JOURNAL OF Evolutionary Biology

doi: 10.1111/jeb.12863

Influence of oxidative homeostasis on bacterial density and cost of infection in *Drosophila–Wolbachia* symbioses

D. MONNIN*, N. KREMER*, C. BERNY*, H. HENRI*, A. DUMET†, Y. VOITURON†, E. DESOUHANT* & F. VAVRE*

*Laboratoire de Biométrie et Biologie Evolutive, Univ Lyon, Université Claude Bernard, CNRS, UMR 5558, F-69622 Villeurbanne, France
†Laboratoire d'Ecologie des Hydrosystèmes Naturels et Anthropisés, Univ Lyon, Université Claude Bernard, CNRS, UMR 5023, F-69622 Villeurbanne, France

Keywords:

coevolution;
immunity;
oxidative homeostasis;
symbiosis;
tolerance;
Wolbachia.

Abstract

The evolution of symbioses along the continuum between parasitism and mutualism can be influenced by the oxidative homeostasis, that is the balance between reactive oxygen species (ROS) and antioxidant molecules. Indeed, ROS can contribute to the host immune defence to regulate symbiont populations, but are also toxic. This interplay between ROS and symbiosis is notably exemplified by recent results in arthropod–*Wolbachia* interactions. *Wolbachia* are symbiotic bacteria involved in a wide range of interactions with their arthropods hosts, from facultative, parasitic associations to obligatory, mutualistic ones. In this study, we used *Drosophila–Wolbachia* associations to determine whether the oxidative homeostasis plays a role in explaining the differences between phenotypically distinct arthropod–*Wolbachia* symbioses. We used *Drosophila* lines with different *Wolbachia* infections and measured the effects of pro-oxidant (paraquat) and antioxidant (glutathione) treatments on the *Wolbachia* density and the host survival. We show that experimental manipulations of the oxidative homeostasis can reduce the cost of the infection through its effect on *Wolbachia* density. We discuss the implication of this result from an evolutionary perspective and argue that the oxidative homeostasis could underlie the evolution of tolerance and dependence on *Wolbachia*.

Introduction

Symbiosis is defined as a long-term interaction between organisms belonging to different species, typically a host and its symbionts, and encompasses parasitism as well as mutualism (Frank, 1877; de Bary, 1879). The position of the symbiotic relationship along this parasitism–mutualism continuum predominantly depends on the molecular interplay taking place between the partners (Hentschel *et al.*, 2000) and its consequences on each other's life-history traits.

Among the molecules that mediate the interaction between host and symbionts, reactive oxygen species (ROS) play an important role (Moné *et al.*, 2014). In eukaryotes, ROS are mostly produced by mitochondria as a by-product of oxidative phosphorylation, but also, in a more controlled way, by NADPH oxidases such as DUOX (Schieber & Chandel, 2014). ROS are detrimental to many cellular components when in excess, a state known as oxidative stress, causing lipid peroxidation as well as degradations to nucleic acids and proteins (Imlay, 2013). Some molecules prevent these oxidative damages and include enzymatic (e.g. superoxide dismutase, catalase) as well as nonenzymatic antioxidants (e.g. C vitamin). Additionally, ROS function as signalling molecules (Schieber & Chandel, 2014) and are also part of the immune response (Nathan & Cunningham-Bussel, 2013). In insects for instance, DUOX-derived ROS are involved in the control of gut bacterial populations (Ha *et al.*, 2009; Lee *et al.*, 2013; see Douglas, 2014 for a review). ROS therefore affect host–symbiont interactions through their effects on both partner's physiologies. As a consequence, the oxidative homeostasis, that is the balance between ROS and

Correspondence: David Monnin, UMR CNRS 5558 – LBBE 'Biométrie et Biologie Évolutive' UCB Lyon 1 – Bât. Grégor Mendel, 43 bd du 11 novembre 1918, 69622 Villeurbanne Cedex, France
Tel.: 04 72 43 19 21; fax: 04 72 43 13 88;
e-mail: david.monnin@univ-lyon1.fr

antioxidant molecules, is a crucial factor underlying trade-offs among life-history traits (Costantini, 2014) and many aspects of symbiosis (Moné *et al.*, 2014). In addition to the gut bacteria already mentioned, insects also frequently harbour intracellular bacteria, such as the alpha-proteobacterium *Wolbachia*, which may be affected by the oxidative homeostasis as well.

Wolbachia is a maternally transmitted intracellular bacterium infecting many species of nematodes (Taylor *et al.*, 2013) and arthropods (Zug & Hammerstein, 2012). In the latter, although primarily known as a manipulator of host reproduction (Werren *et al.*, 2008), *Wolbachia* sometimes acts as a protective symbiont (Hedges *et al.*, 2008; Teixeira *et al.*, 2008) and can also be needed by its host for nutrition (Hosokawa *et al.*, 2010) or oogenesis (Dedeine *et al.*, 2001). In addition, its effects on the host life-history traits vary greatly and can evolve rapidly (Weeks *et al.*, 2007; Chrostek & Teixeira, 2015). Survival, in particular, can be positively (Dobson *et al.*, 2004; Alexandrov *et al.*, 2007) or negatively affected by the infection (Fleury *et al.*, 2000; Vasquez *et al.*, 2011). The wide variety of host–*Wolbachia* interactions raises two questions: (i) What are the mechanisms underlying such differences? and (ii) How do transitions between different kinds of symbiosis occur? Studies of the parasitoid wasp *Asobara tabida* suggest that the oxidative homeostasis could be involved in a dramatic evolutionary transition from facultative to obligatory symbiosis (Kremer *et al.*, 2009, 2010). Other studies give support to this hypothesis and suggest a general interaction between *Wolbachia* and the oxidative homeostasis.

First, *Wolbachia* infection can impact the oxidative homeostasis. Individuals or cells infected by *Wolbachia* exhibit a higher level of oxidative stress compared to uninfected ones, as shown in different hosts (Brennan *et al.*, 2008; Pan *et al.*, 2012; Wong *et al.*, 2015). In the mosquito *Aedes aegypti*, the oxidative stress is due to an increase in the activity of DUOX (Pan *et al.*, 2012). This suggests that *Wolbachia* disrupts the oxidative homeostasis by triggering an immune response, even though other mechanisms could contribute. Indeed, based on genomic and transcriptomic data, Darby *et al.* (2012) suggest that *w*Oo (the *Wolbachia* strain infecting the nematode *Onchocerca ochengi*) can perform oxidative phosphorylation, another potential source of ROS. On the opposite, *Wolbachia* may be able to limit oxidative stress by producing antioxidant enzymes, such as bacterioferritin (Brennan *et al.*, 2008; Kremer *et al.*, 2009).

Second, the oxidative homeostasis affects *Wolbachia* density. The treatment of cultured cells of the mosquito *Aedes albopictus* with a pro-oxidant (paraquat) eliminates *Wolbachia* (Fallon *et al.*, 2013) whereas in *Drosophila simulans* infected by its native strain *w*Ri, the treatment of flies with a nonenzymatic antioxidant (glutathione) leads to an increase in *Wolbachia* density (Brennan *et al.*, 2012). These results suggest that the oxidative stress could contribute to the control of the *Wolbachia* density, a parameter that partly determines the position of the interaction along the parasitism–mutualism continuum. Indeed, the density of *Wolbachia* can be positively correlated with the fitness cost that *Wolbachia* imposes on its host (McGraw *et al.*, 2002; Mouton *et al.*, 2004; Chrostek *et al.*, 2014), as most spectacularly exemplified by *w*MelPop, a *Wolbachia* strain initially found in a *Drosophila melanogaster* laboratory stock, whose excessive replication at high temperature is detrimental to its host (Min & Benzer, 1997; Reynolds *et al.*, 2003; Strunov *et al.*, 2013). It should be noted, however, that the relationship between *Wolbachia* density and fitness cost is probably not linear, and therefore not observable within the whole range of possible densities, notably at densities at which *Wolbachia* is not costly.

