The Bacterial Symbiont Wolbachia Induces Resistance to RNA Viral Infections in Drosophila melanogaster

Luís Teixeira, Álvaro Ferreira, Michael Ashburner

Department of Genetics, University of Cambridge, Cambridge, United Kingdom

Wolbachia are vertically transmitted, obligatory intracellular bacteria that infect a wide number of species of arthropods and nematodes. In insects, they are mainly known for disrupting the reproductive biology of their hosts in order to increase their transmission through the female germline. In Drosophila melanogaster, however, a strong and consistent effect of Wolbachia infection has not been found. Here we report that a bacterial infection renders D. melanogaster more resistant to Drosophila C virus, reducing the load of viruses in infected flies. We identify these resistance-inducing bacteria as Wolbachia. Furthermore, we show that Wolbachia also increases resistance of Drosophila to two other RNA virus infections (Nora virus and Flock House virus) but not to a DNA virus infection (Insect Iridescent Virus 6). These results identify a new major factor regulating D. melanogaster resistance to infection by RNA viruses and contribute to the idea that the response of a host to a particular pathogen also depends on its interactions with other microorganisms. This is also, to our knowledge, the first report of a strong beneficial effect of Wolbachia infection in D. melanogaster. The induced resistance to natural viral pathogens may explain Wolbachia prevalence in natural populations and represents a novel Wolbachia–host interaction.

Introduction

Wolbachia are obligatory, intracellular α-proteobacteria that infect a wide range of arthropods and filarial nematodes. They are found in 17% to 76% of surveyed arthropods and have recently been estimated to be present in 66% of all arthropod species [1–3]; therefore Wolbachia are one of the most widespread intracellular bacteria. Although the phylogenies of Wolbachia strains and their arthropod hosts show horizontal transmission of the bacteria on an evolutionary time-scale [4], these endosymbionts are mainly transmitted maternally. Consequently, Wolbachia strains and the species they infect form long-term associations.

Wolbachia were first discovered infecting the mosquito Culex pipiens in 1924 [5], but interest in these bacteria mainly arose when it was shown that infected mosquito males do not successfully breed with noninfected females [6]. This phenomenon is termed cytoplasmic incompatibility (CI) and has, since then, been found in many other insect species infected with Wolbachia [7]. In some hosts, Wolbachia can also cause feminization, male killing, or parthenogenesis [7]. All these mechanisms profoundly alter the reproductive biology of their hosts and are thought to increase the success of bacterial transmission through the female germline. In the majority of known cases, Wolbachia behave like reproductive parasites of their hosts.

Interestingly, in the parasitic wasp Asobara tabida, a Wolbachia strain is required for the inhibition of apoptosis in the germline and, consequently, normal oogenesis [8,9]. Similarly, Wolbachia is required for normal development and fertility in many filarial nematodes [10–13]. In all these cases, the endosymbionts are obligatory mutualists—they are essential for the survival of their host species. Curiously, examples of Wolbachia infections that are facultative and provide a fitness benefit are rare (e.g., [14,15]). One would, however, expect them to be frequent, since these are long-term symbioses, and Wolbachia fitness ultimately depends on the host fitness.

The model organism Drosophila melanogaster can also be infected with Wolbachia. In fact, they are detected in a large proportion of flies of natural populations and laboratory stocks [16–18]. Interestingly the presence of wMel, the Wolbachia strain associated with D. melanogaster, does not seem to cause a strong phenotype. wMelPop, a Wolbachia variant from a laboratory stock, does cause tissue degeneration and significantly shortens the lifespan of its carriers [19]. The appearance of this strain may be an artifact of conditions in which laboratory stocks are kept, because no wMel variant from natural populations with these characteristics has been discovered. Wolbachia also rescues the sterility of Sex-lethal hypomorphic mutants [20]. However, it is not known how this translates to the interaction of Wolbachia with wild-type flies. How natural variants of Wolbachia affect wild-type D. melanogaster has been extensively addressed. wMel only induces a weak and transient CI phenotype in D. melanogaster

Academic Editor: Laurent Keller, University of Lausanne, Switzerland

Received August 13, 2008; Accepted November 13, 2008; Published December 23, 2008

Copyright: © 2008 Teixeira 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.

Abbreviations: CI, cytoplasmic incompatibility; DCV, Drosophila C virus; FHV, Flock House virus; IV-6, Insect Iridescent Virus 6; RT-PCR, reverse-transcription PCR; TCID50, median tissue culture infective dose

* To whom correspondence should be addressed. E-mail: l.teixeira@gen.cam.ac.uk
Many symbiotic bacteria confer fitness benefits to the organisms that they infect. *Wolbachia* are one of the most widespread intracellular bacteria, infecting a great number of species of insects. Here we show that in the fruit fly *Drosophila melanogaster*, infection with *Wolbachia* increases resistance to a natural pathogen of *Drosophila*, an RNA virus called *Drosophila C* virus. Furthermore, we show that *Wolbachia* also increases resistance of *Drosophila* to two other RNA viruses (Nora and Flock House virus) but not to a DNA virus (Insect Iridescent Virus 6). These results identify a significant new factor that regulates *D. melanogaster* resistance to infection by RNA viruses. Our results add to a growing body of literature showing that the response of an organism to a particular pathogen is modulated by prior or contemporaneous interactions with other microorganisms. That the fruit fly clearly benefits from increased resistance to viruses may provide a solution to the longstanding puzzle as to why *Wolbachia* is so common in natural populations of *D. melanogaster*.

Although some *Wolbachia* strains induce strong CI in the closely related *D. simulans* [24]. This low CI cannot explain how *Wolbachia* spreads and is maintained in wild-type populations, especially considering that, in the wild, infection is not vertically transmitted with 100% fidelity [25]. A strong hypothesis to explain *Wolbachia* presence in natural populations is that *Wolbachia* gives a fitness benefit to *D. melanogaster* [26,27]. Several studies have either been unable to find differences in fitness parameters or found only slight beneficial or detrimental effects of *Wolbachia* infection [25,27–31]. Moreover, even when effects were observed, they were dependent on the *Wolbachia* variant or the fly’s genetic background. A clear strong beneficial effect of *Wolbachia* infection in *D. melanogaster* has still not been shown, and it remains a puzzle why these bacteria are so prevalent in natural populations.

