Characterization of infection in *Drosophila* following oral challenge with the Drosophila C virus and Flock House virus

Aleksej Stevanovic
Bachelor of Science (Honours)

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School of Biological Sciences*
Abstract

Understanding antiviral processes in infected organisms is of great importance when designing tools targeted at alleviating the burden viruses have on our health and society. Our understanding of innate immunity has greatly expanded in the last 10 years, and some of the biggest advances came from studying pathogen protection in the model organism *Drosophila melanogaster*. Several antiviral pathways have been found to be involved in antiviral protection in *Drosophila* however the molecular mechanisms behind antiviral protection have been largely unexplored and poorly characterized. Host-virus interaction studies in *Drosophila* often involve the use of two model viruses, Drosophila C virus (DCV) and Flock House virus (FHV) that belong to the *Dicistroviridae* and *Nodaviridae* family of viruses respectively.

The majority of virus infection assays in *Drosophila* utilize injection due to the ease of manipulation, and due to a lack of routine protocols to investigate natural routes of infection. Injecting viruses may bypass the natural protection mechanisms and can result in different outcome of infection compared to oral infections. Understanding host-virus interactions following a natural route of infection would facilitate understanding antiviral protection mechanisms and viral dynamics in natural populations. In the 2nd chapter of this thesis I establish a method of orally infecting *Drosophila* larvae with DCV to address the effects of a natural route of infection on antiviral processes in *Drosophila*. To confirm productive infection, I designed a single-stranded RT-qPCR assay. Using this assay I show that larvae that survive beyond 24 h post-contamination are not persistently infected.

Establishing a method of orally infecting *Drosophila* flies allowed the subsequent studies to focus on antiviral mechanisms following a natural route of virus infection. While host-derived defence mechanisms, such as immunity, are important for mediating antiviral protection, extrinsic protection mechanisms such those provided by the endosymbiotic bacteria *Wolbachia pipientis* can also confer antiviral protection in *Drosophila*. *Wolbachia* is a maternally transmitted intracellular alpha-proteobacteria found in a large number of arthropods and nematodes which can mediate antiviral protection against a range of viruses including DCV, FHV and Cricket paralysis virus. The 3rd chapter focuses on understanding the antiviral effects of *Wolbachia* on viral tolerance following oral infection of *Drosophila*.
larvae and adults. The results showed that in adults, *Wolbachia* strains that confer viral tolerance following systemic DCV infection do so also following oral DCV infection. Interestingly, *Wolbachia*-mediated protection was life-stage dependent as oral feeding of L1 stage larvae resulted in a loss of *Wolbachia*-mediated protection in 3 out of 4 *Drosophila-Wolbachia* associations shown to be protective at the adult stages. Loss of protection was associated with lower *Wolbachia* densities at larval compared to adult stages in the same three *Drosophila-Wolbachia* associations. These results will aid in understanding the effects of *Wolbachia* on viral dynamics in natural populations and will contribute to our understanding of life-stage susceptibility in *Drosophila*.

In the 4th chapter the role of apoptosis in mediating antiviral protection was studied following both viral injections (systemic infection) and oral infections using FHV as a model. Using altered gene expression of key genes involved in apoptosis I investigated the importance of apoptosis on antiviral protection. Knocking-out a pro-apoptotic transcription factor dP53 in adult flies lead to an increase in viral titers following a systemic FHV infection and resulted in earlier mortality of infected individuals compared to wild type flies. Contrary to systemic infection, oral FHV infection of the same fly line showed no effect on viral accumulation but led to earlier mortality of infected individuals. Over-expressing a pro-apoptotic gene *reaper* lead to a reduction in FHV viral titers following both systemic and oral infections, however while a reduction in viral titers lead to a delay in virus-induced mortality in systemically infected flies, no differences in mortality was observed between wild type and mutant flies following oral infection. Oral infection with FHV caused 30 % mortality in both wild type and P53 and *reaper* over-expressing flies. The similarities in the number of flies succumbing to oral infection indicates that apoptosis does not impact the outcome of initial challenge, but that it likely functions in protection following primary infection. Depending on whether apoptosis was suppressed or enhanced lead to differences in the effects of apoptosis on resistance (ability of the host to control virus accumulation) and tolerance (ability of the host to endure infection), which lead to the idea that viral tissue tropism could be responsible for the differences in resistance versus tolerance.

