Wolbachia-mediated antiviral protection in Drosophila larvae and adults following oral infection

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Short title: Wolbachia antiviral protection in larvae and adults
Abstract

Understanding viral dynamics in arthropods is of great importance when designing models to describe how viral spread will influence arthropod populations. The endosymbiotic bacterium *Wolbachia*, which is present in up to 40% of all insect species, has the ability to alter viral dynamics in both *Drosophila* and mosquitoes, a feature that in mosquitoes may be utilised to limit spread of important arboviruses. To understand the potential effect of *Wolbachia* on viral dynamics in nature, it is important to consider the impact of natural routes of virus infection on *Wolbachia* antiviral effects. Using adult *Drosophila* we show that *Drosophila*-*Wolbachia* associations that have previously been shown to confer antiviral protection following systemic viral infection also confer protection against viral-induced mortality following the oral route of Drosophila C virus infection in adults. Interestingly, a different pattern was observed when the same fly lines were challenged with virus as larvae. Analysis of the four *Drosophila*-*Wolbachia* associations that were protective in adults, indicated that only the *w*$_{1118}$-*wMelPop* conferred protection in larvae following oral delivery of the virus. Analysis of *Wolbachia* density using qPCR showed that high *Wolbachia* density was congruent with antiviral protection in both adults and larvae. This study indicates that *Wolbachia*-mediated protection may vary between larval and adult stages of a given *Wolbachia*-host association, and that the variation in life-stages susceptibility corresponds with *Wolbachia* density. The differences in the outcome of virus infection is likely to influence viral dynamics in *Wolbachia*-infected insect populations in nature, and could also have important implications for the transmission of arboviruses in mosquito populations.
Introduction

Arthropods harbour a wide range of viruses that can be transmitted between individuals or populations of the same species, or can bridge the interspecies gap to infect plants or other animals. The outcome of viral infections can be modulated by tripartite interactions between arthropods, viruses and bacteria (1). One such interaction is the tripartite interaction between insects, viruses and the endosymbiotic bacteria *Wolbachia pipientis*.

*Wolbachia* has gained much attention due to the antiviral effects it confers to its host. The impact of *Wolbachia* on virus infection was first described in the *Drosophila melanogaster* host, where it was shown to protect against mortality induced by diverse viruses including *Drosophila* C virus (DCV), Cricket paralysis virus and Flock House virus (2, 3). Since that discovery, *Wolbachia*-mediated antiviral effects have been demonstrated in a number of insect hosts, and are being investigated as a way of limiting spread of arboviruses (reviewed in 1, 4, 5, 6). Notably, *Wolbachia*-mediated antiviral effects have been demonstrated in adult mosquitoes artificially infected with *Wolbachia*, where *Wolbachia* can interfere with accumulation and transmission of important human pathogens including dengue and Chikungunya viruses (7-17). While in many cases *Wolbachia* confers antiviral effects to its host organism, in some cases the presence of *Wolbachia* can enhance viral susceptibility (18-23). The impact of the presence of *Wolbachia* on virus infection can include two main effects: 1) interference with viral replication/accumulation, and/or 2) protection against viral-induced mortality. In mosquitoes, *Wolbachia* interferes with viral replication/accumulation, while in *Drosophila* *Wolbachia* can interfere with viral replication/accumulation and/or protect flies from viral-induced mortality.
In this paper we will focus on the effect of Wolbachia on the survival of the host, and will define protection as a reduction/delay in viral-induced mortality.

The mechanisms involved in Wolbachia-mediated antiviral effects have not yet been fully elucidated. There is some evidence that miRNAs (24, 25), competition for host-derived resources (26) and elevated reactive oxygen species (27, 28) may influence antiviral effects. Drosophila-Wolbachia associations can be subdivided into two groups, protective and non-protective. The Drosophila-Wolbachia pairings CO-wAu, DSR-wRi, w1118-wMel and the over-replicating and life-shortening wMelPop in w1118 show a delay in DCV induced mortality when DCV is injected into adult flies, while N7NO-wNo and DSH-wHa do not (2, 3, 11, 29). A feature that all protective Wolbachia strains share is high density within their respective host organism, indicating that high Wolbachia density may serve as a prerequisite for antiviral protection (7, 12, 29-34).

