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Cite this article: Zélé F, Nicot A, Berthomieu A, Weill M, Duron O, Rivero A. 2014 *Wolbachia* increases susceptibility to *Plasmodium* infection in a natural system. *Proc. R. Soc. B* **281**: 20132837.
http://dx.doi.org/10.1098/rspb.2013.2837

Received: 30 October 2013
Accepted: 7 January 2014

Subject Areas:
evolution

Keywords:
symbiont-mediated protection, vectorial competence, infection prevalence, infection intensity, oocysts, sporozoites

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Electronic supplementary material is available at http://dx.doi.org/10.1098/rspb.2013.2837 or via http://rspb.royalsocietypublishing.org.

Royal Society Publishing

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

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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 centred 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.

1. Introduction

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

A few years ago, two seminal papers showed that *Wolbachia*, a maternally transmitted bacterial endosymbiont of arthropods, protects *Drosophila* flies from several viral infections [8,9]. This stimulated a great deal of research into *Wolbachia*-mediated parasite interference in other insect systems (see the electronic supplementary material, table S1), and raised the exciting possibility of using *Wolbachia* to control or limit the spread of mosquito-transmitted diseases, such as dengue and malaria. Interestingly, although neither *Aedes aegypti* (vector of the dengue virus) nor *Anopheles gambiae* or *Anopheles stephensi* (vectors of *Plasmodium falciparum*) are naturally infected by *Wolbachia*, they can

be successfully transfected in the laboratory using bacteria isolated from other insect species [10–12], although not always stably (in *An. gambiae* the infections are somatic and do not transmit vertically to the offspring [13,14]). As a consequence, in the past few years, a large number of studies have been conducted using transfected mosquitoes. These studies have largely confirmed the results obtained in naturally infected *Drosophila*: transfected *Wolbachia* exhibit considerable pathogen-interference properties against a wide range of parasite taxa (e.g. [12,13,15–17]; see also the electronic supplementary material, table S1). By contrast, studies of natural *Wolbachia* infections in mosquitoes have been much less conclusive; some studies have shown no effect of *Wolbachia* on pathogen development [17–19], while others have shown that *Wolbachia* facilitates [20] or blocks [21] pathogen replication (see the electronic supplementary material, table S1 for a summary). This raises the question of whether the *Wolbachia*-mediated parasite protection observed in recently transfected mosquitoes can be maintained across generations. *Wolbachia*–mosquito combinations with a long evolutionary history may be key for understanding what will happen with *Wolbachia*-transfected mosquitoes several generations down the line if, as has been shown in other systems [22,23], the novel *Wolbachia*–mosquito interactions evolve rapidly.

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

We used an entirely natural system, consisting of the avian malaria parasite *P. relictum*, its natural vector, the mosquito *Cx. pipiens*, and its native (wPip) *Wolbachia* strain. The aim was to establish whether the infection with *Wolbachia* decreases the prevalence and/or intensity of *Plasmodium* infection. In contrast to most previous studies, which have been exclusively centred on the quantification of oocysts in the midgut of mosquitoes 7 days after the infection (but see [12]), we aimed to obtain a measurement of parasitaemia that would relate more directly to transmission by following the infections all the way to day 14, when the sporozoites have infected the salivary glands of the female. Indeed, the epidemiological significance of having more or fewer oocysts in the gut remains to be demonstrated: a single oocyst produces thousands of sporozoites, but as few as 10 of these sporozoites suffice to initiate a new infection in a host [26]. Thus, despite earlier studies showing a difference in *Plasmodium* oocystaemia in *Wolbachia*-infected mosquitoes, the question of whether natural *Wolbachia* infections can interfere with *Plasmodium* transmission in mosquitoes has not been entirely resolved.