Taken together the results enhance our understanding of antiviral mechanisms in *Drosophila* and show that both the route of infection and life-stage can have important impacts on antiviral mechanisms. The methods used to orally infect flies with both DCV and FHV will be valuable in facilitating research focused on *Drosophila*-pathogen interactions.
following the oral route of infection and will hopefully encourage more research to focus on understanding the effects of oral infections on host-virus interactions.
Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

Peer-reviewed papers


Preparing for submission

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Conference abstract

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Chapters 2 and 3 have been published in peer-reviewed journals, and chapter 4 is written as papers for publication to Plos ONE.


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<td>Designed experiments (80%)</td>
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<tr>
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<td>Performed experiments (100%)</td>
</tr>
<tr>
<td></td>
<td>Wrote the paper (80%)</td>
</tr>
<tr>
<td>Pieter Arnold</td>
<td>Prepared figures 1, 2 and 3 using data</td>
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<tr>
<td></td>
<td>Wrote the paper (85%)</td>
</tr>
<tr>
<td>Karyn Johnson</td>
<td>Designed experiments (20%)</td>
</tr>
<tr>
<td></td>
<td>Wrote the paper (10%)</td>
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Contributor Statement of contribution

Aleksej Stevanovic (Candidate)
- Designed experiments (80%)
- Performed experiments (100%)
- Wrote the paper (80%)

Karyn Johnson
- Designed experiments (20%)
- Wrote and edited paper (20%)
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<td>Designed experiments (95%)</td>
</tr>
<tr>
<td></td>
<td>Performed experiments (100%)</td>
</tr>
<tr>
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<td>Wrote the paper (90%)</td>
</tr>
<tr>
<td>Author Karyn Johnson</td>
<td>Designed experiments (5%)</td>
</tr>
<tr>
<td></td>
<td>Wrote and edited paper (10%)</td>
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List of abbreviations

DCV  Drosophila C virus
FHV  Flock House virus
CrPV  Cricket paralysis virus
IIV-6  Invertebrate iridescent virus 6
SINV  Sindbis virus
CuniNPV  Autographa californica multiple nucleopolyhedrovirus
AcMNPV  Culex nigripalpus virus
ss  single-stranded
ds  double-stranded
ORF  open reading frame
dpi  Days post infection
siRNA  small interfering RNA
miRNA  micro RNA
RNAi  RNA interference
siRNA  short interfering RNA
piRNA  piwi-interacting RNA
dsRNA  double stranded RNA
JAK-STAT  Janus kinase signal transducer and activator of transcription pathway
Imd  Immune deficiency pathway
PAMPs  pathogen-associated molecular patterns
PRRs  pattern recognition receptors
AMP  antimicrobial peptides
IAP  inhibitor of apoptosis
BIR  baculovirus IAP repeat domain
IRER  irradiation-responsive enhancer region
°C  degrees centigrade
µg  microgram
µl  microliter
µM  micromolar
mL  millilitre
h

hour

rpr

reaper
Chapter 1: General introduction
Viruses that infect arthropod species can carry a heavy burden on society. Insects like mosquitoes can transmit a diverse range of human viruses such as dengue, West Nile and chikungunya viruses. The cost to the economy for dengue virus alone is up to $1.8 billion US per year, and poses a significant risk to human health (1). While viruses can be detrimental to human health and the economy, they can also be used to our advantage. Viruses have started gaining ever greater interested for their use as insecticides to control pests affecting several economically important crops (2). Whether our aim is to restrict or enhance viral spread, understanding viral dynamics is essential.

