High Anti-Viral Protection without Immune Upregulation after Interspecies *Wolbachia* Transfer

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Abstract

Wolbachia, endosymbionts that reside naturally in up to 40–70% of all insect species, are some of the most prevalent intracellular bacteria. Both *Wolbachia* wAu, naturally associated with *Drosophila simulans*, and wMel, native to *Drosophila melanogaster*, have been previously described to protect their hosts against viral infections. wMel transferred to *D. simulans* was also shown to have a strong antiviral effect. Here we directly compare one of the most protective wMel variants and wAu in *D. melanogaster* in the same host genetic background. We conclude that wAu protects better against viral infections, it grows exponentially and significantly shortens the lifespan of *D. melanogaster*. However, there is no difference between wMel and wAu in the expression of selected antimicrobial peptides. Therefore, neither the difference in anti-viral effect nor the life-shortening could be attributed to the immune stimulation by exogenous *Wolbachia*. Overall, we prove that stable transinfection with a highly protective *Wolbachia* is not necessarily associated with general immune activation.

Introduction

Wolbachia, intracellular bacteria inhabiting up to 40–70% of known insect species [1,2], have been initially described as powerful manipulators of arthropods reproduction [3]. *Wolbachia* are maternally transmitted and, in some hosts, provide infected females with a relative fitness advantage by cytoplasmic incompatibility, male killing or other forms of reproductive manipulation. Recently, *Wolbachia* have been attracting widespread attention due to their ability to protect their hosts against viral infections. This phenomenon has been initially reported in *Drosophila melanogaster* carrying its natural wMel *Wolbachia* strain [4,5]. Interestingly, antiviral protection was the first phenotype of *Wolbachia* discovered in *D. melanogaster* that could explain high prevalence of the symbiont in natural populations of fruit flies [6–13].

The ubiquity of *D. melanogaster* in research has placed wMel *Wolbachia* strain among the most extensively studied insect symbionts. Based on the molecular markers it has been shown that wMel strain consists of five polymorphic variants, namely: wMel, wMel2, wMel3, wMelCS and wMelCS2 [14]. Our previous work [15] has placed these variants in the context of a recent wMel phylogenetic analysis [16] and shown that they cluster into two monophyletic groups: wMel-like and wMelCS-like. The wMelCS-like variants reach higher densities in the host and provide more antiviral protection than the wMel-like variants. Moreover, some wMelCS-like variants shorten the lifespan of their hosts [15], including the extreme example of the pathogenic wMelPop [17].

wAu is a *Wolbachia* native to *D. simulans* that used to be present at low frequencies in Australia and does not induce cytoplasmic incompatibility [18,19]. Based on the analyses employing molec-

ular markers different authors concluded that wMel of *D. melanogaster* and wAu of *D. simulans* are closely related and both belong to the *Wolbachia* supergroup A [20–24].

wAu and one of the most protective *Wolbachia* wMel variants - wMelCS_b, the two strains used in this study, have been previously described as protective against *Drosophila* C virus (DCV) and flock house virus (FHV) [4,5,25,26]. Moreover, wMel has been previously transferred from *D. melanogaster* to *D. simulans* [27] and protection in this new *Drosophila*-host association was similar to the protection provided by wAu in its natural host [25]. However, different *Wolbachia* lines were studied in different *D. simulans* genetic backgrounds, preventing direct comparison of the protective abilities of wAu and wMel.

This study compares the antiviral protection and other phenotypes provided by wMelCS_b and wAu in genetically identical *D. melanogaster* hosts. In mosquitoes recently transinfected with *Wolbachia* the antiviral effect is frequently associated with activation of the host immune system [29–35], while in natural co-evolved *D. melanogaster* – *Wolbachia* associations antiviral protection is strong but expression of immune genes remains unchanged [31,36–38]. Therefore we also evaluated general activation of the fly immune system by wMelCS_b and wAu transinfected to *D. melanogaster*.