2. Material and methods

(a) Mosquito lines

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

(b) *Plasmodium* strain and bird infections

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

(c) Mosquito experimental infections and dissections

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

To count oocysts in the mosquito gut, 20 blood-fed females of each line were haphazardly chosen from each cage 7–8 days post blood meal (dpbm) and dissected under a binocular microscope

in 100 µl of 0.01 M phosphate-buffered saline (PBS). One wing was also extracted and measured along its longest axis as an estimate of female size. The dissected midguts were stained with a 5% mercurochrome solution to assess infection rate (oocysts present/absent) and oocyst burden (number of oocysts) under a phase contrast microscope. The dissected abdomens (minus the midguts) were individually frozen at −20°C for the subsequent *Wolbachia* quantification. A similar procedure was carried out at day 14 pbm, when the sporozoites have migrated to the salivary glands. At this time, 40 blood-fed females from each mosquito line were haphazardly sampled from each of the cages. Females were first dissected to get rid of the midgut (at this stage, all oocysts in the midgut are expected to have burst), and then the mosquito was severed to separate the thorax (containing the salivary glands) and the abdomen, both of which were individually frozen at −20°C for the subsequent quantification of *Plasmodium* and *Wolbachia* infections, respectively.

(d) *Wolbachia* and *Plasmodium* sporozoite quantification

Real-time quantitative PCR was used to estimate the relative density of *Wolbachia* (abdomen) and *Plasmodium* sporozoites (thorax) in each mosquito. We carried out two PCRs on each of the body segments: one was specific for the *Culex ace-2* locus [30], and the other was either specific for the *Wolbachia wsp* locus [31] or for the mtDNA *cytb* gene of *Plasmodium*. For the latter, we used the primers CytSPO7F (5′-AGTTTCATGGATATGTGGTGGA-3′) and CytSPO10R (5′-AAAGATTTGGATAGAAGGGTATTT-3′). For each of the genes under study, the 5 µl reaction mixture contained 1 µl of template DNA (thorax at 5 ng µl^{-1} and abdomens at 10 ng µl^{-1}), 2.5 µl of 2X LightCycler DNA Master SYBR Green I (Roche Applied Science), 0.25 µl of primers at 10 µM and 1 µl of RNase-Free Water (QIAGEN). Amplification conditions were 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 s. Standard curves were plotted using dilutions of a pBluescriptKS vector containing one copy of each of the *ace-2*, *wsp* and *cytb* gene fragments. Each abdomen (or thorax) DNA template was analysed in triplicate for *ace-2* and *wsp* (or *cytb*) quantification. Assuming that each gene is present in a single copy per haploid genome, the ratio between the *wsp* (or *cytb*) and *ace-2* provides the number of *Wolbachia* (or *Plasmodium*) genomes relative to the *Culex* genomes.

(e) Statistical analysis

Analyses were carried out using the R statistical package (v. 2.12.0). The different statistical models built to analyse the data are described in the electronic supplementary material, table S2. The general procedure for building the statistical models was as follows: mosquito lines (w^{SL} and $w^{(-)}$), dissection day (7–8 days pbm) and mosquito wing size were fitted as fixed explanatory variables, whereas bird and qPCR plate were fitted as random explanatory variables. *Plasmodium* infection prevalence (proportion of mosquitoes containing at least one parasite; models 1–5, electronic supplementary material, table S2) was analysed using generalized linear mixed models with a binomial error distribution (lmer, lme4 package). *Plasmodium* infection intensity (oocyst and sporozoite loads) was analysed by including only individuals that became infected. As found in other systems [32], oocyst count data were greatly overdispersed. One way of handling this overdispersion is by using negative binomial pseudo distributions [32]. However, to our knowledge, it is not currently possible to account for negative binomial distributions within a mixed model *lmer* procedure. For this reason, we used instead a *glm* model with a negative binomial error distribution (*glm.nb*, MASS package; models 6 and 8; electronic supplementary material, table S2) and we fitted bird and qPCR plate as fixed factors, next to our variables of interest (i.e. mosquito strain, dissection day, mosquito wing size). Using fixed rather than mixed models results in some loss of statistical power, but the results are likely to be conservative [33]. Sporozoite load data were analysed using a *glm* model with a quasi-error distribution and a log link with a variance equal to μ^2 to correct for overdispersion (models 7 and 9). *Wolbachia* density was Box-Cox transformed [34] (models 10 and 11) and subsequently analysed using linear mixed-effect models (lme, nlme package). Differences in wing size between the lines were analysed using an ANOVA (aov). Maximal models, including all higher-order interactions, were simplified by sequentially eliminating non-significant terms and interactions to establish a minimal model [34]. The significance of the explanatory variables was established using a likelihood ratio test (LRT), which is approximately distributed as a χ^2 distribution [33]. The significant χ^2 values given in the text are for the minimal model [34]. Full dataset has been deposited in the Dryad Digital Repository (doi.org/10.5061/dryad.m3752).

