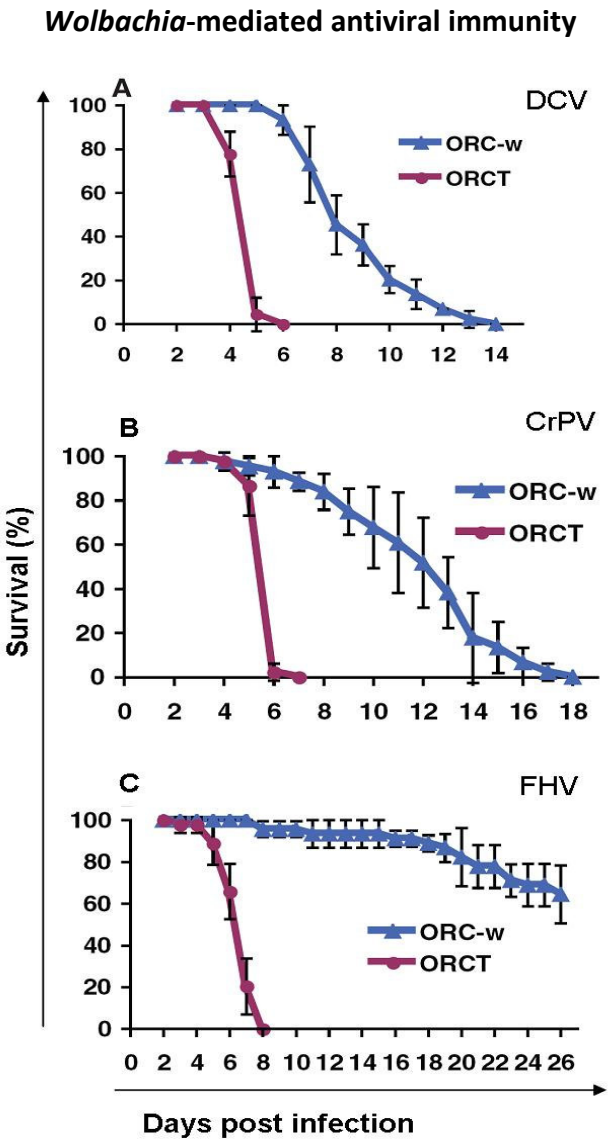
Exploring the mechanism behind *Wolbachia*-mediated antiviral immunity in *Drosophila melanogaster*

Daphne Stapels



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Abstract

The intracellular bacterium *Wolbachia pipientis* is widely spread amongst insect populations, like *Drosophila*. Recently, *Wolbachia* infection was found to reduce virus-induced mortality and lower the viral load in dually infected *Drosophila melanogaster*. Unravelling the mechanism behind this *Wolbachia*-induced survival might further elucidate the high prevalence of *Wolbachia* amongst insect species; but above all it might give new insights in antiviral immunity of insects that spread vector-borne diseases and it might help to better understand antiviral immunity in general. Even thought *Wolbachia* infection does not induce a clear phenotype in *D. melanogaster*, it does result in profound changes in transcription, for example of signaling molecules in the Toll, Imd, and JNK pathways. In addition, heat shock proteins, autophagy, and other processes – possibly induced by secreted Ankyrin-repeat-containing proteins of *Wolbachia* – might be active during *Wolbachia* infection. *Wolbachia* was shown to protect against positive sense (+) single-stranded RNA viruses from different families that replicate in association with cellular membranes. These viruses normally promote antiviral RNA interference, Imd and Jak/STAT signaling, autophagy, phagocytosis, and/or apoptosis in *Drosophila*. Remarkably, *Wolbachia* does not protect against infection with a double-stranded DNA virus. Unfortunately, little is known about the reactions induced upon double-stranded DNA-virus infection. All together, *Wolbachia* might protect *Drosophila* by priming the antiviral immune response, for example by activating Imd and Toll signaling. Importantly, all examined RNA viruses show overlapping cell tropism with *Wolbachia*, thus *Wolbachia* might also directly affect viral replication. For example, *Wolbachia* could slow down viral replication by competing for cellular nutrients, affect vesicle transport, or secrete proteins detrimental to the viruses. Additional research will unravel the mechanism behind the *Wolbachia*-induced antiviral immunity in *Drosophila* and the occurrence of this phenomenon amongst other species.

Front page figure. *Wolbachia*-mediated antiviral immunity. Survival of the *Drosophila melanogaster* (Oregon strain) with (ORC-w) and without (ORCT) *Wolbachia* infection upon super infection with [A] Drosophila C virus (DCV), [B] Cricket Paralysis virus (CrPV) or [C] Flock House virus (FHV). Similar results were obtained using the w^{1118} *D. melanogaster* strain[2].

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Introduction

At the beginning of the 20th century, Thomas H. Morgan used plain fruit flies *(Drosophila melanogaster)* on a large scale for his Nobel-prize winning genetic studies. *Drosophila* is an optimal model organism because it is easy to grow and has a short life cycle[1]. For immunologists *Drosophila* have the additional advantage that they solely exhibit an innate immune system, which enables researchers to investigate innate immunity apart from adaptive immunity. Currently, with access to the full genome sequence and new cell-biologic techniques, *Drosophila* regained popularity as a model system in genetic, developmental biology and immunologic studies.