Taken together, these results suggest that the regulation of the oxidative homeostasis is an important driver of host–*Wolbachia* coevolution (Moné *et al.*, 2014; Zug & Hammerstein, 2015a). In particular, the modulation of the oxidative homeostasis could be involved in the reduction in the cost of *Wolbachia*. To test this hypothesis, we performed experimental manipulations of the oxidative homeostasis, using pro-oxidant (paraquat) and antioxidant (glutathione) treatments, to determine (i) whether the cost of infection, here measured as reduced survival, is modulated by the oxidative homeostasis, and (ii) whether this effect is due to variations in the *Wolbachia* density. These experiments were performed on lines of *D. melanogaster* infected by a highly virulent *Wolbachia* strain, *w*MelPop, a commensal strain, *w*Mel, or uninfected. Three independent experiments were performed (i) to evaluate the reliability of the results, (ii) to perform measurements of the effect of treatments on oxidative homeostasis and (iii) to include a *D. simulans* line infected with its native *w*Ri strain, making possible the comparison with results from the literature. We show that experimental manipulations of the oxidative homeostasis generally affected *Wolbachia* density, but not in the same way in the different associations. In addition, the reduction in the density of *w*MelPop led to a lower cost of infection on the host survival. Insofar as our experimental manipulations can be thought to mimic endogenous oxidative homeostasis modulations, these results suggest that the oxidative homeostasis underlies part of the cost of infection in natural conditions and may therefore be involved in shifts along the parasitism–mutualism continuum through host–symbiont coevolution.

Material and methods

Biological systems

We used three lines of *D. melanogaster*^{w1118}, sharing the same nuclear background: one uninfected by *Wolbachia*, one infected by the virulent strain *w*MelPop (Min &

Benzer, 1997) and one infected by the natural, roughly commensal, *w*Mel. These lines were provided by Scott O'Neill (Monash University, Australia). As they were maintained in the laboratory for a long time, their nuclear background was homogenized by two generations of crossing between females from both infected lines and uninfected males before the experiments. To compare our results with those already published by Brennan *et al.* (2012), we also included one line of *D. simulans* naturally infected by *w*Ri, which was established from individuals captured in 2012 in Botswana and provided by Michel and Jocelyne Boulétreau. Flies were maintained under controlled rearing conditions (18 °C, 12 LD cycle).

Experimental manipulations of the oxidative homeostasis: paraquat and glutathione treatments

Drosophila larvae were fed on standard medium (David & Clavel, 1965) unsupplemented (control condition) or supplemented with either paraquat or L-glutathione reduced (Sigma-Aldrich, St. Louis, MO, USA). The concentration of glutathione was 0.22 mM, as in Brennan *et al.* (2012). The concentration of paraquat, 1 mM, was determined during a preliminary experiment to ensure that the dose would neither be lethal, nor biologically negligible (data not shown). The number of developing larvae was controlled by depositing 100 eggs on 1.5 g of supplemented or unsupplemented medium.

This oxidative treatment procedure was repeated in three independent experiments. In the first experiment, we assessed on the three *D. melanogaster* lines the effects of glutathione and paraquat on symbiotic density and survival.

As Brennan *et al.* (2012) have shown that glutathione increases *w*Ri density in *D. simulans*, and as we did not observe such effects on neither *w*Mel nor *w*MelPop in *D. melanogaster*, we included a line of *w*Ri-infected *D. simulans* in the second experiment. We also aimed to assess the physiological impact of the treatments on oxidative homeostasis by measuring the antioxidant capacity and the lipid peroxidation.

Given some discrepancies between our survival data in experiments 1 and 2, we then performed a third experiment to assess the robustness of our results. This experiment was conducted at 29 °C instead of 25 °C to increase *w*MelPop virulence, providing better conditions to test the effects of the treatments on survival. In this last experiment, we also aimed to assess the physiological impact of our treatments by measuring the expression level of genes known to be affected by oxidative stress.

Survival assays

Right after emergence, 50 female flies per modality (*Drosophila* line × treatment) were transferred on a medium consisting of agar supplemented with sugar. Five replicate vials of 10 females were made in exp. 1, and 10 replicates of five females in exp. 2 and exp. 3. Surviving individuals were then counted each day.

Wolbachia density

In exp. 1, *Wolbachia* density was measured on 3-day-old flies to allow the full maturation of the ovaries. In the following experiments, density was measured at different time points to get access to the intra-host population dynamics of *Wolbachia*. Density was measured at emergence (i.e. 0-day-old flies) and then on 7-day-, 14-day- and 21-day-old flies in exp. 2, and on 6-day- and 10-day-old flies in exp. 3. For each experiment, 4–6 females per modality were collected at emergence and either frozen directly or transferred on sugar-supplemented agar (10% sugar) to be later collected at the desired age and frozen at −80 °C. Flies were individually crushed using a TissueLyser (Qiagen, Venlo, the Netherlands). DNA was extracted using the NucleoSpin 96 Tissue Kit (Macherey Nagel, Düren, Germany) following the instructions from the manufacturer, eluted in 100 μL of elution buffer and stored at −20 °C. Relative *Wolbachia* density was measured in each extract by quantitative PCR using a probe-based detection method on 2 monocopy genes: one in *Wolbachia* (*FtsZ*) and one in *Drosophila* (*RP49*) (see Supporting information for details).

Antioxidant capacity and lipid peroxidation assays (exp. 2)

In studies involving insects, glutathione and paraquat are often used as antioxidant (Bonilla *et al.*, 2006; Brennan *et al.*, 2012) and pro-oxidant molecules (Bonilla *et al.*, 2002, 2006; Rzezniczak *et al.*, 2011; Jumbo-Lucioni *et al.*, 2013), respectively. Their effects on oxidative homeostasis are more rarely assessed. We thus characterized those effects by quantifying the lipid peroxidation and the antioxidant capacity.

Flies were collected as third-stage larvae, emerging adult, or 14-day-old adults that had been transferred on sugar-supplemented agar at emergence. Flies were weighted and then frozen in liquid nitrogen, to prevent any physiological response to the cold, and stored at −80 °C.

The antioxidant capacity stands for the amount of ROS that can be detoxified by all the antioxidants present in the organism. It was measured using the antioxidant assay kit (Cayman Chemical, Ann Arbor, MI, USA). Individual emerging female flies were crushed in 150 μL of antioxidant assay buffer using a TissueLyser (Qiagen). After 15 min of centrifugation at 10 000 *g* and 4 °C, 15 μL of supernatant was diluted in the same volume of buffer. The assay was then performed following the instructions of the manufacturer. The results

are expressed as millimolar Trolox equivalents per mg. Seven to eight replicates per modality were performed.

Lipid peroxidation, a deleterious outcome of an excess of ROS in the cell, was estimated via thiobarbituric acid assay for malondialdehyde (MDA). This assay was performed based on the method developed by Yagi (1976) (see Supporting information for details), using chemicals from Sigma-Aldrich. Each replicate consisted in either four adult females (emerging or 14 days old) or three-third-stage larvae (only for *D. melanogaster*). The results are expressed as nmoles of MDA per mg. Two to 4 replicates per modality were performed for the larvae and 14-day-old adults; 4–8 replicates per modality were performed for the adults at emergence.

Gene expression (exp. 3)

To assess the effects of the treatments on fly oxidative homeostasis, we also measured the expression levels of *HSP83, Dorsal* and *RP49* by quantitative RT-PCR. *HSP83* encodes for a heat-shock protein playing a role in response to various stresses, including oxidative stress (Doğanlar *et al.*, 2014). *Dorsal* is part of the Toll immune pathway, whose expression has been shown to be positively affected by ROS in the mosquito *A. aegypti* (Pan *et al.*, 2012). *RP49* was used as the housekeeping gene. Expression was measured on 2-day-old and 8-day-old females, *w*Mel- and *w*MelPop-infected, which were transferred on sugar-supplemented agar at emergence and were frozen at −80 °C at the appropriate age. Eight replicates per modality were performed. The quantitative RT-PCR protocol follows the MIQE guideline (Bustin *et al.*, 2009). See Supporting information for specifics concerning RNA extraction, reverse transcription, and quantitative RT-PCR.

