Can *Wolbachia* modulate the fecundity costs of *Plasmodium* in mosquitoes?

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Abstract

Vertically transmitted parasites (VTPs) such as *Wolbachia* are expected not only to minimize the damage they inflict on their hosts, but also to protect their hosts against the damaging effects of coinfecting parasites. By modifying the fitness costs of the infection, VTPs can therefore play an important role in the evolution and epidemiology of infectious diseases.

Using a natural system, we explore the effects of a *Wolbachia–Plasmodium* co-infection on mosquito fecundity. While *Plasmodium* is known to frequently express its virulence by partially castrating its mosquito vectors, the effects of *Wolbachia* infections on mosquito fecundity are, in contrast, highly variable. Here, we show that *Plasmodium* drastically decreases the fecundity of mosquitoes by ca. 20%, and we provide the first evidence that this decrease is independent of the parasite's burden. *Wolbachia*, on the other hand, increases fecundity by roughly 10%, but does not alter the tolerance (fecundity–burden relationship) of mosquitoes to *Plasmodium* infection.

Although *Wolbachia*-infected mosquitoes fare overall better than uninfected ones, *Wolbachia* does not confer a sufficiently high reproductive boost to mosquitoes to compensate for the reproductive losses inflicted by *Plasmodium*. We discuss the potential mechanisms and implications underlying the conflicting effects of these two parasites on mosquito reproduction.

Introduction

In nature, parasites are usually found sharing the host's resources with an array of other symbionts, including parasites, pathogens, mutualists and microbiota. These co-infections can result in complex interactions, with potentially important implications for the evolution and epidemiology of diseases, as well as for the fitness and wellbeing of the hosts (Haine *et al.* 2005; Mideo, 2009; Jones *et al.* 2010). One case of particular interest is when co-infecting parasites have conflictive evolutionary interests, as is the case when horizontally transmitted parasites (HTPs) and vertically transmitted parasites (VTPs) co-occur in the same host (Ben-Ami *et al.* 2011; reviewed by Haine *et al.* 2005; Haine, 2008; Jones *et al.* 2010). VTPs and HTPs are both selected to minimize host mortality, as both benefit of the extended period over which transmission may occur. Conflicts are however expected to arise when it comes to the host's fecundity (Jones *et al.* 2010). VTPs are transmitted from mother to offspring and are thus under strong selection to minimize the damage to the host (Bull *et al.* 1991; Lipsitch *et al.* 1996). Instead, as vertical transmission is most efficient when host fecundity is high, VTP parasites may be expected to maximize their fitness by boosting the reproductive output of their hosts (Zug and Hammerstein, 2015; but see Baton *et al.* 2013). By contrast, HTPs are typically transmitted between unrelated hosts and are therefore not directly concerned by an increase in host fecundity. Instead, they will be selected to castrate their hosts if the host's energy is redirected to maximize parasite transmission (Jaenike, 1996; O'Keefe and Antonovics, 2002; Jones *et al.* 2010). Recent theoretical work has shown that such conflicts of interest can have important consequences for the evolutionary trajectory of both types of parasites, as well as for the fitness of the host (Jones *et al.* 2010).

The mosquito *Culex pipiens* is naturally infected by both parasite types: *Wolbachia*, a maternally inherited endosymbiotic bacterium whose prevalence in *Cx. pipiens* populations is close to 100% (Rasgon and Scott, 2003; Duron *et al.* 2005; Dumas *et al.* 2013), and the avian malaria parasite *Plasmodium relictum* SGS1 which is found infecting ca. 4% of field-collected *Cx. pipiens* mosquitoes in the South of France (Zélé *et al.* 2014*b*). *Wolbachia* spreads rapidly and efficiently in mosquito populations through cytoplasmic incompatibility (CI), a form of reproductive manipulation whereby infected females have a reproductive advantage over uninfected ones (Werren *et al.* 2008; Engelstadter and Hurst, 2009). In most cases CI is sufficient by itself to ensure the spread of non-mutualist *Wolbachia* variants through the host populations (Zug and Hammerstein, 2015). However, both models and field observations have shown that selection may favour variants that also increase the relative fecundity of the infected females (Turelli, 1994). These 'Jekyll and Hyde' infections, so-called because *Wolbachia* acts both as a beneficial symbiont and a reproductive parasite (Jiggins and

Hurst, 2011), are expected to be particularly efficient at invading and spreading across host populations (Zug and Hammerstein, 2015).

Plasmodium, on the other hand, is one of the HTPs that frequently expresses its virulence by partially castrating its vectors: several species of malaria parasites have been shown to significantly reduce the fecundity (number of eggs) and fertility (number of hatched larvae) of mosquitoes (reviewed in Hurd, 2003, 2009). The effects of *P. relictum* on *Cx. pipiens* fecundity are particularly acute, with highly infected mosquitoes laying up to 40% fewer eggs than their uninfected counterparts (Vézilier *et al.* 2012). Although neither the mechanistic nor the putative adaptive nature of these fecundity reductions have been entirely resolved, the reproductive curtailment induced by *Plasmodium* has been widely assumed to be an adaptive strategy of the parasite aimed at increasing mosquito survival (Schwartz and Koella, 2001; Ferguson *et al.* 2003; Hurd, 2003).