Results and Discussion

wAu provides stronger antiviral protection than wMelCS_b in *D. melanogaster*

It was previously shown that wAu provides strong protection against viruses in its native *D. simulans* host [25]. We have

discovered that among *Wolbachia* endosymbionts of *Drosophila melanogaster* wMelCS_b is one of the most potent in viral interference [15]. In order to directly compare these two strains in *Drosophila melanogaster*, we used *Wolbachia*-infected lines in a genetically identical DrosDel w^{1118} isogenic background [39]. wMelCS_b was naturally associated with this background while wAu was introduced from *D. simulans* to *D. melanogaster* [28] and subsequently placed in this background by chromosome replacement using balancers. A *Wolbachia*-free line, designated "*iso*", was used as a control in all experiments. All flies were virus-free and had homogenized gut microbiota (see [15]).

To compare antiviral properties of wMelCS_b and wAu, we challenged the flies carrying the respective *Wolbachia* strains and *iso* controls with two viruses: DCV (Figure 1A), a natural pathogen of *Drosophila*, and FHV (Figure 1B), initially isolated from a coleopteran host, but now widely used in studies on dipteran immune response. We observed that wAu significantly prolongs the survival of the infected flies in comparison with both *iso* and wMelCS_b carrying flies (Figure 1A, S1A, 1B and S1B; Tukey's test on the mixed effects Cox model fit, wAu versus both, wMelCS_b and *iso*, for DCV: $p < 0.001$; for FHV: $p < 0.001$). This effect is almost completely abolished in tetracycline-treated flies derived from *Wolbachia*-positive stocks (Figures 1C, S1C, 1D and S1D; DCV infected wAu tet *vs iso* tet, $p = 0.0774$ and wAu tet *vs* wMelCS_b tet, $p = 0.0161$; FHV infected wAu tet *vs iso* tet, $p = 0.1147$ and wAu tet *vs* wMelCS_b tet, $p = 0.8881$). The difference between wAu tet and wMelCS_b tet is very small in the case of DCV infection (compare Figures S1A and S1C).

The strong inhibition of virus-induced mortality in wAu carrying flies could be either due to the direct reduction of pathogen load (resistance) or due to neutralization of negative impact of the pathogen on the fly's health without direct influence on the virus titres (tolerance or resilience). To distinguish between these two possibilities we tested the levels of each virus in whole flies either 3 and 6 days post infection (dpi) for DCV or 3 dpi for FHV (Figures 1E and 1F). Consistent with previous reports both *Wolbachia* strains reduce the DCV load. However, this effect is much stronger for wAu, which is approximately 4.5 times more efficient 3 dpi (pairwise Wilcoxon rank sum test, $p = 0.03$) and over 13 times more efficient 6 dpi (pairwise Wilcoxon rank sum test, $p < 0.001$) in reducing the DCV titres than wMelCS_b. Flies carrying wAu have also 5.8 times less FHV 3 days after infection in comparison with wMelCS_b (pairwise Wilcoxon rank sum test, $p = 0.003$). All these data allow us to conclude that wAu protects better against viral infections than one of the most protective wMel variants and this can be, at least partially, explained by the reduction of the viral titres.

wAu reduces the lifespan of *D. melanogaster* and grows exponentially

We have previously reported the cost of antiviral protection in terms of reduced longevity for some wMelCS-like *Wolbachia* variants [15]. Here we have also tested the longevity of the *Wolbachia* infected flies in the absence of viral challenge (Figure 2A). We observed that wAu shortens the lifespan of flies by 20 days (31% difference in median time to death) in comparison with wMelCS_b (Figure 2A, S1E; Tukey's test on the mixed effects Cox model fit, wAu versus wMelCS_b and *iso*, $p < 0.001$) demonstrating that harbouring this protective endosymbiont is associated with a cost in the absence of infection. After elimination of *Wolbachia* from our fly stocks the flies derived from the wAu line also live shorter, but there is only a 5 days difference (9% in median time to death) between them and wMelCS_b derived flies (Figure 2B). Despite being smaller, this effect is also significant (Figure S1F; Tukey's test