Understanding viral dynamics requires knowledge of the molecular pathways involved in regulating host-virus interactions. Genetic techniques used to dissect the molecular pathways involved in protection against virus invasion can be challenging to apply. Drosophila melanogaster has emerged as a model organism to study host-virus interactions in arthropods for several reasons. As one of the original genetic models, Drosophila has well established tools and procedures, and is easy and inexpensive to culture in the lab. Genetic manipulation in Drosophila is well characterized and easily achieved. An extensive selection of Drosophila flies are available that possess null mutations in a range of genes, which can be used to study gene function and molecular pathways. As well as null mutants, a large collection of flies exists, where genes can be knocked-down or over-expressed in specific tissues at different developmental stages. Furthermore, Drosophila is a good model organism to study innate immune system regulation, due to the conservation of innate immunity throughout evolution.

Pathogens often infect arthropod populations, making it essential for the host to possess protection mechanisms to fight infection. Defence against pathogen entry and invasion have ancient origins, as genetic studies have found that pathogen defence mechanisms existed in the ancestors of plants and animals before they diverged (3). Due to the importance of the defence system, immunity has remained very well conserved between organisms through evolution. In Drosophila, the molecular mechanisms required for protection against bacterial and fungal infections have been well characterized, while antiviral molecular mechanisms remain poorly characterized (4). A wide range of viruses from diverse families have been used to study Drosophila-virus interactions, some of which infect natural Drosophila populations, while others are artificially delivered (Table 1). Availability of a variety of viruses that can successfully infect Drosophila allows for a comparative analysis to identify both virus-specific and broad antiviral responses in Drosophila. Expanding our knowledge of antiviral mechanisms in Drosophila will allow us to determine the role they play in protection, and could provide us with the opportunity to understand and control viral dispersion.
Table 1: Commonly studied viruses in *Drosophila*

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<th>Name</th>
<th>Abbreviation</th>
<th>Family</th>
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<tr>
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<td>Togaviridae</td>
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**Host – virus interaction**

**Drosophila C virus (DCV)**

DCV is the most well studied *Drosophila* virus, and has been used extensively to study *Drosophila*’s antiviral immune responses (5). DCV was first isolated from a *D. melanogaster* strain Charolles in 1972 (6), and has since been shown to infect numerous *Drosophila* species in natural populations (7). Different DCV isolates exist depending on their geographical location (8). The first DCV isolate was isolated from the French Charolles flies, and was named DCV<sub>C</sub>, while the strain used throughout all the experiments in this thesis is the Australian isolate DCV<sub>EB</sub> (8). DCV is a member of the *Dicistroviridae* family and is a single stranded + sense RNA virus, with a genome comprising 9264 nucleotides (9). The genome contains two open reading frames (ORF); ORF1 encodes a helicase, protease and a RNA-dependent RNA polymerase, while ORF2 encodes a 100 kDa polyprotein, which is processed to form the capsid proteins (9). During infection, the virus replicates its genome by synthesizing the – strand RNA, which is then used to make more + strand RNA. The – strand RNA is not encapsidated in virus particles and can be detected only in cells with actively replicating virus. The icosahedral viral capsid is composed of 4 major proteins VP1 (33 kDa), VP2 (29 kDa), VP3 (29 kDa) and VP4 (8.5 kDa), however it is also possible to detect the protein VP0 (37 kDa) which is the precursor of VP3 and VP4 (10).
DCV infection

Studies on antiviral responses in *Drosophila* often use DCV as a model virus due to its high pathogenicity. Following DCV injection into the hemocoel, the virus becomes internalized by clathrin-mediated endocytosis (11). Once inside the cells, DCV remodels the Golgi apparatus and forms a vesicle on which viral replication occurs (12, 13). Vesicle formation requires the coat protein complex I (COPI) and fatty acid biosynthesis, both of which are essential for DCV replication (13). Once DCV becomes established inside the cells, it kills its host within 4-6 days post injection (dpi) (6, 14). While DCV infection is extremely pathogenic following viral injection, infection of adult flies through feeding on contaminated media is much less pathogenic and causes ~15% mortality within 4-8 days post ingestion (15).