Wolbachia is estimated to infect 40% of all insects (35), therefore the effect it exerts on natural viral dynamics could be pronounced. The understanding of natural tripartite Drosophila-virus-Wolbachia interactions is very limited at present, partially due to a lack of a method of orally delivering virus. Recently, three methods for oral infection of larvae and adults were described, which will allow us to study the effects of the oral route of infection on antiviral protection mechanisms in Drosophila (36-38).

To investigate the effects of Wolbachia on viral-induced mortality following the oral route of infection, we used DCV; a natural Drosophila pathogen and the most
widely studied *Drosophila* virus (39). DCV is a positive-sense RNA virus belonging to the *Dicistroviridae* family (40). When injected into flies, DCV is pathogenic, causing mortality within 4-6 days post-injection (41). Injecting DCV is a useful method to study *Wolbachia*-DCV interactions, however injection bypasses the fly’s natural immune barriers present within the midgut, and can cause a differential immune response compared to virus feeding alone (37). DCV infection by ingestion is less pathogenic compared to injection (36, 37) and represents a more natural *Drosophila*-DCV interaction. While *Wolbachia*-mediated protection has been extensively studied in adult flies following a systemic infection, it is not yet clear whether the *Drosophila*-*Wolbachia* associations that are protected from viral-induced host mortality following viral injection exhibit a similar protective characteristic following the oral route of infection. Ingesting infected cadavers is thought to be one of the mechanisms through which DCV transmission occurs naturally within a population (42), therefore *Wolbachia*-mediated antiviral protection following the oral route of infection could have a direct impact on viral transmission and maintenance of the virus within a population.

Understanding the potential of *Wolbachia* to effect viral dynamics in natural populations will be facilitated by insight into the impact of antiviral protection on susceptibility throughout the life cycle of the host following a natural route of infection. Both *Drosophila* and mosquitoes are holometabolous insects, undergoing metamorphosis between larval and adult stages. A wide range of genes coordinate the disintegration of larval structures, where some larval organs are histolysed and major new growth takes place, altering the morphology and in some cases pathogen susceptibility (43-45). Pathogen susceptibility is often age or life-stage dependent and
can have a large effect on population dynamics, viral spread and maintenance of the virus within the population (45-48). Studies focusing on the antiviral effects of *Wolbachia* have to date been conducted solely on adult flies and mosquitoes, without consideration of the other developmental stages.

Here we investigate the effect of *Wolbachia* on viral-induced mortality of *Drosophila* larvae and adults following oral challenge with DCV. By using four *Drosophila-Wolbachia* associations that have previously been shown to be protective in adults following viral injection, we show that the *Drosophila-Wolbachia* associations that are protected against viral-induced mortality following injection, are also protected following oral infection of adults. In contrast, *Wolbachia* protection at the adult stages is not indicative of protection at larval stages, as only one out of four *Drosophila-Wolbachia* associations that are protective at the adult stages show protection at the larval stages.

### Materials and methods

#### *Drosophila and Wolbachia*

Two *D. melanogaster* and three *D. simulans* fly lines were reared on a standard cornmeal media at a constant temperature of 25°C with a 12-hour light/dark cycle. Paired populations of flies were used that either contained *Wolbachia* (*w*\(^{1118}\)-wMel, *w*\(^{1118}\)-wMelPop, N7NO-wNo, DSR-wRi, Co-wAu), or have been cured of *Wolbachia* by tetracycline treatment (*w*\(^{1118}\)-T, N7NO-T, DSR-T and CO-T), and maintained on a standard cornmeal media for at least five generations before use. Gut flora was reconstituted and normalised across fly lines using standardised methods (31). Briefly,
Drosophila embryos were transferred to vials containing 150 μl of a bacterial inoculum, which was prepared by adding 2 g of 10 days old food containing w^{1118-} wMelPop flies to 5 ml of sterile water and strained through a fine sterile mesh to remove larvae and embryos. The newly treated flies were checked for the presence of Wolbachia using PCR, to make sure that no cross-contamination had occurred.