on the mixed effects Cox model fit, wAu tet versus wMelCS_b tet and *iso* tet, $p < 0.001$). This difference and the one we observed for the DCV-infected tetracycline treated wAu and wMelCS_b lines may be due to differences in mitochondria between wAu and wMelCS_b fly stocks (see [40,41]) or to a mutation in the nuclear genetic background that could have arisen since the lines were separated. Given these results we cannot completely rule out an interaction between these possible mitochondrial or nuclear variation and *Wolbachia* as the cause of the differential phenotypes seen in the presence of *Wolbachia*.

The association between *Wolbachia* densities and the strength of antiviral-protection is well established. Various experimental approaches, i.e. treatment of *Wolbachia*-infected flies with increasing antibiotic concentrations or examining natural variation in endosymbiont density, have shown that the higher the *Wolbachia* density, the stronger the antiviral protection [15,25,26,42,43]. In order to assess if wAu titres were also higher than wMelCS_b titres, we tested the densities of these symbionts throughout their host's lifespan (Figure 2C). We observed that the *Wolbachia* densities at adult emergence are the same for both strains (log-linear model, intercept difference: 0.165027, $p = 0.352$), but wAu grows much faster than wMelCS_b (slope difference between wAu and wMelCS_b: 0.046097, $p < 0.001$). The exponential growth of the symbiont may be the cause of the life-shortening, either by direct tissue damage or by constituting a significant metabolic burden compromising the insect's health. This is reminiscent of host life-shortening by the exponentially growing wMelPop strain [15,17,44].

wAu does not stimulate *D. melanogaster* immune system despite recent transfer from *D. simulans*

Immune upregulation has been shown to occur after transfer of *Wolbachia* into a new insect species [29–35]. Stimulation of the insect immune system by *Wolbachia* is one of the proposed mechanisms explaining *Wolbachia*-mediated antiviral protection in mosquitoes [29,30,32,35]. On the other hand, chronic immune activation was also proven to be responsible for lifespan reduction in *Drosophila melanogaster* [45].

To test if chronic immune activation could be responsible for the high antiviral protection and life-shortening by wAu we examined the expression of genes encoding antimicrobial peptides (AMPs). We chose AMPs that were previously shown to be highly induced by the presence of exogenous *Wolbachia* [29–35], and that represent targets of the two main *Drosophila* immune pathways: Toll and Imd (Figure 3). Quantitative RT-PCR showed that there is no difference between wMel, wAu and *iso* in the expression of *Defensin*, *Cecropin A1* and *Drosomycin* (Figure 3). There is also no significant difference between wMelCS_b and wAu in the expression of *Diptericin*. The lack of an induction of these AMPs by wAu indicates that the Toll and Imd pathways are not activated in transinfected *Drosophila melanogaster*. As the expression of the four AMPs is the same in the wAu and the wMelCS_b infected flies, we could not attribute either the difference in antiviral effect or the lifespan-shortening to the immune activation by exogenous *Wolbachia*. The only statistically significant difference emerging from our analysis was in *Diptericin* gene expression between *iso* and wMelCS_b ($p = 0.006$). However, this effect was not observed in the previous studies [31,36–38] and the three other AMPs are not regulated by the presence of wMelCS_b.