DCV is often detected in natural *Drosophila* populations (7), however the mechanism through which infection occurs is not fully understood. Studies on DCV infection have primarily been performed in *Drosophila* cell cultures and through intra-thoracic injections of adult flies. Neither of these infection mechanisms are representative of the way DCV infection occurs in nature. In natural *Drosophila* populations DCV infection is thought to occur through ingestion of virus-contaminated food (16). Experiments performed in the mid 80’s by Thomas-Orillard and colleagues claim to have achieved natural DCV infection by feeding DCV infected fly homogenates to first instar larvae (17). To confirm that larvae were successfully infected, the flies suspected of being infected with DCV were ground in a buffered solution and injected into DCV free flies to score daily mortality. When DCV infected fly homogenates were injected into DCV free flies, 93% of flies died within 3-4 dpi, which led the authors to conclude that flies feeding on DCV contaminated media become infected with DCV (17). While the above-mentioned method is suitable for detecting viral contamination, it cannot distinguish between viral contamination and infection. When larvae feed on DCV contaminated media, the virus settles on the cuticle and/or on the surface of the digestive tract, without necessarily entering the internal organs and causing an infection. Furthermore, because the flies were pooled before the homogenates were injected into DCV free flies, it is not possible to analyse the percentage of flies that became infected after feeding on contaminated media.

Other studies focused on natural DCV infection found that DCV can form both mutualistic and parasitic relationships with its host, depending on the environmental conditions (18). When flies were exposed to DCV through the rearing media, it was reported that the virus can provide several advantages to the fly, such as increased fertility, increased adult weight and a decreased
developmental time (17). Although the interpretation that DCV can form mutualistic relationships with *Drosophila* were consistent with the results, an alternative interpretation could not be discounted. Because DCV increases egg to adult mortality (16), it is reasonable to postulate that larvae with the lowest fitness will be the ones to succumb to DCV infection before adult emergence. Elimination of the weakest individuals before adult emergence would make the remaining adult population appear fitter, and therefore more likely to show an increase in adult weight, fertility and a decrease in developmental time, therefore skewing the results and leading to the conclusion that DCV infection increases the fitness of the individual. Due to experimental techniques used in the abovementioned experiments, it is hard to determine whether DCV can indeed form mutualistic relationships with *Drosophila*.

Studying the natural route of infection is interesting for several reasons. DCV pathogenicity is much lower following ingestion of the virus compared to injection (15), suggesting that natural barriers to infection exist that are bypassed through injection. Evidence suggests that DCV tissue tropism can be affected by the route of DCV infection. When DCV is injected intra-thoracically, all infected flies show DCV localization in the crop, midgut, hindgut, fat body, testis and trachea, while oral DCV infection in most cases shows DCV to be confined only to some of these tissues (15). The route of virus infection does not only affect viral tissue tropism, but can also affect the ability of the host to mount an immune response against pathogen invasion (15). A recent study showed that the Toll immune pathway in *Drosophila* is responsible for limiting replication of both DCV and Flock House virus (FHV) when the viruses are ingested, however it does not limit viral replication following viral injection, pointing to the importance of the route of viral entry on viral dynamics (15).

The study of viral dynamics following the natural route of virus infection has been impeded by the lack of robust tools available to induce DCV infection through the oral route of infection, and the ability to distinguish between virus infection and contamination. Designing methods capable of inducing oral DCV infection in both larvae and adult, and designing tools able to detect virus infection would be essential to advance our knowledge of the host’s defence mechanisms in natural insect populations. Using the oral route of virus infection would furthermore allow us to study viral dynamics and how it relates to natural insect populations, and would allow us to study *Drosophila*’s natural protective barriers involved in limiting viral entry and replication.
**Flock House virus**

Antiviral responses in *Drosophila* may vary depending on the virus infecting the organism. Flock House virus (FHV) has often been used to study immune responses in *Drosophila* and it belongs to the *Nodaviridae* family of viruses (19). FHV is a non-enveloped, icosahedral single stranded + sense RNA virus, and can replicate in insects, yeast, plants and mammalian cells (19-23). The genome is divided into two RNAs, RNA1 (3.1 kb) and RNA2 (1.4 kb) (19, 24, 25). RNA1 encodes for protein A, which is an RNA-dependent RNA polymerase, and is required for transcription of both genomic strands and the subgenomic RNA3 (25-27). The subgenomic region contains two open reading frames, which encode the proteins B1 and B2, while the genomic RNA2 encodes the precursor of the coat protein (28). The function of the protein B1 is unknown, however it is known that the protein B2 suppresses the cleavage of double-stranded RNA, responsible for the inhibition of small interfering RNA (siRNA) formation, therefore suppressing the RNA silencing pathway (29-31).

