Wolbachia-Mediated Antibacterial Protection and Immune Gene Regulation in Drosophila

Zhee Sheen Wong1, Lauren M. Hedges1, Jeremy C. Brownlie2, Karyn N. Johnson1*

1 School of Biological Sciences, The University of Queensland, Brisbane, Queensland, Australia; 2 School of Biomolecular and Physical Sciences, Griffith University, Brisbane, Queensland, Australia

Abstract

The outcome of microbial infection of insects is dependent not only on interactions between the host and pathogen, but also on the interactions between microbes that co-infect the host. Recently the maternally inherited endosymbiotic bacteria Wolbachia has been shown to protect insects from a range of microbial and eukaryotic pathogens. Mosquitoes experimentally infected with Wolbachia have upregulated immune responses and are protected from a number of pathogens including viruses, bacteria, Plasmodium and filarial nematodes. It has been hypothesised that immune upregulation underpins Wolbachia-mediated protection. Drosophila is a strong model for understanding host-Wolbachia-pathogen interactions. Wolbachia-mediated antiviral protection in Drosophila has been demonstrated for a number of different Wolbachia strains. In this study we investigate whether Wolbachia-infected flies are also protected against pathogenic bacteria. Drosophila simulans lines infected with five different Wolbachia strains were challenged with the pathogenic bacteria Pseudomonas aeruginosa PA01, Serratia marcescens and Erwinia carotovora and mortality compared to paired lines without Wolbachia. No difference in mortality was observed in the flies with or without Wolbachia. Similarly no antibacterial protection was observed for D. melanogaster infected with Wolbachia. Interestingly, D. melanogaster Oregon RC flies which are naturally infected with Wolbachia showed no upregulation of the antibacterial immune genes TepIV, Defensin, Diptericin B, PGRP-SD, Cecropin A1 and Attacin D compared to paired flies without Wolbachia. Taken together these results indicate that Wolbachia-mediated antibacterial protection is not ubiquitous in insects and furthermore that the mechanisms of antibacterial and antiviral protection are different. We suggest that the immune priming and antibacterial protection observed in Wolbachia-infected mosquitoes may be a consequence of the recent artificial introduction of the symbiont into insects that normally do not carry Wolbachia and that antibacterial protection is unlikely to be found in insects carrying long-term Wolbachia infections.

Citation: Wong ZS, Hedges LM, Brownlie JC, Johnson KN (2011) Wolbachia-Mediated Antibacterial Protection and Immune Gene Regulation in Drosophila. PLoS ONE 6(9): e25430. doi:10.1371/journal.pone.0025430

Editor: Dipishkha Chakravortty, Indian Institute of Science, India

Received July 10, 2011; Accepted September 5, 2011; Published September 29, 2011

Copyright: © 2011 Wong et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This research was supported by funds from the Australian Research Council (grant DP1092492). The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

*E-mail: karynj@uq.edu.au

Introduction

The interaction between two microbes within a host can impact on the outcome of infection for the host. Wolbachia are a maternally transmitted endosymbiotic bacteria that is predicted to infect up to 70% of insect species [1,2]. Wolbachia can protect insects from infection by a range of microbes and parasites [3–10]. Where the microbes are pathogens of the insect, this protection has the potential to greatly influence the ecology of the host, pathogen and Wolbachia [11–13]. In addition, it has been widely suggested that Wolbachia-mediated pathogen protection could be harnessed in biological control programs to interfere with the transmission of human diseases that are vectored by insects, including dengue and malaria. However, the molecular mechanisms involved in protection are yet to be determined.

Mosquitoes that are experimentally infected with Wolbachia are protected from a range of viruses, bacteria and parasites. A number of mosquito species that are important human disease vectors are not naturally found to be infected with Wolbachia, for example Aedes aegypti and Anopheles species. However, utilising transfection techniques A. aegypti has been experimentally infected with Wolbachia strains wMelPop-CLA or wAlbB [14,15]. The mosquitoes stably infected with Wolbachia accumulate and transmit RNA viruses such as Dengue and Chikungunya less readily than Wolbachia-free mosquitoes [3,8]. Infection of Ae. aegypti with the D. melanogaster derived Wolbachia strain wMelPop-CLA also reduced the prevalence of the filarial nematode Brugia pahangi, impaired the ability to transmit the avian malarial parasite Plasmodium gallinaceum. In addition reduced mortality induced by infection with the Gram-negative bacterium Erwinia carotovora but not the Gram-positive bacteria Micrococcus luteus was observed in these mosquitoes [7]. In addition, whilst stable transinfection of Anopheles mosquitoes is yet to be achieved, An. gambiae that were somatically infected with Wolbachia showed reduced accumulation of Plasmodium oocysts [5,6]. Thus in mosquitoes, artificially introduced Wolbachia induces broad ranging antipathogen protection.