*Wolbachia* is a valuable tool in the study of resistance to pathogens, with many components of innate immunity signaling pathways conserved between *Drosophila* and mammals [32]. The epitome of its utility was the discovery of the involvement of Toll-like receptors in innate immunity. Toll was first discovered to be important in the resistance of *Drosophila* to fungi [33], later Toll-like receptors were shown to have fundamental functions in mammalian innate immunity [34]. Moreover, Toll-like receptors are important in the activation and modulation of mammalian adaptive immunity.

The responses of *Drosophila* to systemic infection by fungi and bacteria are increasingly well known [35]. Although less extensively, *Drosophila* has also been used as a model system to study resistance to viruses. Recent research has shown conservation between flies and mammals in their immune response to viruses. Mutations in *hopscotch*, the gene that encodes the kinase of the JAK-STAT pathway, reduce resistance to *Drosophila C* virus (DCV) infection and increases viral titers [36]. Interestingly, in mammals, JAK-STAT pathways are involved in cytokine signaling, including anti-viral type I interferon [37]. Work in *Drosophila* has also been important in showing that RNA interference is involved in anti-viral resistance in animals. Flies mutant in genes that encode components of this pathway, *Dicer-2, Argonaute-2*, and *r2d2*, are more sensitive to infection by several RNA viruses and have higher titers of viruses than the wild type [38–41].

To identify new genes involved in *Drosophila* resistance to viruses, we initiated a screen for DCV-sensitive flies. In doing so, we found that flies infected with intracellular bacteria were much more resistance to DCV infection than those that were uninfected. We identified these bacteria as *Wolbachia* and show that DCV titers are much lower in *Wolbachia*-infected flies. Moreover, resistance to infection extends to two other RNA viruses but not to a DNA virus. These results identify a new major factor involved in *Drosophila* resistance to RNA viruses, and the first strong beneficial effect associated with *Wolbachia* infection in *D. melanogaster*.

**Results**

**Tetracycline Treatment Reduces Resistance to DCV**

In order to identify new genes involved in *D. melanogaster* resistance to viruses, we are conducting a genetic screen for virus-sensitive mutants (LT, AF, MA, unpublished data). We have generated a collection of mutant lines by P-element insertional mutagenesis using the set of w1118 iso isogenic lines described in Ryder et al., 2004 [42]. We chose an isogenic background to minimize variability in the response to viral infection and, for the same reason, cleaned the initial set of lines of potential chronic viral infections using the protocol described in Brun and Plus, 1978 [43]. We test the resistance of each insertion line to DCV infection. DCV is a small, non-enveloped virus with a single-stranded, positive-sense RNA genome that belongs to the Dicistroviridae family, an insect specific family of viruses very similar to picornaviruses [44]. This virus is a natural pathogen of *D. melanogaster*, it is sequenced and relatively well characterized, and its infection has an easily scored lethal phenotype [43,44].

In the initial screen, we assayed adult survival after intrathoracic DCV injection and realized that, unexpectedly, the control w1118 iso line was much more sensitive to DCV than most of the tested P-element insertion lines (Figure 1 and unpublished data). When injected with a dose of 500 times the median tissue culture infective dose (TCID$_{50}$), all the w1118 iso males died within 12 d, whereas very little death was observed in the males of the P-element insertion lines (Figure 1A). Moreover, a large proportion of males of the P-element insertion line survived until 21 d after infection. Preliminary analysis showed that the P-element was not responsible for virus resistance (unpublished data). We then tested the hypothesis that a previous treatment of the w1118 iso line with tetracycline could have rendered it more sensitive to DCV. We raised flies of a P-element insertion line, VF-0058–3, on tetracycline-containing medium or control medium and compared the adults’ resistance to DCV infection (Figure 1A). The tetracycline treatment made the flies die much faster upon DCV infection, with a sensitivity similar to that of w1118 iso line. This result strongly suggested that a tetracycline-sensitive bacteria, associated with the resistant stocks, conferred resistance to the viral infection. We discarded the possibility that the effect was an artifact of the tetracycline per se because raising w1118 iso flies on medium with tetracycline did not make them more sensitive to DCV (Figure 1B). We also treated the VF-0058–3 and VF-0097–5 P-element insertion lines with tetracycline for two generations and moved them back to normal medium for at least five generations in order to negate any side effects of tetracycline itself (these stocks will be referred as VF-0058–3t and VF-
We then repeated the assay, comparing resistance to \( D \)CV of these treated stocks to the non-treated stocks (Figure 1C). The tetracycline treatment makes the \( P \)-element insertion lines stably more sensitive to \( D \)CV than non-treated lines and equally sensitive to \( D \)CV as the \( w^{1118} \) iso line. A similar result was obtained when females of these lines were injected with \( D \)CV, with the difference that females are less sensitive to \( D \)CV than males (unpublished data). Importantly, in the timeframe of this assay, the survival of tetracycline-treated and non-treated stocks do not differ when only injected with buffer (Figure 1D).

In summary, these results show that tetracycline-sensitive bacteria, not easily acquired from the laboratory environment, confer on \( D. \) melanogaster resistance to \( D \)CV.

The increased resistance to \( D \)CV could be due to increased resistance to the damage caused by the viral infection or decreased viral proliferation. To test this, we probed by Western blot the levels of viral proteins in VF-0058–3 and VF-0058–3t adult flies after infection with \( D \)CV (Figure 1E). While viral proteins were not detectable on extracts of \( D \)CV-infected VF-0058–3 flies, they were clearly detectable on extracts of \( D \)CV-infected VF-0058–3t flies, and their levels increased from 3 to 6 d post-infection. We extended this analysis by quantifying, in cell culture, the viral titer in these flies after \( D \)CV infection (Figure 1F). \( D \)CV is detected after infection in flies from both stocks and slightly increases from 3 to 6 d post-infection. However, \( D \)CV levels are considerably higher, by approximately 10,000 times, in VF-0058–3t flies. These experiments show that the bacteria that confer resistance to \( D \)CV infection interfere with the virus proliferation.