FHV is not a natural *Drosophila* pathogen, however it can replicate inside *Drosophila* cells. Injecting FHV into the haemocoel of adult *Drosophila* causes death of the host within 7-8 dpi (14, 32), while oral FHV infection is much less pathogenic, and causes 10-15 % mortality within 13 days of ingestion (15).

**Antiviral defence mechanisms in *Drosophila***

Viruses are obligate intracellular parasites and require the host’s cellular machinery for replication. Conversely, the host tries to prevent the establishment of viruses within the cellular compartments by mounting a defence response. The constant struggle between viruses and hosts has led to the evolution of several well-conserved antiviral pathways, of which we are only beginning to understand the molecular mechanisms. While humans have evolved both the adaptive and innate immune system, *Drosophila* lacks the adaptive immune system, and uses the innate immune system as the primary tool to control viral infections (33). *Drosophila*’s innate immune system uses the pattern recognition receptors (PRRs) to recognize pathogen-specific pathogen associated molecular patterns (PAMPs) to mount an immune response (reviewed in 34). Several distinct viral defence mechanisms have evolved in *Drosophila*, providing protection against a diverse range of viruses (Figure 1). What follows is an overview of the known antiviral defence mechanisms in *Drosophila*,...
in order to summarise and discuss the advances made in this field, and identify areas where further research is required.

Figure 1. Antiviral defence pathways in Drosophila. The figure summarizes the immune pathways involved in antiviral protection in Drosophila. Shown are the sensing molecules for each pathway. For the JAK-STAT, Toll, Imd and Autophagy pathways, the sensing molecules are cellular receptors. For the RNAi pathway the sensing molecule is Dicer-2 found in the intracellular department, while the sensing molecule for virus-induced apoptosis has not yet been described, therefore is marked with a question mark. For each pathway the viruses against which it confers antiviral protection are shown.

RNA interference pathway (RNAi)

The RNAi pathway is one of most well characterized antiviral pathways in Drosophila. The effectors of the RNAi pathway are small non-coding RNAs, which function in chromosome segregation, chromatin structure, RNA processing and stability, transcription, and immunity (35). The three major pathways are: micro RNA (miRNA), piwi-interacting RNA (piRNA) and small interfering RNA (siRNA), with the latter being predominantly responsible for protection against viruses (34). Recognition of pathogens through the siRNA pathway occurs through recognition of
double-stranded viral RNA intermediates by Dicer-2 (Figure 1) (33). Dicer-2 processes dsRNAs into small siRNAs fragments 21 nucleotides long (36, 37). The siRNAs are subsequently loaded onto the RNA induced silencing complex (RISC). The siRNAs guide the RISC complex to the complementary messenger or virus RNA where Argonaute-2, the catalytic component of RISC, cleaves the target viral RNA. Cleavage of viral RNA limits viral replication and can prevent a systemic infection. *Drosophila* in which Dicer-2 or Argonaute-2 genes have been deleted show hypersensitivity to DCV, FHV and Cricket paralysis virus (CrPV) infections (38-40). Co-evolution between *Drosophila* and viruses has led the viruses such as DCV, FHV, CrPV and the invertebrate iridescent virus 6 (IIV-6) to evade the RNAi system by expressing RNAi suppressors, which target Dicer-2 or other components of the machinery to avoid processing (29, 31, 38, 41, 42).