A previous work in this system has shown the existence of significant interactions between *Wolbachia* and *P. relictum* in mosquitoes: *Wolbachia* increases the susceptibility of *Cx. pipiens* mosquitoes to *P. relictum* (*Wolbachia*-infected mosquitoes have higher parasite burdens, Zélé *et al.* 2014*a*) and *Plasmodium*-infected females suffer lower mortality rates if they are also infected with *Wolbachia* (Zélé *et al.* 2012) suggesting that *Wolbachia* has a protective effect against *Plasmodium*-induced mortality and that, contrary to what has been shown in other systems (Hoffmann *et al.* 2015), *Wolbachia* increases mosquito tolerance to the infection.

Here, we explore the effects of *Wolbachia*–*Plasmodium* co-infections on mosquito fecundity. For this purpose we first carry out a two-way factorial experiment to determine the effect of *Wolbachia* and *Plasmodium,* singly or in co-infections, on the number of eggs laid by *Cx. pipiens* mosquitoes (Experiment 1). We make two specific predictions: (1) as a strictly VTP sharing a long co-evolutionary history with its host (Atyame *et al.* 2011), *Wolbachia* should have a positive effect on the fecundity of mosquitoes in the absence of a *Plasmodium* infection, and (2) in *Plasmodium*-infected mosquitoes the fecundity will be the net result of the balance between the *Wolbachia*-driven gains and the *Plasmodium*-driven losses, with the actual outcome depending on the relative strength of, and interactions between, these opposing physiological forces. In other words, we expect *Wolbachia* to (partially) protect mosquitoes against the *Plasmodium*-driven fecundity reductions.

To deepen our understanding of the protective effect of *Wolbachia* against *Plasmodium*-driven losses, we then carried out a second experiment where we aimed to establish whether there is a negative correlation between fecundity and *Plasmodium* infection intensity quantified as the number of oocysts in the midgut (Experiment 2). We call this our tolerance experiment because the slope of host fitness against infection intensity is also known as disease tolerance (Raberg *et al.* 2007) and, to our knowledge, it has never been quantified for *Plasmodium*-infected mosquitoes. Whether *Plasmodium* has an all-or-nothing (infected/uninfected) effect on mosquitoes, or whether its effects depend on the intensity of the infection has been the subject of some recent discussion (Churcher *et al.* 2017). A previous work has shown that *Plasmodium* burden correlates negatively with mosquito longevity (Dawes *et al.* 2009). We therefore further predict that: (3) the degree of fecundity reduction in mosquitoes will be proportional to the intensity of the infection, and that: (4) *Wolbachia* may be able to increase the tolerance (i.e. reduce the slope of the fitness–burden relationship) of the mosquitoes to a *Plasmodium* infection. We discuss the potential mechanisms and implications underlying the conflicting effects of these two parasites on mosquito reproduction.

Materials and methods

Mosquito and Plasmodium strains

We used two isogenic lines of *Cx. p. quinquefasciatus* that share the same nuclear genome, but differ in their *Wolbachia* infection. The first line, Slab (which, for simplicity reasons will be henceforth termed w^{SL}), was collected in California in 1954 (Georghiou *et al.* 1966) and is naturally infected by the *Wolbachia* wPip(Sl) strain (Duron *et al.* 2006*a*). The second line, w$^{(-)}$, was generated by an antibiotic (tetracycline hydrochloride) treatment of w^{SL} larvae for three consecutive generations to eliminate the *Wolbachia* infection (as described in Duron *et al.* 2006*b*). The w$^{(-)}$ line was initiated from over 10 000 w^{SL} larvae. To eliminate the potential side-effects of the tetracycline, the w$^{(-)}$ line was reared for ca. 25 generations before the experiment took place under standard laboratory, tetracycline-free, conditions.

We used one lineage of *P. relictum* known as SGS1. This *Plasmodium* lineage has been thus far recorded in 29 bird species belonging to eight families of the Passeriformes in Eurasia and Africa (MalAvi database, Bensch *et al.* 2009) and in 4% of the *Cx. pipiens* mosquitoes collected in the Montpellier region (France, Zélé *et al.* 2014*b*). The strain used in the experiments was isolated from wild sparrows collected in 2009 in the region of Dijon (France) and passaged to canaries (*Serinus canaria*) through intraperitoneal injection. The strain has been since maintained in our animal house by carrying out regular passages between our stock canaries every ca. 3 weeks. A diagnostic PCR technique (Waldenstrom *et al.* 2004) was carried out on all experimental canaries prior to the onset of the experiments to ensure that they were free from any previous *Plasmodium* infection. Each canary was tested five times to avoid false negatives.

Experiment 1

Newly hatched (L1) larvae from the two different mosquito lines were placed in plastic trays (34 cm × 23 cm × 7 cm) filled with 1 L of water (Eau de Source, Carrefour, France) at a constant density of 300 larvae per tray (*n* = 10 trays per line). The experiments took place under standard temperature (24 ± 2 °C), humidity (65 ± 5%) and photoperiod (12L:12D) conditions. Larvae were fed *ad libitum* on brewer's yeast on the first day and on ground Tetramin® fish flakes thereafter (2nd and 4th day: 200 mg; 6th day: 400 mg). Water and food were changed every two days. On day 7 post-hatching, each plastic tray was individually placed inside an 'emergence cage' (40 cm × 28 cm × 31 cm) and emerged adults were allowed to feed *ad libitum* on a 10% glucose water solution.

