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 transinfestation 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 molecular 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 [29–33], 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 w1118 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 15 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. aegypti [33]. The contrast between the effects of these transfers on immunity and lack of immune activation by wAu
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. aegypti* 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. aegypti* natural populations are not infected with this endosymbiont. Thus, *D. melanogaster* may have evolved not to.
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 *wMel* explain the different phenotypes. *wAu* genome is not sequenced, however, several differences between the genome of *wAu* and *wMel* are described. *wAu* lacks a 21.86 kb genomic region present in *wMel*, 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 *wMel*). 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 *w1118* 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 *w1118* isogenic chromosomes using a first and third double balancer line. Next, a second chromosome balancer line was used to replace 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. doi:10.1371/journal.pone.0099025.g002

![Figure 2. wAu shortens the lifespan of the flies and grows exponentially within the hosts.](image-url)
the 2nd chromosome. As both Wolbachia and mitochondria are maternally transmitted the \( wAu, w_{MelCS_b} \) 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       | TATCGGTTTGGTCTGCTGG            | TGTGGTTCCAGTCCACCTTG          |
| Diptericin     | ACCGAGTACCCAATC               | CCAATGGTCTCCTCCAAGTG         |
| Cecropin A1    | CATCACGTGGCTGACGCTG          | TTCTCAGCCACAGCCGCTTC         |
| Drosomycin     | TACCAAGGGTGGGGAAGGCC         | CAGGGACCTTCTGTACCTCC         |

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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 the specificity of the reactions. Data was analysed in R [50] 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 wMelCS_b carrying line and wAu line for: (A) DCV infection, (B) FHV infection, (E) uninfected flies. (C,D,F) Hazard ratios between either iso or wMelCS_b tetracycline-treated line and wAu 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. wAu tet for DCV infection (C) and both iso tet and wMelCS_b tet vs. wAu tet for FHV infection (D). (TIF)

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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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