**Janus kinase signal transducer and activator of transcription pathway (JAK-STAT)**

The JAK-STAT pathway has several roles and is essential in embryonic segmentation, formation of the eye, cell differentiation, cell movement and immunity (43, 44). In *Drosophila*, the JAK-STAT pathway is involved in protection against both viruses and bacteria (44). Recognition of viral particles leads to dimerization of the Jak tyrosine kinase Hopscotch and a single Stat transcription factor (Stat92E). Dimerization of Stat92E causes nuclear translocation, where transcriptional activation of genes containing Stat-binding sites occurs (45, 46). Genetic studies have shown that mutants in the Hopscotch receptor show increased susceptibility to infection with DCV, FHV and CrPV viruses, suggesting the importance of the JAK-STAT pathway in the antiviral response in *Drosophila* (4). While clear evidence exists for the involvement of the JAK-STAT pathway in immunity, it remains to be determined how transcriptional activation of genes containing Stat-binding sites leads to inhibition of viral replication.

**Immune deficiency pathway (Imd)**

The Imd pathway forms part of the innate immune system in *Drosophila*, and has been implicated mainly in protection against fungi and bacteria (47). Following bacterial infection, the pathogens are recognized through the pathogen-associated molecular patterns (PAMPS) present on the surface of bacteria. The activation of the Imd pathway requires the activation of the transcription factor Relish, which once activated translocates into the nucleus and facilitates the activation of antimicrobial peptides (AMPs) (48). AMPs have a large spectrum of antimicrobial activity and can inhibit replication of fungi, bacteria and viruses through a range of mechanisms (49). While the molecular mechanisms controlling the host’s antibacterial response are well characterized, very
little is known about how activation of the Imd pathway leads to inhibition of viral replication. In genetic studies where the transcription factor *Relish* has been deleted, a reduction in Sindbis virus (SINV) titer was observed (50). Furthermore, null mutants in several genes involved in the Imd pathway (*peptidoglycan recognition proteins, transforming growth factor-activated kinase 1, immune response deficient 5, Kenny and Relish*) show increased susceptibility to CrPV and SINV infection, suggesting the involvement of the pathway in antiviral protection (51).

Whether the Imd pathway suppresses viral replication through the activation of AMPs or whether an alternative mechanism exists is not yet clear. Evidence for the existence of an alternative mechanism comes from studies focusing on CrPV infection, where the Imd pathway was shown to confer antiviral protection in an AMP-independent manner (51). No studies to date have determined the involvement of the Imd pathway in protection against DCV infection, however transcriptional profiling of DCV infected *Drosophila* cells has shown up-regulation of genes involved in the Imd pathway, and induction of antimicrobial peptides one hour post-incubation (52).

**Toll pathway**

Compared to other immune pathways, relatively little is known about the involvement of the Toll pathway in response to viral infection. Similarly to the Imd pathway, the Toll pathway is involved in protection against fungi, bacteria and viruses (53-55). The Toll pathway recognizes PAMPs through the PRR receptors referred to as Toll-like receptors (reviewed in 56). Following bacterial and fungal infection, activation of the Toll pathway leads to the production of antimicrobial peptides responsible for limiting fungal and bacterial replication (57). Loss-of-function mutants in two Toll pathway genes (*Dorsal-related immunity factor* and *Dorsal*) are significantly more sensitive to Drosophila X virus (DXV) infection, suggesting the involvement of the Toll pathway in antiviral protection (55). Furthermore, DXV infection induces production of several AMPs (Drosomycin, Diptericin, Defensin, Attacin, Cecropin, Drosocin and Metchnikowin) however constitutive expression of these AMPs in adult flies does not alter DXV titres, suggesting an alternative mechanism of viral inhibition (55). Furthermore, deletion of several key Toll pathway genes (*Spatzle, Toll, Pelle* and *Dorsal*) leads to increased sensitivity to oral infections with FHV, CrPV and Nora virus, but shows no such effect upon viral injection, suggesting that the involvement of the Toll pathway in antiviral protection is dependent on the route of infection (15). The exact molecular mechanism involved in Toll-mediated antiviral protection is still unclear, and further research is required to elucidate the molecular mechanisms.
Autophagy