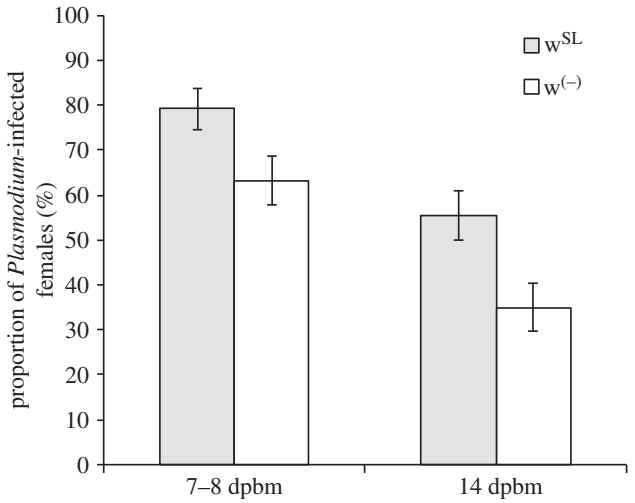

Figure 1. Effect of *Wolbachia* on the prevalence of *Plasmodium* infection 7 days (oocyst stage) and 14 days post blood meal (sporozoite stage). Bars represent means (±s.e.) for *Wolbachia*-carrying females (grey bars) and *Wolbachia*-free ones (white bars). dpbm, days post blood meal.

3. Results

During the blood meal, one infected canary died for an unknown reason, so this replicate was eliminated from all subsequent analyses. The percentages of mosquitoes that did not blood feed or died before the dissections are detailed in the electronic supplementary material, table S3. In the end, a total of 77 w^{SL} and 79 $w^{(-)}$ mosquitoes and 81 w^{SL} and 83 $w^{(-)}$ mosquitoes were dissected at the oocyst (day 7–8 pbm) and sporozoite (day 14 pbm) stages, respectively. Overall, $w^{(-)}$ females were smaller than w^{SL} ones (mean ± se, $w^{(-)}$ 3.52 ± 0.01 mm, w^{SL} 3.62 ± 0.01 mm, $\chi^2_1 = 8347$, $p < 0.0001$).

We first analysed whether *Wolbachia* influences *Plasmodium* prevalence. Our results show that the probability of becoming infected with *P. relictum* is significantly higher when *Wolbachia* is present (w^{SL}). This effect is consistent across the oocyst (probability of infection in w^{SL} is on average 15.9 ± 7.1% higher than in $w^{(-)}$, $\chi^2_1 = 5.42$, $p = 0.02$, model 1) and the sporozoite (20.6 ± 7.7% higher, $\chi^2_1 = 10.74$, $p = 0.001$, model 2) stages (figure 1). The combined analysis of the two measurement times revealed a mean (±s.e.) decrease of 26.2 (± 5.3) % in the *Plasmodium* prevalence between 7–8 and 14 dpbm

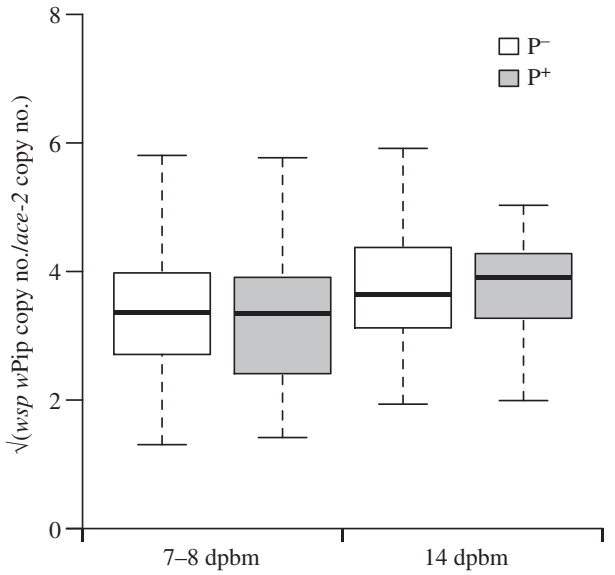

Figure 2. Boxplot of the *Wolbachia* density in w^{SL} females according to the *Plasmodium* infection status at 7–8 days (oocysts) and 14 days (sporozoites) post blood meal. White boxes: *Plasmodium* uninfected mosquitoes (includes females fed on a control bird and females that did not become infected 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.