Experimental canaries (*n* = 10) were haphazardly allocated to one of two treatments: half of them were experimentally infected with our SGS1 *Plasmodium* lineage ('experimental cages'; birds parasitaemia ranged 0.87–2.43%), 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. Parasitaemia was regularly monitored from the tenth day of infection onwards using thin blood smears as described by Valkiūnas (2005). Mosquito blood feeding took place 10 days after the infection, to coincide with the acute phase of the parasitaemia (Vézilier *et al.* 2010). The parasitaemia and haematocrit (quantified as the Packed Cell Volume, PVC) of the bird were quantified immediately prior to the mosquito blood meal.

To explore the effect of *Plasmodium* and of *Wolbachia* on mosquito fecundity, groups of 75 adult *Cx. pipiens* females (6–8-day old) from each line (w^{SL} and w$^{(-)}$) were haphazardly chosen from the 20 emergence cages (10 per mosquito line) ca. 12 days after emergence and placed together to feed overnight inside an experimental cage (*n* = 5 infected cages, *n* = 5 control cages). To simplify the identification of the strains, 2 days before the blood

meal the mosquitoes were marked using a small amount (1 μg/female) of either pink or blue fluorescent powder (RadGlo® JST) applied as a dust storm (Service, 1993). Preliminary trials have shown that at this concentration the dust has no effect on mosquito survival and fecundity or parasite burden (Vézilier *et al.* 2012; Zélé *et al.* 2012), and is only detectable using a binocular microscope. The two colours were used in rotation to mark the two strains, so that the strain-colour code was switched from cage to cage.

On day 1 post-blood meal (pbm), all engorged females were placed individually in numbered plastic tubes (30 mL) covered with a mesh (haematin tubes). Food was provided in the form of a cotton pad soaked in a 10% glucose solution. Five days later (day 6 pbm), all mosquitoes were transferred to a new tube containing 4 mL of mineral water (Eau de Source Carrefour) to allow the females to lay their eggs (oviposition tubes). Haematin tubes were stored at 4 °C for subsequent quantification of the amount of haematin excreted at the bottom of each tube as an estimate of the blood meal size as described in (Vézilier *et al.* 2010). To obtain an estimate of the infection success (the infection status of dead mosquitoes cannot be established), on day 7 pbm, fifteen blood-fed females from each of the infected cages were haphazardly sampled from the tubes, and killed with CO_2. One wing was taken out and measured along its longest axis as an estimate of body size (this parameter is also been shown to positively correlate with female fecundity in *Aedes* mosquitoes; Briegel, 1990; Armbruster and Hutchinson, 2002), and their midguts were dissected in PBS (Standard Phosphate Buffered Saline) and examined under a microscope to assess oocyst prevalence and burden (Vézilier *et al.* 2010).

The oviposition tubes were provided daily with a cotton pad soaked in mineral water placed on top of each tube. In these conditions, 90% of the females lay their eggs in a single day in the form of one or several rafts (Vézilier *et al.* 2012). The oviposition tubes were checked daily for the presence of eggs. Egg rafts were photographed using a binocular microscope equipped with a numeric camera, after which they were put back in the insectary where they were checked daily until the emergence of the larvae. Larvae were killed by adding 5 mL of 100% ethanol to the tube, and tubes were stored at 4 °C for later counting. After oviposition, females were measured (wing length) and allocated to one of the two mosquito lines by examining their colour under a binocular microscope. Egg number was recorded by manually counting the number of eggs on the photographs using the Mesurim Pro freeware (Academie d'Amiens, France). Larvae were counted directly under the binocular microscope.

Experiment 2

To quantify fecundity and oocystaemia simultaneously, the procedure was identical to that of Experiment 1 save for a few differences. First, mosquito larvae were fed with 400 mg (instead of 200 mg) of ground Tetramin® fish flakes on the 4th day after hatching. Second, adult *Cx. pipiens* females from each line (w^{SL} and w$^{(-)}$) were placed to feed inside six experimental cages, each containing an infected bird (parasitaemia ranging from 1.55 to 28.9%; no uninfected birds were used in this experiment). Third, the number of females dissected per mosquito line and experimental cage was increased to 50, and dissections took place on days 8–10 pbm, after oviposition took place. Fourth, egg rafts were photographed for fecundity but not kept for fertility (larval hatching rate) assessment.

Statistical analysis

Analyses were carried out using the R statistical package (version 3.3.2). The different statistical models built to analyse the data are described in the Supplementary Materials Table S1). The general procedure for building the statistical models was as follows. Models were built by including mosquito lines [with, w^{SL}, or without, w$^{(-)}$, *Wolbachia*], parasite treatment (exposed to an infected or a control bird), haematin excreted (a proxy for blood meal size) and mosquito wing size as fixed explanatory variables, and experimental cage as a random explanatory variable. In addition, as egg production has been shown to be a saturating function of haematin quantity (Vézilier *et al.* 2010), the quadratic term haematin^2 was added to the minimal model when it improved significantly the model fit.