The phenomenon of Wolbachia-mediated antiviral protection is well established in the model insect Drosophila. Naturally Wolbachia-infected flies are protected from a diverse range of RNA viruses [4,10]. In the case of the pathogenic viruses Drosophila C virus (DCV), Flock House virus (FHV) and Cricket paralysis virus (CrPV)
**Wolbachia-infected flies survive upwards of twice as long as their uninfected counterparts** [4,10]. This protection has been shown to be consistent across the closely related *Wolbachia* strains that infect *D. melanogaster* (wMel, wMelCS and wMelPop) and across different host backgrounds [4,10]. *Wolbachia* antiviral protection has also been demonstrated in the related species *D. simulans* [9].

Not all *Wolbachia*-host combinations result in antiviral protection. *D. simulans* are naturally infected with diverse *Wolbachia* strains from both supergroup A (wHa, wRi and wMel) and B (wNo) [16,17]. *D. simulans* lines naturally infected with wHa and wRi (line CO and DSR respectively) are protected from DCV and FHV, whereas those naturally infected with wHa and wNo (line DSH and N7NO respectively) are not protected [9]. In these fly lines antiviral protection correlates with both phylogegetic relatedness to wMel and also high density of *Wolbachia* in the host [9]. The *D. simulans* line Me29, which was transinfected with wMel in 1996 [18], is also protected from both DCV and FHV infection. The mechanism(s) of *Wolbachia*-mediated protection have not been determined. The correlation between density and distribution of *Wolbachia* in flies and mosquitoes supports the hypothesis that *Wolbachia* and pathogens may be in competition for limited host resources [8,9]. Alternatively several studies have demonstrated that antipathogen protection in experimentally infected mosquitoes is concomitant with *Wolbachia* induced upregulation of a range of host immune genes [3–5]. Genes involved in the antimicrobial IMD and Toll pathways are upregulated, with the effector genes such as cecropins and other antimicrobial peptides (AMPs) showing the highest upregulation. These observations led to the hypothesis that *Wolbachia* infection primes the immune system so that when *Wolbachia*-infected insects are challenged with a pathogen the insect is protected from the pathogen. Little direct evidence is available in support of this hypothesis, although in somatically infected *Ae. gambiæ* upregulation of Tep1 has been experimentally linked with protection against parasite *P. berghei* [6] and Dengue virus is somewhat impacted by upregulation of the Toll pathway [19].

In contrast to mosquitoes, it is less clear if *Wolbachia* stimulates immune priming in *Drosophila*. Cultured *D. melanogaster* cells (S2 cell line) showed upregulation of genes from the Toll and IMD pathways as well as AMP effector molecules when experimentally infected with the *Wolbachia* wRi strain (which naturally infects *D. simulans*) [20]. In contrast, an early study using Northern blot analysis found no difference in cecropin or diptericin RNA levels in *D. simulans* line DSR with and without wRi infection and similarly no difference was recorded for Defensin expression in *Ae. auberti* with and without *Wolbachia* infection [21]. It remains to be confirmed whether *Wolbachia*-mediated immune priming is linked to the antiviral protection that has been documented in *Drosophila*.

In mosquitoes *Wolbachia* mediates protection against a range of pathogens, yet it is unclear whether a single molecular mechanism underpins protection against this diverse group of microbes and parasites. In *Drosophila* not all host-*Wolbachia* combinations protect against virus infection [9]. If the mechanism that underlies protection were the same for bacteria and viruses we would predict that those *Drosophila*: *Wolbachia* combinations with reduced virus-induced mortality would similarly reduce pathogenic bacterial infection.

In this study we investigated whether antibacterial protection occurs in flies infected with *Wolbachia*. To do this five *D. simulans*: *Wolbachia* lines were utilised that we previously used to investigate antiviral protection. Bacterial pathogens *Pseudomonas aeruginosa* PA01, *Serratia marcescens* and *E. carotovora* were used to challenge the flies. *P. aeruginosa* PA01 and *S. marcescens* are the opportunistic and natural pathogens of *Drosophila* respectively [22,23] and *Wolbachia* mediates protection in *Ae. aegypti* against mortality induced by *E. carotovora* [7]. Further, using reverse transcription and quantitative PCR to assay expression of six AMPs and immune genes there was no evidence of antibacterial immune priming in *D. melanogaster* naturally infected with *Wolbachia*.