(*Plasmodium stage* effect: $\chi^2_1 = 24.15$, $p < 0.0001$, model 3), irrespective of the presence of *Wolbachia* (*Wolbachia* × *Plasmodium stage* interaction: $\chi^2_1 = 0.02$, $p = 0.88$, model 3; figure 1). In w^{SL} females, the probability of becoming infected by *Plasmodium* when exposed to an infected bird is independent of the density of *Wolbachia* (oocysts: $\chi^2_1 = 0.21$, $p = 0.64$, model 4; sporozoites: $\chi^2_1 = 1.18$, $p = 0.28$, model 5). Reciprocally, the *Wolbachia* density in female abdomens did not differ between mosquitoes fed on a *Plasmodium*-infected or uninfected bird either at 7–8 dpbm ($\chi^2_1 = 2.84$, $p = 0.09$, model 10) or at 14 dpbm ($\chi^2_1 = 0.01$, $p = 0.91$, model 11; figure 2).

We then analysed whether *Wolbachia* influences intensity of the *Plasmodium* infection. The number of oocysts that successfully developed in the mosquito midgut is significantly higher in w^{SL} than in $w^{(-)}$ females ($\chi^2_1 = 4.95$, $p = 0.03$, model 6, figure 3*a*). w^{SL} females have on average three more oocysts than $w^{(-)}$ ones (mean ± s.e., 8.4 ± 1.4 and 5.7 ± 0.8 oocysts, respectively). By contrast, the relative quantity of sporozoites present in infected mosquito thoraxes is independent of the presence of *Wolbachia* ($\chi^2_1 = 0.69$, $p = 0.55$, model 7; figure 3*b*). As above, neither oocyst nor sporozoite load are correlated with *Wolbachia* density (oocyst: $\chi^2_1 = 2.64$, $p = 0.10$, model 8; sporozoite: $\chi^2_1 = 0.06$, $p = 0.84$, model 9; figure 4).

4. Discussion

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 number of *Plasmodium* oocysts in the midgut of mosquitoes. By contrast, and probably because of the difficulty in finding natural *Wolbachia* infections in epidemiologically significant malaria vectors, the role of natural *Wolbachia* infections in *Plasmodium* development has either been ignored entirely or been given only cursory attention. *Wolbachia*–mosquito combinations with a long evolutionary history may, however, be key for understanding what will happen with *Wolbachia*-transfected mosquitoes several generations down the line if, as has been shown in other systems [22,23], the novel *Wolbachia*–host interaction evolves rapidly. The number of generations needed for such evolutionary change can be between 20 [22] and 200 [23,35]. To our knowledge, the only previous studies carried out using natural *Wolbachia* infections involve the mosquito *Aedes fluviatilis* and the Asian avian malaria parasite *P. gallinaceum*. This work has shown that, far from decreasing parasitaemia, *Wolbachia* either has no effect [17,19] or increases [19] the number of *Plasmodium* oocysts in the midgut of the mosquito. *Aedes fluviatilis* is, however, a South American mosquito that serves as a convenient laboratory host for *P. gallinaceum*, but it is not its natural vector. Previous work has indeed shown that *Wolbachia* can render contrasting results on natural [23] and artificial [17,19] *Plasmodium* combinations, so the question that is relevant for the long-term success of malaria control programmes—of whether *Wolbachia* can interfere with *Plasmodium* transmission in an entirely natural system—is still unresolved.

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

Irrespective of the underlying mechanism, we believe that the epidemiological significance of having more or fewer *Plasmodium* parasites in the gut or even in the salivary glands remains to be demonstrated. As stated above, a single oocyst can produce between 2000 and 8000 sporozoites [37], and as few as 10 sporozoites suffice to start a new infection [26]. There is also no consistent evidence that the density of sporozoites in the salivary glands correlates with the number of infecting sporozoites [38], or that this correlates with the probability of a successful infection in the host (but see [39]). Mosquito infection intensity is, indeed, conspicuously absent from current models of malaria transmission and epidemiology [26,40]. Infection intensity may, however, bear on epidemiology if it correlates negatively with key life-history traits of the vector, such as longevity, but the evidence for this is sparse and comes from unrealistically high infections [41]. By contrast, infection prevalence, i.e. the number of infectious mosquitoes in a population, is the keystone of

