodium stage* effect:  $\chi_1^2 = 24.15$ ,  $p < 0.0001$ , model 3), irrespective of the presence of



201 *Wolbachia* (*Wolbachia* x *Plasmodium* stage interaction:  $\chi_1^2 = 0.02$ ,  $p = 0.88$ , model 3; Figure  
202 1). In  $w^{SL}$  females, the probability of becoming infected by *Plasmodium* when exposed to an  
203 infected bird is independent of the density of *Wolbachia* (oocysts:  $\chi_1^2 = 0.21$ ,  $p = 0.64$ , model  
204 4; sporozoites:  $\chi_1^2 = 1.18$ ,  $p = 0.28$ , model 5). Reciprocally, the *Wolbachia* density in female  
205 abdomens was not different between mosquitoes fed on a *Plasmodium*-infected or uninfected  
206 bird either at 7-8 dpbm ( $\chi_1^2 = 2.84$ ,  $p = 0.09$ , model 10) or at 14 dpbm ( $\chi_1^2 = 0.01$ ,  $p = 0.91$ ,  
207 model 11; Figure 3).

208 We then analysed whether *Wolbachia* influences intensity of the *Plasmodium*  
209 infection. The amount of oocysts that successfully developed in the mosquito midgut is  
210 significantly higher in  $w^{SL}$  than in  $w^{(-)}$  females ( $\chi_1^2 = 4.95$ ,  $p = 0.03$ , model 6, Figure 2a).  $w^{SL}$   
211 females have on average 3 more oocysts than  $w^{(-)}$  ones (mean  $\pm$  se,  $8.4 \pm 1.4$  and  $5.7 \pm 0.8$   
212 oocysts, respectively). By contrast, the relative quantity of sporozoites present in infected  
213 mosquito thoraxes is independent of the presence of *Wolbachia* ( $\chi_1^2 = 0.69$ ,  $p = 0.55$ , model 7;  
214 Figure 2b). As above, neither oocyst nor sporozoite load are correlated with *Wolbachia*  
215 density (oocyst :  $\chi_1^2 = 2.64$ ,  $p = 0.10$ , model 8; sporozoite :  $\chi_1^2 = 0.06$ ,  $p = 0.84$ , model 9;  
216 Figure 4).

217

218

## 219 Discussion

220 Current views about the impact of *Wolbachia* on *Plasmodium* infections are almost entirely  
221 based on data regarding artificially transfected mosquitoes. This work has shown that  
222 *Wolbachia* reduces the number of *Plasmodium* oocysts in the midgut of mosquitoes. In  
223 contrast, and probably because of the difficulty in finding natural *Wolbachia* infections in  
224 epidemiologically significant malaria vectors, the role of natural *Wolbachia* infections on  
225 *Plasmodium* development have either been ignored entirely or been given only cursory  
226 attention. *Wolbachia*-mosquito combinations with a long evolutionary history may, however,  
227 be key for understanding what will happen with *Wolbachia*-transfected mosquitoes several  
228 generations down the line if, as has been shown in other systems [22, 23], the novel  
229 *Wolbachia*-host interaction evolves rapidly. The number of generations needed for such  
230 evolutionary change can be between 20 [22] and 200 [23, 35]. To our knowledge, the only  
231 studies carried out using natural *Wolbachia* infections involve the mosquito *Aedes fluviatilis*  
232 and the Asian avian malaria parasite *P. gallinaceum*. This work has shown that, far from  
233 decreasing parasitaemia, *Wolbachia* either has no effect [17, 19] or increases [19] the number

234 of *Plasmodium* oocysts in the midgut of the mosquito. *Ae. fluviatilis* is, however, a South  
235 American mosquito that serves a convenient laboratory host for *P. gallinaceum*, but it is not  
236 its natural vector. Previous work has indeed shown that *Wolbachia* can render contrasting  
237 results on natural [23] and artificial [17, 19] *Plasmodium* combinations so the question,  
238 which is relevant for the long term success of malaria control programs, of whether  
239 *Wolbachia* can interfere with *Plasmodium* transmission in an entirely natural system is still  
240 unresolved.

241 Here we used an entirely natural mosquito-*Wolbachia-Plasmodium* combination to  
242 investigate whether *Wolbachia* increases or decreases the parasitaemia of mosquitoes. In  
243 contrast to most previous studies, which have been centered on the quantification of oocysts  
244 in the midgut of mosquitoes, we aimed to obtain a measurement of parasitaemia that would  
245 relate more directly to transmission by following the infections all the way to the sporozoites  
246 stage as recently done in *An. stephensi* [12]. We found that *Wolbachia* increases marginally,  
247 albeit statistically significantly, the oocyst load of mosquitoes. However, the difference in  
248 oocyst load found in the midguts on day 7 was not sufficiently marked to translate into a  
249 difference in sporozoite load in the salivary glands seven days later. One potential  
250 explanation for these results is that since a single oocyst can produce thousands sporozoites,  
251 beyond a certain oocyst threshold the salivary glands of mosquitoes may have become  
252 saturated by sporozoites [36]. Alternatively, the drastic loss of parasites that inevitably takes  
253 place between the midgut and the salivary stages in any *Plasmodium* infection [32] may  
254 upstage the marginal differences in oocystaemia that exist early on. Proof of the inefficient  
255 migration from the midgut to the salivary glands is the significant (26%) decrease in  
256 *Plasmodium* prevalence we observed between the oocyst and the sporozoite stages, which  
257 was independent of the presence of *Wolbachia*.

258 Irrespective of the underlying mechanism, we believe that the epidemiological  
259 significance of having more or less *Plasmodium* parasites in the gut or even in the salivary  
260 glands remains to be demonstrated. As stated above, a single oocyst can produce between  
261 2000 and 8000 sporozoites [37], and as few as ten sporozoites suffice to start a new infection  
262 [26]. There is also no consistent evidence that the density of sporozoites in the salivary glands  
263 correlates with the number of infecting sporozoites [38], or that this correlates with the  
264 probability of a successful infection in the host (but see [39]). Mosquito infection intensity is,  
265 indeed, conspicuously absent from current models of malaria transmission and epidemiology  
266 [26, 40]. Infection intensity may, however, bear on epidemiology if it correlates negatively  
267 with key life history traits of the vector, such as longevity, but the evidence for this is sparse

268 and comes from unrealistically high infections [41]. In contrast, infection prevalence, ie the  
269 number of infectious mosquitoes in a population, is the keystone of epidemiological models  
270 [26]. The proportion of infectious mosquitoes in a population, sometimes called the  
271 sporozoite rate, is a key determinant of the rate at which hosts are bitten in a population [26,  
272 40]. Here we show that the presence of *Wolbachia* increases sporozoite prevalence by as  
273 much as 21%. *Wolbachia* does therefore play a major role in the transmission of *Plasmodium*  
274 in the avian malaria system.