**Results**

**Wolbachia does not protect *D. simulans* from pathogenic bacteria**

The impact of *Wolbachia* on the outcome of virus infection varies in *D. simulans* lines challenged with RNA viruses. *D. simulans* lines CO, DSR and Me29 are protected against DCV and FHV infections by *Wolbachia* strains wAu, wRi and wMel respectively. In contrast, *D. simulans* lines DSH and N7NO are not protected against DCV and FHV infections by wHa and wNo respectively [9]. In order to investigate whether these *Wolbachia* strains confer protection to *D. simulans* lines challenged with pathogenic bacteria, *D. simulans* CO, DSR, Me29, DSH and N7NO lines with *Wolbachia* and paired lines that had been cured of *Wolbachia* infection were challenged with three pathogenic Gram-negative bacteria (*P. aeruginosa* PA01, *S. marcescens* and *E. carotovora*) and mortality recorded for up to 36 hours.

Mortality of CO flies challenged with pathogenic bacteria was similar regardless of *Wolbachia* infection status (Figure 1). Flies both with and without *Wolbachia* challenged with *P. aeruginosa* PA01 died within 25 hours of infection and there was no significant difference in the survival curves (Figure 1 A; p = 0.2). In this and all other experiments there was negligible mortality of mock-infected flies during the time course. *S. marcescens* and *E. carotovora* are more virulent than *P. aeruginosa* PA01. After infection, flies with and without *Wolbachia* died within 15 hours (Figure 1 B and C). Statistical analysis showed that there was no significant difference in survival of flies with and without *Wolbachia* (p = 0.3 for *S. marcescens* infection, p = 0.0533 for *E. carotovora* infection). Each survival bioassay was independently repeated at least three times with similar results (data not shown). These results indicate that while wAu infection of CO flies protects the flies from viral-induced mortality there is no protection against pathogenic bacteria mediated by wAu.

To investigate whether lack of protection in *D. simulans* was limited to this particular host: *Wolbachia* combination we challenged four other fly lines with and without *Wolbachia* with the three pathogenic bacteria. DSR, Me29, DSH and N7NO that were challenged with *P. aeruginosa* PA01 died within 25 to 30 hours post infection (Figure 2 A, 3 A, 4 A and 5 A). *D. simulans* DSR, Me29, DSH and N7NO when challenged with *S. marcescens* and *E. carotovora*, died within 10 to 25 hours post infection (Figure 2 B and C, 3 B and C, 4 B and C, 5 B and C). In each assay there was no difference between the survival curves of flies with and without *Wolbachia* (p > 0.05). Results shown are representative of at least three independent bioassays, each with similar results obtained. The survival bioassays were also repeated with at least two independent experiments with a lower concentration of bacterial culture (O.D.₆₀₀ₙ₉ = 0.1–0.5) (data not shown). At this lower concentration of bacteria, 100% mortality was not achieved in most cases, however, there was still no protection against bacterial-induced mortality. Taken together these results give strong evidence that *Wolbachia* does not confer protection in *D. simulans* against bacterial-induced mortality.
Analysis of Wolbachia-mediated antibacterial protection in D. melanogaster

In contrast to Ae. aegypti mosquitoes [7] our results show that there is no antibacterial protection mediated by Wolbachia in D. simulans. To investigate whether lack of protection was restricted to this single species we utilised the D. melanogaster line ORC in protection bioassays. This species was chosen as Wolbachia has been shown to protect D. melanogaster from a number of different viral pathogens [4,9,10]. ORC flies with and without wMelCS were challenged with P. aeruginosa PA01, S. marcescens and E. carotovora and survival was monitored (Figure 6). There was no difference in the survival of flies with and without Wolbachia in response to S. marcescens challenge (p = 0.3). ORC flies with Wolbachia were somewhat more susceptible to P. aeruginosa PA01 and E. carotovora than flies without Wolbachia (p<0.05) in the results shown in Figure 6. However, this small difference was only observed in one out of three experiments, suggesting that if
there is a biological difference it is negligible. These results show that natural infection with *Wolbachia* does not confer protection against pathogenic bacteria-induced mortality in *D. melanogaster*.