Identification of the Viral Resistance–Inducing Bacteria

The fact that the tetracycline treatment permanently renders the flies sensitive to \( D \)CV shows that the bacteria are not easily acquired from the laboratory environment. To test if the resistance could be horizontally acquired, we raised together the progeny of \( w^{1118} \) iso females (without resistant-inducing bacteria) with the progeny of VF-0058–3 females (with resistant-inducing bacteria) and then assayed the levels of viral proteins in infected flies (Figure 2A). The progeny of these females can be distinguished by their eye color, due to

---

**Figure 1.** Tetracycline Treatment Increases Flies Sensitivity to \( D \)CV

(A, B, and C) Fifty 3–6-d-old males, per sample, were injected with \( D \)CV, and their survival was followed daily. (A) Flies \( w^{1118} \) iso, VF-0058–3, and VF-0058–3 raised on tetracycline for one generation were injected with 500 TCID\(_{50} \) \( D \)CV. (B) Flies \( w^{1118} \) iso and \( w^{1118} \) iso raised on tetracycline were injected with 50 TCID\(_{50} \) \( D \)CV. (C) Flies \( w^{1118} \) iso, VF-0058–3, VF-0058–3t, VF-0097–3, and VF-0097–3t were injected with 500 TCID\(_{50} \) \( D \)CV. Each assay was repeated once with males and twice with females with similar results.

(D) Fifty 3–6-d-old males, per sample, of VF-0058–3 and VF-0058–3t lines were injected with 50 mM Tris-HCl, pH 7.5, kept at 18°C and their survival was followed daily.

(E) Extracts of VF-0058–3 and VF-0058–3t flies 3 and 6 d after injection with 500 TCID\(_{50} \) \( D \)CV or not injected were probed in a Western blot with anti-\( D \)CV. Anti-tubulin was used as a loading control.

(F) Titration, in cell culture, of \( D \)CV levels per fly of VF-0058–3 and VF-0058–3t flies 3 and 6 d after injection with 500 TCID\(_{50} \) \( D \)CV. Squares are replicates (four per sample), lines are geometric means of replicates. Virus titres in VF-0058–3 and VF-0058–3t are significantly different on both days post-infection (Mann-Whitney test, \( p = 0.0287 \) for both comparisons).

doi:10.1371/journal.pbio.1000002.g001
the presence of a functional white gene, in the Rs3 transposon, only in the progeny of VF-0058–3 flies. w1118 iso flies do not acquire the resistance to DCV when raised mixed with the progeny of VF-0058–3 flies. Therefore, the bacteria that confer resistance to DCV are not acquired horizontally. We then tested if the resistance was vertically transmitted by crossing males and females from VF-0058–3 and VF-0058–3t stocks in all four possible combinations and assaying the survival of the adult progeny after DCV infection (Figure 2B). The results clearly show that the determinant factor of the progeny resistance is the mother’s resistance; therefore, the bacteria in question are maternally transmitted, which strongly suggests they are intracellular. However, the bacteria could, in theory, be only transmitted by the mother but not be intracellular (e.g., they could be deposited on the egg surface). This did not seem to be the case, because flies that were raised from VF-0058–3 surface-sterilized embryos did not become more sensitive to DCV (Figure 2C). Moreover, we could visualize the presence of intracellular bacteria by DNA staining in embryos from VF-0058–3 and VF-0097–3 stocks but not from VF-0058–3t, VF-0097–3t, or w1118 iso stocks (Figure 2D). We can therefore conclude that the viral resistance is mediated through maternally transmitted intracellular bacteria.

To identify the intracellular bacteria in question, we extracted DNA from surface-sterilized embryos of resistant-to-DCV flies (VF-0058–3) and performed PCR amplification using prokaryotic 16S rRNA universal primers. We analysed the product of this amplification by cloning it and sequencing over 100 independent clones. All the 104 sequences of inserts in the cloning plasmid we obtained were at least 99.5% identical to the sequence of a fragment of the 16S rRNA gene of Wolbachia (GenBank accession number EU096232; http://www.ncbi.nlm.nih.gov/Genbank/). Therefore, these embryos, which carry the resistance to DCV inducing bacteria, are most probably only infected with Wolbachia. To verify the presence of Wolbachia, we performed PCR amplification using primers for the Wolbachia specific genes wsp and wspB [45,46] (Figure 2E). Wolbachia is present in VF-0058–3 and VF-0097–3 and absent from the VF-0058–3t and VF-0097–3t embryos' extracts. The sequence of the wsp-specific primers' PCR amplification product from the VF-0058–3 flies is identical to the wsp sequence of wMel (GenBank accession number DQ235407), the only Wolbachia strain known to infect D. 
melanogaster. In a recent survey in 35 different Drosophila species, that screened over 4,500 individuals, only two kind of heritable endosymbiotic bacteria were found: Wolbachia and Spiroplasma [47]. We specifically tested for the presence of Spiroplasma in the VF-0058–3 line (and the wt-1 to -6 lines used in Figure 3B) using primers specific for the 16S rRNA gene of Spiroplasma [18]. We detect Spiroplasma in a positive control, RED-67 [48], but not in any of the other tested lines. Therefore it is not Spiroplasma that confers resistance to viruses. This result and the sequencing results strongly suggest that the maternally inherited intracellular bacteria that confer resistance to DCV are Wolbachia.