275 In several host species *Wolbachia* density can fluctuate both between individuals [31,  
276 42] and within individuals over time [42, 43], and several *Wolbachia*-induced phenotypes,  
277 such as cytoplasmic incompatibility [42] (but see [43]), longevity curtailment [44] or host  
278 resistance to viruses [45], have been shown to depend on the density of infecting bacteria.  
279 The correlation between *Wolbachia* density and parasite density can provide interesting  
280 insights as to the mechanisms underlying the interaction. For example, a strong negative  
281 correlation was found between *Wolbachia* density and dengue virus load in *Ae. aegypti* and  
282 *Ae. albopictus* cell lines [45], whereas in *Ae. albopictus* infected with the chikungunya virus,  
283 the intensive phase of the viral replication is concomitant with a significant decrease in  
284 *Wolbachia* load [20, 46, 47], leading the authors to suggest immune competition and resource  
285 competition respectively, as the mechanisms driving the interaction between these two  
286 players. Here, however, neither the probability nor the intensity of *Plasmodium* infection at  
287 either the oocyst or sporozoite stages are explained by the density of *Wolbachia*. It would  
288 therefore appear that it is the presence of *Wolbachia*, irrespective of its density, that  
289 determines the increase in prevalence and intensity observed, as previously found in *An.*  
290 *gambiae* with both *P. falciparum* and *P. berghei* [13, 14]. In addition, the density of bacteria  
291 did not differ depending on whether the mosquitoes were infected by *Plasmodium* or not,  
292 suggesting that the *Wolbachia-Plasmodium* interaction only works one way.

293 With this in mind, several different, but non-exclusive, mechanisms may be envisaged  
294 to explain our results. First, we found that *Wolbachia*-infected mosquitoes were significantly  
295 bigger than *Wolbachia*-free ones and may thus have simply taken larger blood meals, thereby  
296 increasing their intake of *Plasmodium* gametocytes (the stage which is transmissible to  
297 mosquitoes). We have previously shown that the number of *P. relictum* oocysts is  
298 significantly correlated with the amount of blood ingested by the mosquitoes, albeit in a non-  
299 linear way [29]. Second, *Wolbachia* may facilitate the successful establishment of  
300 *Plasmodium* within the mosquito tissues. One obvious way in which this could happen is  
301 through a *Wolbachia*-induced down-regulation of the nonspecific arm of the mosquito

302 immune system, a form of self-protection that has been observed both in pill bugs (or  
303 woodlice) [48] and parasitoids [49]. In this respect, these natural *Wolbachia* infections would  
304 behave in a drastically different way to artificial infections, which are often found to up-  
305 regulate the immune system when introduced into a novel host [12, 13, 15, 17, 45].

306 Third, the differences observed between our *Wolbachia*-infected and -free mosquito  
307 lines could be mediated by differences in their midgut microbiota, which have been recently  
308 shown to play a key role in mosquito resistance to *Plasmodium* infection [50, 51]. Using  
309 tetracycline to eliminate *Wolbachia* is standard practice, the consensus being that mosquitoes  
310 recover their microbial flora over a certain number of generations, a premise that, to our  
311 knowledge has never been explicitly tested. Therefore, the possibility that the antibiotic  
312 treatment may have irreversibly altered the midgut microbiota of mosquitoes, and therefore  
313 the resistance to *Plasmodium* infection, cannot be totally eliminated. More interesting from a  
314 biological point of view, but to our knowledge also hitherto unexplored, is the possibility that  
315 *Wolbachia* itself may modify (through competition, or facilitation), the density and  
316 composition of the microbial flora of their hosts.

317 Finally,  $w^{(-)}$  was reared for ca. 30 generations before the experiment to eliminate side  
318 effects of the tetracycline. Although the  $w^{SL}$  and  $w^{(-)}$  were kept throughout under identical  
319 culturing conditions, we cannot entirely exclude the possibility that the two lines may have  
320 diverged and that the results we obtain are due to different genetic backgrounds. Further work  
321 should replicate these results with, if possible, several *Wolbachia*-infected and uninfected  
322 lines.

323 Previous work in this system has shown that *Plasmodium*-infected females suffer  
324 lower mortality rates if they are also infected with *Wolbachia* [27]. We had originally  
325 advanced two potential explanations for these results: *Wolbachia* infected mosquitoes could  
326 be either more resistant or more tolerant to a *Plasmodium* infection. Under the first  
327 (resistance) scenario, *Wolbachia* would limit or inhibit parasite development, thereby  
328 reducing overall parasitaemia. Dawes et al [41] have indeed shown that in rodent malaria the  
329 number of oocysts in the mosquito midgut is correlated with mosquito longevity, but the  
330 evidence comes from extremely high (100-2000) oocyst burdens. Under the second  
331 (tolerance) scenario, *Wolbachia* would limit or compensate for the damage incurred by the  
332 parasite, without necessarily altering the within-host growth rate of the parasite [52]. An  
333 increase in tolerance to pathogens has been previously observed with native *Wolbachia* strain  
334 of *Drosophila* flies when challenged with viruses [9, 53]. Elucidating which of these  
335 mechanisms is at play is essential from a transmission perspective because parasite-resistant

336 vectors are expected to be worse vectors of diseases, while the opposite will be true for  
337 parasite-tolerant ones (the "tragedy of tolerance" [54]). The results of the present experiments  
338 show that *Wolbachia*-infected mosquitoes are in fact less resistant to *Plasmodium*, leaving a  
339 higher *Wolbachia*-associated tolerance to *Plasmodium* as the only potential explanation for  
340 the longevity results, the mechanisms underlying which remain to be explored.

341 In conclusion, we show that *Wolbachia* increases the susceptibility of *Cx. pipiens*  
342 mosquitoes to *P. relictum*, significantly increasing the prevalence of salivary gland stage  
343 infections. Previous work on this same system has shown that *Wolbachia* also protects  
344 mosquitoes against a *Plasmodium*-induced mortality [27]. As both mosquito mortality and  
345 infection prevalence are two key determinants of *Plasmodium* epidemiology, these results  
346 suggest that naturally *Wolbachia*-infected mosquitoes may, in fact, be better vectors of  
347 malaria than *Wolbachia*-free ones.