The response variables used in the analyses were either linearized using a Box-Cox transformation (Crawley, 2007) then analysed using a linear model with normal error distributions (fixed effects model: *lm*; mixed effects model: *lme*, package nlme), or analysed using a generalized linear mixed effect model (*glmer*, lme4 package) using the appropriate family of error distribution (e.g. binomial when the response variable was a proportion). Count (e.g. oocysts number) and proportion (e.g. hatching rate) data were often greatly over-dispersed. Several methods to deal with overdispersion are currently available such as using 'quasi' families in GLMs (Generalized Linear Models; Crawley, 2007), or explicitly modelling the source of extra-variation in the data (e.g., β-binomial or negative-binomial models). However, to our knowledge, it is not currently possible to account for quasi-, negative-binomial, or β-binomial distribution within the usual mixed model *glmer* procedure. For this reason, we used instead a mixed model glmmadmb procedure (glmmADMB package) from AD Model Builder (Fournier *et al.* 2012) with the appropriated error structure (Table S1).

Maximal models, including all higher-order interactions, were simplified by sequentially eliminating non-significant terms and interactions to establish a minimal model (Crawley, 2007). The significance of the explanatory variables was established using *F*-tests or a LRT (likelihood ratio test), which is approximately distributed as a χ^2 distribution (Bolker, 2008). The significant values given in the text are for the minimal model, while non-significant values correspond to those obtained before deletion of the variable from the model.

Ethical statement

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).

Results

Experiment 1

To explore the effect of *Plasmodium* and of *Wolbachia* on mosquito fecundity, groups of *Wolbachia*-infected (w^{SL}) and uninfected (w$^{(-)}$) adult *Cx. pipiens* females were fed on either *Plasmodium*-infected ($n = 5$) or uninfected birds ($n = 5$). Mosquitoes fed on four of the five infected birds showed a very high infection rate (% of infected mosquitoes in each of these cages ± s.e.: 61.5 ± 1.9, 88.0 ± 1.3, 89.3 ± 1.1 and 80.8 ± 1.5) whereas, for unknown reasons, in the fifth cage only 24.2 ± 1.3% of the mosquitoes became infected. For this reason, this cage was eliminated from further analyses. There was no significant difference in size between mosquitoes from the two different lines [adult wing size, mean ± s.e. 3.27 ± 0.01 and 3.29 ± 0.01 for

w$^{(-)}$ and w^{SL}, respectively; model 1, $F_{1,1087} = 2.97$, $P = 0.09$, Supplementary Materials Fig. S1.A; for this and all other analyses see Supplementary Materials Table S1]. Overall, w$^{(-)}$ females excreted marginally (albeit significantly) less haematin than w^{SL} ones (mean ± S.E., $15.47 ± 0.25$ μg and $16.94 ± 0.26$ μg, respectively, model 2, $\chi_1^2 = 7.31$, $P < 0.0001$, Fig. S1.B). In both lines, the amount of excreted haematin depended on both the haematocrit of the bird and on the presence of *Plasmodium* (*Plasmodium* × *haematocrit* interaction, model 2, $\chi_1^2 = 21.13$, $P < 0.0001$, Fig. 1; Fig. S2). There were, however, no significant differences between the two mosquito lines either in the prevalence (probability of containing at least one oocyst, model 3, $\chi_1^2 = 0.79$, $P = 0.37$) or in the intensity (number of oocytsts; model 4, $\chi_1^2 = 0.02$, $P = 0.89$) of the infection (Supplementary Materials Table S2).

Once the females were transferred to the oviposition tube, it took them on average 3 days to lay their eggs. The number of eggs laid by females (henceforth fecundity) was strongly dependent on whether the females were infected or not by *Wolbachia*: egg rafts of w^{SL} females contained on average $17 ± 3$ eggs more than rafts from w$^{(-)}$ (model 5: $\chi_1^2 = 37.48$, $P < 0.0001$). Fecundity was also strongly dependent on whether the females were infected or not by *Plasmodium*: egg rafts of *Plasmodium*-infected females contained on average $32 ± 3$ fewer eggs than rafts from their uninfected counterparts (model 5, $\chi_1^2 = 7.76$, $P = 0.005$, Table S2). There was, however, no significant interaction between the infection by both parasites: the *Plasmodium*-induced fecundity reduction was not buffered by the presence of *Wolbachia* (model 5, $\chi_1^2 = 2.32$, $P = 0.13$, Fig. 2A). As expected, haematin, its quadratic term haematin^2, and mosquito size were found to be strong predictors of the amount of eggs laid (model 6, haematin: $\chi_1^2 = 87.80$, $P < 0.0001$, Fig. 3A; haematin^2: $\chi_1^2 = 29.01$, $P < 0.0001$; wing length: $\chi_1^2 = 10.08$, $P = 0.001$). However, neither haematin, haematin^2, nor mosquito size affect the significance of *Wolbachia*, *Plasmodium* or their interaction in the model (model 5 *vs* model 6).

On average, 97% of the egg rafts laid by *Cx. pipiens* females were viable (i.e. they showed non-null hatching rates; Table S2).

This proportion was positively correlated with the number of eggs contained in the raft (model 7, $\chi_1^2 = 58.92$, $P < 0.0001$) but was not influenced by either *Plasmodium* or *Wolbachia* (model 7, $\chi_1^2 = 0.02$, $P = 0.88$ and $\chi_1^2 = 0.34$, $P = 0.56$, respectively). The proportion of larvae hatched in each raft (hatching rate, Table S2) was strongly dependent on the number of eggs contained in the raft, but also on the amount of excreted haematin (model 8, $\chi_1^2 = 98.82$, $P < 0.0001$, and $\chi_1^2 = 46.66$, $P < 0.0001$, respectively). However, there was no effect of either *Wolbachia*, *Plasmodium*, or their interaction on hatching rate (model 8, $\chi_1^2 = 0.14$, $P = 0.71$, $\chi_1^2 = 1.80$, $P = 0.18$ and $\chi_1^2 = 1.68$, $P = 0.19$, respectively; Fig. 2B).