Analysis of *in vivo* bacterial growth in *D. melanogaster*

Taken together, results obtained from survival bioassays of all *D. simulans* and *D. melanogaster* challenged with pathogenic bacteria indicate that *Wolbachia* does not mediate protection against mortality induced by pathogenic bacteria in *Drosophila*. In order to investigate whether *Wolbachia* affects the *in vivo* accumulation of pathogenic bacterial load in *Drosophila* the *D. melanogaster* ORC line was challenged with *P. aeruginosa* PA01. Samples were collected at 0 and 12 hours post infection and bacterial load was analysed. In flies that were not challenged with *P. aeruginosa* PA01 no bacterial colonies grew on the selective LB-Ampicillin plates, indicating that all colonies identified in challenged flies arose from antibiotic

![Figure 3. Survival of *D. simulans* Me29 flies challenged with pathogenic bacteria. Graphs show the survival of *D. simulans* Me29 with (cross) and without (triangle) wMel challenged with (A) *P. aeruginosa* PA01, (B) *S. marcescens* and (C) *E. carotovora*. Flies were infected with pathogenic bacteria (black line) or mock infected with LB (grey line). Error bars represent SEM calculated from three replicate vials. doi:10.1371/journal.pone.0025430.g003](#)

![Figure 4. Survival of *D. simulans* DSH flies challenged with pathogenic bacteria. Graphs show the survival of *D. simulans* DSH infected with (cross) and without (triangle) wHa challenged with (A) *P. aeruginosa* PA01, (B) *S. marcescens* and (C) *E. carotovora*. Flies were infected with pathogenic bacteria (black line) or mock infected with LB (grey line). Error bars represent SEM calculated from three replicate vials. doi:10.1371/journal.pone.0025430.g004](#)
resistant *P. aeruginosa* PA01 bacteria. At time 0 bacterial counts were 1.0–3.5 × 10^3 CFU/fly, indicating that this was the dose with which the flies were challenged (Figure 7). At 12 hours post infection the bacterial load had increased by 3 orders of magnitude to approximately 1.0 × 10^6 CFU/fly. Similar bacterial loads were observed in both flies with and without *Wolbachia* indicating that there was no difference in the accumulation of the *P. aeruginosa* PA01.

**Figure 5.** Survival of *D. simulans* N7NO challenged with pathogenic bacteria. Graphs show the survival of *D. simulans* N7NO with (cross) and without (triangle) wNo challenged with (A) *P. aeruginosa* PA01, (B) *S. marcescens* and (C) *E. carotovora*. Flies were infected with pathogenic bacteria (black line) or mock infected with LB (grey line). Error bars represent SEM calculated from three replicate vials.

doi:10.1371/journal.pone.0025430.g005

**Figure 6.** Survival of *D. melanogaster* Oregon RC challenged with pathogenic bacteria. Graphs show the survival of *D. melanogaster* ORC with (cross) and without (triangle) wMelCS challenged with (A) *P. aeruginosa* PA01, (B) *S. marcescens* and (C) *E. carotovora*. Flies were infected with pathogenic bacteria (black line) or mock infected with LB (grey line). Error bars represent SEM calculated from three replicate vials.

doi:10.1371/journal.pone.0025430.g006

Regulation of immune genes in *D. melanogaster* in response to *Wolbachia* infection

*Wolbachia* has been shown to stimulate different immune gene responses in different *Wolbachia*-host combinations [6–8,20,21,24,25] and it has been suggested that immune priming stimulated by *Wolbachia* may be causally linked to *Wolbachia*-mediated antipathogen protection [3,6–8]. Given no antibacterial protection was mediated in the six host-*Wolbachia* combinations utilised in this study we wanted to investigate whether antibacterial immune
genes were upregulated by the presence of Wolbachia in our system. The regulation of six immune genes in D. melanogaster ORC flies with and without Wolbachia was investigated using RT-qPCR. These genes were chosen as they were homologous to genes that were upregulated by Wolbachia presence in Aedes aegypti [7]. None of the six immune genes that were investigated in Drosophila (TepIV, Defensin, Diptericin B, PGRP-SD, Cecropin A1 and Attacin D) were differentially regulated in the presence of Wolbachia ($p<0.05$; Figure 8).