**Virus**

Plaque-purified DCV isolate EB (49, 50) was propagated and purified from Schneider’s Drosophila Line 2 cells (51), and virus titres were determined by tissue culture infective dose (TCID₅₀) as described previously (29, 49).

**DNA extraction**

Thirty 0-4 hour old larvae or ten newly emerged male adult flies were pooled to perform DNA extraction. The flies were homogenized using a pestle in 180 μl of extraction buffer and 20 μl of proteinase K. The DNeasy Blood & Tissue kit (Qiagen) was used to extract the DNA as per the manufacturer’s protocol. Three replicates on independent cohorts were performed for each treatment.

**Quantitative PCR**

The abundance of Wolbachia was determined by qPCR by quantifying the abundance of the Wolbachia surface protein (WSP) relative to either the D. melanogaster RrpL32 or D. simulans Act5C genes. Platinum SYBR® Green qPCR SuperMix-UDG (Invitrogen) was used as per manufacturer’s instruction using the WSP specific primer pair 5’- GCATTTGGTTAYAAAATGGACGA-3’ and 5’-
GGAGTGATAGGCATATCTTCAAT-3’ (producing a 185 bp PCR product) (29),  
Rpl32 specific 5’- GACGCTTCAAGGGACAGTATCTG-3’ and 5’- 
AAACGCGTTCTGCATGAG-3’ (producing a 141 bp PCR product) (49) and  
Act5C 5’-GACGAAGAAGTTGCTGCTCTGGTT  
G-3’ and 5’-TGAGGATACCACGCTTGCTGC-3’ (producing a 192 bp PCR  
product) (30). The Rotor-Gene 6000 thermal cycler (Corbett Life Sciences, Qiagene)  
was used with the following profile: 95˚C 2 minutes, followed by 40 cycles of 95 ºC  
10 seconds, 52 ºC 10 seconds and 72 ºC for 20 seconds. This was followed by a  
standard melt analysis to assess specificity of the amplified product. Two technical  
replicates (separate qPCR reactions on the same DNA) were performed for each  
sample (with a third been done where necessary) and DNA extracted from flies  
without Wolbachia was used as a negative control. Mean normalized WSP:Rpl32  
DNA ratios were calculated using qGENE software (52), and statistical analysis  
included a two-tailed Student’s t-test to compare differences of the means.  

Survival bioassay  

Virus for larval and adult feeding assays was prepared by injecting flies with either  
5000 infectious units (IU) of DCV, or an equivalent volume of phosphate buffered  
saline (PBS) which acted as a control. Live flies were collected at 4 days post-  
injection and stored at -20 ºC until further use. Thirty PBS or DCV injected flies were  
pooled, homogenized in 300 μl of PBS, and the supernatant filter sterilized using the  
Millex GV 22 μm filter (Merck Millipore). Homogenates prepared in this way were  
used for both adult and larval bioassays. The titre of DCV-injected fly homogenates  
were measured on four occasions and ranged between 4.4 x 10^{10} and 2 x 10^{11} IU/ml.
For adult infections a modified version of a previously described method was used (37). A 250 μl of a mix containing 75% of the above described fly homogenate (DCV or PBS) and 25% of dry yeast was applied to a 1.5 x 1.5 cm filter paper and placed in a vial containing ten 4–7 day old male flies. Flies were incubated with the media for 24 h at 25°C with high humidity to prevent the food from drying out. Following this period, the flies were transferred to standard cornmeal media and daily mortality was scored for 15 days. Three replicates on independent cohorts were performed for each treatment.

Larval infections were performed by spreading DCV or mock infected fly homogenates onto petri dishes containing 10 ml of standard cornmeal media (36). 100 eggs were collected for each treatment on a wet piece of sterile filter paper, and transferred onto petri dishes containing homogenates from either PBS or DCV injected flies. Larvae were maintained on the treatment media until adult emergence, when they were counted 3 days post-emergence. Egg to adult survival was determined as a proportion of adults post-emergence compared to the initial number of eggs at the start of the treatment, and each survival bioassay was replicated 3 times on independent cohorts of insects.