Statistical analyses

We used the R software (version 3.1.3) for all analyses (R Core Team, 2015).

Survival data were analysed by mixed generalized linear models [gamma distribution and inverse link; *lme4* package (Bates *et al.*, 2014)]. This method allows to take into account the potential pseudo-replications due to the flies surviving in the same vial. The *treatment* and *Drosophila line* were included as fixed explanatory factors and the replicate *vial* as a random factor. We used the method of contrasts to test the effect of the different infection statuses and treatments.

All the other data (*Wolbachia* density, antioxidant capacity, lipid peroxidation and gene expression) were analysed by linear models. Normality and homoscedasticity were checked graphically for each fitted model. For the *Wolbachia* density data, a different model was fitted for each strain of *Wolbachia*: *w*Mel, *w*MelPop and *w*Ri. The explanatory variables were as follows: the factor *treatment* (exps 1–3) and *age* (quantitative variable; exps 2 and 3). In exps 2 and 3, we focused on the overall effect of the *treatment*, rather than doing two-by-two comparisons for each age. For the antioxidant capacity data, one model was fitted for each *Drosophila* species. For *D. melanogaster*, *treatment* and *line* were used as factors. For *D. simulans*, *treatment* was used as factor. Concerning the lipid peroxidation data, one model was fitted for each *Drosophila* species. For *D. melanogaster*, *treatment*, *line* and *age* were used as explanatory factors. For *D. simulans*, *treatment* and *age* were included as factors. Age was therefore not included as a quantitative variable here, as our samples included larvae that cannot be given a time to emergence value. *F* statistics were given for global effects of factors; the method of contrasts was used for side-by-side comparisons. Expression data for *HSP83* and *Dorsal* were normalized by the expression level of the housekeeping gene *RP49* and analysed separately. *Treatment*, *infection status* and *age* were used as explanatory factors. See the Supporting information for the complete output (additive effects and interactions) of each model. All the means are given with their standard error.

Results

Impact of infection status and treatment on *Drosophila* survival

To clarify the relationships between the infection status, the oxidative homeostasis and the life-history traits of the host, we determined the effects of *w*Mel and *w*MelPop on *D. melanogaster* survival in control and manipulated conditions.

In the control condition (no treatment), the infection by *w*MelPop decreased *Drosophila* survival (Fig. 1a). The difference in survival between uninfected and *w*MelPop-infected flies was statistically significant in exp. 1 ($z = 4.067$, $P = 4.76 \times 10^{-5}$, mean difference: 4 ± 0.93 days) and exp. 3 ($z = 4.759$, $P = 1.95 \times 10^{-6}$, mean difference: 4.4 ± 0.66 days) but, surprisingly, not in exp. 2 ($z = 0.607$, $P = 0.544$). By contrast, no significant effect of the infection by *w*Mel was observed (exp. 1: $z = -1.398$, $P = 0.162$; exp. 2: $z = 0.931$, $P = 0.350$; exp. 3: $z = 1.026$, $P = 0.305$). Therefore, as already shown (Min & Benzer, 1997; Reynolds *et al.*, 2003; Strunov *et al.*, 2013), *w*MelPop induced a reduction in the *Drosophila* survival, whereas *w*Mel did not.

We also studied the effect of glutathione and paraquat treatments on fly survival (Fig. 1b,d). Paraquat had no effect on the survival of uninfected flies (exp. 1: $z = 0.644$, $P = 0.519$; exp. 2: $z = 0.337$, $P = 0.736$; exp. 3: $z = 0.567$, $P = 0.571$). By contrast, it increased the survival of *w*MelPop-infected flies (exp. 1: $z = -4.494$, $P = 7.01 \times 10^{-6}$; exp. 2: $z = -2.169$, $P = 0.030$; exp. 3: $z = -2.078$, $P = 0.038$). The paraquat treatment increased the mean longevity of the *w*MelPop-infected

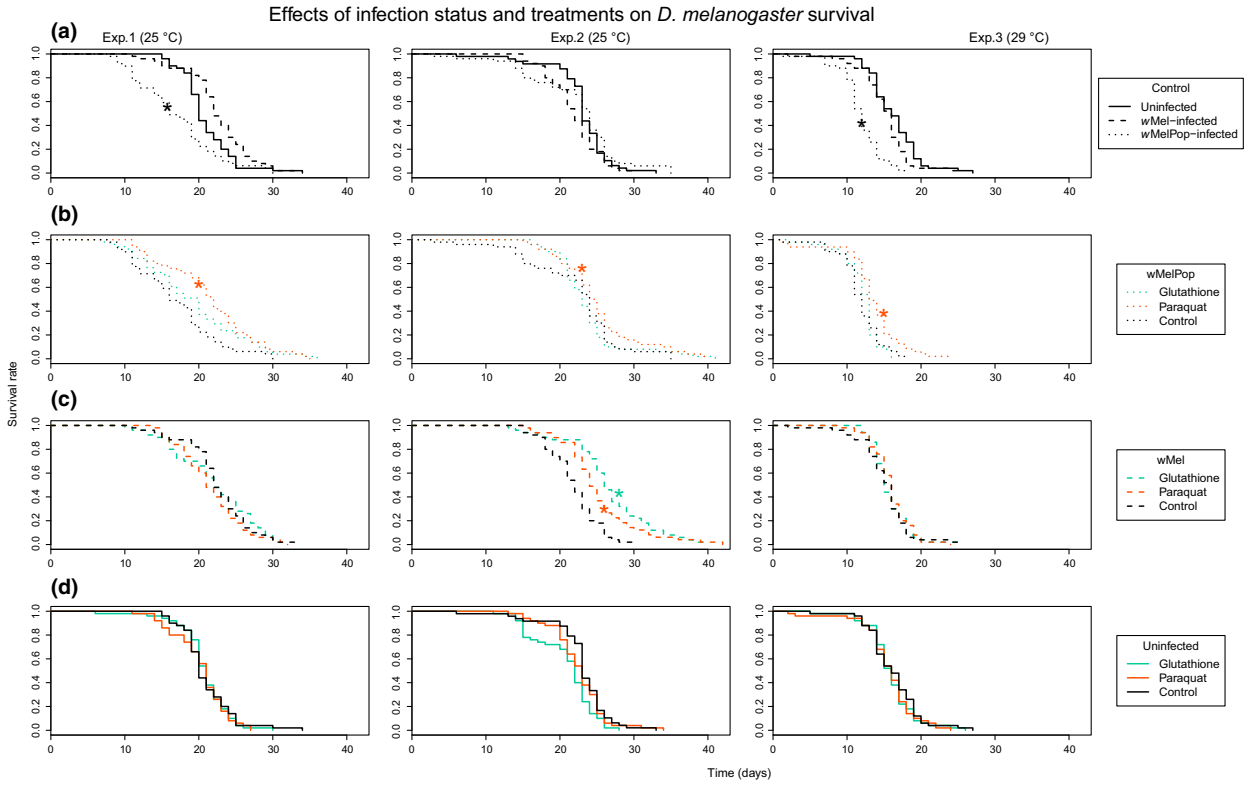

Fig. 1 *Drosophila melanogaster* survival. Effect of the infection status in control condition (a) and of the oxidative treatments on *w*MelPop-infected (b), *w*Mel-infected (c) and uninfected flies (d). Black asterisks indicate a significant effect of the infection by *Wolbachia* compared to uninfected flies ($P < 0.05$). Coloured asterisks indicate a significant effect of treatments compared to control ($P < 0.05$). Survival curves were constructed using Kaplan–Meier survival estimates with the survfit function of the *survival* package.

flies by 4.5 ± 1.18 days in exp. 1, by 2.9 ± 1.21 days in exp. 2 and by 1.7 ± 0.72 days in exp. 3. The pattern is less clear concerning the *w*Mel-infected flies: paraquat had no effect on survival in exp. 1 ($z = 0.830$, $P = 0.406$) and exp. 3 ($z = -0.725$, $P = 0.469$), but a positive effect in exp. 2 ($z = -2.533$, $P = 0.011$). There was no effect of paraquat on the *D. simulans* line (exp. 2: $z = -0.380$, $P = 0.704$; Fig. S1).