**Discussion**

The lack of Wolbachia-mediated antibacterial protection in Drosophila differs from studies in mosquitoes where Wolbachia has been shown to mediate broad-spectrum anti-microbe and -parasite protection [3,5–8]. In mosquitoes experimentally infected with Wolbachia there is upregulation of a number of genes for immune effector molecules and those involved in antimicrobial pathways [3,7,8]. It has been suggested that this Wolbachia induced “immune priming” may be the mechanism underlying protection and some evidence has been presented for this for anti-Plasmodium protection [6]. We reasoned that as Wolbachia-infected Drosophila were not protected from pathogenic Gram-negative bacteria, this may be because Wolbachia was not stimulating an immune response in these flies. To assess this we analysed the expression of six antimicrobial immune genes whose homologues were upregulated in Wolbachia-infected mosquitoes [7]. In D. melanogaster Wolbachia infection did not stimulate expression of the antimicrobial response genes.

It is interesting that Wolbachia differentially stimulates immune responses in different hosts. The host:Wolbachia examples discussed above differ in two ways. Firstly, the hosts are from different insect families: mosquitoes and flies. But perhaps more importantly each analysis of immune regulation has been performed using mosquitoes that have been recently experimentally infected with Wolbachia. In contrast the D. melanogaster ORC line used here, is naturally infected with Wolbachia. As a

![Figure 7. In vivo bacterial growth of P. aeruginosa PA01 D. melanogaster ORC adults with and without Wolbachia infection.](image)

Graph shows the number of bacteria per fly (CFU/fly) of P. aeruginosa PA01 in D. melanogaster without (triangle) and with wMelCS (cross) at time 0 and 12 hours post infection. doi:10.1371/journal.pone.0025430.g007

![Figure 8. Immune gene expression in response to Wolbachia infection.](image)

The relative expression (RE) of immune genes of D. melanogaster with and without Wolbachia was analysed using RT-qPCR. The box and whisker plots show the ratio of immune to reference gene (RpL32) expression from the indicated genes: (A) Attacin D, (B) Diptericin B, (C) Cecropin A1, (D) Defensin, (E) PGRP-SD and (F) TepIV. Boxes represent medians, 25 (bar below median) and 75 (above median) percentiles of 10 individual male flies. None of the medians were significantly different (Mann Whitney-U test; $p>0.05$). doi:10.1371/journal.pone.0025430.g008
maternally inherited endosymbiont, *Wolbachia* remains in close association with its host from generation to generation. Co-evolution of the bacteria and host is postulated to eventuate in commensal or mutualistic associations. Experimental introduction of *Wolbachia* into a new host can lead to over-replication of the bacteria and pathogenicity, although these effects can be ameliorated in later generations [5,26,27]. This raises the possibility that *Wolbachia* induced immune priming is a consequence of maladapted interactions following experimental introduction of *Wolbachia* into a new host. This premise is supported by previous studies that show upregulation of immune genes in *D. melanogaster* cell line S2 experimentally infected with *D. simulans* derived *Wolbachia* strain wRi [20], but not in *D. simulans* flies which were naturally infected with wRi or *Ae. albopictus* naturally infected with wAlbB [21]. Further some natural *Wolbachia* infections can depress antibacterial immunity [25]. It should be noted that the *D. simulans* Me29 line used in the present study is artificially infected with the *D. melanogaster* derived *Wolbachia* strain wMel. We suggest that the lack of antibacterial protection in the Me29 line may be a consequence of adaptation that has occurred during the 13 or more years since this line was established [18]. Thus both the data presented here and previously is consistent with *Wolbachia*-mediated immune priming being important for antibacterial and antiparasite protection in hosts artificially infected with *Wolbachia*.

This study investigated *Wolbachia*-mediated antibacterial protection by utilising three Gram-negative bacterial challenge models. It is possible that the protection response upon challenge with a Gram-positive bacterium may differ from that observed in this study. However, we consider this to be unlikely given the lack of protection against Gram-positive bacteria in mosquitoes [7] and lack of *Wolbachia*-mediated immune stimulation observed in naturally *Wolbachia*-infected *Drosophila*.

The immune pathways Toll, IMD and AMPs showed no evidence of antibacterial immune priming by *Wolbachia* in *D. simulans* [21] or *D. melanogaster*, and no protection against bacterial infection was observed. Given that antiviral protection has been demonstrated using the same *Drosophila* lines as used in the current study [4,9], our results indicate that stimulation of the Toll, IMD or AMP pathways are not necessary for *Wolbachia* stimulated antiviral mechanisms. This leaves other immune pathways, such as Vago and vir-1 [28,29], the major insect viral-defence RNA silencing pathway [30] or competition for host resources as potential *Wolbachia*-mediated antiviral responses. As *Wolbachia*-mediated antiviral protection occurs in both naturally and experimentally infected hosts [3,4,8,10,31] its likely to occur through a conserved mechanism that is independent of that involved in protection against other pathogens.