**Statistical analysis of the survival bioassay**

We used Generalized Linear Mixed Effects Regression (GLMER) models based on a binomial distribution to examine the effect of feeding treatment and co-infection on the mortality of five *D. melanogaster* and *simulans* larvae using the lme4 R package in R 2.15.3 (53) (R Foundation for Statistical Computing, Vienna, Austria). The
mortality response, as the binomial count of flies that survived or died for each line, was determined by fitting the feeding treatment (PBS, DCV) and co-infection treatment (-wol, Wolbachia strain), as well as the interaction between the two factors. The interaction term compares across the mortality values of each of the feeding treatments across the absence (-wol) or presence (+Wolbachia strain) of Wolbachia. Each model included experimental replicate as a random factor, included as replicate variance component in each model. For adult survival bioassays, the survival curves were compared using Kaplan-Meier analysis and log-rank statistics using GraphPad Prism.

Results

Wolbachia protection in adult flies following oral challenge with DCV

Initially, we tested the protective effects of the Wolbachia strain wAu in CO fly background (CO-wAu) due to a strong antiviral protection observed previously following a systemic DCV infection (29). Wolbachia-free CO flies challenged with DCV by oral infection showed 40% mortality within 15 days post-feeding. In contrast, CO-wAu flies showed a significant reduction in mortality during the same time period to 7% (Fig. 1A, Kaplan-Meyer analysis, P < 0.05). We investigated an additional three Drosophila-Wolbachia associations DSR-wRi, w^{1118}-wMel, w^{1118}-wMelPop, all of which have previously been shown to confer protection against DCV-induced mortality in adult flies following a systemic infection (3, 29, 49), and the results indicate that all three Drosophila-Wolbachia associations conferred protection against DCV-induced mortality following the oral route of infection (Fig.
Because not all Drosophila-Wolbachia associations protect against systemic viral infections, we tested a non-protective association N7NO-wNo to see whether protection would occur following oral virus challenge (29). Feeding the non-protective N7NO-wNo flies with DCV lead to a non-significant difference in viral-induced mortality compared to Wolbachia-free flies (Fig. 1C, Kaplan-Meyer analysis, P > 0.05). Taken together these results indicate Wolbachia-mediated protection against virus-induced mortality in adults infected through the oral route was consistent with what was previously reported following injection of virus.

Wolbachia protection in larvae following oral challenge with DCV

To determine whether the presence of Wolbachia protects larvae from virus-induced mortality, we orally challenged CO-wAu larvae with DCV. We found that in Wolbachia-free flies, larval to adult mortality increased from about 37% in mock-infected flies, to about 46% in DCV-infected flies and that the presence of Wolbachia had no significant effect on DCV-induced mortality (Fig. 2A and Table 1). This suggests that the Wolbachia strain wAu may not protect its host against DCV-induced mortality following this route of infection at the larval developmental stages.

As no protection was observed in CO-wAu larvae, we then investigated whether the lack of protection was specific to this Drosophila-Wolbachia association. We investigated other protective Drosophila-Wolbachia associations DSR-wRi and w118-wMel, and one non-protective association N7NO-wNo. None of these associations showed significant differences in DCV-induced mortality between larvae.
with and without Wolbachia (Fig. 2B-D and Table 1), suggesting that the lack of Wolbachia-mediated protection at the larval stages is not confined to CO-wAu flies.

The Wolbachia strain wMelPop has a strong protective effect in both adult flies and mosquitoes, so we investigated whether w^{1118}-wMelPop larvae exhibit a protective phenotype. In this Drosophila-Wolbachia association there was a statistically significant difference in DCV-induced mortality between flies with and without Wolbachia (25% and 37% mortality, respectively) (Fig. 2E and Table 1).

Unlike the other Drosophila-Wolbachia associations, wMelPop provided complete protection against DCV induced mortality (Fig. 2E). Because the ability to confer antiviral effects is strongly associated with Wolbachia density in adult flies and mosquitoes, and because wMelPop is known to be an over-replicative strain, we investigated whether the observed differences in Wolbachia protection were associated with differences in Wolbachia densities.