Our findings add to previous reports on high AMPs expression not only after *Drosophila* - mosquitoes transfers [29–35] but also on *Wolbachia* transferred within the same genus, i.e. wAlbB from *A. albopictus* to *A. aegyptii* [33]. The contrast between the effects of these transfers on immunity and lack of immune activation by wAu
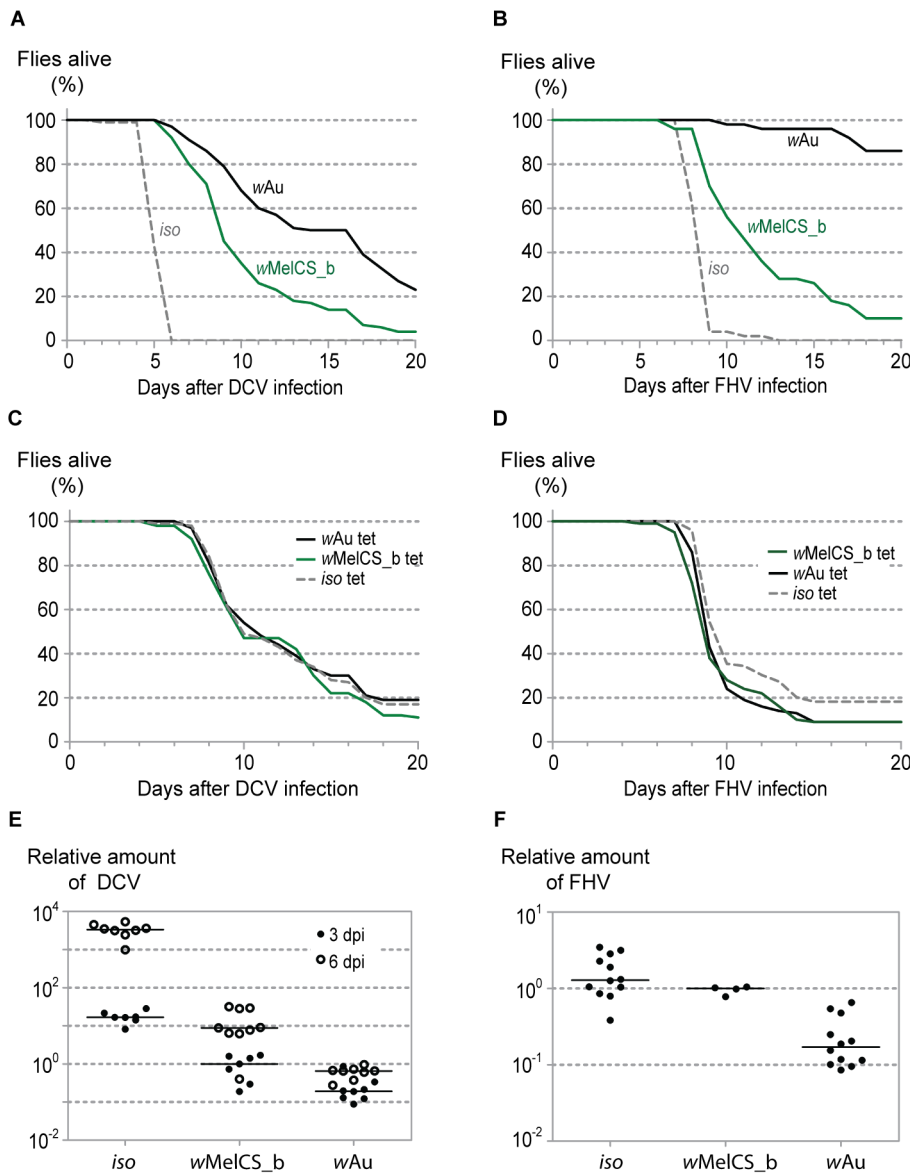
Figure 1. wAu provides more antiviral resistance than wMelCS_b in *D. melanogaster*. (A) One hundred *Wolbachia*-free *iso*, wAu and wMelCS_b infected male flies were pricked with DCV (10^9 TCID$_{50}$/ml) and survival was followed daily. This experiment was repeated twice and statistical analysis was performed on the data from all 3 repetitions (Figure S1A). (B) Fifty *Wolbachia*-free *iso*, wAu and wMelCS_b infected male flies were pricked with FHV (10^8 TCID$_{50}$/ml) and survival was followed daily. This experiment was repeated and statistical analysis was performed on the data from both repetitions (Figure S1B). (C) One hundred males from wAu, wMelCS_b and *iso* tetracycline-treated lines were pricked with DCV (10^7 TCID$_{50}$/ml) and survival was followed daily. This experiment was repeated and statistical analysis was performed on the data from both repetitions (Figure S1C). (D) One hundred males from wAu, wMelCS_b and *iso* tetracycline-treated stocks were pricked with FHV (10^8 TCID$_{50}$/ml) and survival was followed daily. For data analysis see Figure S1D. (E) *Wolbachia*-free *iso*, wAu and wMelCS_b carrying male flies were pricked with DCV (10^9 TCID$_{50}$/ml) and collected 3 and 6 days later for RNA extraction and RT-qPCR. Relative amounts of DCV were calculated using host Rpl32 mRNA as a reference and presented values are relative to median of wMelCS_b samples 3 dpi. Each point represents a replicate (ten males per replicate, 8 replicates per *Drosophila* line per time point), and lines are medians of the replicates. DCV loads are significantly different between the lines with wAu and wMelCS_b both 3 dpi (pairwise Wilcoxon rank sum test, *p* = 0.03) and 6 dpi (pairwise Wilcoxon rank sum test, *p*<0.001). (F) *Wolbachia*-free *iso*, wAu and wMelCS_b carrying male flies were pricked with FHV (10^9 TCID$_{50}$/ml) and collected 3 days post infection for RNA extraction and RT-qPCR. Relative amount of virus were calculated using host Rpl32 mRNA as a reference and presented values are relative to median of wMelCS_b samples. Each point represents a replicate (ten males per replicate), and lines are medians of the replicates. FHV loads are significantly lower in flies with wAu comparing to flies with wMelCS_b (pairwise Wilcoxon rank sum test, *p* = 0.003).
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transferred to *D. melanogaster* could be explained in various ways. The first possible explanation may be the phylogenetic distances between the source and target host insect species; the most recent common ancestor of *A. albopictus* and *A. aegyptii* dates to ∼34–42 million years ago [46], while *D. melanogaster* and *D. simulans*