*Wolbachia*-mediated antiviral protection in *Drosophila* has been shown to be robust. Antiviral protection is observed against many different RNA viruses including both natural pathogens of *Drosophila* and viruses that normally infect mosquito vectors or other insects [4,9,10,32]. In addition, several different *Wolbachia* strains have been shown to protect flies from viruses in a number of different lines of both *D. simulans* and *D. melanogaster* [4,9]. In stark contrast, we show here that *Wolbachia* does not mediate protection against pathogenic bacteria in *Drosophila*. We have demonstrated this using three different Gram-negative bacterial pathogens and six different *Wolbachia*- *Drosophila* combinations, including both *D. simulans* and *D. melanogaster* hosts. These results have implications both for the potential mechanisms of and uses for *Wolbachia*-mediated protection.

### Materials and Methods

**Flies and Wolbachia**

All fly lines were maintained on standard cornmeal diet at 25°C with 12 hours light/dark cycle and were sourced from the lab fly collection. The *D. melanogaster* line Oregon RC (ORC) is naturally infected with *Wolbachia* strain wMelCS [33]. The *D. simulans* lines CO, DSR, N7NO and DSH are naturally infected with *Wolbachia* strains wAu [34], wRi [35], wNo [36] and wHa [37] respectively. The *D. simulans* line Me29 was experimentally infected with wMel over a decade ago by transinfection of NAA TC embryos with wMel from *D. melanogaster* embryos [18]. *Wolbachia*-free lines were generated from each of the fly lines as previously described [4,9].

**Pathogenic bacteria**

Three Gram-negative bacteria that are pathogens of *Drosophila* were used in challenge experiments. *S. marcescens* and *E. carotovora* were grown in LB medium [23]. *P. aeruginosa* PA01 carries an ampicillin resistance gene and was grown in LB medium supplemented with 100 µg/ml ampicillin [38]. To prepare bacteria for challenge bioassays, LB broth was inoculated with bacteria from single colonies on agar plates and incubated for 16 hours at 37°C. Bacteria were then pelleted by centrifugation at 1,500 x g for 10 minutes at room temperature. Fresh bacterial pellets were prepared for each infection bioassay.

**Survival bioassays**

To analyse the susceptibility of flies with and without *Wolbachia* to bacterial induced mortality, 4–7 day old adult male *Drosophila* were challenged with each of the pathogenic bacteria. Flies were anaesthetised with CO₂ prior to infection. A thin needle (diameter = 0.193 mm) was dipped into the undiluted bacterial pellet and used to prick into the thoracic region of each fly. For each fly line, two groups of flies were challenged with pathogenic bacteria: flies with *Wolbachia* and flies without *Wolbachia*. For each group of flies three vials of ten flies were challenged with one of the pathogenic bacteria and one vial of ten flies was mock infected with LB medium. Following challenge flies were maintained in a 25°C incubator and survival of the flies was monitored every 2–6 hours. Mortality within the first 6 hours was deemed to be due to needle injury. At least three independent survival bioassays were done for each bacteria/fly line combination. Survival curves of the two groups of flies were compared using Kaplan-Meier analysis and log-rank statistics reported (GraphPad Prism).

**In vivo bacterial growth**

To analyse the impact of *Wolbachia* on the accumulation of pathogenic bacteria in flies, 4–7 day old adult male ORC flies with and without *Wolbachia* were infected with *P. aeruginosa* PA01 as described above. At 0 and 12 hpi, three live flies were collected individually into 1.5 ml tubes. After addition of 200 µL of LB medium supplemented with 100 µg/ml ampicillin and two 3 mm glass beads (Sigma-Aldrich) to each individual, flies were homogenised for 90 s using a TissueLyser II (Qiagen). Fly homogenates were serially diluted and spread on LB agar plates containing 100 µg/ml ampicillin. Colony forming units per fly (CFU/fly) were calculated after overnight incubation of plates. The experiment was replicated on two independent cohorts of flies and the data pooled.