Wolbachia density

Wolbachia densities have previously been determined for different Drosophila-Wolbachia associations in adults but not in larvae. Using quantitative PCR (qPCR) we determined Wolbachia densities at both larval and adult stages for all five Drosophila-Wolbachia associations used in this study (Fig. 3). In adults, the densities of the protective Wolbachia strains wAu, wRi, wMel and wMelPop are significantly higher compared to the non-protective wNo strain, providing an association between Wolbachia density and protection. In contrast, Wolbachia strains wAu, wRi and wMel show lower abundance at the larval compared to adult stages (two-tailed Student’s t-test, \( p < 0.05 \), Fig. 3A, B), while the Wolbachia strain wMelPop shows high densities
at both larval and adult stages (Fig. 3A). *Wolbachia* density in the non-protective N7NO-wNo larvae remained at lower densities compared to both wRi and wAu at both developmental stages (Fig. 3B), consistent with lack of protection.

**Discussion**

The importance the route of pathogen entry has on the outcome of infection has been well-documented following bacterial infections in *Drosophila*. Injecting bacteria into the hemocoel induces a systemic immune response (54-57), while oral infections often lead to a localized immune induction in the gut, often making them less pathogenic (58-60). A recent paper showed the involvement of the Toll immune pathway in mediating resistance to oral infections with DCV, Flock House virus, Cricket paralysis virus and Nora virus, however showed no involvement of the pathway following a systemic infection (37), indicating that the route of viral entry can have an affect on the host’s response to viral infection.

We used a natural route of DCV infection through oral feeding, to investigate the effect of *Wolbachia* on protection against viral-induced mortality to investigate whether *Wolbachia*-mediated protection is confined to systemic viral infections in *Drosophila*. By examining *Wolbachia*-mediated protection in adult flies across four *Drosophila-Wolbachia* associations that have previously been shown to be protective following systemic infection, we show that oral DCV infections lead to a reduction in viral-induced mortality in adult flies with *Wolbachia* compared to *Wolbachia*-free flies (Fig. 1). These findings are consistent with a recently published report (37) and support the idea that *Wolbachia*-mediated protection extends beyond systemic viral...
infections and could be used in future experiments to better understand the effects of Wolbachia on viral dynamics in natural insect populations.

While in adults, Wolbachia-mediated reduction in viral-induced mortality is comparable between systemically and orally infected flies, the same is not always true in larvae. Out of the four Drosophila-Wolbachia associations that show protection following DCV infection in adults, only the w1118-wMelPop flies showed protection against DCV-induced mortality during the larval stages (Figure 2E). These results suggest that Wolbachia-mediated protection may vary between different life stages of the same Drosophila-Wolbachia associations, although it is possible that the amount of virus ingested by larvae and adults is different. Since Wolbachia density has previously been shown to be important for mediating antiviral effects, we measured Wolbachia density in adults and found that there was congruence between Wolbachia density and protection against DCV-induced mortality following the oral route of infection. Similarly to adults, Wolbachia-protection in larvae was associated with Wolbachia density, however interestingly high Wolbachia density was only observed in w1118-wMelPop larvae, which was also the only association to show protection against DCV-induced mortality at the larval stages. The wMelPop strain causes a life-shortening phenotype and is present in relatively high densities in both mosquitoes and Drosophila (7, 61-63). The relatively high density and the life-shortening effects of the wMelPop strain have been reported to be due to the high copy number of 8 Wolbachia genes referred as the Octomom region (31, 62). It remains to be seen whether other strains will be protective in larvae and what controls the differences in density between larvae and adults. The finding that Wolbachia-protection correlates with Wolbachia density is consistent with previous findings in
adult flies following a systemic infection (29-32). Gradually reducing *Wolbachia* density in both *Drosophila* adults and mosquito cell culture using tetracycline leads to a dose-dependent loss of antiviral protection (12, 30).