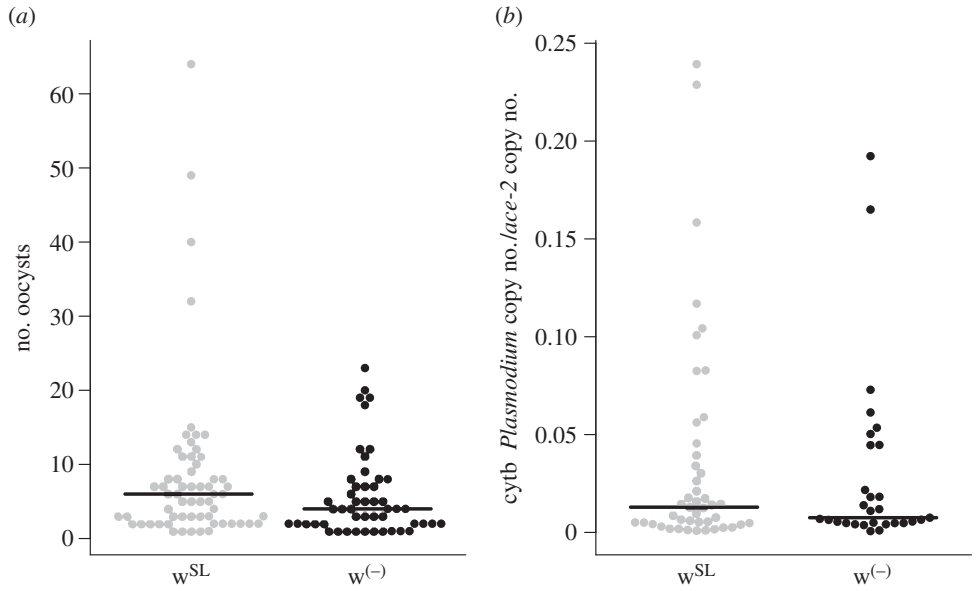

Figure 3. Effect of the presence of *Wolbachia* on *Plasmodium* burden in mosquitoes. (*a*) Distribution of the number of oocysts in the midgut of *Plasmodium*-infected females 7–8 days post blood meal, and (*b*) distribution of the relative quantity of sporozoites in the thorax of *Plasmodium*-infected females 14 days post blood meal, for *Wolbachia*-carrying females (grey circles) and *Wolbachia*-free ones (black circles). Horizontal lines represent medians.