The glutathione treatment had no positive effect on the survival of uninfected flies (exp. 1: $z = 0.206$, $P = 0.837$; exp. 2: $z = 1.827$, $P = 0.067$; exp. 3: $z = 0.295$, $P = 0.768$). In exp. 2, glutathione had a tendency not to increase, but to reduce survival. By contrast, it had a positive effect on *w*MelPop-infected flies survival in exp. 1 ($z = -2.418$, $P = 0.016$, mean difference: 2.3 ± 1.23 days) but not in exp. 2 ($z = -1.093$, $P = 0.275$) and exp. 3 ($z = -0.296$, $P = 0.768$). In *w*Mel-infected flies, glutathione had no effect in exp. 1 ($z = 0.455$, $P = 0.649$) and exp. 3 ($z = -0.576$, $P = 0.565$), but a positive effect in exp. 2 ($z = -3.451$, $P = 5.59 \times 10^{-4}$, mean difference: 4.8 ± 0.95 days). There was no effect of glutathione on the *D. simulans* strain (exp. 2: $z = -1.210$, $P = 0.226$).

See the Table S1 in Supporting information for the complete output of these models.

To summarize, paraquat had a positive effect on *Drosophila* survival, but this effect was specific to infected lines. Indeed, even though the paraquat treatment had no effect on the survival of uninfected *Drosophila*, it reduced the cost of the infection by *w*MelPop. In exp. 2, paraquat had also a positive effect on *w*Mel-infected flies. The lack of replicability between experiments prevents us from reaching strong conclusions concerning the impact of glutathione on survival. However, as well as paraquat, glutathione can have a positive effect on the survival of *Wolbachia*-infected flies, but not on uninfected flies.

Impact of treatments on *Wolbachia* density

To determine whether the treatments impact survival by affecting the symbiotic population size, we studied the effect of glutathione and paraquat on the density of *w*MelPop, *w*Mel and *w*Ri (Figs 2, S2 and S3).

In all three experiments, paraquat reduced *w*MelPop (exp. 1: $t_{19} = -5.916$, $P = 1.07 \times 10^{-5}$; exp. 2: $t_{44} = -3.204$, $P = 2.52 \times 10^{-3}$; exp. 3: $t_{33} = -3.169$, $P = 3.29 \times 10^{-3}$) and *w*Mel density (exp. 1: $t_{18} = -3.496$, $P = 2.58 \times 10^{-3}$; exp. 2: $t_{44} = -4.726$, $P = 2.37 \times 10^{-5}$; exp. 3: $t_{33} = -9.637$, $P = 4.05 \times 10^{-11}$). In exp.

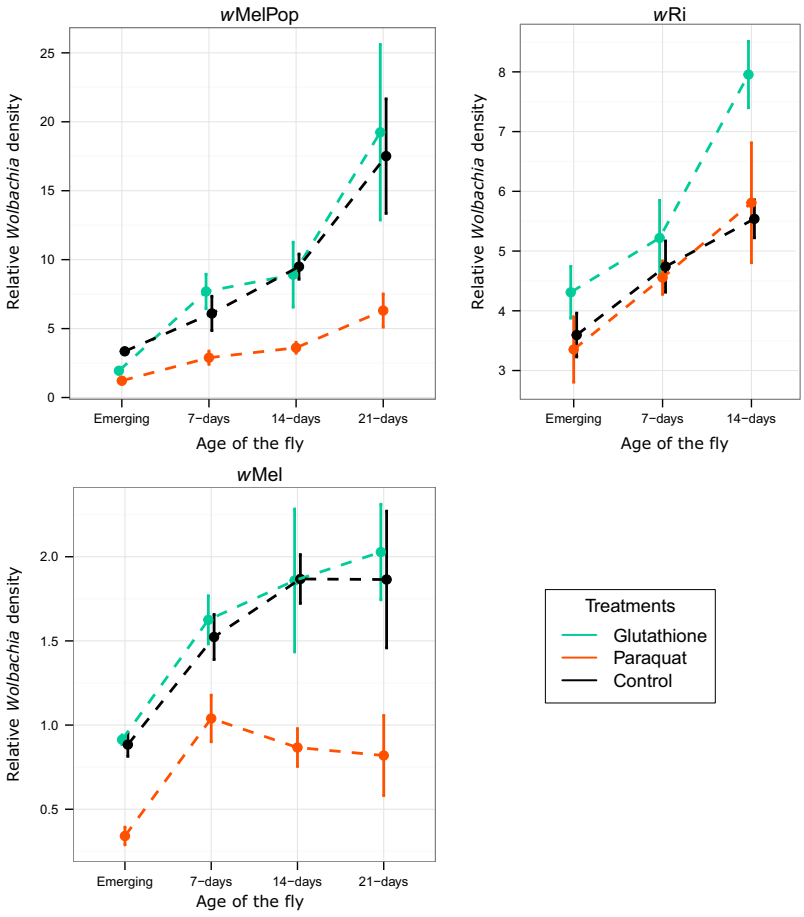

Fig. 2 Effect of oxidative treatments on *Wolbachia* density (mean ± SE). *w*MelPop and *w*Mel infect *Drosophila melanogaster* and *w*Ri infects *Drosophila simulans*.

2, at 21 days, the paraquat treatment reduced *w*MelPop density by 64% and *w*Mel density by 56%. By contrast, paraquat had no effect on *w*Ri density in the *D. simulans* line (exp. 2: $t_{32} = -0.120$, $P = 0.906$).

Contrary to paraquat, glutathione had no effect on *w*MelPop (exp. 1: $t_{19} = 0.042$, $P = 0.967$; exp. 2: $t_{44} = 0.190$, $P = 0.850$; exp. 3: $t_{33} = -0.959$, $P = 0.345$) and *w*Mel density (exp. 1: $t_{18} = 1.927$, $P = 0.070$; exp. 2: $t_{44} = 0.439$, $P = 0.663$; exp. 3: $t_{33} = -0.587$, $P = 0.562$). However, it increased *w*Ri density in *D. simulans* (exp. 2: $t_{32} = 2.728$, $P = 0.010$).

See the Table S2 in Supporting information for the complete output of these models.

To summarize, *w*MelPop and *w*Mel densities in *D. melanogaster* were therefore reduced by paraquat and unaffected by glutathione. In *D. simulans*, *w*Ri density was increased by glutathione, a result in accordance with Brennan *et al.* (2012), but unaffected by paraquat.

Impact of treatments on oxidative homeostasis

To check whether paraquat and glutathione have an impact on the oxidative homeostasis, we measured the antioxidant capacity and the quantity of oxidative damages in exp. 2, and the expression level of oxidative stress-induced genes (*HSP83* and *Dorsal*) in exp. 3.