These results show that resistance to DCV is associated with the presence of Wolbachia, however it could be possible that other cryptic intracellular bacteria were responsible for the virus resistance, and Wolbachia would merely be present in these flies by chance. Wolbachia cannot be cultured and therefore we cannot infect a sensitive stock with a pure cultured isolate and verify acquired resistance to DCV. Wolbachia can be artificially transferred from an infected host to a new host. However, if we did transfer Wolbachia from infected flies to non-infected flies and show concomitant transfer of resistance to DCV, we could not discard the possibility that we were also transferring the hypothetical cryptic bacteria. We addressed this problem by treating the Wolbachia-infected stock VF-0058–3 with a suboptimal dose of tetracycline for one generation and then establishing isofemale lines from the progeny. We expected to obtain lines that kept the Wolbachia infection and other lines that lost it. The segregation of Wolbachia should be independent of the segregation of any hypothetical other bacteria. From two independent sets, one set of ten lines and another set of 23 lines, we established, in total, three lines that conserved Wolbachia infection and 30 lines that lost it. We then tested these lines for resistance to viruses (Figure 3A). Wolbachia presence and viral resistance fully segregate with each other; the probability that the presence of Wolbachia and resistance to DCV are independent is very low (Fisher’s exact test, p = 0.0002). These data strongly indicate that it is Wolbachia infection that induces DCV resistance.

To corroborate that it is Wolbachia infection that protects D. melanogaster from DCV, we analyzed this interaction in other independent fly stocks. We screened, by PCR, for Wolbachia presence in a collection of wild-type stocks kept in our laboratory and we found six infected lines. After establishing tetracycline-treated stocks derived from these
We can conclude that not statistically significant (Mann-Whitney test, p). Determining, in cell culture, the viral titer per infected fly, 6 proteins in the infected VF-0058–3t flies, compared with the Surprisingly, we only detected a slight increase in FHV day 21. We can detect, by Western blot, increase in FHV flies die by day 13, while only 40% of VF-0058–3 flies die by day 21. We can detect, by Western blot, increase in FHV flies. This probably represents just a cumulative effect of the deleterious effects of Wolbachia and IIV-6 infection. In fact, Wolbachia infection has a long-term deleterious effect that results in a shorter lifespan in the absence of viral infection (Figure 4E). In accordance with this interpretation, the average IIV-6 titer, 10 d after infection, is only 1.8-fold higher in Wolbachia-infected flies compared with Wolbachia-free flies, and not significantly different (Mann-Whitney test, p = 0.08118) (Figure 4G). In conclusion, Wolbachia presence does not protect D. melanogaster from IIV-6 infection.

Discussion

We have shown that Wolbachia infection in D. melanogaster induces resistance to DCV infection. Several lines of evidence lead to this conclusion. The resistance to DCV was maternally transmitted and sensitive to tetracycline, as is Wolbachia; in embryos infected with bacteria inducing resistance to DCV, we can only detect the presence of Wolbachia; all tested D. melanogaster lines that carried Wolbachia became more sensitive to DCV after tetracycline treatment; lines that did not carry Wolbachia did not become more sensitive to DCV after tetracycline treatment. Finally, when transmission to the next generation was imperfect, due to treatment of larvae with a low dose of tetracycline, Wolbachia and resistance to DCV co-segregated. Following Occam’s razor principle—Pluralitas non est ponenda sine necessitate. “Plurality should not be posited without necessity.” —the simplest and most plausible hypothesis is that Wolbachia is the causative agent of resistance to DCV.

Infection by Wolbachia considerably increased the lifespan of DCV-infected flies. This is due to a strong reduction in viral titers, as observed by Western blot and titration by cell culture. At 3 d post infection, the DCV titer in Wolbachia-infected flies was 10,000 times less than that in Wolbachia-free flies. This difference is larger than that reported between the wild type and mutants in the anti-viral resistance genes Der-2,
ago-2, and hop [36,38,40]. Wolbachia is clearly a major factor affecting Drosophila resistance to DCV.

Wolbachia and DCV are common symbionts of D. melanogaster. However, the interaction is not specific to DCV; we found that Wolbachia also induced resistance to two other RNA viruses. In the case of Nora virus, there was also reduction in the viral titer of infected flies. FHV infection, in terms of mortality, was also much less severe in the presence of Wolbachia, to a degree similar to that seen with DCV. But, with this virus, Wolbachia only slightly affected viral titer. The resistance to FHV is most probably an increase in resistance to the damage caused by the viral infection rather than an ability to inhibit virus proliferation. However, we cannot exclude the possibility that there is strong inhibition of FHV proliferation in certain essential adult tissues or that a small decrease in viral titer is enough to significantly increase the lifespan of infected individuals.

DCV and Nora virus differ from FHV in two ways: they are both natural pathogens of Drosophila and both are picornavirus-like. An endogenous virus and its host could be co-adapted so that a small advantage, in this case provided by the bacteria to the host, would profoundly tilt the equilibrium between virus and host, whereas an exogenous pathogen may be less sensitive to bacterial infection of its host. On the other hand, Wolbachia could interfere with the life cycle of picornavirus-like viruses but not of FHV, a nodavirus. We cannot distinguish between these possibilities with such a small sample of viruses; it would be interesting to extend the analysis to other RNA viruses that infect D. melanogaster (e.g., Sigma [a rhabdovirus] [43], Drosophila X virus [a birnavirus] [55], and Drosophila A virus [picornavirus-like]) [43].

We have also tested the interaction of Wolbachia with a DNA virus, IVV-6. Wolbachia did not protect Drosophila from this virus; it actually decreased the lifespan of infected flies. We think this is due to the cumulative effect of Wolbachia and IVV-6 infection, since, in the genetic background of the flies we were using, Wolbachia had a negative effect on long-term survival. It would be interesting to also extend the analysis to other DNA viruses, however there are no DNA viruses known to infect D. melanogaster. To our knowledge, this is the first report of a DNA virus proliferating in adults of D. melanogaster.