Experiment 2

In this experiment, fecundity and oocystaemia were quantified simultaneously for w^{SL} and w$^{(-)}$ adult *Cx. pipiens* females fed on six different *Plasmodium*-infected bird (no uninfected birds were used in this experiment). For unknown reasons, one of the birds resulted in extraordinary high number of oocysts in mosquitoes (up to 1825 oocysts; mean ± S.E. $487 ± 49$) compared to all the other ones combined (mean ± S.E. $25 ± 2$ oocysts). Therefore, we decided to exclude this bird from the analysis. Despite being kept under identical conditions, and in several rearing trays, w$^{(-)}$ mosquitoes were significantly larger than w^{SL} mosquitoes (mean ± S.E. $3.66 ± 0.01$ and $3.62 ± 0.01$ for w$^{(-)}$ and w^{SL}, respectively; model 9, $F_{1,306} = 5.04$, $P = 0.03$; Fig. S1.A; Table S2). As was the case in Experiment 1, the amount of haematin excreted by the two mosquito lines depended on the haematocrit of the bird (model 10, $\chi_1^2 = 98.56$, $P < 0.0001$, Fig. 1). In both strains, the number of eggs laid is strongly correlated with haematin (and its quadratic term haematin^2, model 14, haematin: $\chi_1^2 = 26.42$, $P < 0.0001$; haematin^2: $\chi_1^2 = 10.48$, $P = 0.001$, Fig. 3B) and mosquito size ($\chi_1^2 = 13.94$, $P = 0.0002$). As a result, w$^{(-)}$ females laid a significantly higher number of eggs than w^{SL} ones (mean ± S.E. $188 ± 4$ and $166 ± 3$ for w$^{(-)}$ and w^{SL}, respectively; model 14, $\chi_1^2 = 14.40$, $P = 0.0001$, Fig. 4).
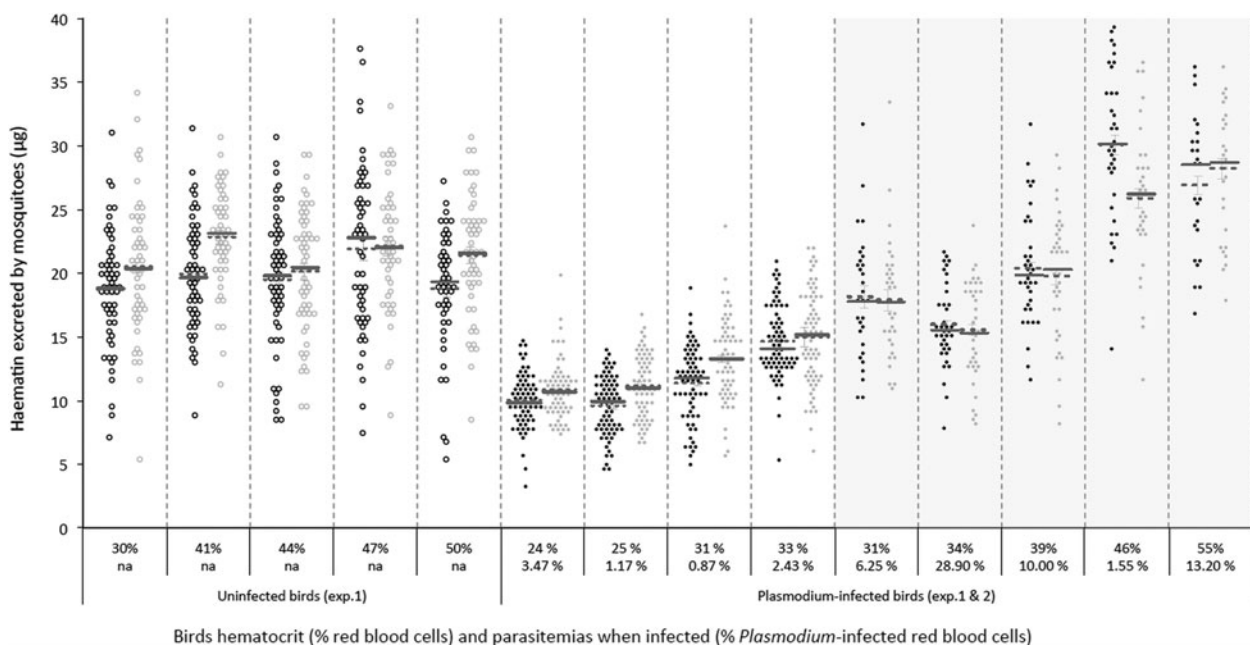
Fig. 1. Distribution plot of the amount of haematin excreted by both *Cx. pipiens* lines according to the haematocrit (quantified as % Packed Cell Volume; upper level x-axis labels) and the *Plasmodium* infection intensity (quantified as % red blood cells infected; second level x-axis labels) of the birds they fed on. *Wolbachia*-infected (w^{SL}; grey circles) and uninfected (w$^{(-)}$; black circles) mosquitoes exposed (full circles) or not (empty circles) to *Plasmodium* infected blood in Experiment 1 (light background) and 2 (shaded background; this experiment only includes *Plasmodium*-infected females). Horizontal lines represent means (dotted line) and medians (solid lines).
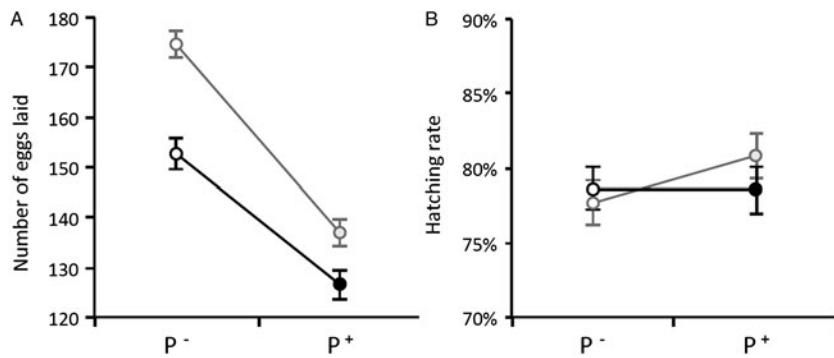
Fig. 2. *Wolbachia* and *Plasmodium* infection effects on *Cx. pipiens* fecundity (A) and egg hatching rate (B) in Experiment 1. Figures represent means ± s.e. Grey circles: w^{SL} females, black circles: $w^{(-)}$ females. Empty circles: females fed on a control bird (P^-), full circles: females fed on a *Plasmodium*-infected bird (P^+). Only mosquitoes whose rafts were productive (i.e. from which at least one larva emerged) were included in the analysis.