The antioxidant capacity was higher in the *D. simulans* line than in any of the *D. melanogaster* lines (Fig. 3a). In *D. melanogaster*, the antioxidant capacity was neither affected by the paraquat treatment ($t_{62} = -0.185$, $P = 0.854$) nor by the glutathione treatment ($t_{62} = 0.470$, $P = 0.640$). Compared to uninfected flies, neither *w*MelPop-infected ($t_{62} = 1.167$, $P = 0.248$) nor *w*Mel-infected flies ($t_{62} = 1.325$, $P = 0.190$) showed different levels of antioxidant capacity. By contrast, the paraquat-treated *D. simulans* exhibited a higher level of antioxidant defences than the untreated flies ($t_{19} = 2.434$, $P = 0.025$), but there was no effect of glutathione ($t_{19} = -0.844$, $P = 0.409$). A higher production of antioxidant is consistent with a reaction of *D. simulans* against the oxidative stress induced by paraquat.

Oxidative damages on lipids were higher in the *D. simulans* line than in any of the *D. melanogaster* lines (Fig. 3b). In *D. melanogaster*, lipid peroxidation was neither affected by the paraquat treatment ($t_{124} = 1.395$, $P = 0.165$), nor by the glutathione treatment ($t_{124} = 0.882$, $P = 0.379$). It was, however, significantly

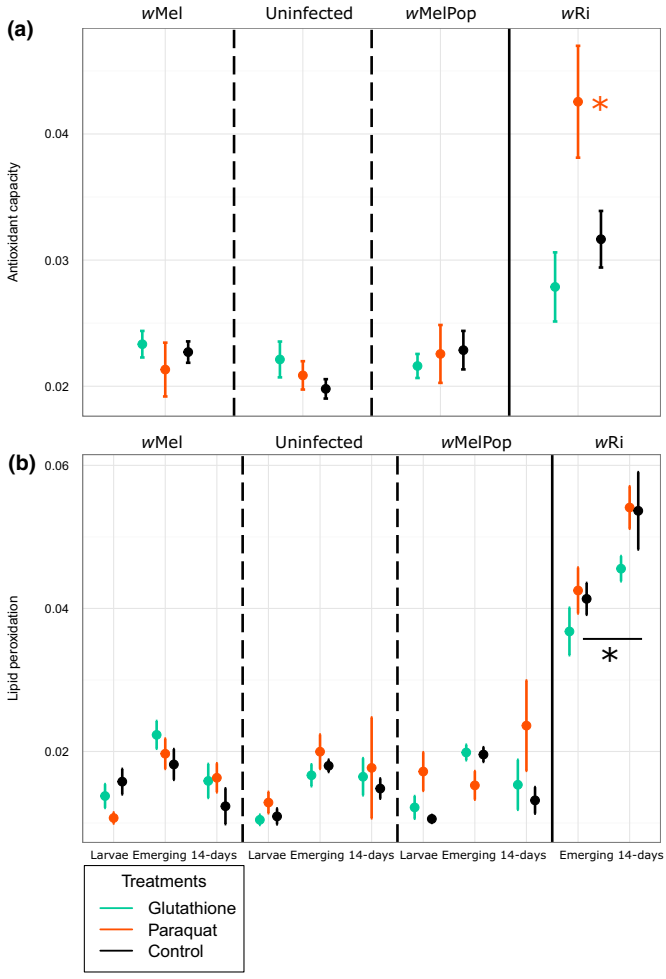

Fig. 3 Effect of infection status and oxidative treatment on (a) antioxidant capacity and (b) lipid peroxidation (mean ± SE). Black asterisks indicate a significant difference between untreated flies ($P < 0.05$). Coloured asterisks indicate a significant effect of treatments compared to control ($P < 0.05$).

affected by age ($F_{2,126} = 15.091$, $P = 1.33 \times 10^{-6}$). Compared to uninfected flies, neither *w*Mel-infected ($t_{124} = 1.067$, $P = 0.288$) nor *w*MelPop-infected ($t_{124} = 0.799$, $P = 0.426$) flies showed significantly different levels of lipid peroxidation. In *D. simulans*, we observed more damages in 14-day-old flies than in emerging flies. At both ages, we observed a nonsignificant tendency of glutathione to decrease the amount of damages ($t_{30} = -1.680$, $P = 0.103$). In *D. simulans*, the lipid peroxidation assay gives therefore a weak evidence that glutathione decreased oxidative damages and no evidence that paraquat increased oxidative damages ($t_{30} = 0.269$, $P = 0.790$). As the basal levels of both lipid peroxidation and antioxidant capacity were lower in *D. melanogaster*, the lack of observable effect of the treatments in this species may be due to a low resolution of the protocols used. Indeed, the expression data for genes classically involved in oxidative stress response, such as *HSP83* and *Dorsal* (Pan *et al.*, 2012; Doğanlar *et al.*, 2014), suggest that the paraquat did increase ROS levels in *D. melanogaster* (Fig. 4). At 2 days after emergence, paraquat increased *HSP83* expression in *w*Mel-infected flies ($t_{84} = 2.907$, $P = 0.005$, fold change: 1.51) and *Dorsal* expression in both *w*Mel- and *w*MelPop-infected flies ($t_{88} = 2.371$, $P = 0.020$, fold change: 1.34 for *w*Mel-infected flies and 1.24 for *w*MelPop-infected flies). Given the involvement of *Dorsal* in the immune response (Pan *et al.*, 2012), this overexpression may partly explain the paraquat-induced reduction in the *Wolbachia* density. By contrast, there was no significant effect of any treatment on gene expression of 8-day-old flies, regardless of their infection status. This may be explained by the time elapsed since the interruption of the treatment, which is only ingested by larvae.

See the Tables S3–5 in Supporting information for the complete output of these models.

Discussion

We hypothesized that the oxidative homeostasis mediates the interaction between *Wolbachia* and its *Drosophila* hosts. We tested this hypothesis by studying the effects of both a pro-oxidant and an antioxidant

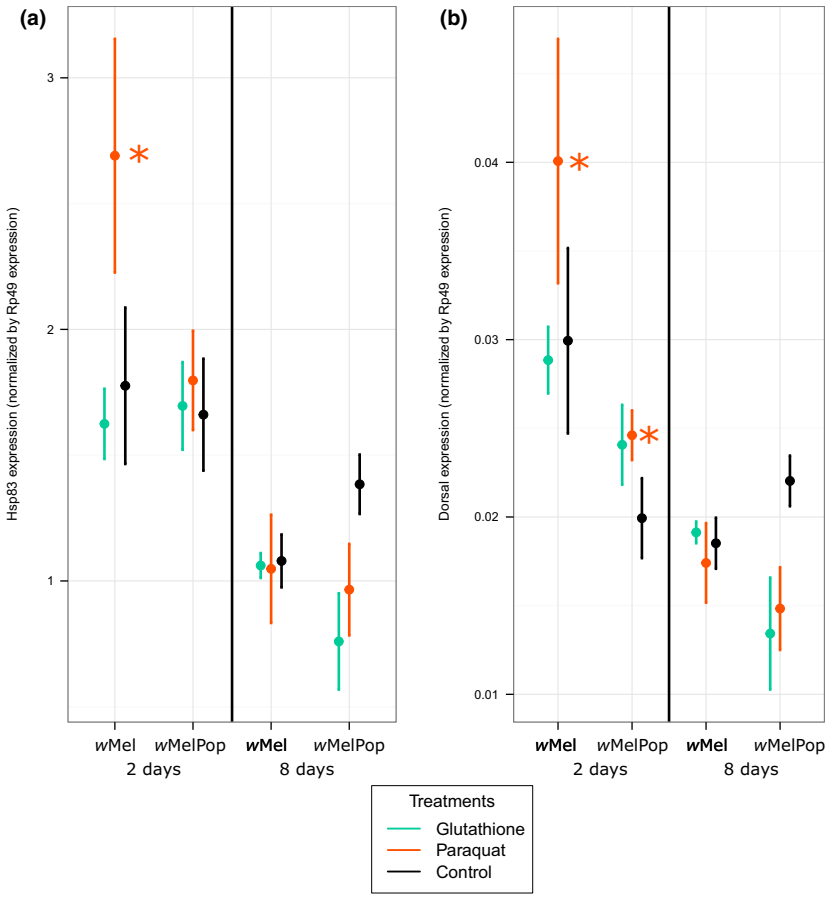

Fig. 4 Effect of infection status, age and oxidative treatment on (a) *HSP83* and (b) *Dorsal* expression (mean ± SE). Asterisks indicate significant effect of treatments compared to control ($P < 0.05$).

treatment on fly survival and *Wolbachia* density. We showed that *Wolbachia* density is affected by these treatments and that this impacts the cost of the infection.