Nine known Toll-like receptors exist in Drosophila, and not all of them activate the Toll pathway, but some are involved in activating a different immune pathway known as autophagy. Autophagy in Drosophila plays an important role in development and is involved in hematopoiesis and cell death during morphogenesis at the larval developmental stages (58, 59). Furthermore, autophagy forms an essential part of the innate immune system, and acts as a protective mechanism against viral and bacterial infections (60). Autophagy is a catabolic process involved in degradation of cellular components. The process begins by the formation of the pre-autophagic structure that acts as a nucleation point for the formation of a double membrane called the autophagosome, which contains the cellular material destined for degradation (61). Following the engulfment of the cellular material by the double membrane, the autophagosome fuses with lysosomes where degradation of cellular materials occurs, and culminates in the death and removal of the cell (61). In Drosophila, the Toll-like receptor Toll-7 is required for the recognition and induction of autophagy following Vesicular stomatitis virus (VSV) infection. Silencing Toll-7 and Toll-2 during VSV infection in Drosophila S2 cells leads to an increase in the number of infected cells (62), suggesting the involvement of the pathway in antiviral protection. Inhibition of autophagy in Drosophila cells or adult flies has no effect on DCV replication (13), while it is yet to be determined whether this mechanisms is involved in controlling FHV infection. Removal of infected cells through autophagy likely inhibits viral replication due to the destruction of the host cell machinery components required for viral replication, however no direct evidence exist to confirm this hypothesis.

Apoptosis

Apoptosis is a regulated mode of cell death, and although is mainly known for its importance during development, it forms an important part of the innate immune response against viral infections (63-66). Relatively little research has focused on understanding the effects of apoptosis on viral infections in Drosophila. Apoptosis has been implicated in the protection against two DNA viruses (Autographa californica multiple nucleopolyhedrovirus (AcMNPV) and Culex nigripalpus virus (CuniNPV)) and one RNA virus (FHV) (further details given below) (65-67). Although to date apoptosis has been implicated in protection only against AcMNPV, CuniNPV and FHV viruses, studies performed in human cells, shrimps, mosquitoes and Lepidoptera have shown the importance of apoptosis to extend beyond insects (68-71).
Antiviral mechanisms conclusion

The study of antiviral responses in *Drosophila* is still relatively new and as we gain a better understanding of antiviral protection, we are likely to uncover new pathways responsible for protection against viral invasion. What is evident from studying antiviral responses in *Drosophila* is that in some cases multiple pathways are activated upon viral infection (Figure 1), and it is likely that antiviral pathways act in synergy to combat viral infection.

Due to the recent evidence showing the impact of the route of viral entry on immune system regulation (15), importance should be given to try to understand the effects of the route of pathogen entry on the ability of the host to mount an immune response. Since the vast majority of studies focused on antiviral responses in *Drosophila* have been performed by injection, a considerable gap in knowledge exists in how the innate immune response is regulated in naturally infected populations. In the following section I focus on the importance of apoptosis as an innate immune mechanism. I will summarize and discuss the molecular mechanisms governing apoptosis in *Drosophila* in order to identify gaps in knowledge and introduce the reasoning behind the decision to expand on the scientific knowledge necessary to better understand the importance of apoptosis in antiviral defence.

Apoptosis-mediated antiviral protection

Apoptosis is a highly specific and regulated mode of cell death and inappropriate regulation of apoptosis can lead to a variety of diseases including autoimmune diseases, cancers and neurodegenerative disorders, demonstrating that it is essential for the organism to exert a high degree of control over apoptosis to ensure proper function (72). During apoptosis, a cell undergoes a physical transformation involving cytoplasmic shrinkage, blebbing of the plasma membrane, chromatin condensation, DNA fragmentation and swelling of the outer mitochondrial membrane, and culminates in the packaging of the cell for removal by phagocytosis (73, 74).
**Apoptosis in Drosophila**

Apoptosis is essential for normal *Drosophila* development, it occurs as early as the 11th embryonic stage, and continues throughout embryogenesis (75). During pupation, apoptosis is essential for normal development of the eye, removal of the hindgut, larval muscles and salivary glands (76-78).