The regained popularity probably explains why last year two independent research groups (Teixeira *et al.* and Hedges *et al.*) discovered that *Drosophilae* benefit from infection with the obligatory intracellular bacterium *Wolbachia pipientis* when challenged with viral infections[2, 3]. In short, time of survival increased and viral load decreased if virally infected *D. melanogaster* were also infected with *Wolbachia*. This observation might explain the high prevalence of *Wolbachia* amongst insect species. However, the mechanism behind the *Wolbachia*-mediated immunity is not yet known. In this review possible mechanisms will be examined to acquire new insights in insect-antiviral immunity – which plays an important role in spreading of vector-borne diseases – and to acquire better understanding of antiviral immunity in general.

Trinity: *Drosophila*, *Wolbachia*, viruses

Key players in the *Wolbachia*-induced immunity are *Drosophila*, *Wolbachia* and viruses. As said, in *Drosophila* all immune components are marked as innate immunity. To conquer bacterial and fungal infections, *Drosophila* possess systemic immunity, which is mediated by secreted anti-microbial peptides (AMPs) produced by the fat body; and cellular immunity, which is mediated by hemocytes. Immune responses are initiated by pattern recognition receptors which transduce their signals via the Toll and Imd signaling pathways[4]. On the contrary, most viral infections trigger RNA interference (RNAi) as typical immune response[5]. Recently, also autophagy has been implicated in *Drosophila* anti-viral immunity[6].

The second pillar of this phenomenon is formed by *Wolbachia*. This obligatory intracellular, Gram negative bacterium has a broad host range; it infects all insect genera, mites, spiders, crustaceans and filarial nematodes[7, 8]. *Wolbachia* is known for its vertical transmission, although horizontal transmission has been reported as well[9]. *Wolbachia* has developed several strategies to maintain itself in a host population. For example, it commonly interferes with the host reproductive system to increase the likelihood of infected females to generate offspring over uninfected females[10]. In addition, it is speculated that *Wolbachia* protects insects against other pathogens[11], conferring an advantage to *Wolbachia*-infected insects, although only few data have been published[2, 3, 12].

The third pillar of the observed immunity is formed by viruses. Remarkably, the *Wolbachia*-mediated protection described by Teixeira *et al.* and Hedges *et al.* was not universal: only lethality induced by the positive-sense (+) single stranded (ss)RNA viruses Drosophila C virus (DCV), Cricket Paralysis virus (CrPV), Flock House virus (FHV), and Nora virus (NV) was lowered in presence of *Wolbachia*, but not the lethality induced by the double stranded (ds) DNA virus Insect Iridescent virus-6 (IIV-6)[2, 3]. It is intriguing to find out what determines the difference in susceptibility to RNA and DNA viruses in combination with *Wolbachia* infection.

Hypotheses for *Wolbachia*-induced immunity

Logically, there are two ways in which *Wolbachia* could promote antiviral immunity in *Drosophila*. First of all, the *Drosophila* immune response may be influenced. For example, the immune system could be activated or primed by the initial *Wolbachia* infection, allowing for faster or more robust response upon viral infection. Secondly, *Wolbachia* could directly influence viral replication. For example, if the bacterium and virus infect the same cell, they could compete for available nutrients or *Wolbachia* could produce molecules that directly affect viral integrity. In order to understand which of these possibilities is most likely, the immune reactions induced by *Wolbachia* and viruses will be examined separately before speculating about *Wolbachia's* interference.

Chapter 1: *Drosophila* and *Wolbachia*

Wolbachia infection characteristics

The symbiosis of *Wolbachia* with its numerous host species ranges from mutualistic to purely parasitic[13], depending both on the *Wolbachia* strain and the host genotype[14]. In order for parasitic, vertically transmitted *Wolbachia* to maintain their prevalence they have to confer fitness to infected hosts. Therefore, *Wolbachia* that infect *Drosophila* species most commonly interfere with the host's reproductive system by inducing cytoplasmatic incompatibility (CI). CI is a process in which embryos from an infected male and uninfected female are not viable, whereas infected females are able to inhibit the CI induced by infected males[10, 13]. Furthermore, to establish a persistent infection *Wolbachia* escapes the host immune response by residing inside host-derived vesicles in the cytoplasm[15].

The most prevalent *Wolbachia* strain endogenous to *D. melanogaster* is *wMel*[10, 16]. This strain shows vertical transmission and low levels of horizontal transfer[14], but also induces low levels of CI[17, 18]. Therefore, *wMel* must confer additional advantage to infected flies to be maintained amongst the *Drosophila* population[17, 18]. Since the genotype of both the *Wolbachia* strain and the host are so important seemingly contradictory reports have been published; ranging from beneficial effects of *Wolbachia* infection on *D. melanogaster* to no effects or even detrimental effects of this infection[17-20]. In order to better understand the infection the key immune events will be examined.