348

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## References

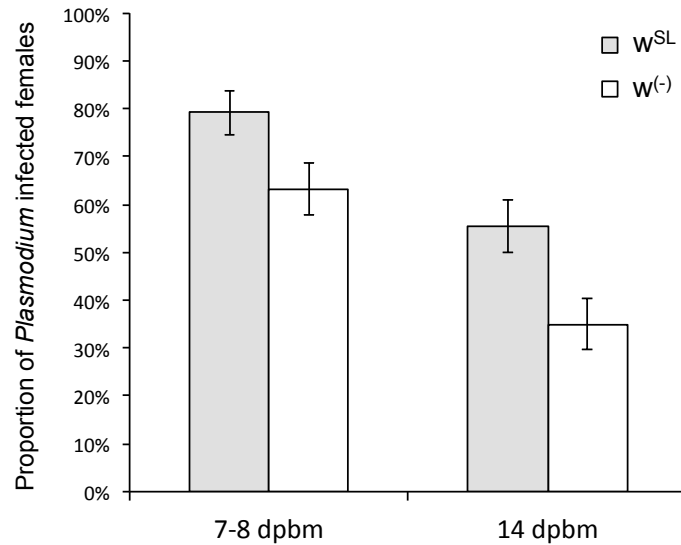
1. Alizon S., van Baalen M. 2008 Multiple infections, immune dynamics, and the evolution of virulence. *Am Nat* **172**(4), E150-E168. (doi:10.1086/590958).
2. Pedersen A.B., Fenton A. 2007 Emphasizing the ecology in parasite community ecology. *Trends Ecol Evol* **22**(3), 133-139. (doi:10.1016/j.tree.2006.11.005).
3. Graham A.L. 2008 Ecological rules governing helminth-microparasite coinfection. *Proc Natl Acad Sci USA* **105**(2), 566-570. (doi:10.1073/pnas.0707221105).
4. Riley M.A., Wertz J.E. 2002 Bacteriocins: evolution, ecology, and application. *Annual Review of Microbiology* **56**, 117-137. (doi:10.1146/annurev.micro.56.012302.161024).
5. Swanson S.J., Neitzel D., Reed K.D., Belongia E.A. 2006 Coinfections acquired from Ixodes ticks. *Clin Microbiol Rev* **19**(4), 708-727. (doi:10.1128/cmr.00011-06).
6. Hughes T., Irwin P., Hofmeister E., Paskewitz S.M. 2010 Occurrence of avian *Plasmodium* and West Nile virus in *Culex* species in Wisconsin. *J Am Mosq Control Assoc* **26**(1), 24-31.
7. Vazeille M., Mousson L., Martin E., Failloux A.-B. 2010 Orally co-infected *Aedes albopictus* from La Reunion Island, Indian Ocean, can deliver both dengue and chikungunya infectious viral particles in their saliva. *PLoS Negl Trop Dis* **4**(6). (doi:10.1371/journal.pntd.0000706).
8. Hedges L.M., Brownlie J.C., O'Neill S.L., Johnson K.N. 2008 *Wolbachia* and virus protection in insects. *Science* **322**(5902), 702-702. (doi:10.1126/science.1162418).
9. Teixeira L., Ferreira A., Ashburner M. 2008 The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. *PLoS Biol* **6**(12), 2753-2763. (doi:10.1371/journal.pbio.1000002).
10. Xi Z.Y., Khoo C.C.H., Dobson S.L. 2005 *Wolbachia* establishment and invasion in an *Aedes aegypti* laboratory population. *Science* **310**(5746), 326-328. (doi:10.1126/science.1117607).
11. McMeniman C.J., Lane R.V., Cass B.N., Fong A.W.C., Sidhu M., Wang Y.F., O'Neill S.L. 2009 Stable introduction of a life-shortening *Wolbachia* infection into the mosquito *Aedes aegypti*. *Science* **323**(5910), 141-144. (doi:10.1126/science.1165326).
12. Bian G., Joshi D., Dong Y., Lu P., Zhou G., Pan X., Xu Y., Dimopoulos G., Xi Z. 2013 *Wolbachia* invades *Anopheles stephensi* populations and induces refractoriness to *Plasmodium* infection. *Science* **340**, 748-751. (doi:10.1126/science.1236192).
13. Hughes G.L., Koga R., Xue P., Fukatsu T., Rasgon J.L. 2011 *Wolbachia* infections are virulent and inhibit the human malaria parasite *Plasmodium falciparum* in *Anopheles gambiae*. *PLoS Pathog* **7**(5), e1002043. (doi:10.1371/journal.ppat.1002043).
14. Hughes G.L., Vega-Rodriguez J., Xue P., Rasgon J.L. 2012 *Wolbachia* strain wAlbB enhances infection by the rodent malaria parasite *Plasmodium berghei* in *Anopheles gambiae* mosquitoes. *Appl Environ Microbiol* **78**(5), 1491-1495. (doi:10.1128/aem.06751-11).
15. Kambris Z., Blagborough A.M., Pinto S.B., Blagrove M.S.C., Godfray H.C.J., Sinden R.E., Sinkins S.P. 2010 *Wolbachia* stimulates immune gene expression and inhibits *Plasmodium* development in *Anopheles gambiae*. *PLoS Pathog* **6**(10), e1001143. (doi:10.1371/journal.ppat.1001143).