*Wolbachia*-mediated antiviral protection is not limited to *Drosophila*, and since *Wolbachia* infects up to 40% of all arthropod species (35) it may be important to consider the impact of life-stage susceptibility on arthropod population dynamics and viral transmission. Similarly to *Drosophila*, mosquitoes also undergo metamorphosis, a change that can result in life-stage dependent differences in viral susceptibility. Mosquitoes are known to form natural associations with *Wolbachia*, however it is the artificial *Wolbachia* transinfections that have shown promise as a tool for limiting spread of human pathogenic viruses (5, 6). Commonly, there is a focus on transmission of arboviruses that occurs between the mosquitoes and human hosts. While this horizontal transmission is responsible for the major health concerns in humans, vertical transmission of arboviruses within mosquito populations can affect the maintenance of the virus within the population (64, 65). Viruses such as dengue and Chikungunya can be vertically transmitted from an infected adult female to its offspring. Dengue virus can spread vertically in both natural (66-69) and laboratory conditions (70-72). Furthermore transovarially infected female mosquitoes can transmit dengue virus orally (73). Chikungunya is also capable of vertical transmission in laboratory conditions, which would suggest that a similar transmission is possible in nature (64).

Various models have been applied to try to understand the impact of *Wolbachia* on the transmission of dengue in its mosquito host (74-76). These models
do not consider the effects of vertical transmission on the maintenance of dengue within a population, which has been suggested to be an important factor affecting the ability of the virus to persist within the population in rural areas with low population densities (65). Furthermore vertical transmission could allow the survival of arboviruses during adverse climatic conditions, and has been suggested to be an important mechanism of maintenance of the virus during inter-epidemic periods (64).

Given the importance of vertical transmission on virus dynamics, and the possible life-stage-dependent variations in *Wolbachia*-mediated protection, it is important to consider the impact of *Wolbachia* antiviral protection, or the lack of thereof on the maintenance of the virus within the population. Understanding the impact of *Wolbachia* antiviral protection at different life stages is likely to be an important consideration when designing programs to minimize the spread of insect borne viruses.

**Acknowledgments**

We thank David Merritt and members of the Johnson lab for useful comments, and Craig White for help with statistical analysis.
References


Figure Legends

**Fig. 1.** Survival of adult flies following oral challenge with DCV. Each fly line contained a different *Wolbachia* strain (+wol) or was tetracycline treated to remove *Wolbachia* (-wol). Adult flies were exposed to either homogenates from DCV or mock infected (PBS) flies for 24 hours before being transferred to vials containing standard cornmeal media. Survival of flies is shown from 3 biological replicates of 10 flies or one replicate of 10 flies for PBS controls. Differences in survival were determined statistically using the log rank test on Kaplan-Meier curves.

**Fig. 2.** The impact of *Wolbachia* on virus-induced mortality in DCV infected *Drosophila* larvae. Each fly line (shown in title of each graph) contained a different *Wolbachia* strain or was tetracycline treated to remove *Wolbachia* (-wol) as indicated on the x-axis. Larvae were exposed to either homogenates from DCV or mock infected (PBS) flies. Graphs display means and standard errors from three replicates of 100 individuals per line. * indicates a significant interaction (p < 0.05) between the feeding treatment and presence or absence of *Wolbachia* on mortality.

**Fig. 3.** The density of six different *Wolbachia* strains during larval and adult stages of development. (A) Relative abundance of the *Wolbachia* surface protein (*WSP*) gene in *D. melanogaster* using *Rpl32* as a reference gene. (B) Relative abundance of the *WSP* gene in *D. simulans* using *Act5C* as a reference gene.

Tables
Table 1. Analysis of mortality in response to DCV feeding in *Drosophila* larvae either with or without *Wolbachia*. Generalised linear mixed effects regression (GLMER) analysis of five *Drosophila* lines mortality (%) in response to a feeding treatment of Phosphate-Buffered Saline (PBS) or *Drosophila* C Virus (DCV). Each line was either co-infected with a *Wolbachia* strain (*w*Au, *w*Ri, *w*No, *w*MelPop, *w*Mel) or *Wolbachia*-free (-*wol*).
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<td>-0.594</td>
<td>0.553</td>
</tr>
<tr>
<td>Treatment × -wol: wMel</td>
<td>0.137</td>
<td>0.254</td>
<td>0.537</td>
<td>0.591</td>
</tr>
<tr>
<td>Replicate variance component</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
A. CO-wAu

B. DSR-wRi

C. N7NO-wNo

D. $w^{1118}$-wMel

E. $w^{1118}$-wMelPop

Survival (%) vs. Days post feeding

- $-\text{wol}$ PBS
- $+\text{wol}$ PBS
- $-\text{wol}$ DCV
- $+\text{wol}$ DCV