diverged only 2.3 million years ago [47]. Therefore, wAu could be better pre-adapted to infect *D. melanogaster* inconspicuously. Another explanation is that *D. melanogaster* has co-evolved with *Wolbachia* while *A. aegyptii* natural populations are not infected with this endosymbiont. Thus, *D. melanogaster* may have evolved not to
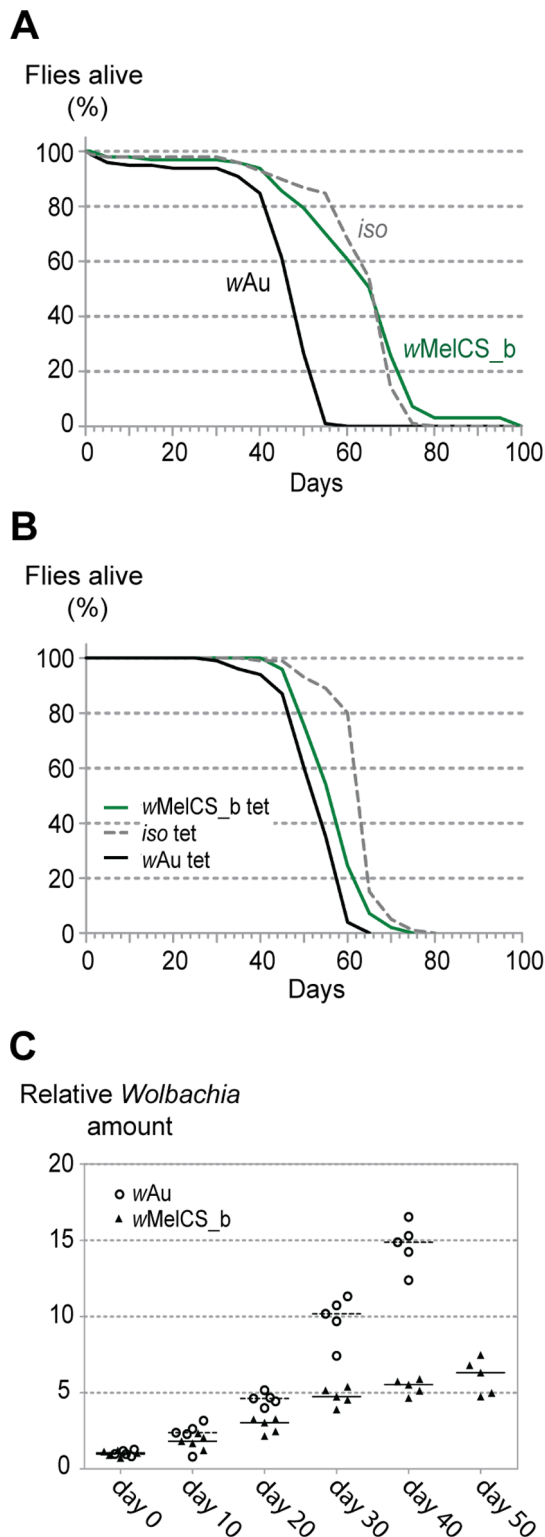
A