**Analysis of immune gene regulation**

RT-qPCR was used to compare the expression of six immune genes in ORC flies with and without *Wolbachia*. Genes were chosen on the basis of homology to genes that were upregulated in *Ae.
**References**

1. Hülge-Brecker K, Hammenrieder P, Schlattmann P, Teschner A, Werren JH (2000) How many species are infected with Wolbachia? - A statistical analysis of current data. FEMS Microbiol Lett 201: 215–220.

2. Werren JW, Windsor DM (2000) Wolbachia infection frequencies in insects: evidence of a global equilibrium? Proc Biol Sci 267: 1277–1283.

3. Bnan GW, Xu Y, Lu P, Xie Y, Xi ZY (2010) The endosymbiotic bacterium Wolbachia induces resistance to Dengue virus in Aedes aegypti. PLoS Pathogens 6.

4. Hedges LM, Brownlie JC, O'Neill SL, Johnson KN (2008) Wolbachia and virus protection in insects. Science 322: 702.

5. Hughes GL, Koga R, Xue P, Fukatsu T, Rasgon JL (2011) *Wolbachia* infections are virulent and inhibit the human malaria parasite *Plasmodium falciparum* in *Anopheles gambiae*. PLoS Pathog 7: e1002043.

6. Kambris Z, Blagbrough AM, Pinto SB, Blagrove MSC, Godfray HCJ, et al. (2010) *Wolbachia* stimulates immune gene expression and inhibits plasmodium development in *Anopheles* mosquitoes. PLoS Pathog 6.

7. Kambris Z, Cook PE, Phuc HK, Sinkins SP (2009) Immune activation by life-shortening Wolbachia and reduced filarial competence in mosquitoes. Science 326: 134–136.

8. Moreira LA, Iurie-Ormaetxe I, Jeffery JA, Lu GJ, Pyke AT, et al. (2009) A *Wolbachia* symbiont in *Aedes aegypti* limits infection with Dengue, Chikungunya, and *Plasmodium*. Cell 139: 1208–1278.

9. Osborne SE, Leong SY, O'Neill SL, Johnson KN (2009) Variation in antiviral protection mediated by different *Wolbachia* strains in *Drosophila simulans*. PLoS Pathog 5: e1000656.

10. Tixeira I, Ferreira A, Ashburner M (2008) The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. PLoS Biology 6: e1000002. doi: 10.1371/journal.pbio.1000002.

11. Brownlie JC, Johnson KN (2008) *Wolbachia* and virus protection in insects. Science 322: 702.

12. McMeniman CJ, Lane RV, Cass BN, Fong AWC, Sidhu M, et al. (2009) *Stable Drosophila* male flies from the ORC flies with and without frozen and homogenised in Ribozol (Amresco) with two 3 mm glass beads using TissueLyser II (Qiagen) for 90 seconds with the frequency of 30 shakes/s. Total RNA was extracted and treated with DNase (Promega) for 30 minutes at 37°C to eliminate DNA contamination. 1 μg of total RNA was reverse transcribed using random primers (Promega) and SuperScript III reverse transcriptase (Invitrogen) after which the manufacturer’s protocol. Quantitative PCR was performed in duplicate reactions using Platinum SYBR® green qPCR supermix as per manufacturers instructions (Invitrogen). The temperature profile for the qPCR was 95°C for 2 min, 50°C for 2 min and 40 cycles of 95°C for 10 s, 60°C for 10 s and 72°C for 20 s. qPCR was performed using a Rotor-Gene 6000 (Qiagen). Expression of the target genes was normalised using reference genes Actin 79b (GenBank accession no. NM_079486) and ribosomal protein L 32 (Rpl 32) (Genbank accession no. NM_001144636.2) [41]. Target to reference gene ratios were obtained using QGene v 2.42 [42] and treatment effects on the expression ratios were assessed using Mann Whitney-U tests in STATISTICA V8 (StatSoft).

**Supporting Information**

**Table S1** RT-qPCR primers. (DOCX)

**Acknowledgments**

We thank Elizabeth McGraw for pathogenic bacterial strains and the Johnson lab for critical review of the manuscript.

**Author Contributions**

Conceived and designed the experiments: ZSW LMH JCB KNJ. Performed the experiments: ZSW LMH. Analyzed the data: ZSW LMH KNJ. Contributed reagents/materials/analysis tools: ZSW LMH KNJ. Wrote the paper: ZSW JCB KNJ.

---

13. Xi Z, Gavotte L, Xie Y, Dobson SL (2008) Genome-wide analysis of the interaction between the endosymbiotic bacterium *Wolbachia* and its *Drosophila* host. BMC Genomics 9.