Wolbachia density and the oxidative homeostasis

Intracellular symbionts such as *Wolbachia* are isolated from many environmental factors to which free living bacteria are exposed. However, intracellular environments can still differ in their hospitability to symbionts and our results confirm that the oxidative homeostasis is an important factor in this respect. As already shown by Fallon *et al.* (2013), paraquat can reduce *Wolbachia* density. We also confirm the result of Brennan *et al.* (2012) that glutathione can increase *Wolbachia* density. These effects, however, are context dependent. Although paraquat decreased *Wolbachia* density in the *D. melanogaster* lines, it had no effect in the *D. simulans* line. Conversely, glutathione increased *Wolbachia* density in the *D. simulans* line but not in the two infected *D. melanogaster* lines. This may be due to differences in the control of oxidative homeostasis in the two *Drosophila* species, as *D. simulans* exhibits both a higher level of oxidative damages on lipids and a higher antioxidant capacity than *D. melanogaster*. However, our data cannot rule out a *Wolbachia* strain effect (or an interaction between host and bacteria), as the species and strain factors are confounded. In *D. melanogaster*, the two strains *w*Mel and *w*MelPop react in the same way to the oxidative treatments, but as they are more closely related to each other than to *w*Ri, this reaction could be explained by homology as well as by distinctive features of the *Drosophila* species. In any case, these results suggest that differences in oxidative homeostasis regulation can explain part of the variability of *Wolbachia* density in different hosts. The reduction in the *Wolbachia* density due to the paraquat likely results from the production of ROS, either through direct toxicity or through the induction of a compensatory host response. Furthermore, as the oxidative homeostasis can be adjusted by the host, in particular through the production of ROS by the NADPH oxidases, it could be involved in the regulation of the symbiotic population. ROS production may have a direct effect on *Wolbachia* density, and/or an indirect one, through the induction of the Toll immune pathway (Pan *et al.*, 2012), as also suggested by our data on *Dorsal* expression.

The oxidative homeostasis and the cost of infection

We showed that paraquat consistently increased the survival of *w*MelPop-infected flies, without having any positive effect on uninfected individuals. We cannot exclude the hypothesis that the two lines reacted differentially to paraquat not because of their *Wolbachia* infection status, but rather to other cytoplasmic differences, possibly related to mitochondria. However, this seems unlikely for two reasons. First, the positive effect of paraquat on the survival of *w*MelPop-infected flies is consistent with the observed reduction in the density and the results from the literature linking *w*MelPop virulence to its density (Min & Benzer, 1997; Reynolds *et al.*, 2003; Strunov *et al.*, 2013). Second, the lipid peroxidation and antioxidant assays do not show any differences associated with the *D. melanogaster* lines, suggesting a relative homogeneity in terms of the impact of mitochondria on oxidative homeostasis. As a result, it is very likely that paraquat reduces the cost of *w*MelPop through the reduction in its density. Moreover, neither the larval development duration nor the size at emergence of the *w*MelPop-infected flies was affected by the paraquat treatment (data not shown). It is therefore unlikely that this effect on survival is a by-product of the paraquat treatment on another trait. This specific effect suggests that the oxidative homeostasis determines in part the cost of the infection in host–*Wolbachia* symbioses. Glutathione also tends to increase the survival of infected flies, but this effect has not been observed in all experiments. Contrary to the effect of paraquat, this potential effect of glutathione cannot be attributed to a reduction in the *Wolbachia* density. If *Wolbachia* causes an oxidative stress, the glutathione, through its antioxidant effect, may reduce its deleterious effects.

The oxidative homeostasis and the evolution of host–*Wolbachia* symbioses

The oxidative homeostasis appears to be a mediator of the *Drosophila–Wolbachia* symbioses influencing the cost of infection. Even though we used pharmacological manipulations, our results open the possibility that endogenous variations for the control of oxidative homeostasis may be a target of selection to reduce the cost of infection in host–*Wolbachia* symbioses. For example, mutations causing higher ROS concentration could become advantageous in infected individuals. However, the facts that (i) an appropriate modulation of the oxidative homeostasis can reduce *Wolbachia* density and (ii) the cost associated with the presence of *Wolbachia* is positively correlated with its density do not guarantee that this modulation of oxidative homeostasis will be selected for. Even though the modulation of oxidative homeostasis reduces *Wolbachia*-associated costs, it may have other costs by itself through the deleterious side effects of ROS, which will select against the modulation. However, in our study, the paraquat treatment had a net positive impact on the survival of *w*MelPop-infected flies, suggesting that the benefit associated with the reduction in the *Wolbachia* density is not outweighed by other costs on this trait.

The fact that *w*MelPop is exceptionally virulent among *Wolbachia* strains questions the relevance of our results for the evolution of host–*Wolbachia* symbioses. However, the level of virulence observed in host–*Wolbachia* symbioses is usually the outcome of generations of coevolution, with an expected attenuation of the cost of infection (Turelli, 1994). As new associations originate from horizontal transfer, they can exhibit a much higher level of virulence, as can be shown by artificial transfer experiments (Le Clec'h *et al.*, 2012). Oxidative homeostasis could thus be one of the primary targets of selection in newly formed associations.

Adaptation and dependence

If hosts can adapt to *Wolbachia* through a modulation of their oxidative homeostasis, such adaptations would allow the evolution of a costly symbiosis towards a more commensal one. Moreover, through the fixation of mutations that are adaptive only in the context of symbiosis, this could lead to a maladaptation in the absence of the symbiont and therefore to the dependence of the host on its symbiont. This is what may have occurred in the case of the parasitoid *A. tabida* (Hymenoptera). *Wolbachia* is fixed in natural populations of *A. tabida*, and females are no longer able to produce fertile eggs in the absence of the symbiont (Dedeine *et al.*, 2001). The dependence of *A. tabida* to *Wolbachia* is hypothesized to be a side effect of its adaptation to *Wolbachia*. More precisely, the cost of the infection could have triggered the evolution of compensatory mechanisms by the host, consequently making it unable to produce fertile eggs in the absence of *Wolbachia*. Results coming from transcriptomic data (Kremer *et al.*, 2010, 2012) suggest that a deregulation of the oxidative homeostasis is involved in the dependence; the failure of oogenesis being an outcome of the apoptosis (Pannebakker *et al.*, 2007) triggered by oxidative stress. Indeed, the removal of *Wolbachia* alters the expression of oxidative stress-related genes, such as ferritin and transferrin. Moreover, the supplementation of diet with iron, which is known to increase oxidative stress, has a similar effect on oogenesis than the removal of *Wolbachia* (Kremer *et al.*, 2009). In the present study, by focusing on a biological system in which *Wolbachia* is facultative rather than obligatory for its host, we were able to test one aspect of this scenario of the evolution of dependence, namely the fact that a modulation of the oxidative homeostasis is likely to be involved in the reduction in the cost of *Wolbachia*.