Antimicrobial peptides and signaling

Upon bacterial infection the *Drosophila* fat body normally starts producing several of the seven AMPs. Toll signaling results in Drosomycin synthesis, whereas Imd signaling results in Attacin, Cecropin and Diptericin synthesis[21, 22]. Most likely a combination of Imd and Toll signaling is important for synthesis of Metchnikowin, Defensin and Drosocin. In addition to Toll and Imd, JNK signaling is known to be activated by Gram-negative bacteria as well. Although the JNK pathway is triggered by the same receptors as the Imd signaling, it does not lead to AMP production[23,24] (see Figure 1).

Since *Wolbachia* express diaminopimelic acid (DAP)-type peptidoglycan on their cell wall[25, 26] the intracellular peptidoglycan recognition protein (PGRP)-LE and the extracellular PGRP-LC are most likely triggered upon infection. If so, Imd signaling would be induced in cells of the fat body[27, 28]. This prediction partially corresponds with an *in vitro* study that examined the transcriptional activity in *Wolbachia*-infected *Drosophila* S2 cells. Several signaling molecules were upregulated during infection, amongst which Relish, Attacin (A, B, C, D) and Diptericin B from the Imd pathway and dJun and Puc from the JNK pathway: all downstream of PGRP-LE. However, also Spätzle and Dorsal from the Toll pathway were upregulated[29]. As earlier reported by an *in vivo* study in a sister species (*Drosophila simulans*) the expression of Defensin, Diptericin (A) and Cecropin showed to be unaffected during *Wolbachia* infection in *D. melanogaster* cells[29, 30].

Heat-shock proteins

In addition to synthesis of Attacin and Diptericin B, and JNK signaling, eleven out of twenty-seven *Drosophila* heat-shock proteins (HSPs) were downregulated in *Wolbachia*-infected *Drosophila* S2 cells and expression of the other sixteen HSPs did not change[29]. Since HSPs are normally upregulated under stress conditions such as infections, *Wolbachia* probably actively suppresses this transcription. Another indication that *Wolbachia* alters HSP expression comes from studies in *D. simulans*, which express a different subset of HSPs in sperm cells once infected with *Wolbachia*[31]. In addition, upregulation of HSPs by applying natural heat shock rescued *D. simulans* from CI, which normally is very high in this species[31]. The question remains why HSP expression is lowered in *D. melanogaster* and still low amounts of CI are seen.

Autophagy

Another process induced by several intracellular bacteria is autophagy. During autophagy long-lived, cytosolic proteins are randomly degraded to serve as nutrient source under stress conditions[32]. The process is shown to be triggered by intracellular sensing of both peptidoglycan (PGN) types, which suggests that *Wolbachia* could induce autophagy in *Drosophila*. Even though PGN also triggers AMP secretion, induction of bacterial-triggered autophagy was shown to be independent of Toll and Imd signaling. Therefore, additional signaling pathways should play a role in triggering autophagy[33].