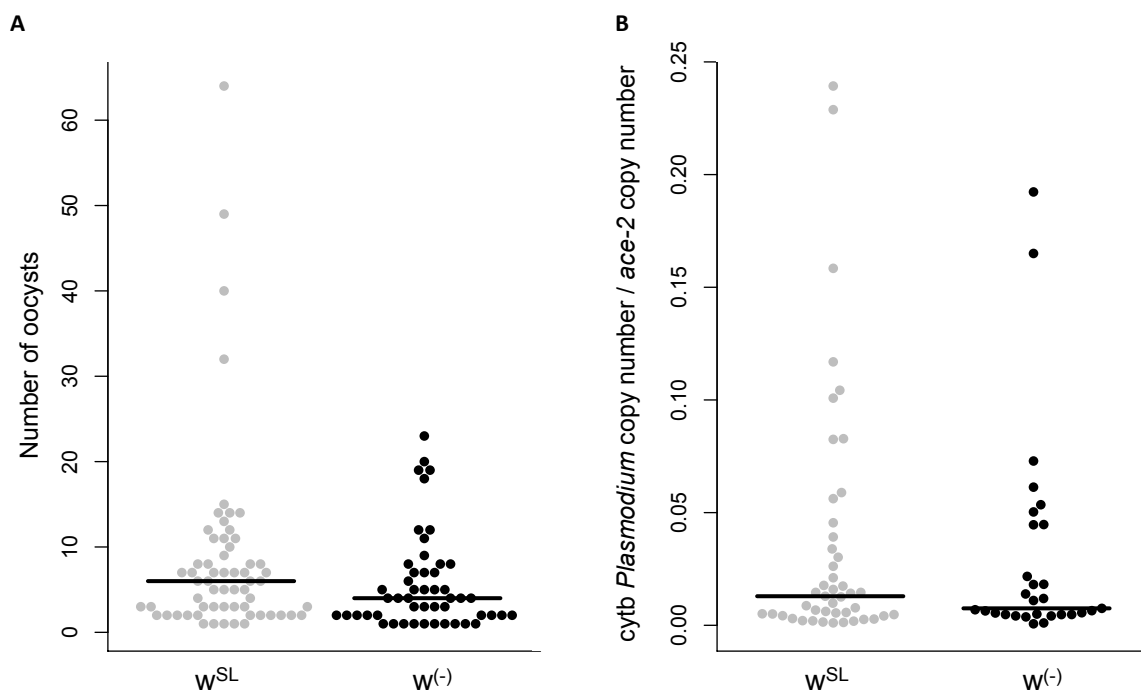
16. Kambris Z., Cook P.E., Phuc H.K., Sinkins S.P. 2009 Immune activation by life-shortening *Wolbachia* and reduced filarial competence in mosquitoes. *Science* **326**(5949), 134-136. (doi:10.1126/science.1177531).
17. Moreira L.A., Iturbe-Ormaetxe I., Jeffery J.A., Lu G.J., Pyke A.T., Hedges L.M., Rocha B.C., Hall-Mendelin S., Day A., Riegler M., et al. 2009 A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, Chikungunya, and *Plasmodium*. *Cell* **139**(7), 1268-1278. (doi:10.1016/j.cell.2009.11.042).
18. Blagrove M.S.C., Arias-Goeta C., Failloux A.-B., Sinkins S.P. 2012 *Wolbachia* strain wMel induces cytoplasmic incompatibility and blocks dengue transmission in *Aedes albopictus*. *Proc Natl Acad Sci USA* **109**(1), 255-260. (doi:10.1073/pnas.1112021108).
19. Baton L.A., Pacidonio E.C., Goncalves D.d.S., Moreira L.A. 2013 wFlu: Characterization and evaluation of a native *Wolbachia* from the mosquito *Aedes fluviatilis* as a potential vector control agent. *PLoS One* **8**(3), e59619-e59619. (doi:10.1371/journal.pone.0059619).
20. Mousson L., Martin E., Zouache K., Madec Y., Mavingui P., Failloux A.B. 2010 *Wolbachia* modulates Chikungunya replication in *Aedes albopictus*. *Mol Ecol* **19**(9), 1953-1964. (doi:10.1111/j.1365-294X.2010.04606.x).
21. Glaser R.L., Meola M.A. 2010 The native *Wolbachia* endosymbionts of *Drosophila melanogaster* and *Culex quinquefasciatus* increase host resistance to West Nile virus infection. *PLoS One* **5**(8), e11977. (doi:10.1371/journal.pone.0011977).
22. McGraw E.A., Merritt D.J., Droller J.N., O'Neill S.L. 2002 *Wolbachia* density and virulence attenuation after transfer into a novel host. *Proc Natl Acad Sci USA* **99**(5), 2918-2923. (doi:10.1073/pnas.052466499).
23. Weeks A.R., Turelli M., Harcombe W.R., Reynolds K.T., Hoffmann A.A. 2007 From parasite to mutualist: Rapid evolution of *Wolbachia* in natural populations of *Drosophila*. *PLoS Biol* **5**(5), 997-1005. (doi:10.1371/journal.pbio.0050114).
24. Tripet F. 2009 Ecological immunology of mosquito-malaria interactions: Of non-natural versus natural model systems and their inferences. *Parasitology* **136**(14), 1935-1942. (doi:10.1017/s0031182009006234).
25. Cohuet A., Osta M.A., Morlais I., Awono-Ambene P.H., Michel K., Simard F., Christophides G.K., Fontenille D., Kafatos F.C. 2006 *Anopheles* and *Plasmodium*: from laboratory models to natural systems in the field. *EMBO Rep* **7**(12), 1285-1289. (doi:10.1038/sj.embor.7400831).
26. Smith D.L., McKenzie F.E. 2004 Statics and dynamics of malaria infection in *Anopheles* mosquitoes. *Malaria J* **3**, 13. (doi:10.1186/1475-2875-3-13).
27. Zélé F., Nicot A., Duron O., Rivero A. 2012 Infection with *Wolbachia* protects mosquitoes against *Plasmodium*-induced mortality in a natural system. *J Evol Biol* **25**(7), 1243-1252. (doi:10.1111/j.1420-9101.2012.02519.x).
28. Bensch S., Hellgren O., Perez-Tris J. 2009 MalAvi: a public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome b lineages. *Mol Ecol Res* **9**(5), 1353-1358. (doi:10.1111/j.1755-0998.2009.02692.x).