Resistance and tolerance

Evolution towards a more commensal symbiosis (Roy & Kirchner, 2000) or dependence (Zug & Hammerstein, 2015b) is usually associated with tolerance strategies, defined as strategies that reduce the fitness consequences of the infection without reducing the infection itself (Roy & Kirchner, 2000; Råberg, 2014). By contrast, the reduction in the cost through a reduction in the density is, strictly speaking, a resistance strategy. Antagonistic coevolution, rather than a reduction in the antagonism, is usually thought to be the outcome of resistance strategies. In antagonistic coevolution, adaptations in one partner have negative effects on the other, which lead to counter-adaptations and therefore to arms races or red queen dynamics (Svensson & Råberg, 2010). However, the important factor underlying the evolutionary consequences of resistance and tolerance is not whether symbiont density is reduced, but whether symbiont fitness is lowered. As *Wolbachia* is a vertically transmitted symbiont, its fitness may not always be strongly correlated with its replication. Indeed, vertical transmission generate both a high kinship between *Wolbachia* cells within a host, and a convergence of interest between both partners for offspring production, so that there is strong selection on the symbiont itself to reduce the infection cost, as long as the efficiency of transmission is not affected too much (Turelli, 1994). Consequently, if a high symbiotic density reduces the fitness of the host, selection on *Wolbachia* may not oppose the reduction in the density. Notably, many *Wolbachia* do not contribute to the transmission to host offspring, because of their localization in their host body, which is highly variable between host–*Wolbachia* interactions (e.g. Veneti *et al.*, 2004). The efficiency of transmission can indeed be expected to depend upon the symbiotic density in ovaries rather than in the whole insect. Depending on the extent to which density is differentially regulated in different host tissues, the conflict between *Wolbachia* and its host may be negligible. Given that the reduction in the cost by the host through a reduction in the density may have the same evolutionary outcome as typical tolerance evolution, it would be preferable to use 'tolerance' in a broader sense, to include all cases where the host reduces the cost of symbiosis but not the symbiont fitness. We propose therefore that the oxidative homeostasis can be a crucial factor underlying the evolution of 'tolerance' in host–*Wolbachia* symbioses.

Acknowledgments

We thank Gladys Mialdea for technical assistance with the design of the oxidative treatment protocol, Christophe Plantamp for providing the *RP49* RT-PCR primers and adjusting the reaction conditions, and Nicole Lara for providing the *Drosophila* media. This work was supported by the Agence Nationale de la Recherche (programme 'ImmunSymbArt' ANR-2010-BLAN-170101) and by the Marie Curie Actions grant FP7-PEOPLE-2010-IOF/272684/SymbiOx. This work was performed within the framework of the LABEX ECOFECT (ANR-11-LABX-0048) of Université de Lyon, within the programme 'Investissements d'Avenir' (ANR-11-IDEX-0007) operated by the French National Research Agency (ANR).

Conflict of interest

The authors declare that they have no conflict of interest.

References

Alexandrov, I.D., Alexandrova, M.V., Goryacheva, I.I., Rochina, N.V., Shaikevich, E.V. & Zakharov, I.A. 2007. Removing endosymbiotic *Wolbachia* specifically decreases lifespan of females and competitiveness in a laboratory strain of *Drosophila melanogaster*. *Russ. J. Genet.* **43**: 1147–1152.

de Bary, A. 1879. Die Erscheinung der Symbiose. Vortrag aufder Versammlung der Naturforscher und Artze zu Cassel 1–30; 21–22. Quoted in Sapp, J. 1994. *Evolution by association: a history of symbiosis.* Oxford University Press, USA.

Bates, D., Maechler, M., Bolker, B. & Walker, S. 2014. *lme4: Linear Mixed-Effects Models Using Eigen and S4*. R package version 1.1-7. http://CRAN.R-project.org/package=lme4

Bonilla, E., Medina-Leendertz, S. & Díaz, S. 2002. Extension of life span and stress resistance of *Drosophila melanogaster* by long-term supplementation with melatonin. *Exp. Gerontol.* **37**: 629–638.

Bonilla, E., Medina-Leendertz, S., Villalobos, V., Molero, L. & Bohórquez, A. 2006. Paraquat-induced oxidative stress in *Drosophila melanogaster*: effects of melatonin, glutathione, serotonin, minocycline, lipoic acid and ascorbic acid. *Neurochem. Res.* **31**: 1425–1432.

Brennan, L.J., Keddie, B.A., Braig, H.R. & Harris, H.L. 2008. The endosymbiont *Wolbachia pipientis* induces the expression of host antioxidant proteins in an *Aedes albopictus* cell line. *PLoS One* **3**: e2083.

Brennan, L.J., Haukedal, J.A., Earle, J.C., Keddie, B. & Harris, H.L. 2012. Disruption of redox homeostasis leads to oxidative DNA damage in spermatocytes of *Wolbachia*-infected *Drosophila simulans*. *Insect Mol. Biol.* **21**: 510–520.

Bustin, S., Venes, B., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M. *et al.* 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* **55**: 611–622.

Chrostek, E. & Teixeira, L. 2015. Mutualism breakdown by amplification of *Wolbachia* genes. *PLoS Biol.* **13**: e1002065.

Chrostek, E., Marialva, M.S.P., Yamada, R., O'Neill, S.L. & Teixeira, L. 2014. High anti-viral protection without immune upregulation after interspecies *Wolbachia* transfer. *PLoS One* **9**: e99025.

Costantini, D. 2014. *Oxidative Stress and Hormesis in Evolutionary Ecology and Physiology*. Springer, Berlin.

Darby, A.C., Armstrong, S.D., Bah, G.S., Kaur, G., Hughes, M.A., Kay, S.M. *et al.* 2012. Analysis of gene expression from the *Wolbachia* genome of a filarial nematode supports both metabolic and defensive roles within the symbiosis. *Genome Res.* **22**: 2467–2477.

David, J.R. & Clavel, M.F. 1965. Interaction entre le génotype et le milieu d'élevage. Conséquences sur les caractéristiques du développement de la Drosophile. *Bull. Biol. Fr. Belg.* **99**: 369–378.

Dedeine, F., Vavre, F., Fleury, F., Loppin, B., Hochberg, M.E. & Boulétreau, M. 2001. Removing symbiotic *Wolbachia* bacteria specifically inhibits oogenesis in a parasitic wasp. *Proc. Natl. Acad. Sci. USA* **98**: 6247–6252.

Dobson, S.L., Rattanadechakul, W. & Marsland, E.J. 2004. Fitness advantage and cytoplasmic incompatibility in *Wolbachia* single- and superinfected *Aedes albopictus*. *Heredity* **93**: 135–142.

Doğanlar, Z.B., Doğanlar, O. & Tabakçıoğlu, K. 2014. Genotoxic effects of heavy metal mixture in *Drosophila melanogaster*: expressions of heat shock proteins, RAPD profiles and mitochondrial DNA sequence. *Water Air Soil Pollut.* **225**: 2104.

Douglas, A.E. 2014. The molecular basis of bacterial-insect symbiosis. *J. Mol. Biol.* **426**: 3830–3837.

Fallon, A.M., Kurtz, C.M. & Carroll, E.M. 2013. The oxidizing agent, paraquat, is more toxic to *Wolbachia* than to mosquito host cells. *In Vitro Cell. Dev. Biol. Anim.* **49**: 501–507.

Fleury, F., Vavre, F., Ris, N., Fouillet, P. & Bouletreau, M. 2000. Physiological cost induced by the maternally-transmitted endosymbiont *Wolbachia* in the *Drosophila* parasitoid *Leptopilina heterotoma*. *Parasitology* **121**: 493–500.

Frank, A.B. 1877. Uber die biologischen Verhaltnisse des Thallus eineger Krustenflechten. *Beitrage zur Biologic der Pflanzen*, **2**: 123–200, 195. Quoted in Sapp, J. 1994. *Evolution by association: a history of symbiosis.* Oxford University Press, USA.

Ha, E.M., Lee, K.A., Seo, Y.Y., Kim, S.H., Lim, J.H., Oh, B.H. *et al.* 2009. Coordination of multiple dual oxidase-regulatory pathways in responses to commensal and infectious microbes in drosophila gut. *Nat. Immunol.* **10**: 949–957.