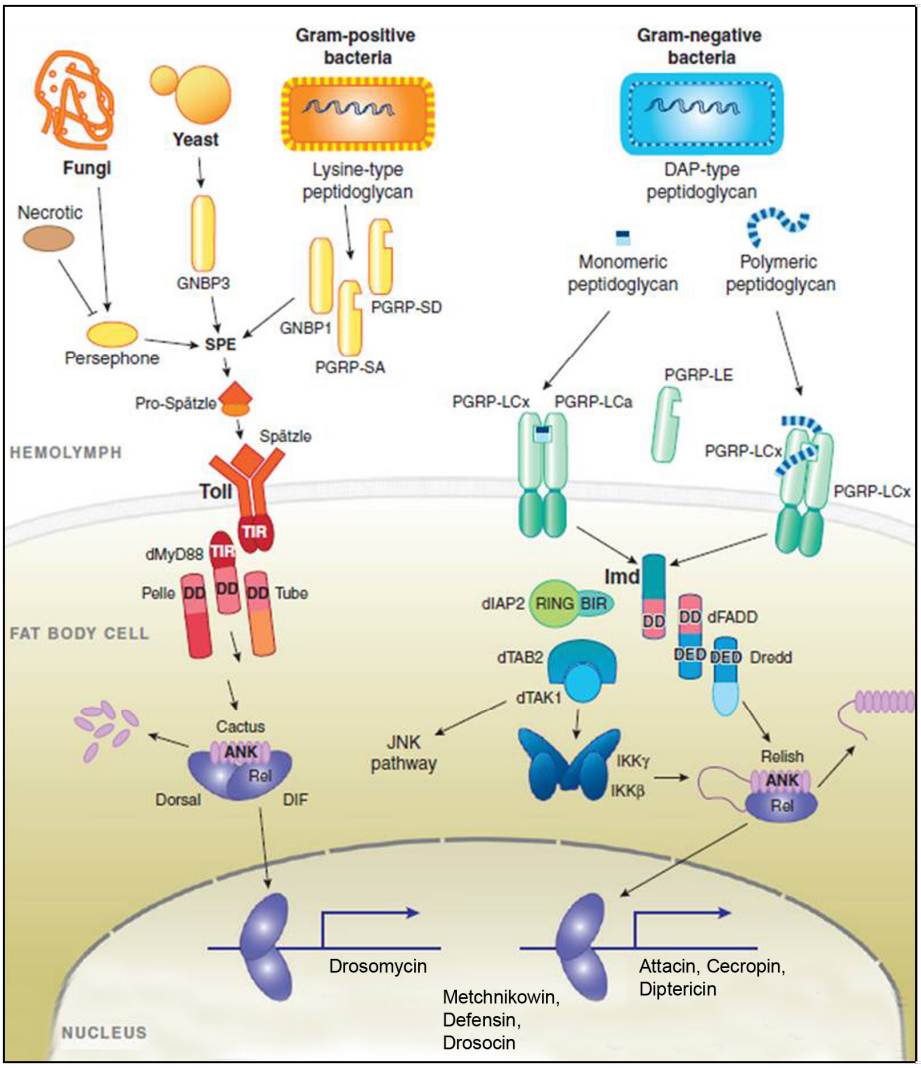

Figure 1. Overview of *Drosophila* immune signaling in cells from the fat body[4].

On the left the Toll pathway, activated by fungi, yeasts and Gram-positive bacteria.

On the right the Imd pathway, activated by Gram-negative bacteria, thus most likely also by *Wolbachia*.

Note: the pattern-recognition receptor of the Imd pathway PGRP-LE has two splice variants: the full-length protein, which functions intracellular and the truncated protein, which functions extracellular (i.e. the form that is depicted here)[27].

Wolbachia manipulating the *Drosophila* immune system

Since *Wolbachia* are persistent amongst *Drosophila* populations they must escape the guarding immune system. The recent unraveling of the *Wmel* genome sequence revealed two groups of proteins which might play a role therein[13]. First of all, a Type IV secretion system was identified, via which immune regulating proteins might be secreted[13]. Secondly, unusually many Ankyrin-repeat-containing proteins were identified. Ankyrin-repeats are commonly used to form protein-protein interactions. Therefore, they are likely candidates to interact with *Drosophila* immune mediators. Of all identified Ankyrin-repeat-containing proteins few contain a direct secretion signal, others could be secreted via the Type IV secretion system[13]. Noteworthy, in absence of an activating stimulus the proteins Relish and Dif/Dorsal are prevented from entering the nucleus by Ankyrin-repeat-containing proteins[4] (see figure 1). In this regard it might be interesting to examine the affinity of *Wolbachia* Ankyrin-repeat-containing proteins for Relish and Dif/Dorsal to see if an interaction could occur. However, since Imd-dependent transcription is still present in *Wolbachia*-infected *Drosophila*, the *Wolbachia* proteins will not fully block Relish.

Another manner for *Wolbachia* to influence immunity is by manipulating intracellular vesicles, so that the *Wolbachia*-containing vesicles will not be degraded. The bacterium is already known to interact with dynein and kinesin to maintain correct cellular localization[10]. Additionally, this might distort the normal routing of the vesicles and ensure *Wolbachia* persistence.

Table 1: Characteristics of the viruses tested by Teixeira *et al*. and Hedges *et al*.

(+)ssRNA viruses (Picorna-like viruses)[34]

Dicistroviridae [35, 36]

Cripa virus

Drosophila C virus (DCV)

Natural *Drosophila* pathogen

Replication on Golgi-derived vesicles[37]

Teixeira *et al.* and Hedges *et al.*

Cricket paralysis virus (CrPV)

Natural *Drosophila* pathogen

Replication most likely on Golgi-derived vesicles

Hedges *et al.*

Nodaviridae [38]

Alphanodavirus

Flock House virus (FHV) = Black Beetle virus

Non-natural *Drosophila* pathogen

Replication on outer mitochondrial membrane[39, 40]

Teixeira *et al.* and Hedges *et al.*

Unassigned [41]

Noravirus

Nora virus (NV)

Natural *Drosophila* pathogen

Replication site unknown

Teixeira *et al.*

DNA viruses

Iridoviridae

Iridovirus

Insect Iridescent virus-6 (IIV-6) = Chilo Iridescent virus

Non-natural *Drosophila* pathogen

Replication inside the nucleus and viroplasm[42,43]

Teixeira et al.

Phylogenetic classification, natural association with Drosophila, site of replication and research group that examined the effect of Wolbachia infection on the virus are summarized. Numbers indicate used references in addition to Teixeira *et al.* (2008)[3] and Hedges *et al.* (2008)[2].

Chapter 2: *Drosophila* and viruses

Virus characteristics

As can be seen in Table 1, the examined viruses against which *Wolbachia* induces protection show remarkable resemblances. They are all small, non-enveloped, positive-sense (+) single-stranded (ss) RNA viruses, belonging to the order of the Picornavirales[2, 3, 34]. Based on the sequence of their RNA-dependent RNA polymerase and helicase proteins Drosophila C virus (DCV) and Cricket Paralysis virus (CrPV) belong to the same family of the *Dicistroviridae*[35, 36], Flock House virus (FHV) belongs to the *Nodaviridae* family[38] and Nora virus (NV) forms a yet unnamed family[41]. The only infection not affected by the presence of *Wolbachia* was with the double-stranded (ds) DNA virus Insect Iridescence virus-6 (IIV-6), which belongs to the *Iridoviridae*. It is noteworthy that besides an increased survival a decrease in viral titer was observed during DCV and NV infections in *Wolbachia*-infected *Drosophila*, but not during FHV viral infection[3]. Unfortunately, the CrPV titer was not determined[2].