An obvious question is how Wolbachia induces resistance to

Figure 4. Wolbachia Interaction with Other Viruses
(A) RT-PCR was done with Nora virus primers on RNA of VF-0058–3, VF-0058–3t, and Oregon R flies (left). PCR with RpL32 was done as control. Three–six–d-old males of VF-0058–3 and VF-0058–3t lines were injected with a virus extract of Oregon R flies and collected 3 d later. RNA was extracted and RT-PCR done with primers for Nora virus and RpL32. The same number of PCR cycles was done for both samples. The assay was repeated three more times, from infection of flies with virus extract, with similar results.
(B) Fifty 3–6-d-old males, per sample, of VF-0058–3 and VF-0058–3t lines were injected with 50 TCID50 FHV, and their survival was followed daily. The assay was repeated twice with males and once with females with similar results.
(C) Extracts of VF-0058–3 and VF-0058–3t flies 3, 6, and 9 d after injection with 50 TCID50 FHV or not injected were probed in a Western blot with anti-FHV. Anti-tubulin was used as a loading control.
(D) Titration, in cell culture, of FHV levels per fly of VF-0058–3 and VF-0058–3t flies 6 d after injection with 50 TCID50 FHV. Squares are replicates (10 per sample), lines are geometric means of replicates. Virus titres in VF-0058–3 and VF-0058–3t are not significantly different (Mann-Whitney test, p = 0.05764).
(E) Fifty 3–6-d-old males, per sample, of VF-0058–3 and VF-0058–3t lines were injected with 1,000 TCID50 IIV-6 or buffer, and their survival followed. The assay was repeated once with males and the IIV-6 injected flies survival curves were also repeated with females, with similar results.
(F) Iridescent-infected male 20 d after injection with 1000 TCID50 IIV-6 (right) and not infected same age male (left) are shown.
(G) Titration, in cell culture, of IIV-6 levels per fly of VF-0058–3 and VF-0058–3t flies 10 days after injection with 1000 TCID50 IIV-6. Squares are replicates (10 per sample), lines are geometric means of replicates. Virus titres in VF-0058–3 and VF-0058–3t are not significantly different (Mann-Whitney test, p = 0.08118).
doi:10.1371/journal.pbio.1000002.g004
RNA viruses. The different effect on DCV/Nora virus and FHV raises the possibility that this effect is multifactorial; interfering with virus replication in some cases and increasing resistance of Drosophila to viral infection damage in others. One important question to address is whether the effect is cell-autonomous or systemic. Wolbachia is widespread throughout tissues of the infect host [18,56], so both hypotheses are possible. This could be investigated in tissue culture with Wolbachia-infected cells. If the effects are cell autonomous, one explanation for increased resistance to viruses could just be competition for resources, since both microorganisms occupy the same niche, the host’s cytoplasm. For example, Wolbachia is thought to acquire much of its energy from the metabolism of amino acids imported from the host cytoplasm [46]. DCV, on the other hand, is very sensitive to perturbations in host translation [57]. The presence of Wolbachia could reduce the pool of cytoplasmic amino acids to a point that interferes with translation of viral proteins. Another possibility is that Wolbachia infection could trigger cell-autonomous mechanisms of resistance to intracellular pathogens, such as a reduction in cellular metabolism. A further explanation for a cell-autonomous effect would be that Wolbachia has been selected to actively interfere with virus replication in co-infected cells. Wolbachia has a complete type IV secretion system [46], which many bacteria use for translocation of effector molecules into host cells (e.g., Legionella and Agrobacterium). Genes encoding proteins with ankyrin repeats, involved in protein–protein interactions, are over-represented in the Wolbachia genome [46,58] and are good candidates for mediators of anti-viral resistance.

If the effect is systemic, a strong hypothesis is that Wolbachia could alter the host–immune response, increasing resistance to viral infection. The pre-activation of the host immune system, for example, could allow for a faster response upon viral infection. This would be similar to what happens in a herpesvirus-induced resistance to Listeria in mice, due to the production of cytokines [59]. It was also reported recently that the presence of gut flora slightly increases the resistance of Aedes aegypti to Dengue virus, presumably through activation of the Toll pathway [60]. In tissue culture of D. melanogaster cells, infection with Wolbachia slightly increases the expression of innate immune genes [61]. There is also a report that Wolbachia increases resistance of D. melanogaster to the pathogenic fungus Beauveria bassiana [62]. All these reports support a model of general activation of innate immunity. However it has also been shown that in adult D. simulans and Aedes albopictus Wolbachia does not activate the expression of anti-microbial peptides [63], in D. simulans, Wolbachia infection does not alter sensitivity to Beauveria and renders the host more sensitive to parasitoid wasps [64], and in D. melanogaster, Wolbachia presence does not affect Spiroplasma levels [65]. In summary, it is not clear if there is a general activation of innate immunity in adult D. melanogaster infected with Wolbachia that would render them more resistant to other pathogens. It would be interesting to identify immune pathways involved in anti-viral resistance activated by Wolbachia infection. It would also be important to analyze Wolbachia-induced resistant to other microorganisms, including pathogenic bacteria.

A different hypothesis would be that Wolbachia infection actually inhibits some of the immune responses against viral infection and that increases the lifespan of infected D. melanogaster. This may be true if the host response to infection damages the host itself, as in the case of septic shock in mammals. This could explain the increased resistance to FHV infection without a strong effect on viral titers. Finally, a similar hypothesis would be that Wolbachia inhibits, cell-autonomously or systemically, apoptosis induced upon viral infection. Some published data support this hypothesis; FHV induces apoptosis in tissue culture cells [66], Wolbachia inhibits apoptosis in the germline of Asobara tabida [8,9], and the Wolbachia protein Wsp inhibits apoptosis in human cells [67].

This new host-microorganism-microorganism interaction adds to the perception that the response of a host to a particular pathogen also depends on its interactions with other microorganisms. Other examples are herpesvirus latency-induced protection to Listeria in mice mentioned above [59], the suppression of HIV-1 infection by human herpesvirus 6 in human cells [68], symbiotic bacteria protection against fungi in a shrimp and an aphid [69,70], symbiotic bacteria protection against parasitic wasps in an aphid [71], and symbiotic bacteria protection against fungal infection in a wasp [72]. As also mentioned above, there is a recent report that gut flora has a protective role against Dengue virus in A. aegypti [60]. However, this is, to our knowledge, the first report where bacteria that confer protection against viruses have been identified.