29. Vézilier J., Nicot A., Gandon S., Rivero A. 2010 Insecticide resistance and malaria transmission: infection rate and oocyst burden in *Culex pipiens* mosquitoes infected with *Plasmodium relictum*. *Malaria J* **9**, 379. (doi:10.1186/1475-2875-9-379).
30. Weill M., Berticat C., Raymond N., Chevillon C. 2000 Quantitative polymerase chain reaction to estimate the number of amplified esterase genes in insecticide-resistant mosquitoes. *Anal Biochem* **285**(2), 267-270. (doi:10.1006/abio.2000.4781).
31. Berticat C., Rousset F., Raymond M., Berthomieu A., Weill M. 2002 High *Wolbachia* density in insecticide-resistant mosquitoes. *Proc R Soc Lond B* **269**(1498), 1413-1416. (doi:10.1098/rspb.2002.2022).
32. Vaughan J.A. 2007 Population dynamics of *Plasmodium* sporogony. *Trends Parasitol* **23**(2), 63-70. (doi:10.1016/j.pt.2006.12.009).
33. Bolker B.M. 2008 *Ecological models and data in R* New Jersey, Princeton University Press.
34. Crawley M.J. 2007 *The R Book* Chichester, England, John Wiley & Sons, Ltd; 942 p.
35. Carrington L.B., Hoffmann A.A., Weeks A.R. 2010 Monitoring long-term evolutionary changes following *Wolbachia* introduction into a novel host: the *Wolbachia* popcorn infection in *Drosophila simulans*. *Proc R Soc B* **277**(1690), 2059-2068. (doi:10.1098/rspb.2010.0166).
36. Sinden R.E., Dawes E.J., Alavi Y., Waldock J., Finney O., Mendoza J., Butcher G.A., Andrews L., Hill A.V., Gilbert S.C., et al. 2007 Progression of *Plasmodium berghei* through *Anopheles stephensi* is density-dependent. *PLoS Pathog* **3**(12), 2005-2016. (doi:10.1371/journal.ppat.0030195).
37. Wang Q., Fujioka H., Nussenzweig V. 2005 Exit of *Plasmodium* sporozoites from oocysts is an active process that involves the circumsporozoite protein. *PLoS Pathog* **1**(1), e9. (doi:10.1371/journal.ppat.0010009).
38. Beier J.C. 1998 Malaria parasite development in mosquitoes. *Ann Rev Entomol* **43**, 519-543. (doi:10.1146/annurev.ento.43.1.519).
39. Kebaier C., Voza T., Vanderberg J. 2009 Kinetics of Mosquito-Injected Plasmodium Sporozoites in Mice: Fewer Sporozoites Are Injected into Sporozoite-Immunized Mice. *PLoS Pathog* **5**(4). (doi:10.1371/journal.ppat.1000399).
40. Smith D.L., Dushoff J., Snow R.W., Hay S.I. 2005 The entomological inoculation rate and *Plasmodium falciparum* infection in African children. *Nature* **438**(7067), 492-495. (doi:10.1038/nature04024).
41. Dawes E.J., Churcher T.S., Zhuang S., Sinden R.E., Basanez M.G. 2009 *Anopheles* mortality is both age- and *Plasmodium*-density dependent: implications for malaria transmission. *Malaria J* **8**, 228. (doi:10.1186/1475-2875-8-228).
42. Clark M.E., Veneti Z., Bourtzis K., Karr T.L. 2003 *Wolbachia* distribution and cytoplasmic incompatibility during sperm development: the cyst as the basic cellular unit of CI expression. *Mech Dev* **120**(2), 185-198. (doi:10.1016/s0925-4773(02)00424-0).
43. Duron O., Fort P., Weill M. 2007 Influence of aging on cytoplasmic incompatibility, sperm modification and *Wolbachia* density in *Culex pipiens* mosquitoes. *Heredity* **98**(6), 368-374. (doi:10.1038/sj.hdy.6800948).

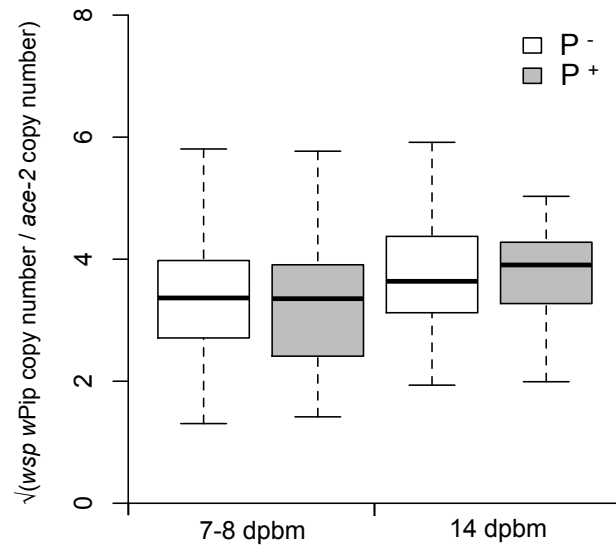
44. Min K.T., Benzer S. 1997 *Wolbachia*, normally a symbiont of *Drosophila*, can be virulent, causing degeneration and early death. *Proc Natl Acad Sci USA* **94**(20), 10792-10796. (doi:10.1073/pnas.94.20.10792).
45. Lu P., Bian G., Pan X., Xi Z. 2012 *Wolbachia* induces density-dependent inhibition to dengue virus in mosquito cells. *PLoS Negl Trop Dis* **6**(7), e1754. (doi:10.1371/journal.pntd.0001754).
46. Tortosa P., Courtiol A., Moutailler S., Failloux A.B., Weill M. 2008 Chikungunya-*Wolbachia* interplay in *Aedes albopictus*. *Insect Mol Biol* **17**(6), 677-684. (doi:10.1111/j.1365-2583.2008.00842.x).
47. Zouache K., Michelland R.J., Failloux A.-B., Grundmann G.L., Mavingui P. 2012 Chikungunya virus impacts the diversity of symbiotic bacteria in mosquito vector. *Mol Ecol* **21**(9), 2297-2309. (doi:10.1111/j.1365-294X.2012.05526.x).
48. Sicard M., Chevalier F., De Vlehouwer M., Bouchon D., Greve P., Braquart-Varnier C. 2010 Variations of immune parameters in terrestrial isopods: a matter of gender, aging and *Wolbachia*. *Naturwissenschaften* **97**(9), 819-826. (doi:10.1007/s00114-010-0699-2).
49. Fytrou A., Schofield P.G., Kraaijeveld A.R., Hubbard S.F. 2006 *Wolbachia* infection suppresses both host defence and parasitoid counter-defence. *Proc R Soc B* **273**(1588), 791-796. (doi:10.1098/rspb.2005.3383).
50. Dong Y.M., Manfredini F., Dimopoulos G. 2009 Implication of the mosquito midgut microbiota in the defense against malaria parasites. *PLoS Pathog* **5**(5), e1000423. (doi:10.1371/journal.ppat.1000423).
51. Cirimotich C.M., Dong Y.M., Clayton A.M., Sandiford S.L., Souza-Neto J.A., Mulenga M., Dimopoulos G. 2011 Natural microbe-mediated refractoriness to *Plasmodium* infection in *Anopheles gambiae*. *Science* **332**(6031), 855-858. (doi:10.1126/science.1201618).
52. Raberg L., Sim D., Read A.F. 2007 Disentangling genetic variation for resistance and tolerance to infectious diseases in animals. *Science* **318**(5851), 812-814. (doi:10.1126/science.1148526).
53. Osborne S.E., Leong Y.S., O'Neill S.L., Johnson K.N. 2009 Variation in antiviral protection mediated by different *Wolbachia* strains in *Drosophila simulans*. *PLoS Pathog* **5**(11), e1000656. (doi:10.1371/journal.ppat.1000656).
54. Vale P.F., Wilson A.J., Best A., Boots M., Little T.J. 2011 Epidemiological, evolutionary, and coevolutionary implications of context-dependent parasitism. *Am Nat* **177**(4), 510-521. (doi:10.1086/659002).