Like the sequence of their replication proteins, replication strategies of these RNA viruses differ slightly. Most likely, all associate with cellular membranes for replication: DCV (thus most likely also CrPV) replication is associated with Golgi-derived vesicles, formation of which depends on COP-I activity[37], and FHV replication is associated with mitochondrial outer membranes[39, 40]. Membrane association of NV during replication is unknown. On the other hand, IIV-6 is thought to start its replication in the nucleus, after which it may be finished in the cytoplasm[42]. Other reports also describe *Iridoviridae* replication entirely in the viroplasm, i.e. the part of the cytoplasm where viral components are synthesized and viruses assemble[43]. Unfortunately, the behavior of IIV-6 and other dsDNA viruses in *Drosophila* have not yet been analyzed, probably since no dsDNA virus is known as natural *Drosophila* pathogen[3].

RNA interference

Like in all other arthropods, the most extensively described immune response against RNA viruses is RNA interference (RNAi). In this process, dsRNA guides sequence specific cleavage of mRNA, preventing its translation. In *Drosophila*, the dsRNA is cleaved by the endoribonuclease Dicer-2 into 21-23 nucleotide long molecules. Subsequently, the cleaved RNA is bound by the RNA induced silencing complex (RISC) and one of the two strands is released. The remaining single-stranded RNA molecule (siRNA) guides RISC to complementary mRNA, which will be degraded by Argonaute-2, a component of RISC[5]. Since dsRNA is an obligatory intermediate of (+)ssRNA-virus replication, siRNAs of viral origin (viRNAs) will be generated during replication, leading to degradation of viral RNAs and decreased viral replication[5, 44] (see Figure 2).

For most (+)ssRNA *Drosophila* viruses indeed RNAi has shown to be an important defense mechanism: Dicer-2, R2D2 and Argonaute-2 mutant flies suffered significantly more from DCV, CrPV and FHV infections than WT flies[46-48]. However, there seems to be no role for RNAi during NV infection[49] (see Table 2). In addition, it is unlikely that IIV-6 will evoke an RNAi response, since dsRNA is not an intermediate in DNA virus replication.

As a reaction to the siRNA machinery of *Drosophila*, viruses express viral suppressors of RNAi (VSRs). Such proteins have been identified in DCV, CrPV and FHV[47]. For FHV, the B2 protein inhibits dsRNA cleavage by Dicer[50, 51]. The VSR identified in DCV, protein 1A, inhibits Dicer by means of an RNA-binding domain (RBD), not found in B2 of FHV and the VSR of CrPV[47]. This convergent evolution emphasizes the importance of RNAi as an antiviral immune reaction.

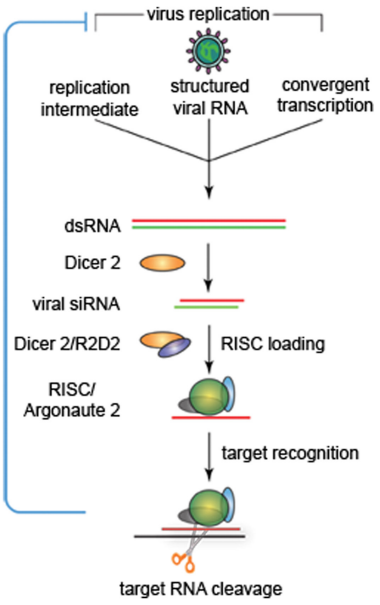

Figure 2. Silencing of viral RNA in *Drosophila* cells[45]. Double stranded (ds)-DNA, which is an essential intermediate in the replication of RNA viruses, is cleaved by Dicer-2. The formed viral siRNA guides the RNA induced silencing complex (RISC) to complementary viral (target) RNA, which will be degraded by the RISC component Argonaute-2.

Table 2. Knock-out effect on the severity of viral infection

	Dicer-2	R2D2	Argonaute-2
DCV	yes[48]	-	yes[47]
CrPV	yes[46]	yes[46]	yes[47]
FHV	yes[46, 48]	yes[46]	yes[46]
NV	no[49]	no[49]	no[49]
IIV-6	-	-	-

"Yes" indicates a detrimental effect of the inactivating mutation during the viral infection, "No" indicates no effect of the mutation and "-" means that no report has been found. DCV: Drosophila C virus; CrPV: Cricket Paralysis virus; FHV: Flock House virus; NV: Nora virus; IIV-6: Insect Iridescent virus-6.