This interaction has some practical consequences. Researchers working on Drosophila immunity against viruses should take in consideration the presence of Wolbachia in the stocks they are analyzing. On the other hand, researchers working on Wolbachia should consider that any observed effects of Wolbachia could be mediated through effects on viral infections. A practical application of this discovery would be, if possible, to induce resistance to viruses, by infection with Wolbachia, in insects that are beneficial to humans (e.g., honeybee) or transmit arboviruses (e.g., mosquitoes). However, introducing Wolbachia to virus-transmitting vectors could be a double-edged sword. If the interaction Wolbachia-vector-virus were similar to the one seen in this report with DCV, then it would be beneficial because it could decrease the probability of the vector being infected or transmitting the disease. If, however, it were similar to the interaction with FHV, then there would be the risk of having healthier infected vectors with high titers of viruses, therefore increasing disease transmission. This latest possibility should be taken into account in proposed strategies of introducing Wolbachia in vectors of arboviruses [73,74].

Finally, this is, to our knowledge, the first report of a strong beneficial effect of Wolbachia infection in D. melanogaster. The induced resistance to natural viral pathogens may explain the prevalence of Wolbachia in natural populations. It also indicates that the fitness benefit of having Wolbachia is dependent on the viral infection status of the population. This may explain differences in Wolbachia infection frequencies between populations [17,25] and variable fitness effects in different D. melanogaster lines [28,30]. It would be interesting to broaden the analysis to other Wolbachia strains and to other Wolbachia–host combinations. If Wolbachia induces resistance to viruses in other hosts, this would have major implications for our understanding of the very widespread presence of this endosymbiont in arthropods and filarial nematodes.

After the submission of this manuscript, an independent report with similar findings to ours was published [75]. In
agreement with our data, Hedges et al. show that the treatment of Wolbachia-infected flies with tetracycline renders them more sensitive to three RNA viruses: DCV, Cricket Paralysis virus, and FHV. Moreover, they also show that the levels of DCV increase in infected Wolbachia-free flies.

Materials and methods

Fly strains and husbandry. The set of w^{118} iso isogenic flies were obtained from the DrosDel collection in our laboratory [42]. These lines were cleaned of viruses similarly to the protocol in Brun and Plus, 1978 [43]. Flies were aged to 30 d at 25 °C and their eggs were collected in agar plates, treated with 50% bleach for 10 min, washed with water, and transferred to fresh vials.

The wild-type laboratory lines used in Figure 3B have the origins described in Table 1.

Stocks were treated with tetracycline (cleaned of Wolbachia infection) by raising them for two generations in ready-mix dried food (Philip Harris) with 0.05 mg/ml of tetracycline hydrochloride (Sigma). Sub-optimal tetracycline treatment was done by raising flies, for one generation, in food with 0.00625 mg/ml of tetracycline hydrochloride. Virgin adult females, emerging from these vials, were collected and individually crossed with males to establish isofemale lines.

Sensitive and resistant-to-viruses flies were raised together by placing in a vial one w^{118} iso female, one VF-0058–3 female, and two w^{118} iso males. The progeny of the two different females could be distinguished by the eye color, because only the progeny of VF-0058–3 females have a functional white gene. Flies were only collected from vials that had adults of both phenotypes.

| Number on Figure 3B | Ashburner Lab Identification | Origin | Via |
|---------------------|------------------------------|--------|-----|
| 1                   | W-10                         | Hanover, MA, USA, 1967 | unknown |
| 2                   | W-12                         | Jerusalem, Israel, 1970 | Stock number W520, Umeå DSC, Sweden |
| 3                   | W-20                         | Oregon, USA, 1925 or earlier | Oregon-R stock, NIG, Mishima, Japan |
| 4                   | W-23                         | Tai, Ivory Coast, 1983 | Stock number 255.1, CNRS, GIF, France |
| 5                   | W-30                         | Sengwa Wildlife Reserve, Zimbabwe, 1990 | NIG, Mishima, Japan |
| 6                   | Aljezur 1                    | Aljezur, Portugal, 2005 | Collected by L. Teixeira |
| 7                   | W-16                         | Crete, Greece, 1975 | Stock number W570, Umeå DSC, Sweden |
| 8                   | W-29                         | Staket, Sweden | Stock number W830, Umeå DSC, Sweden |
| 9                   | W-19                         | Villeurbanne, France | CNRS, GIF, France |
| 10                  | W-26                         | Novosibirsk, Russia | Glasgow, UK |
| 11                  | W-35                         | Line 2b, as described in [76] | Lund, Sweden |
| 12                  | Oregon R                     | Oregon, USA 1925 or earlier | Unknown |

FHV was kindly provided by Dr. J.-L. Imler [38] and dilutions of this aliquot were used in this work.

Virus injection. Three-to-six-d-old flies were injected with a Nanoject II injector (Drummond). Viruses were re-suspended or diluted in 50 mM Tris-HCl, pH 7.5, and 69 ml of virus solution was injected, per fly, in the thorax, between the mesopleura and the metapleura. Flies were injected while anesthetized with CO2. Fifty flies were injected per sample, ten flies were placed per vial, and vials were changed twice a week. DCV injected flies were kept at 18 °C. N. corona–-, FHV–, and IV–6–injected flies were kept at 25 °C. Flies were counted daily for all survival curves except for CV-0058–3 and CV-0058–3t injected with buffer, as shown in Figure 4E, which were counted at least twice a week.

Virus titration. Five flies were pooled per sample. Flies were squashed in 50 mM Tris-HCl, pH 7.5, frozen, thawed, and centrifuged for 10 min at 20,000 g and supernatant was collected (DCV and FHV). For IV–6–, centrifugation was done twice at 600 g and the supernatant was passed through a 0.45-μm filter before the assay. Viruses titers were determined in cell culture and calculated by the Reed and Muench end-point calculation method [80]. DL2 cells in 96-well plates were infected with the serial dilutions of virus suspensions. DCV and FHV infection was scored by the presence of cell death. IV–9 was scored by non-proliferation of cells and presence of very large cells. Extracts of non-infected VF-0058–3 or VF-0058–3t flies did not cause any cytopathic effect in tissue culture cells.