Hedges, L., Brownlie, J., O'Neill, S. & Johnson, K. 2008. *Wolbachia* and virus protection in insects. *Science* **322**: 2008.

Hentschel, U., Steinert, M. & Hacker, J. 2000. Common molecular mechanisms of symbiosis and pathogenesis. *Trends Microbiol.* **8**: 226–231.

Hosokawa, T., Koga, R., Kikuchi, Y., Meng, X.Y. & Fukatsu, T. 2010. *Wolbachia* as a bacteriocyte-associated nutritional mutualist. *Proc. Natl. Acad. Sci. USA* **107**: 769–774.

Imlay, J.A. 2013. The molecular mechanisms and physiological consequences of oxidative stress: lessons from a model bacterium. *Nat. Rev. Microbiol.* **11**: 443–454.

Jumbo-Lucioni, P.P., Hopson, M.L., Hang, D., Liang, Y., Jones, D.P. & Fridovich-Keil, J.L. 2013. Oxidative stress contributes to outcome severity in a *Drosophila melanogaster* model of classic galactosemia. *Dis. Model. Mech.* **6**: 84–94.

Kremer, N., Voronin, D., Charif, D., Mavingui, P., Mollereau, B. & Vavre, F. 2009. *Wolbachia* interferes with ferritin expression and iron metabolism in insects. *PLoS Pathog.* **5**: e1000630.

Kremer, N., Dedeine, F., Charif, D., Finet, C., Allemand, R. & Vavre, F. 2010. Do variable compensatory mechanisms explain the polymorphism of the dependence phenotype in the *Asobara tabida-Wolbachia* association? *Evolution* **64**: 2969–2979.

Kremer, N., Charif, D., Henri, H., Gavory, F., Wincker, P., Mavingui, P. *et al.* 2012. Influence of *Wolbachia* on host gene expression in an obligatory symbiosis. *BMC Microbiol.* **12** (Suppl 1): S7.

Le Clec'h, W., Braquart-Varnier, C., Raimond, M., Ferdy, J.B., Bouchon, D. & Sicard, M. 2012. High virulence of *Wolbachia* after host switching: when autophagy hurts. *PLoS Pathog.* **8**: e1002844.

Lee, K.A., Kim, S.H., Kim, E.K., Ha, E.M., You, H., Kim, B. *et al.* 2013. Bacterial-derived uracil as a modulator of mucosal immunity and gut-microbe homeostasis in *Drosophila*. *Cell* **153**: 797–811.

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**: 2918–2923.

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**: 10792–10796.

Moné, Y., Monnin, D. & Kremer, N. 2014. The oxidative environment: a mediator of interspecies communication that drives symbiosis evolution. *Proc. Biol. Sci.* **281**: 20133112.

Mouton, L., Dedeine, F., Henri, H., Boulétreau, M., Profizi, N. & Vavre, F. 2004. Virulence, multiple infections and regulation of symbiotic population in the *Wolbachia-Asobara tabida* symbiosis. *Genetics* **168**: 181–189.

Nathan, C. & Cunningham-Bussel, A. 2013. Beyond oxidative stress: an immunologist's guide to reactive oxygen species. *Nat. Rev. Immunol.* **13**: 349–361.

Pan, X., Zhou, G., Wu, J., Bian, G., Lu, P., Raikhel, A.S. *et al.* 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**: E23–E31.

Pannebakker, B.A., Loppin, B., Elemans, C.P.H., Humblot, L. & Vavre, F. 2007. Parasitic inhibition of cell death facilitates symbiosis. *Proc. Natl. Acad. Sci. USA* **104**: 213–215.

R Core Team 2015. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/

Råberg, L. 2014. How to live with the enemy: understanding tolerance to parasites. *PLoS Biol.* **12**: 00–00.

Reynolds, K.T., Thomson, L.J. & Hoffmann, A.A. 2003. The effects of host age, host nuclear background and temperature on phenotypic effects of the virulent *Wolbachia* strain popcorn in *Drosophila melanogaster*. *Genetics* **1034**: 1027–1034.

Roy, B.A. & Kirchner, J.W. 2000. Evolutionary dynamics of pathogen resistance and tolerance. *Evolution* **54**: 51–63.

Rzezniczak, T.Z., Douglas, L.A., Watterson, J.H. & Merritt, T.J.S. 2011. Paraquat administration in *Drosophila* for use in metabolic studies of oxidative stress. *Anal. Biochem.* **419**: 345–347.

Schieber, M. & Chandel, N.S. 2014. ROS function in redox signaling and oxidative stress. *Curr. Biol.* **24**: R453–R462.

Strunov, A., Kiseleva, E. & Gottlieb, Y. 2013. Spatial and temporal distribution of pathogenic *Wolbachia* strain wMelPop in *Drosophila melanogaster* central nervous system under different temperature conditions. *J. Invertebr. Pathol.* **114**: 22–30.

Svensson, E.I. & Råberg, L. 2010. Resistance and tolerance in animal enemy-victim coevolution. *Trends Ecol. Evol.* **25**: 267–274.

Taylor, M.J., Voronin, D., Johnston, K.L. & Ford, L. 2013. *Wolbachia* filarial interactions. *Cell. Microbiol.* **15**: 520–526.

Teixeira, L., Ferreira, A. & Ashburner, M. 2008. The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. *PLoS Biol.* **6**: e2.

Turelli, M. 1994. Evolution of incompatibility-inducing microbes and their hosts. *Evolution* **48**: 1500–1513.

Vasquez, C.J., Stouthamer, R., Jeong, G. & Morse, J.G. 2011. Discovery of a CI-inducing *Wolbachia* and its associated fitness costs in the biological control agent *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae). *Biol. Control* **58**: 192–198.

Veneti, Z., Clark, M.E., Karr, T.L., Savakis, C. & Bourtzis, K. 2004. Heads or tails: host-parasite interactions in the *Drosophila-Wolbachia* system. *Appl. Environ. Microbiol.* **70**: 5366–5372.

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**: e114.

Werren, J.H., Baldo, L. & Clark, M.E. 2008. *Wolbachia*: master manipulators of invertebrate biology. *Nat. Rev. Microbiol.* **6**: 741–751.

Wong, Z.S., Brownlie, J.C. & Johnson, K.N. 2015. Oxidative stress correlates with *Wolbachia*-mediated antiviral protection in *Wolbachia-Drosophila* associations. *Appl. Environ. Microbiol.* **81**: 3001–3005.

Yagi, K. 1976. A simple fluorometric assay for lipoperoxide in blood plasma. *Biochem. Med.* **15**: 212–216.

Zug, R. & Hammerstein, P. 2012. Still a host of hosts for *Wolbachia*: analysis of recent data suggests that 40% of terrestrial arthropod species are infected. *PLoS One* **7**: 7–9.

Zug, R. & Hammerstein, P. 2015a. *Wolbachia* and the insect immune system: what reactive oxygen species can tell us about the mechanisms of *Wolbachia*-host interactions. *Front. Microbiol.* **6**: 1201.

Zug, R. & Hammerstein, P. 2015b. Bad guys turned nice? A critical assessment of *Wolbachia* mutualisms in arthropod hosts. *Biol. Rev.* **90**: 89–111.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Appendix S1 Material and methods.

Table S1–S5 Output of the statistical models.

Figure S1 Effect of oxidative treatments on *Drosophila simulans* survival.

Figure S2. Effect of oxidative treatments on *Wolbachia* density (mean ± SE) of 3-days old *Drosophila melanogaster*.

Figure S3 Effect of oxidative treatments on *Wolbachia* density (mean ± SE) of 6-days old and 10-days old *Drosophila melanogaster*.

Data deposited at Dryad: doi: 10.5061/dryad.f1170

Received 8 December 2015; revised 25 March 2016; accepted 8 March 2016