B

C

Figure 2. wAu shortens the lifespan of the flies and grows exponentially within the hosts. (A) The survival of one hundred *Wolbachia*-free iso, wAu and wMelCS_b carrying male flies was checked every five days. The experiment was repeated once with comparable results and analysis was performed on both repetitions (Figure S1E). (B) The survival of one hundred males derived from iso, wAu and wMelCS_b tetracycline-treated stocks was checked every five days. The experiment was repeated once with comparable results and analysis was performed on both repetitions (Figure S1F). (C) qPCR on

DNA isolated from males of wMelCS_b and wAu lines, collected every 10 days. Day 0 corresponds to 3–6 days-old flies, after day 40 the wAu carrying flies were not collected due to the high mortality. Each point represents a sample (each sample consisted of ten males), and lines are medians of the samples. Relative amount of *Wolbachia* genomic DNA was calculated using host Rpl32 as a reference gene and all values are relative to median of samples of wMelCS_b at day 0.
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respond to *Wolbachia* infection. This may also explain why *A. albopictus* has a provisional or no immune response to *Wolbachia* somatic transient infection [34]. Finally, wAu and wMel might be so similar that the insect's immune system does not perceive wAu as foreign.

It would be interesting to know which genetic differences between the closely related wAu and wMelCS explain the different phenotypes. wAu genome is not sequenced, however, several differences between the genome of wAu and wMelCS are described. wAu lacks a 21.86 kb genomic region present in wMelCS, named Octomom, which includes genes from WD0506 to WD0518 [15,21]. This fragment contains genes with domains homologous to eukaryotic proteins (putative *Wolbachia* effector proteins) and many proteins possibly involved in DNA repair and processing. The amplification of this region has been recently proposed to be responsible for the over-replicative phenotype of wMelPop *Wolbachia* variant [15], although alternative explanations have been suggested [48]. There are also many other differences in the number or coding sequences of ankyrin repeat genes between wMel strain genomes and wAu [21,49] (see also [15] and [48] for sequence of wMelCS). All the above analyses were based on PCR amplification, gene sequencing and DNA hybridization and only the sequencing of the whole wAu genome would allow to complete the comparison.

Our study uses wAu and one variant of *Wolbachia* wMel – wMelCS_b – in the same *D. melanogaster* genetic background and provides a direct comparison of the protective capabilities of the two strains. We conclude that wAu protects better against viral infections – it increases lifespan of virus-infected flies and significantly limits viral replication. Additionally, we have discovered that wAu grows exponentially within this host and significantly shortens its lifespan in the absence of viral infection, demonstrating that harbouring this protective endosymbiont is associated with a fitness cost. Testing the expression of selected antimicrobial peptides showed that there is no difference between wMel and wAu. Therefore, we could not attribute either the difference in anti-viral effect or the lifespan-shortening to the immune activation by exogenous *Wolbachia*. Our work provides evidence that interspecies *Wolbachia* transfer is not always associated with general immune up-regulation in the recipient host.