Contrary to expectations, we found no negative relationship between mosquito fecundity and the intensity of the *Plasmodium* infection, quantified as the number of oocysts in the mosquito midgut (model 15, $\chi_1^2 = 0.90$, $P = 0.34$), and no effect of *Wolbachia* on the slope of the fecundity-intensity regression (model 15, oocysts*Wolbachia interaction: $\chi_1^2 = 1.53$, $P = 0.22$; Fig. 4). Similarly, no significant differences between the two mosquito lines were found either in the prevalence (probability of containing at least one oocyst, model 11, $\chi_1^2 = 0.04$, $P = 0.83$) or in the intensity of infection (number of oocysts; model 12, $\chi_1^2 = 0.46$, $P = 0.50$; Table S2; Fig. S2).

Discussion

Multiple infections can have many different outcomes on the host's life history traits. Most often than not, these co-infections have cumulative deleterious effects on the host, which are mediated by a higher parasite diversion of host resources or to cumulative damages to the hosts due to a greater overall parasite load (Morand, 2011). However, in certain situations, co-infections may lead to lower virulence than single infections, most notably when parasites competitively suppress each other within the host (Balmer et al. 2009; Rodrigues et al. 2016). Co-infections involving HTPs and VTPs are also expected to have non-cumulative effects on the host because of the conflicting evolutionary interests associated with these two different transmission strategies. HTPs may be selected to castrate their hosts if this leads to increased transmission (Jaenike, 1996; O'Keefe and Antonovics, 2002), while VTPs are strongly predicted to have a protective effect on host fecundity (Smith and Dunn, 1991; Ebert and Herre, 1996). Our results show that *Plasmodium* drastically decreases the fecundity of *Cx. pipiens* females by about 20% (Fig. 2A), and that this decrease is all-or-nothing effect which is independent of the parasite's burden (Fig. 4). *Wolbachia*, on the other hand increases fecundity by roughly 10%, irrespective of whether the mosquito is infected or not by *Plasmodium* (Fig. 2A) but does not alter the tolerance (quantified as the slope of the fecundity-burden relationship) of mosquitoes to a *Plasmodium* infection (Fig. 4). Overall, although *Wolbachia*-infected mosquitoes fare better than uninfected ones, *Wolbachia* does not confer a sufficiently higher reproductive boost to mosquitoes to compensate for the large reproductive losses inflicted by *Plasmodium*. Below, we discuss the potential mechanisms and implications underlying the conflicting effects of these two symbionts on mosquito reproduction.

Plasmodium effects on fecundity

Drastic reductions in mosquito fecundity are a common outcome of *Plasmodium* infections (reviewed in Hurd, 2003, 2009), and agree with previous results obtained in our laboratory with this same experimental system (Vézilier et al. 2012). The reproductive curtailment induced by *Plasmodium* has been widely assumed to be an adaptive strategy of the parasite aimed at increasing mosquito survival (Schwartz and Koella, 2001; Ferguson et al. 2003; Hurd, 2003). This appealing hypothesis rests on the crucially important role of mosquito longevity for *Plasmodium* transmission (Vézilier et al. 2012). To date, however, the issue of whether the *Plasmodium*-driven fecundity reduction is an adaptive strategy of the parasite aimed at increasing longevity or whether it is simply the result of a competition for nutrients between the parasite and the mosquito remains unresolved.