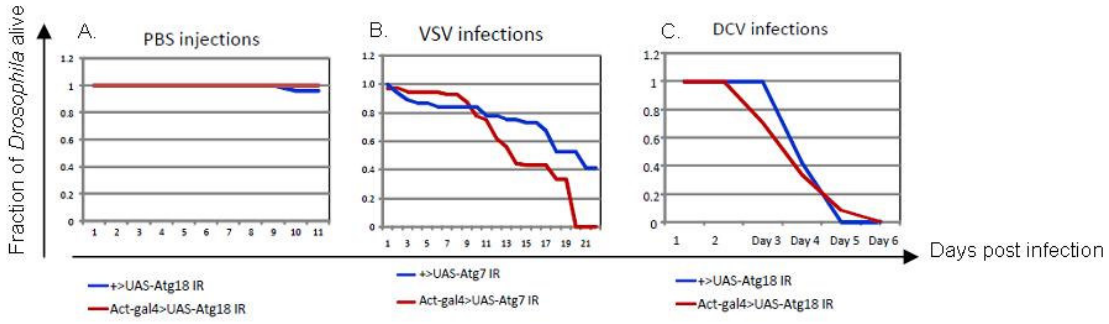

Figure 3. The role of autophagy in antiviral immunity. Blue lines reflect infection in negative control flies; red lines reflect infection in Atg knockdown flies. [A] PBS injection control. [B] When infected with VSV, increased mortality is seen in absence of functional Atg. [C] Although the authors state that during DCV infection there is no difference between control flies and Atg knockdown flies, a clear increase in mortality can be seen during the first days of infection[57]. DCV: Drosophila C virus; VSV: Vesicular Stomatitis virus.

Signaling and transcriptional activity

Whereas it has been long known that fungi and bacteria induce AMPs in *Drosophila* via Toll and Imd signaling, transcriptional activity upon viral infections has only recently been described. Even though DCV and CrPV infection do not induce AMP production[52, 53], *Drosophilae* without functional Imd signaling succumb faster to CrPV infection. Apparently, Imd signaling plays a role in antiviral immunity next to its well-known role in AMP production[53]. On the other hand, AMP production might also be influenced by the route of infection, since injected viruses do not induce AMP production[52, 53], whereas flies soaked in infected medium do show Attacin A, Cecropin A1 and A2, and Drosomycin expression[54]. Additional experiments should clarify these contradictory findings.

The other extensively described signaling pathway Jak/STAT has recently been reported to function during *Drosophila* anti-DCV immunity as well[52]. One of gene products resulting from Jak/STAT signaling is *vir-1* (virus induced RNA 1). It is transcribed by the Janus kinase (Jak) Hopscotch, but overexpression of Hopscotch alone is insufficient to induce *vir-1* transcription, indicating that additional stimuli are needed for the Jak/STAT promotor to transcribe *vir-1*[49, 52].

Another transcript specifically upregulated upon viral infection is *Vago* (CG2081)[52]. *Vago* mutant *Drosophilae* show higher viral titers than WT *Drosophilae*, exclusively in the fat body. Unlike transcription of *vir-1*, *Vago* is no longer upregulated upon DCV infection in Dicer-2-mutant *Drosophilae*. In addition, presence of FHV B2 protein also reduced the upregulation of *Vago* while leaving enhanced *vir-1* transcription intact. Both findings point to a role for Dicer-2 in antiviral immunity next to its role in RNAi[55].

In the same experiment, two other transcripts (*CG12780* and *CG9080*) were shown to be upregulated in a virus-specific manner[52]. This response was not triggered by empty DCV capsids, thus structural proteins (helicase, protease and RdRp) or a special feature of the DCV ssRNA are the most likely triggers[56]. Possibly additional stress is needed for induction of these transcripts, since a different route of administration (i.e. without injection) did not show upregulated *CG12780* transcription[54].

Table 3. Immune components induced by viruses

	Imd signaling	AMPs	Jak/STAT (*vir-1*)	*Vago*	Autophagy	HSP	Phagocytosis	Apoptosis
DCV	yes, [A]	yes[54] / no[52]	yes[52]	yes[55]	yes[57]	-	-	yes[52]
CrPV	yes[53]	no [53]	-	-	-	-, [B]	yes[53]	-
FHV	-	-	yes[52]	no[55], [C]	-	-, [B]	-	yes[60]
NV	-	-	possibly[49], [D]	-	-	-	-	-
IIV-6	-	-	-	-	-	-	-	-

"Yes" indicates a role during antiviral immunity, "No" indicates no role during antiviral immunity and "-" means that no report has been found. In addition to the named processes, all viruses except for NV induce RNAi in Drosophila. AMP: antimicrobial peptides; HSP: heat shock proteins; DCV: Drosophila C virus; CrPV: Cricket Paralysis virus; FHV: Flock House virus; NV: Nora virus; IIV-6: Insect Iridescent virus-6.

[A] Imd signaling must be induced if Attacin and Cecropin are transcribed[54]; [B] no reports are found that heat shock proteins are induced by these viral infections, but CrPV infection is temperature-dependent[61] and FHV replication depends on correct HSP90 expression[62]; [C] during FHV infection no upregulation of Vago is measured. However, due to the importance of Dicer-2 during this upregulation FHV B2 protein possibly inhibits this process; suggesting that Vago upregulation originally was triggered by (+)ssRNA viruses, but no longer measurable in FHV infection[55]; [D] overexpression of the Jak/STAT transcription factor Hopscotch did not affect virus titers[49], but that does not exclude Jak/STAT signaling importance, as reported for DCV and CrPV[52].