Materials and Methods

The data for iso and wMelCS_b in the Figures 1D, 1F, 2C are already published in Chrostek *et al.* 2013. All the remaining data, all statistical analysis and all conclusions are original.

Fly strains and husbandry

D. melanogaster with wMelCS_b DrosDel w^{1118} *isogenic* flies and the matching controls without *Wolbachia* were described before [4,39]. *D. melanogaster* with wAu from *D. simulans* Coffes Harbour (CO) was described before [28]. The 1st and 3rd chromosome of the *D. melanogaster* stock with wAu were replaced with DrosDel w^{1118} *isogenic* chromosomes using a first and third double balancer line. Next, a second chromosome balancer line was used to replace
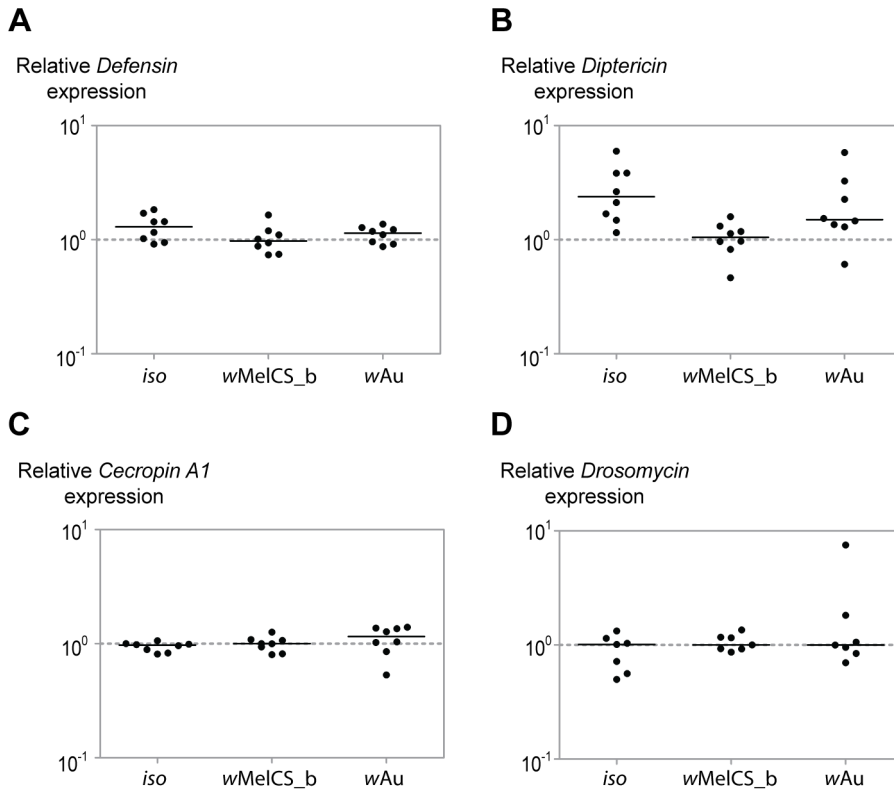
Figure 3. Expression of antimicrobial peptide genes in *Wolbachia*-free *iso*, *w*Au and *w*MelCS_b harbouring flies. qRT-PCR on the RNA collected from 3–6 days-old whole flies performed with the primers specific for *Defensin* (A), *Diptericin* (B) *Cecropin A1* (C) and *Drosomycin* (D). Relative expression of the host antimicrobial peptide genes was calculated using host Rpl32 as a reference. Values are relative to median of samples of *w*MelCS_b. The only statistically significant difference is in *Diptericin* gene expression between *iso* and *w*MelCS_b (pairwise Wilcoxon rank sum test, $p = 0.006$).