Western blots. Five to eight males were pooled per sample. Rabbit polyclonal antibodies raised against purified DCV was kindly given by Dr. Peter Christian. Rabbit polyclonal antibodies raised against FHV capsids was kindly given by Dr. Jean-Luc Imler [38]. Specificity of antibodies was verified by lack of signal on Western blot lanes of non-infected flies (Figures 1E and 4C). E7 mouse monoclonal anti-b- tubulin was acquired from Developmental Studies Hybridoma Bank [81].

Propidium iodide staining. Embryos 0–2-h-old were collected, treated with 50% commercial bleach for 10 min, fixed for 30 min in 4% formaldehyde, 50% heptane, the vitelline membranes were removed by vortexing the embryos in 50% heptane, 50% methanol, then embryos were washed briefly in methanol and for 10 min in 50% methanol, 50% PBS and finally placed in PBS 0.1% tween-20. Embryos were treated with RNAse H 0.25 μg/ml for 30 min at 37 °C, washed in PBS 0.1% tween-20, stained with PBS 0.1% tween-20 and 1 μg/ml propidium iodide (Sigma) for 30 min, washed in PBS 0.1% tween-20 and mounted in Vectorshield. Images were taken in a confocal microscope.

PCR, sequence analysis, and RT-PCR. A fragment of bacterial 16S rRNA gene was amplified from DNA of Drosophila embryos surface sterilized by treatment with 50% commercial bleach for 10 min. DNA was extracted using Wizard Genomic DNA purification kit (Promega). Primers used were 27f (5′-GGCGATTGCTAACTGCTG-3′) and 1495r (5′-CTATCCAGGTTGTTTACGGC-3′). The PCR program was: 94 °C for 4 min; 25 cycles of 94 °C for 30 s, 58 °C for 1 min, and 72 °C for 2 min; 72 °C for 10 min. The PCR product was ligated into pCR 2.1 TOPO vector (Invitrogen) and transformed into DH5α cells. Nineteen plasmid DNA preparations and 96 bacteria cultures were sent for sequencing. Three sequencing reactions failed and 8 clones did not carry an insertion in the cloning plasmid. From all the other
104 sequences, we selected a sequence of at least 600 bp with good quality and aligned it with a fragment of the 16S rRNA gene of Wolbachia (GenBank accession number EU096252) using Clustal W 2 [82].

PCR amplification of Wolbachia-specific genes was done either on the DNA extracts of embryos as described above (Figure 2E) or on DNA extracts of infected flies (Figure 3A) using primers described before. PCR amplification was carried out with an initial denaturation step at 94°C for 4 min, followed by 30 or 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, 20 cycles of 94°C for 1 min; 54°C for 1 min, and 72°C for 1 min, or 20 cycles of 94°C for 30 s, 54°C for 1 min, and 72°C for 1 min. The reaction mixture contained 10 mM Tris-HCl pH 8.0, 50 mM KCl, 1.5 mM MgCl2, 0.2 mM dNTPs, 50 ng template DNA, 1 unit an appropriate unit of Taq DNA polymerase (Invitrogen), 10 pmol of each primer, and 1.25 units of Taq polymerase (Invitrogen). The PCR program was: 94°C for 4 min; 50 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; 20 cycles of 94°C for 30 s, 54°C for 1 min, and 72°C for 1 min. The wsp primers amplification product from VF-0058–3 embryos was purified, as described above, and sequenced. The sequence obtained was identical to a fragment of the wsp sequence of WmEl (GenBank accession number DQ235407).

PCR amplification with primers specific for Spiroplasma 16S rRNA gene was done on DNA extracts of adult flies. Primers used were SpwF (5′-GCTTTGCGGACGATTTC-3′) and SpwR (5′-CGTTGGGTCCTCTGAT-3′) [48]. The PCR program was: 94°C for 4 min; 30 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min; 72°C for 1 min. As a positive control for cytoplasmic DNA extraction we used the primers for mitochondrial 12S rRNA, 12SAI (5′-AACTACGGATTAGATACCTATTAT-3′) and 12SB1 (5′-AAGGACGGGGCGGATGTTGT-3′) [4]. The PCR program used was: 94°C for 4 min; 50 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; 72°C for 10 min. The wsp primers amplification product from VF-0058–3 was used as a positive control for cytoplasmic DNA extraction, the amplification was carried out with an initial denaturation step at 94°C for 4 min, followed by 25 cycles of 94°C for 30 s, 54°C for 1 min, and 72°C for 1 min, or 20 cycles of 94°C for 30 s, 54°C for 1 min, and 72°C for 1 min.

Nora virus presence in VF-0058–3, VF-0058–3t, and Oregon R stocks was analyzed by RT-PCR. RNA of 100 flies, per sample, was extracted using Trizol (Invitrogen). cDNA was synthesized from each RNA sample using random hexamers, at 50°C. The Nora primers used for PCR were Nora-F (5′-TAAAGGTGTAGAGACAGACG-3′) and Nora-R (5′-CGTAAAACACCAACTTCTC-3′) [49]. RpL32 primers, used as a positive control for RNA extraction, were RpL32F (5′-TCTCAGCGCCTTAAGATGC-3′) and RpL32R (5′-CAGCCGTGTGACGCGAGAAC-3′). The PCR program used was as follow: 94°C for 4 min; 10 cycles of 94°C for 30 s, 60°C minus 0.6°C per cycle for 1 min; and 72°C for 1 min; 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; 72°C for 10 min. The PCR amplification fragment obtained with the Nora primers was purified, as described above, and sequenced. The sequence was 98% identical to a fragment of Nora virus genome sequence (GenBank accession number DQ292170).

For semi-quantitative analysis of Nora virus in infected flies, the procedure was as above except that 25 flies were used per sample and the PCR amplification for each sample was done with a total of 20, 25, and 30 cycles.