In *Anopheles* mosquitoes, *Plasmodium* seems to reduce fecundity through a combination of an impaired intake of yolk protein by the ovaries (Hogg et al. 1997; Ahmed et al. 2001), and an increase in egg resorption (Carwardine and Hurd, 1997; Hopwood et al. 2001) mediated by follicular cell apoptosis (Ahmed et al. 2001). Whether similar mechanisms operate in
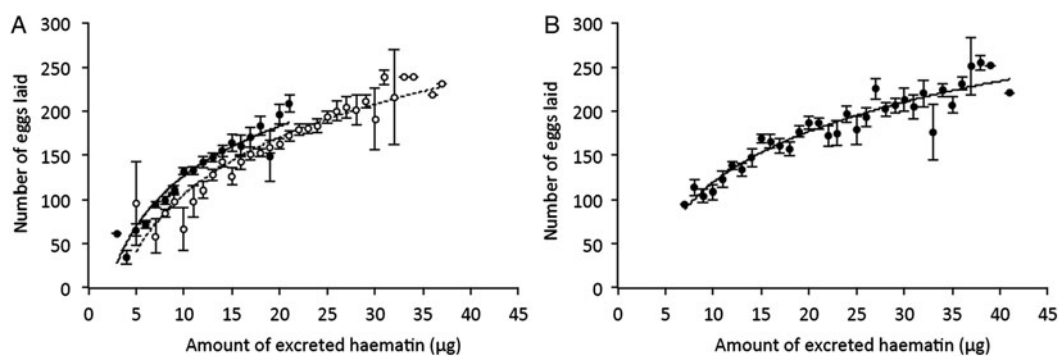
Fig. 3. Relationship between the amount of haematin excreted and the number of eggs laid by *Cx. pipiens* females in Experiments 1 (A) and 2 (B). Figures represent means ± s.e. number of eggs laid for each haematin value (haematin values were rounded up to the nearest integer) for *Plasmodium*-infected (full circles) and – uninfected (empty circles) mosquito females (i.e. independently of their *Wolbachia* infection status; Experiment 2 only includes *Plasmodium*-infected females). The fitted curves correspond to the logarithmic trend of the data plot.
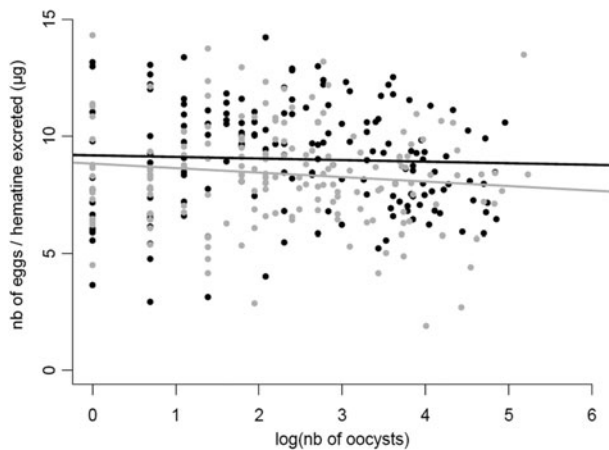
Fig. 4. Relationship between the number of oocysts in the midgut of *Cx. pipiens* females and the number of eggs they laid in Experiment 2. The number of eggs laid has been corrected by the amount haematin excreted (μg) for each mosquito female. The number of oocysts has been log transformed to improve the fit of the model. The fitted lines correspond to the linear trend of the data plots. Grey dots and line: w^{SL} females, black dots and line: w$^{(-)}$ females.

Plasmodium-infected *Cx. pipiens* mosquitoes remains to be established. Reduction in fecundity may also operate through a *Plasmodium*-driven reduction in the quantity or the quality of the blood meal, either because nutrients are scavenged by the parasite or as a host's response to the infection. *Plasmodium* infected hosts are strongly anaemic (Experiment 1, Fig. 1), and anaemia reduces the amount of protein available for egg production. As a result, mosquitoes having fed on a *Plasmodium*-infected bird excreted significantly less haematin than uninfected ones (Fig. S1.B). Comparison of uninfected and infected birds from Experiment 1 (Fig. 1), and a significant interaction between haematocrit and *Plasmodium* infection, however, suggest that haematin excretion is lower in *Plasmodium* infected mosquitoes despite similar haematocrit values, suggesting that, in addition to feeding on poor quality blood, mosquitoes feeding on infected birds may also have taken smaller blood meals (note that such comparison is not possible in Experiment 2 as all birds were infected). Finding a satisfactory method to quantify blood meal size that is independent of the amount of haemoglobin in the blood, the main drawback of haematin, would go a long way towards disentangling the effects of anaemia and clarifying the central role seemingly played by blood meal size in these interactions (Pigeault *et al.* 2015).

Wolbachia effects on fecundity

Although *Wolbachia* is a VTP and therefore would benefit from increasing mosquito fecundity, the ability of this parasite to induce CI allows it to invade host populations even in the absence of a fitness advantage. Invasion has even been observed when *Wolbachia* exerts a negative effect on key host fitness components such as fecundity (Xi *et al.* 2005; Hoffmann *et al.* 2011; Walker *et al.* 2011; Nguyen *et al.* 2015). Our results from Experiment 1, however, show a beneficial effect of *Wolbachia* on *Cx. pipiens* fecundity. The previous work on natural systems has shown either a beneficial effect (Dobson *et al.* 2002; Baton *et al.* 2013) or no effect (Rasgon and Scott, 2003) of *Wolbachia* infection status on mosquito fecundity. These results however contrast with the fecundity reductions found in artificial *Wolbachia*–mosquito combinations (Xi *et al.* 2005; McMeniman *et al.* 2009; Joshi *et al.* 2014; but see Fu *et al.* 2010). Although the *Wolbachia*-induced fecundity boost persists when mosquitoes are infected with a partially castrating parasite such as *Plasmodium*, the

increase is not sufficient to compensate for the drastic *Plasmodium*-driven losses (Fig. 2A).