In stead of directly addressing transcriptional activity, some reports focus on the cellular processes that function in the antiviral immunity. Indications have been found that autophagy, phagocytosis and apoptosis play important roles therein.

Autophagy

In addition to bacteria, recently also the (-)ssRNA virus Vesicular Stomatitis virus (VSV) was shown to induce autophagy in *Drosophila*[57]. Knocking down the autophagy protein Atg18 decreased the survival of *Drosophila* during VSV infection. Even though this mutation was reported not to affect the course of DCV, the reported data do show some differences. Especially the mortality in the first few days post infection is increased (see Figure 3). Therefore, I expect that autophagy plays a role in immunity against both VSV and DCV in *Drosophila*.

Seemingly contradictory, the (+)ssRNA Mouse Hepatitis virus (MHV) is reported to benefit from autophagy[58]. However, this virus is shown to obtain its replication vesicles from autophagic vesicles[58]. Since DCV and FHV replication vesicles are of different origin, it is unlikely that those viruses benefit from autophagy. For DCV,

Golgi-derived vesicles with COP-I were shown to be important for replication and not *COP-II* or *Atg* (autophagy) genes[37]. For FHV, replication takes place in association with the mitochondrial membrane[40], which also decreases the likelihood that autophagic vesicles are involved.

It would be interesting to examine these *Drosophila* viruses for viral suppressors directed against autophagy, which could indicate the importance of autophagy during antiviral immunity.

Phagocytosis

Also phagocytosis is possibly triggered as defense against viral infections. This cellular immunity is executed by hemocytes present in the circulating hemolymph[4]. Blockage of phagocytosis leads to progressed death in adult *Drosophilae*, pointing to a role for phagocytosis in fighting CrPV infection[53].

Apoptosis

Lastly, DCV infection induces transcription of *damm*, one of the seven *Drosophila* caspases[52]. DAMM is suggested to induce apoptosis in *Drosophila* cells[59]. To what extend apoptosis contributes to the antiviral immunity has not yet been described.

Table 3 gives an overview of what immune components are induced by the viruses in addition to RNAi.

Chapter 3: *Wolbachia*-mediated viral protection

The examination of *Wolbachia* and virus infections in *Drosophila* should lead to hypotheses about the molecular basis of the observed *Wolbachia*-mediated viral protection. As mentioned in the introduction, *Wolbachia* could protect *Drosophila* in two ways: by acting upon the immune system or by a direct interaction with the virus.

Does *Wolbachia* modulate the antiviral immune reaction?

If *Wolbachia* would prime the immune system, *Drosophila* might act faster upon viral infections. Even though RNAi is an important antiviral immune response, no indications could be found that *Wolbachia* interferes with RNAi. In stead, Imd signaling might form the bridge between antiviral and antibacterial immunity. Namely, DAP-type peptidoglycan is expressed by the Gram negative *Wolbachia* and mainly triggers Imd signaling. This will lead to secretion of certain antimicrobial peptides and JNK signaling. Although it is not clear yet if AMPs are produced upon viral infection, Imd signaling has shown to be needed for efficient anti-CrPV immunity[52, 53]. Thus indeed, *Wolbachia* might induce Imd signaling, which enables faster immune reaction once super infected with a virus.

Another crosslink between antibacterial and antiviral immunity is found in the gene transcript *CG12780*, which is transcribed after signaling by the ligand Spätzle of the Toll pathway upon *Wolbachia* infection. This transcript is translated into Gram Negative Binding Protein (GNBP)-like protein[63, 64]. Surprisingly, *CG12780* is also upregulated during DCV infection[52]. Thus again, this reaction might already be primed by the *Wolbachia* infection, allowing faster activation upon additional virus infection.

A different hypothesis is that *Wolbachia* skews the immune reaction from purely antiviral – when *Wolbachia*-free *Drosophila* undergo a viral infection – to more antibacterial. This would be beneficial for the flies if they normally die from the adverse effects of their immune reaction to the virus rather than from the virus itself. This possibility remains highly speculative, since also a lowered viral load was seen in *Wolbachia*-infected *Drosophila*[3]. Nonetheless, the mechanism does exist in animals, where helminthes infection in humans leads to a decrease of ongoing allergic or autoimmune reactions[65].

Could *Wolbachia* directly affect the viruses?

The second manner for *Wolbachia* to help fighting the viral infection is by directly affecting viral integrity. To achieve this, *Wolbachia* should show overlapping tissue tropism with the virus. The *Wmel* strain is known to infect the fat body, muscles, wings, nervous tissue, hemolymph, midgut, salivary glands, Malpighian (renal) tubules, testes and ovaries of *D. simulans*[66]. Another strain (*Wolbachia popcorn; Wpop*) infects brain neurons, retina, thoracic muscle, and ovary of *D. melanogaster*[67] (see Table 4). All examined RNA viruses indeed show partial overlap in tropism. Namely, DCV infects the fat body, thoracic muscle fibers, digestive tract, tracheal cells, follicle cells and epithelial cells surrounding the ovaries[68, 69]; CrPV infects the alimentary canal, nerve ganglia and epidermal cells[38]; FHV infects the fat body, muscles and trachea[48]; and Nora virus infects the intestine[70] (see Table 4). Since IIV-6 does not naturally infect *Drosophila* little is reported and information about the tissue tropism is lacking.