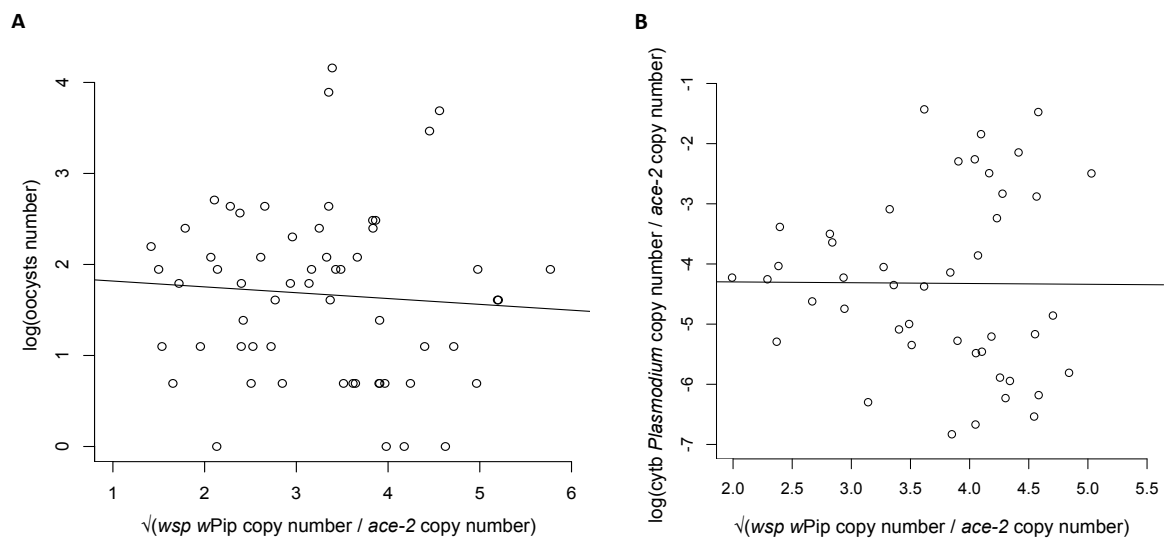
**Figure 1.** Effect of *Wolbachia* on the prevalence of *Plasmodium* infection 7 days (oocyst stage) and 14 days post blood meal (sporozoite stage). Bars represent means ( $\pm$  SE) for *Wolbachia*-carrying females (grey bars) and *Wolbachia*-free ones (white bars).



**Figure 2.** Effect of the presence of *Wolbachia* on *Plasmodium* burden in mosquitoes. Distribution of the number of oocysts in the midgut of *Plasmodium*-infected females 7-8 days post blood meal (A), and of the relative quantity of sporozoites in the thorax of *Plasmodium*-infected females 14 days post blood meal (B), for *Wolbachia*-carrying females (grey circles) and *Wolbachia*-free ones (black circles). Horizontal lines represent medians.



**Figure 3.** Boxplot of the *Wolbachia* density in  $w^{SL}$  females according to the *Plasmodium* infection status at 7-8 days (oocysts) and 14 days (sporozoites) post blood meal. White boxes: *Plasmodium* uninfected mosquitoes (includes females fed on a control bird and females that did not become infected after feeding on a *Plasmodium*-infected bird) and grey boxes: *Plasmodium* infected mosquitoes. *Wolbachia* densities were Box-Cox transformed to linearize the data for the graphic representation.



**Figure 4.** Correlation between the density of *Wolbachia* and the intensity of *Plasmodium* infection at the oocyst (A) and sporozoite (B) stages (7-8 days and 14 days post blood meal respectively). Both *Wolbachia* and *Plasmodium* densities were Box-Cox transformed to linearize the data for the graphic representation.

**Table S1. Summary of the different studies conducted to date on *Wolbachia*-mediated pathogen interference.** <sup>a</sup> nature of the host-*Wolbachia* or host-parasite combination : “natural” when it occurs in nature, “artificial” when it was created in the lab. Lines that refer to complete natural combinations are highlighted in grey. <sup>b</sup> Effect of *Wolbachia* on parasite prevalence and intensity: (+) increase, (–) decrease, (×) no observed effects, (.) not studied. *Abbreviations*: IVV-6: Influenza virus; LACV: La Crosse virus; FHV: Flock House virus; CHIKV: Chikungunya virus; WNV: West Nile virus; DENV-2: Dengue virus. Bacteria: <sup>1</sup> *Pseudomonas aeruginosa*, *Serratia marcescens* and *Erwinia carotovora*; and <sup>2</sup> *Providencia rettgeri*, *Salmonella enterica* and *Listeria monocytogenes*.

Host species	<i>Wolbachia</i> strains	Host- <i>Wolbachia</i> <sup>a</sup>	Parasites	Host-parasite <sup>a</sup>	Parasite prevalence <sup>b</sup>	Parasite intensity <sup>b</sup>	References
<b>Drosophila flies</b>							
<i>D. melanogaster</i>	wMel	natural	DCV, Nora virus	natural	.	–	[1]
	wMel	natural	FHV	artificial	.	×	[1]
	wMel	natural	CHIKV, WNV	artificial	.	–	[2]
	wMel	natural	IVV-6, LACV, Bacteria <sup>1,2</sup>	artificial	.	×	[1-4]
<i>D. simulans</i>	wAu	natural	DCV	natural	.	–	[5]
	wAu	natural	FHV	artificial	.	–	[5]
	wHa / wNO	natural	DCV	natural	.	×	[5]
	wHa / wNO	natural	FHV	artificial	.	×	[5]
	?	natural	<i>Leptopilina heterotoma</i>	natural	×	+	[6]
	wMel	artificial	DCV	natural	.	–	[5]
<b>Mosquitoes</b>							
<i>Aedes aegypti</i>	wAlbB	artificial	DENV-2	natural	.	–	[7-9]
	wMel	artificial	DENV-2	natural	.	–	[10]
	wMelPop-CLA	artificial	CHIKV, DENV-2	natural	–	–	[10, 11]
	wMelPop-CLA	artificial	<i>Brugia pahangi</i>	natural	–	–	[12, 13]
	wMelPop-CLA	artificial	<i>P. gallinaceum</i>	artificial	–	–	[11]
<i>Ae. albopictus</i>	wAlbA&B	natural	CHIKV, DENV-2	natural	×	×	[7, 14-17]
	wMel	artificial	CHIKV, DENV-2	natural	–	–	[16, 18]
<i>Ae. fluventris</i>	wFlu	natural	<i>P. gallinaceum</i>	artificial	.	×	[11]
	wFlu	natural	<i>P. gallinaceum</i>	artificial	.	×+	[19]
<i>Ae. polynesiensis</i>	wPoIA	natural	<i>Brugia pahangi</i>	natural	.	×	[20]
	wAlbB	artificial	<i>Brugia pahangi</i>	natural	.	–	[20]
<i>Culex pipiens quinquefasciatus</i>	wPip	natural	WNV	natural	×	×–	[2]
<i>Anopheles gambiae</i>	wAlbB	artificial	<i>P. falciparum</i>	natural	.	–	[21]
	wAlbB	artificial	<i>P. berghei</i>	artificial	.	+	[22]
	wMelPop-CLA	artificial	<i>P. falciparum</i>	natural	.	–	[21]
	wMelPop-CLA	artificial	<i>P. berghei</i>	artificial	.	–	[12, 22]
<i>An. stephensi</i>	wAlbB	artificial	<i>P. falciparum</i>	natural	–	–	[23]
<b>Other insects</b>							
<i>Spodoptera exempta</i>	wExe	natural	SpexNPV	natural	+	.	[24]