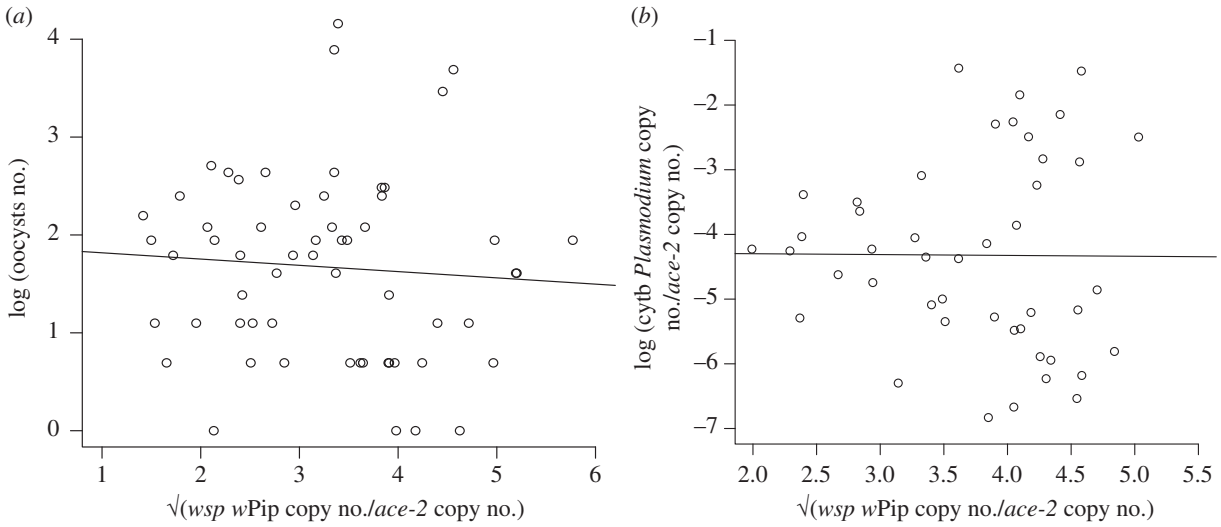

Figure 4. Correlation between the density of *Wolbachia* and the intensity of *Plasmodium* infection at the (*a*) oocyst and (*b*) sporozoite stages (7–8 days and 14 days post blood meal, respectively). Both *Wolbachia* and *Plasmodium* densities were Box-Cox transformed to linearize the data for the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

epidemiological models [26]. The proportion of infectious mosquitoes in a population, sometimes called the sporozoite rate, is a key determinant of the rate at which hosts are bitten in a population [26,40]. Here, we show that the presence of *Wolbachia* increases sporozoite prevalence by as much as 21%. *Wolbachia* does therefore play a major role in the transmission of *Plasmodium* in the avian malaria system.

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

With this in mind, several different, but non-exclusive, mechanisms may be envisaged to explain our results. First, we found that *Wolbachia*-infected mosquitoes were significantly bigger than *Wolbachia*-free ones and may thus have simply

taken larger blood meals, thereby increasing their intake of *Plasmodium* gametocytes (the stage that is transmissible to mosquitoes). We have previously shown that the number of *P. relictum* oocysts is significantly correlated with the amount of blood ingested by the mosquitoes, albeit in a non-linear way [29]. Second, *Wolbachia* may facilitate the successful establishment of *Plasmodium* within the mosquito tissues. One obvious way in which this could happen is through a *Wolbachia*-induced downregulation of the non-specific arm of the mosquito immune system, a form of self-protection that has been observed both in pill bugs (or woodlice) [48] and parasitoids [49]. In this respect, these natural *Wolbachia* infections would behave in a drastically different way to artificial infections, which are often found to upregulate the immune system when introduced into a novel host [12,13,15,17,45].

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

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

Previous work in this system has shown that *Plasmodium*-infected females suffer lower mortality rates if they are also infected with *Wolbachia* [27]. We had originally advanced two potential explanations for these results: *Wolbachia*-infected mosquitoes could be either more resistant or more tolerant to a *Plasmodium* infection. Under the first (resistance) scenario, *Wolbachia* would limit or inhibit parasite development, thereby reducing overall parasitaemia. Dawes *et al*. [41] have indeed shown that in rodent malaria the number of oocysts in the mosquito midgut is correlated with mosquito longevity, but their evidence comes from extremely high (100–2000) oocyst burdens. Under the second (tolerance) scenario, *Wolbachia* would limit or compensate for the damage incurred by the parasite, without necessarily altering the within-host growth rate of the parasite [52]. An increase in tolerance to pathogens has been previously observed with native *Wolbachia* strain of *Drosophila* flies when challenged with viruses [9,53]. Elucidating which of these mechanisms is at play is essential from a transmission perspective because parasite-resistant vectors are expected to be worse vectors of diseases, while the opposite will be true for parasite-tolerant ones (the 'tragedy of tolerance' [54]). The results of the present experiments show that *Wolbachia*-infected mosquitoes are in fact less resistant to *Plasmodium*, leaving a higher *Wolbachia*-associated tolerance to *Plasmodium* as the only potential explanation for the longevity results, the mechanisms underlying which remain to be explored.

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

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

Acknowledgements. We are grateful to S. Alizon and F. Vavre and the three anonymous referees for useful discussions and comments on the manuscript. We also thank N. Barougier, P. Boutinaud, J. Denoyelle, P. Perret and G. Sorci, for their help at different stages of the experiments.

Funding statement. This project is funded by the French ANR program (ANR 'IRMAL') to AR. AN was partly funded by an ERC starting grant to Sylvain Gandon, FZ was funded by a PhD grant from the CNRS and the Languedoc-Roussillon Region. This is contribution ISEM 2013-208 of the Institut des Sciences de l'Evolution de Montpellier (UMR 5554 CNRS – Université Montpellier 2).

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