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the 2nd chromosome. As both *Wolbachia* and mitochondria are maternally transmitted the *w*Au, *w*MelCS_b and *Wolbachia*-free *iso* control lines may have different mitochondria, despite having the same nuclear genetic background. Cleaning the stocks of possible chronic viral infection and gut flora homogenization were performed as in [4,15]. *Drosophila* were maintained at a constant temperature of $25°C$ on standard cornmeal diet. All the experiments were performed on 3–6 days-old male flies.

Long-term survival analysis

The lifespan of different fly lines was tested at $25°C$, with 10 flies per vial, and analysed using Cox hazard models as previously reported [15] with the coxme package in R [50]. We considered genotype and repeat of the experiment fixed and replicate vials within the same experiment random.

Virus production and infection

Viruses were produced, titrated and used to infect flies as before [4,15]. Infections were performed on 3–6 days-old flies. After the infections 10 flies per vial were kept on food without live yeast at $18°C$ for DCV or at $25°C$ for FHV. Survival was monitored daily and vials were changed every 5 days. Statistical analysis was performed the same way as for long-term survival data.

Nucleic acids extractions and real-time qPCR

DNA for the quantification of *Wolbachia* was extracted using standard phenol-chlorophorm protocol. RNA for assessment of viral titres and gene expression was extracted using Trizol (Invitrogen) with an additional DNAse treatment (Promega) of the AMPs RNA samples prior to cDNA synthesis. cDNA was prepared as described previously [15]. Real-time qPCR reactions were carried out in 7900HT Fast Real-Time PCR System

Table 1. Primers used to detect AMPs in real-time quantitative PCR experiments.

Target	Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)
Defensin	TATCGCTTTTGCTCTGCTTG	TGTGGTTCCAGTTCCACTTG
Diptericin	ACCGCAGTACCCACTCAATC	CCATATGGTCCTCCCAAGTG
Cecropin A1	CATCAGTCGCTCAGACCTCAC	TTCTTCAGCCACCCAGCTTC
Drosomycin	TACCAAGCTCCGTGAGAACC	CAGGGACCCTTGTATCTTCC

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(Applied Biosystems) with the iQ SYBR Green supermix (Bio Rad). Each plate contained three technical replicates of every sample for each set of primers. Primers for *Wolbachia*, DCV and FHV were previously described [15], while primers for AMPs are listed in Table 1. For the four antimicrobial peptides the thermal cycling protocol used was: 50°C for 2 min, 10 min at 95°C and 40 cycles of: 95°C for 30 sec, 59°C for 1 min and 72°C for 30 sec. This was followed by the generation of dissociation curve to verify the specificity of the reactions. Data was analysed in R [50] using Wilcoxon rank sum test with Holm correction for FHV levels, DCV levels at each time point and AMPs levels. The increase of *Wolbachia* variants titre over time was analysed using a linear model (lm) in R [50].

Supporting Information

Figure S1   Statistical analysis of survival curves. (A,B,E) Hazard ratios between either *iso* *Wolbachia*-free control or *w*MelCS_b carrying line and *w*Au line for: (A) DCV infection, (B) FHV infection, (E) uninfected flies. (C,D,F) Hazard ratios between either *iso* or *w*MelCS_b tetracycline-treated line and *w*Au tetracycline-treated line for: (C) DCV infection, (D) FHV infection, (F) uninfected flies. In all panels error bars represent standard errors of the estimated hazard ratios. The only non-significant differences in Cox hazard ratios are: *iso* tet vs. *w*Au tet for DCV infection (C) and both *iso* tet and *w*MelCS_b tet vs. *w*Au tet for FHV infection (D).
(TIF)

Author Contributions

Conceived and designed the experiments: EC MSPM LT. Performed the experiments: EC MSPM. Analyzed the data: EC LT. Contributed reagents/materials/analysis tools: RY SLO. Wrote the paper: EC LT.

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