Acknowledgments

We would like to thank Peter Christian for stocks of DCV and IVV-6, antibodies against DCV, invaluable help in the setup of several virus-related protocols, and discussions. We would like to thank Jean-Luc Imler for kindly providing the FHV stock and antibodies against FHV. We would like to thank Gregory Hurst for kindly providing the RED-67 strain. We would also like to thank John Roote for help related with laboratory fly stocks.

Author contributions. LT conceived and designed the experiments. LT performed the experiments. LT analyzed the data. LT and MA wrote the paper. MA also provided support and discussions.

Funding. LT was partially supported by a postdoctoral fellowship from Fundação para a Ciência e Tecnologia (Portugal). The research was supported by a grant from the Biotechnology and Biological Sciences Research Council (UK).

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

References

1. Warren JH, Windsor D, Guo LR (1995) Distribution of Wolbachia among neotropical arthropods. Proc R Soc Lond B Bio 262: 197–204.
2. Jetaprakash A, Hoss MA (2000) Long PCR improves Wolbachia DNA amplification: wsp sequences found in 76% of thirty-six arthropod species. Insect Mol Biol 9: 393–405.
3. Hilgenboecker K, Hammerstein P, Schlattmann P, Telschow A, Werren JH (2007) Wolbachia infection in Aedes albopictus: accelerating cytoplasmic drive. Genetics 160: 1087–1094.
4. O'Neill SL, Giordano R, Colbert AM, Karr TL, Robertson HM (1992) 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility genes. Proc Natl Acad Sci U S A 89: 2699–2702.
5. Hertig M, Wolbach S (1924) Studies on Rickettsia-Like Micro-Organisms in Insects. J Med Res 44: 329–374.
6. H. Yen JH, Barr AR (1973) The etiological agent of cytoplasmic incompatibility in neotropical arthropods. Proc R Soc Lond B Bio 242: 242–250.
7. Shoulong YM, Breesew JA, Hurst GD (1999) Wolbachia pipientis microbial manipulator of arthropod reproduction. Annu Rev Microbiol 53: 71–102.
8. Bedine F, Vavre F, Fleury L, Bopp H, Hochberg ME, et al. (2001) Removing symbiotic Wolbachia bacteria specifically inhibits oogenesis in a parasitic wasp. Proc Natl Acad Sci U S A 98: 6247–6252.
9. Pannenbakker BA, Loppin B, Elemans CP, Humbolt L, Vavre F (2007) Parasitic inhibition of cell death facilitates symbiosis. Proc Natl Acad Sci U S A 104: 213–215.
10. Hoerauf A, Volkman L, Hamelmann C, Adjei O, Autenrieth IB, et al. (2000) Endosymbiotic bacteria in worms as targets for a novel chemotherapy in BLT. Lancet 355: 1242–1243.
11. Hoerauf A, Nissen-Paeth K, Schuetz C, Henke-Duhrsen K, Blaxter ML, et al. (1999) Tetracycline therapy targets intracellular bacteria in the filarial nematode Litomosoides sigmodontis and results in filarial infertility. J Clin Invest 103: 11–18.
12. Carvone M, McCall JW, Simoncini L, Kramer LH, Sacchi L, et al. (2002) Tetracycline treatment and sex-ratio distortion: a role for Wolbachia in the moulting of filarial nematodes. Int J Parasitol 32: 1457–1468.
13. Bandi C, McCall JW, Genchi C, Corona S, Venco L, et al. (1999) Effects of tetracycline on the filarial worms Brugia malayi and Dirofilaria immitis and their bacterial endosymbionts Wolbachia. Int J Parasitol 29: 357–364.
14. Vavre F, Girin C, Boulétreau M (1999) Phylogenetic status of a fecundity-enhancing Wolbachia that does not induce thletychoi in Trichogramma. Insect Mol Biol 8: 67–72.
15. Dobson SL, Marsland EJ, Rattanadechakul W (2002) Mutritional Wolbachia infection in Aedes albopictus: accelerating cytoplasmic drive. Genetcs 160: 1087–1094.
33. Lemaître B, Nicolaes E, Michaut L, Reichhart JM, Hoffmann JA (1996) The dorsoventral regulatory gene cassette spotted/Toll is controlled by the potent antifungal response in Drosophila adults. Cell 86: 973–983.

34. Akira S, Uematsu S, Takeuchi O (2006) Pathogen recognition and innate immunity. Cell 124: 783–801.

35. Lemaître B, Hoffmann J (2007) The host defense of Drosophila melanogaster. Annu Rev Immunol 25: 697–743.

36. Dostert C, Jouangy E, Irving P, Tresler L, Galiana-Arnoux D, et al. (2005) Essential function in vivo for Dicer-2 in host defense against RNA viruses in drosophila. Nat Immunol 7: 590–597.

37. van Rij RP, Saleh MC, Berry B, Foo C, Houk A, et al. (2006) The RNA interference silencing endonuclease Argonaute 2 mediates specific antiviral immunity in Drosophila melanogaster. Genes Dev 20: 2985–2995.

38. Zambon RA, Vakharia VN, Wu LP (2006) RNAi is an antiviral immune response against a dsRNA virus in Drosophila melanogaster. Cell Microbiol 8: 880–889.

39. Ryder E, Blows F, Ashburner M, Bautista-Llacer R, Coulson D, et al. (2004) The Jak-STAT signaling pathway is required but not sufficient for the host response to viruses in Drosophila. Nat Immunol 5: 946–953.

40. Wang XH, Aliyari R, Li WX, Li HW, Kim K, et al. (2006) RNA interference directs innate immunity against viruses in adult Drosophila. Science 312: 452–454.

41. Takasoka A, Yanai H (2006) Interferon signalling network in innate defence. Cell Microbiol 8: 907–922.

42. van Rij RP, Saleh MC, Berry B, Foo C, Houk A, et al. (2006) The RNA silencing endonuclease Argonaute 2 mediates specific antiviral immunity in Drosophila melanogaster. Genes Dev 20: 2985–2995.

43. Zambon RA, Vakharia VN, Wu LP (2006) RNAi is an antiviral immune response against a dsRNA virus in Drosophila melanogaster. Cell Microbiol 8: 880–889.