14. Bourzis K, Pettigrew MM, O'Neill SL (2000) *Wolbachia* neither induces nor suppresses transcripts encoding antimicrobial peptides. Insect Molecular Biology 9: 635–639.

15. Vallet-Gely I, Lemaire B, Boccard F (2008) Bacterial strategies to overcome insect defences. Nature Reviews Microbiology 6: 302–313.

16. Lemaire B, Reichhart JM, Hoffmann JA (1997) *Drosophila* host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. Proc Natl Acad Sci U S A 94: 14614–14619.

17. Siozios S, Sapounidis P, Ioannidis P, Bourriot K (2000) *Wolbachia* symbiosis and insect immune response. Insect Science 15: 89–100.

18. Chevalier F, Herberre-Gabourou J, Bertaux J, Raimond M, Morel F, et al. (2011) The immune cellular effectors of terrestrial isopod *Porcellio scaber* during a first meeting with their invaders, *Wolbachia*. PLoS One 6: e18531.

19. McGraw EA, Merritt DJ, Droller JN, O'Neill SL (2002) *Wolbachia* density and virulence attenuation after transfer into a novel host. Proc Natl Acad Sci U S A 99: 2916–2923.

20. Suh E, Mercere DR, Fu Y, Dobson SL (2009) Pathogenicity of life-shortening *Wolbachia* in *Aedes albopictus* after transfer from *Drosophila melanogaster*. Appl Environ Microbiol 75: 7763–7780.

21. Deddouche S, Matt N, Budd A, Mueller S, Kemp C, et al. (2008) The DEAD/H-box helicase Dicer-2 mediates the induction of antiviral activity in *Drosophila*. Nat Immunol 9: 1425–1432.

22. Dostert C, Jouanguy E, Irving P, Troxler L, Galana-Arnoux D, et al. (2005) The Jak-STAT signaling pathway is required but not sufficient for the antiviral response of *Drosophila*. Nat Immunol 6: 946–955.

23. Kemp C, Imler JL (2009) Antiviral immunity in *Drosophila*. Current Opinion in Immunology 21: 3–9.

24. Mousson L, Martin E, Zouache K, Mader Y, Mavingui P, et al. (2010) *Wolbachia* modulates Chikungunya replication in *Aedes aegypti*. Mol Ecol 19: 1953–1964.

25. Glaser RL, Meola MA (2011) The native *Wolbachia* endosymbionts of *Drosophila melanogaster* and *Culex quinquefaciatus* increase host resistance to West Nile virus infection. PLoS One 6: e19776.

26. Riegler M, Sulho M, Miller WJ, O'Neill SL (2005) Evidence for a global *Wolbachia* replacement in *Drosophila melanogaster*. Curr Biol 15: 1428–1433.

27. Hoffmann AA, Clancy D, Duncan J (1996) Naturally-occurring *Wolbachia* infection in *Drosophila simulans* that does not cause cytoplasmic incompatibility. Heredity 76: 1–8.

28. Hoffmann AA, Turelli M, Simmons GM (1996) Unidirectional incompatibility between populations of *Drosophila simulans*. Evolution 40: 692–701.

29. Mercet H, Poinset D (1998) *Wolbachia* transmission in a naturally bi-infected *Drosophila simulans* strain from New-Caledonia. Entomologia Experimentalis et Applicata 86: 97–103.

30. O'Neill SL, Carr TL (1990) Bidirectional incompatibility between conspecific populations of *Drosophila simulans*. Nature 348: 176–180.

31. Huston WM, Jennings MP, McEwan AG (2002) The multiopper 20xidase of *Pseudomonas aeruginosa* is a ferrioxidase with a central role in iron acquisition. Mol Microbiol 45: 1741–1750.

32. Roosen S, Skalský R, Nevalainen A, Pela J, Wozniak AJ (2005) *Wolbachia* infection in *Drosophila simulans* is a ferrioxidase with a central role in iron acquisition. Mol Microbiol 45: 1741–1750.
40. Nolan T, Hands RE, Bustin SA (2006) Quantification of mRNA using real-time RT-PCR. Nature Protocols 1: 1539–1562.

41. Hedges LM, Johnson KN (2008) The induction of host defence responses by Drosophila C virus. J Gen Virol 89: 1497–1501.

42. Joehanes R, Nelson JC (2008) QGene 4.0, an extensible Java QTL-analysis platform. Bioinformatics 24: 2788–2789.