## References

1. Teixeira L., Ferreira A., Ashburner M. 2008 The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. *PLoS Biol* **6**(12), 2753-2763. (doi:10.1371/journal.pbio.1000002).
2. Glaser R.L., Meola M.A. 2010 The native *Wolbachia* endosymbionts of *Drosophila melanogaster* and *Culex quinquefasciatus* increase host resistance to West Nile virus infection. *PLoS One* **5**(8), e11977. (doi:10.1371/journal.pone.0011977).
3. Wong Z.S., Hedges L.M., Brownlie J.C., Johnson K.N. 2011 *Wolbachia*-mediated antibacterial protection and immune gene regulation in *Drosophila*. *PLoS One* **6**(9), e25430. (doi:10.1371/journal.pone.0025430).
4. Rottschaefer S.M., Lazzaro B.P. 2012 No effect of *Wolbachia* on resistance to intracellular infection by pathogenic bacteria in *Drosophila melanogaster*. *PLoS One* **7**(7), e40500.
5. Osborne S.E., Leong Y.S., O'Neill S.L., Johnson K.N. 2009 Variation in antiviral protection mediated by different *Wolbachia* strains in *Drosophila simulans*. *PLoS Pathog* **5**(11), e1000656. (doi:10.1371/journal.ppat.1000656).
6. Fytrou A., Schofield P.G., Kraaijeveld A.R., Hubbard S.F. 2006 *Wolbachia* infection suppresses both host defence and parasitoid counter-defence. *Proc R Soc B* **273**(1588), 791-796. (doi:10.1098/rspb.2005.3383).
7. Bian G.W., Xu Y., Lu P., Xie Y., Xi Z.Y. 2010 The endosymbiotic bacterium *Wolbachia* induces resistance to dengue virus in *Aedes aegypti*. *PLoS Pathog* **6**(4), e1000833. (doi:10.1371/journal.ppat.1000833).
8. Lu P., Bian G., Pan X., Xi Z. 2012 *Wolbachia* induces density-dependent inhibition to dengue virus in mosquito cells. *PLoS Negl Trop Dis* **6**(7), e1754.
9. Pan X., Zhou G., Wu J., Bian G., Lu P., Raikhel A.S., Xi Z. 2012 *Wolbachia* induces reactive oxygen species (ROS)-dependent activation of the Toll pathway to control dengue virus in the mosquito *Aedes aegypti*. *Proc Natl Acad Sci USA* **109**(1), 13-14. (doi:10.1073/pnas.1116932108).
10. Walker T., Johnson P.H., Moreira L.A., Iturbe-Ormaetxe I., Frentiu F.D., McMeniman C.J., Leong Y.S., Dong Y., Axford J., Kriesner P., et al. 2011 The wMel *Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations. *Nature* **476**(7361), 450-453. (doi:10.1038/nature10355).
11. Moreira L.A., Iturbe-Ormaetxe I., Jeffery J.A., Lu G.J., Pyke A.T., Hedges L.M., Rocha B.C., Hall-Mendelin S., Day A., Riegler M., et al. 2009 A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, Chikungunya, and *Plasmodium*. *Cell* **139**(7), 1268-1278. (doi:10.1016/j.cell.2009.11.042).
12. Kambris Z., Blagborough A.M., Pinto S.B., Blagrove M.S.C., Godfray H.C.J., Sinden R.E., Sinkins S.P. 2010 *Wolbachia* stimulates immune gene expression and inhibits *Plasmodium* development in *Anopheles gambiae*. *PLoS Pathog* **6**(10), e1001143. (doi:10.1371/journal.ppat.1001143).
13. Kambris Z., Cook P.E., Phuc H.K., Sinkins S.P. 2009 Immune activation by life-shortening *Wolbachia* and reduced filarial competence in mosquitoes. *Science* **326**(5949), 134-136. (doi:10.1126/science.1177531).
14. Tortosa P., Courtiol A., Moutailler S., Failloux A.B., Weill M. 2008 Chikungunya-*Wolbachia* interplay in *Aedes albopictus*. *Insect Mol Biol* **17**(6), 677-684. (doi:10.1111/j.1365-2583.2008.00842.x).
15. Mousson L., Martin E., Zouache K., Madec Y., Mavingui P., Failloux A.B. 2010 *Wolbachia* modulates Chikungunya replication in *Aedes albopictus*. *Mol Ecol* **19**(9), 1953-1964. (doi:10.1111/j.1365-294X.2010.04606.x).
16. Blagrove M.S.C., Arias-Goeta C., Failloux A.-B., Sinkins S.P. 2012 *Wolbachia* strain wMel induces cytoplasmic incompatibility and blocks dengue transmission in *Aedes albopictus*. *Proc Natl Acad Sci USA* **109**(1), 255-260. (doi:10.1073/pnas.1112021108).
17. Zouache K., Michelland R.J., Failloux A.-B., Grundmann G.L., Mavingui P. 2012 Chikungunya virus impacts the diversity of symbiotic bacteria in mosquito vector. *Mol Ecol* **21**(9), 2297-2309. (doi:10.1111/j.1365-294X.2012.05526.x).
18. Blagrove M.S.C., Arias-Goeta C., Di Genua C., Failloux A.-B., Sinkins S.P. 2013 A *Wolbachia* wMel transinfection in *Aedes albopictus* is not detrimental to host fitness and inhibits Chikungunya virus. *PLoS Negl Trop Dis* **7**(3), E2152-E2152.
19. Baton L.A., Pacidonio E.C., Goncalves D.d.S., Moreira L.A. 2013 wFlu: Characterization and evaluation of a native *Wolbachia* from the mosquito *Aedes fluviatilis* as a potential vector control agent. *PLoS One* **8**(3), e59619-e59619. (doi:10.1371/journal.pone.0059619).
20. Andrews E.S., Crain P.R., Fu Y., Howe D.K., Dobson S.L. 2012 Reactive oxygen species production and *Brugia pahangi* survivorship in *Aedes polynesiensis* with artificial *Wolbachia* infection types. *PLoS Pathog* **8**(12), e1003075-e1003075. (doi:10.1371/journal.ppat.1003075).
21. Hughes G.L., Koga R., Xue P., Fukatsu T., Rasgon J.L. 2011 *Wolbachia* infections are virulent and inhibit the human malaria parasite *Plasmodium falciparum* in *Anopheles gambiae*. *PLoS Pathog* **7**(5), e1002043. (doi:10.1371/journal.ppat.1002043).
22. Hughes G.L., Vega-Rodriguez J., Xue P., Rasgon J.L. 2012 *Wolbachia* strain wAlbB enhances infection by the rodent malaria parasite *Plasmodium berghei* in *Anopheles gambiae* mosquitoes. *Appl Environ Microbiol* **78**(5), 1491-1495. (doi:10.1128/aem.06751-11).
23. Bian G., Joshi D., Dong Y., Lu P., Zhou G., Pan X., Xu Y., Dimopoulos G., Xi Z. 2013 *Wolbachia* invades *Anopheles stephensi* populations and induces refractoriness to *Plasmodium* infection. *Science* **340**, 748-751. (doi:10.1126/science.1236192).
24. Graham R.I., Grzywacz D., Mushobozi W.L., Wilson K. 2012 *Wolbachia* in a major African crop pest increases susceptibility to viral disease rather than protects. *Ecology Letters* **15**(9), 993-1000. (doi:10.1111/j.1461-0248.2012.01820.x).