The most obvious way in which *Wolbachia* could help antiviral immunity is by outcompeting the virus in the cytosolic niche. Both intracellular agents rely on cellular nutrients[13]. If the presence of *Wolbachia* would slow the replication speed of the virus to some extend, it may be enough for the immune system to deal with the remaining virions.

In addition, both types of infections most likely trigger autophagy in *Drosophila* cells. However, *Wolbachia* must have developed manners to circumvent autophagic lysis of their vesicles, since they are able to persist in *Drosophila*. It seems illogical that *Wolbachia* could selectively suppress autophagy of their surrounding vesicles and at the same moment promote autophagy of viral replication vesicles.

Since heat shock proteins (HSPs) are important under several stress conditions, they might also be responsible for the observed *Wolbachia*-induced antiviral immunity. Whereas HSPs were downregulated in the S2 cell line in presence of *Wolbachia*[29], *in vivo Wolbachia* was shown not to influence resistance against heat shock in adult *Drosophilae*[11]. It is thus unlikely that *Wolbachia* influences HSP expression to an extent that influences the outcome of viral infections.

Table 4. Tissue tropism of the described infectious agents in *Drosophila*

	Wmel in *D. simulans*[66]	*Wpop* in *D. melanogaster*[67]	DCV[68, 69]	CrPV[38] (in crickets)	FHV[48]	NV[70]
fat body	x		x		x	
hemolymph	x					
nervous tissue	x	x		x	x	
(thoracic) muscles	x	x	x			
tracheal cells			x		x	
digestive tract	x		x	x		x
ovaries	x	x	x			
OTHER	renal tubules wings salivary glands testes	retina		epidermal cells		

As can be seen, all mentioned (+)ssRNA viruses infect at least one cell type that will also be infected by Wolbachia. "x" indicates the presence of the virus in that tissue. DCV, FHV and NV tissue tropism is indicated for D. melanogaster. Tissue tropism of IIV-6 is unknown. DCV: Drosophila C virus; CrPV: Cricket Paralysis virus; FHV: Flock House virus; NV: Nora virus; IIV-6: Insect Iridescent virus-6.

Since both the viruses and *Wolbachia* reside inside vesicles and *Wolbachia* is known to interact with vesicular transport via microtubuli, the viral replication complexes could be situated elsewhere when cells are coinfected with *Wolbachia* as opposed to in uninfected cells. This might promote the degradation of the replication complexes. Furthermore, during *Wpop* infection mitochondria appear disintegrated[67]. Under those circumstances, FHV replication will most likely be less efficient. However, *Wpop* and *Wmel* infections induce quite distinct phenotypes in *D. melanogaster*, thus this remains a fragile hypothesis.

A last possibility is that *Wolbachia* directly helps to destroy the viruses. The most likely candidates to execute such a process are again the Ankyrin-repeat-containing proteins and other proteins excreted via the Type IV secretion system.

Indications for a general principle

Intrigued by the finding of *Wolbachia*-induced antiviral immunity in *Drosophila*, an additional study was done to assess the generality of this observation. It appears that also *D. simulans* is protected against DCV and FHV by its natural *Wolbachia* strains, but only the strains that naturally infect with high density[71]. As seen for FHV protection in *D. melanogaster*, DCV protection in *D. simulans* does not always result in lower viral titers. This indicates that the mechanism of *Wolbachia*-induced antiviral protection might vary between viral infections.

To further extent this study, it would be interesting to determine whether *Spiroplasma* infection has the same effect on *Drosophila* antiviral immunity as has *Wolbachia*. *Spiroplasma* is the only other intracellular bacterium naturally occurring in *Drosophila*[72]. Albeit less widespread than *Wolbachia*, *Spiroplasma* was detected in 7 out of 12 tested *Drosophila* species[73]. More importantly, the mechanism by which *Spiroplasma* is able to persist in *Drosophila* is still debated[73], thus the possibility that *Spiroplasma* also induces symbiont-mediated protection is not excluded. Additional experiments should elucidate whether the observed endosymbiont-mediated protection is *Wolbachia* specific, or a more general mechanism is active.

Conclusion

Altogether, *Wolbachia* could help *Drosophila* to survive (+)ssRNA virus infections at two levels: by inducing changes in the immune reaction, or by directly interfering with viral replication. At this stage, both options remain highly speculative, since not all infections are characterized as extensively. In addition, the fact that both host genotype and *Wolbachia* genotype appear very important for the outcome of *Wolbachia* infection makes it difficult to compare studies. Therefore, it would certainly help to better examine the course of infections in *Drosophila*; especially for IIV-6 infection.

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