These results could not be replicated in Experiment 2. In Experiment 2, mosquitoes were significantly larger than those in Experiment 1, and w$^{(-)}$ females were significantly larger than w^{SL} females (Fig. S1.A). One potential explanation for these size differences is that they may have been the result of an unintentional increase in larval food dose on one of the feeding days (see above), although it is less clear to us why this would have given a size advantage to the *Wolbachia*-free line. Irrespective of the underlying mechanism, the result of this experiment is likely to have been biased by this unexplained size differences between the two mosquito lines, as size is positively correlated with both blood meal size and fecundity.

The mechanisms underlying this increase in fecundity are unknown. In *Drosophila mauritiana* flies, *Wolbachia*-infected females produce about four times more eggs than their non-infected counterparts through the combined action of an increased mitotic activity, and a decreased programmed cell death (apoptosis) in the fly's germarium (Fast *et al.* 2011). Inhibition of apoptosis by *Wolbachia* was also found to be necessary for oogenesis completion in the wasp *Asobara tabida* (Bazzocchi *et al.* 2007; Pannebakker *et al.* 2007). Most remarkably, *Wolbachia* have the genetic machinery to influence iron utilization of hosts (Brownlie *et al.* 2009; Kremer *et al.* 2009). Indeed, in *Wolbachia* the expression level of iron metabolism genes is directly influenced by the presence of iron in the diet (Kremer *et al.* 2009) and this regulation can confer fecundity benefit for hosts reared on iron-restricted or -overloaded diets (Brownlie *et al.* 2009). As iron is an abundant compound of vertebrate blood, *Wolbachia* may thus play a pivotal, albeit unrecognized, role as nutritional mutualist in mosquitoes. In this regard, a recent observation in Anopheline mosquitoes is worthy of interest: *Wolbachia* has the capacity to increase the rate at which eggs were laid (Shaw *et al.* 2016), suggesting that it accelerates blood meal digestion and/or egg maturation. Further work needs to be carried out in our system to establish the mechanism underlying the increase in fecundity observed in *Wolbachia*-infected mosquitoes.

Wolbachia effects on tolerance to Plasmodium

The slope of loss of fitness with parasite burden has been termed 'tolerance' and is an essential concept in disease ecology as it can have drastic consequences for both the evolution and epidemiology of diseases (Raberg *et al.* 2007; Read *et al.* 2008). Partial castration is the most common virulence phenotype in mosquitoes infected with *Plasmodium*, with up to 40% of eggs being lost to the infection (Vézilier *et al.* 2012). However, to our knowledge, no study has investigated whether the loss of eggs in mosquitoes is a function of *Plasmodium* burden, although an early report comparing the fecundity of mosquitoes with low and high oocyst burdens showed no significant difference between the two groups (Hogg and Hurd, 1995). Here we show, for the first time, that the intensity of the *Plasmodium* infection is not correlated with the degree of reproductive loss (Fig. 4) which suggests that resource competition may not be a major driver of the fecundity reduction. These results contrast with the correlation found between parasite density and mosquito survival rates, albeit in a non-natural mosquito–*Plasmodium* combination (Dawes *et al.* 2009).

While most studies are entirely focused on tolerance as a genetically determined trait, environmental factors such as resource availability, can also influence tolerance (Sternberg *et al.* 2012). To our knowledge, the role of endosymbiotic bacteria as a factor in tolerance to disease in mosquito vectors has never been explicitly investigated. Here we show that, under our particular set of experimental conditions, *Wolbachia* does not have an effect on

the slope of mosquito fecundity and *Plasmodium* burden. Further work needs to be done to establish whether mosquito tolerance to *Plasmodium* may be modulated by other environmental factors such as temperature (Murdock *et al.* 2014) or nutrient availability (Caragata *et al.* 2016).

Concluding remarks

This is the first study aiming to determine the outcome of the co-infection between *Wolbachia* and *Plasmodium* on mosquito fecundity. We show that, *Wolbachia*-infected mosquitoes fare better in terms of fecundity than their uninfected counterparts but that *Wolbachia* does not protect its host against the detrimental effect of *Plasmodium* on their fecundity. These and previous results showing that *Wolbachia* buffers the effects of *Plasmodium* on mosquito longevity (Zélé *et al.* 2012) support the hypothesis by which *Wolbachia*-carrying females are generally in better condition than those from which *Wolbachia* has been cleared, lending them more resilient against the negative effects of concomitant parasites. This so-called 'Jeckyll and Hide' strategy may contribute to explain why *Wolbachia* is near to or at fixation in worldwide populations of the *C. pipiens* complex (Duron *et al.* 2005). Our results thus open up interesting question about the potential implications of *Wolbachia* on the transmission dynamic of malaria parasites and may have implications for the use of *Wolbachia* as a potential tool to combat malaria.

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