**Table S2. Description of the statistical models used in the analyses.** "Maximal model": model containing all explanatory variables and their interactions. "Minimal model" : model containing only the significant variables and their interactions. Round brackets: variable fitted as a random factor. Square brackets: the error structure used (n: normal, b: binomial errors, nb: negative binomial, quasi: quasipoisson-like with link=log and variance =  $\mu^2$ ).

*Wolb*: mosquito lines ( $w^{SL}$  or  $w^{(-)}$ ), infection: exposed to a *Plasmodium*-infected bird vs. an uninfected bird, size: mosquito wing length, oocyst: number of oocysts in the midgut, sporozoite: relative density of sporozoites in the thorax, density: *Wolbachia* density in the abdomen ( $w^{SL}$  only) , stage: *Plasmodium* developmental stage (oocysts at day 7-8 pbm or sporozoites at day 14 pbm).

Variable of interest	Response variable	Model Nb.	Sample size	Maximal model	Minimal model	R subroutine [err struct.]
<b><i>Effect of Wolbachia presence/absence on Plasmodium prevalence</i></b>						
<i>Plasmodium</i> prevalence at day 7-8 pbm	infection	1	156 <sup>1</sup>	<i>Wolb</i> * size + day + (bird)	<i>Wolb</i> + (bird)	lmer [b]
<i>Plasmodium</i> prevalence at day 14 pbm	infection	2	164 <sup>1</sup>	<i>Wolb</i> * size + (bird)	<i>Wolb</i> + (bird/plate)	lmer [b]
Total <i>Plasmodium</i> prevalence	infection	3	321 <sup>1</sup>	<i>Wolb</i> * stage * size + (bird)	<i>Wolb</i> + stage + (bird)	lmer [b]
<b><i>Effect of Wolbachia density on Plasmodium prevalence</i></b>						
<i>Plasmodium</i> prevalence at day 7-8 pbm	infection	4	75 <sup>4</sup>	density * size + day + (bird)	1 + (bird)	lmer [b]
<i>Plasmodium</i> prevalence at day 14 pbm	infection	5	81 <sup>4</sup>	density * size + (bird)	1 + (bird)	lmer [b]
<b><i>Effect of Wolbachia presence/absence on Plasmodium load</i></b>						
Oocyst load	oocyst	6	111 <sup>2</sup>	<i>Wolb</i> * size + day + bird	<i>Wolb</i> + day	glm [nb]
Sporozoite load	sporozoite	7	74 <sup>2</sup>	<i>Wolb</i> + bird + plate	1	glm [quasi]
<b><i>Effect of Wolbachia density on Plasmodium load</i></b>						
Oocyst load	oocyst	8	59 <sup>5</sup>	density * size + day + bird	bird	glm [nb]
Sporozoite load	sporozoite	9	45 <sup>5</sup>	density * size + bird + plate	1	glm [quasi]
<b><i>Effect of exposure to Plasmodium on Wolbachia density</i></b>						
<i>Wolbachia</i> density at day 7-8 pbm	$\sqrt{\text{density}}$	10	171 <sup>3</sup>	infection + day + (plate)	1 + (plate)	lme [n]
<i>Wolbachia</i> density at day 14 pbm	$\sqrt{\text{density}}$	11	197 <sup>3</sup>	infection + (plate)	1+ (plate)	lme [n]

<sup>1</sup> Includes only females exposed to a *Plasmodium* infected bird.

<sup>2</sup> Includes only females with  $\geq 1$  oocysts.

<sup>3</sup> Includes only  $w^{SL}$  females.

<sup>4</sup> Includes only  $w^{SL}$  females exposed to a *Plasmodium* infected bird.

<sup>5</sup> Includes only  $w^{SL}$  females that became infected by *Plasmodium* (i.e. that contained at least one oocyst or in which sporozoites were detected).

**Table S3.** Percentages of mosquitoes that did not blood feed or died before the dissections took place.

Mosquito line	Did not blood feed		Died before dissection		Died between day 8 and day 14	
	w <sup>(-)</sup>	w <sup>SL</sup>	w <sup>(-)</sup>	w <sup>SL</sup>	w <sup>(-)</sup>	w <sup>SL</sup>
Control birds	-	14.8%	-	4.8 %	-	1.5%
<i>Plasmodium</i> -infected birds	5.6%	2.8%	6.7%	7.8%	3.3%	4.5%