Different signalling pathways exist which are involved in the induction of apoptosis in *Drosophila*, however they all ultimately converge through the transcriptional regulation of *reaper* (*rpr*), *hid*, *grim* and *sickle* (*skl*) (RHG) genes (Figure 2) (79'). Although the RHG genes share similarities, *reaper*, *hid* and *grim* are not functionally identical (80). The *hid* gene is essential for apoptosis of the midgut cells and salivary glands during metamorphosis and apoptosis of polar cells during oogenesis (81, 82). *Grim* is essential for the formation of the central nervous system midline and development of the eye (83), while *rpr* is involved in mitochondrial fusion during apoptosis, and has been implicated in the fly’s immune response to viral infection (65, 84).

![Apoptosis pathway in Drosophila](image)

**Figure 2: Apoptosis pathway in Drosophila.** The core components of the apoptotic pathway in *Drosophila*. Viral infection can induce the transcription factor dP53, which requires the irradiation-responsive enhancer region (IRER) to induce expression of the RHG genes (*rpr, hid, grim* and *skl*). Induction of the RHG genes induces autoubiquination and removal of DIAP1. Autoubiquination of DIAP1 releases the brakes on caspases (Dronc, Drice and Dcp-1), which become activated and lead to the induction of cell death. (Adapted from (85)).
The induction of the RHG genes often occurs through the transcriptional factor dP53, and requires the function of the irradiation-responsive enhancer region (IRER) (Figure 2) (65, 86, 87). Transcriptional activation of the RHG genes results in autoubiquination of the inhibitors of apoptosis (IAPs), which serve as breaks on apoptosis (88, 89).

**Inhibitors of apoptosis**

The first IAP was discovered in a baculovirus, and since its discovery, IAPs have been found in all major organisms (90, 91). IAPs inhibit apoptosis through binding and inhibition of caspases, which are known as the effectors of apoptosis (92, 93). Binding to caspases occurs through the baculovirus IAP repeat domain (BIR), which is required for the interaction with the RHG motif present on the RHG proteins (93, 94). A RING domain present on the IAPs is required for the E3 ubiquitin ligase function and is essential for targeting caspases (95-98), RHG proteins (99, 100) and other IAPs as well as themselves (101, 102) to ubiquitination. Four IAPs are present in Drosophila, Diap1, Diap2, dBruce and Deterin (reviewed in 80). The most widely studied IAP in Drosophila is the inhibitor-of apoptosis protein 1 (DIAP1), which plays a central role in the regulation of apoptosis (88). DIAP1 is an E3 ubiquitin ligase that targets the degradation of caspases, a process necessary for preventing apoptosis from occurring spontaneously (103). Once the cell receives an apoptotic stimuli, an increase in transcription of the RHG genes leads to down-regulation of DIAP1, and activation of caspases.

**Caspases**

Cysteinyl aspartate proteinases, or more commonly known as caspases are a group of proteinases involved in apoptosis in several organisms (104). Drosophila has seven caspases that have been identified so far, DRICE, DRONC, DCP-1, DAMM, DECAY, DREDD and STRICA (for a review see 80). Caspases are synthesized in their inactive precursor form called zymogens, and protein cleavage is usually required for their activation (103, 105, 106). DRONC is known as a caspase activator, a primary initiator of caspase induced cell death, and is essential in regulating cell death during embryogenesis, larval development, metamorphosis and stress-induced apoptosis (85, 107). DRONC is kept in check by DIAP1, which can prevent DRONC processing and activation by ubiquitination (96). Following activation of DRONC, cleavage and activation of executioner caspase drICE, leading to cleavage of other substrates, ultimately culminating in cell death (89, 108, 109).
Apoptosis as a host defence mechanism

Induction of apoptosis during the early stages of pathogen infection can in some circumstances slow down pathogen replication and spread (65). To avoid this, viruses and other pathogens have evolved ways to manipulate apoptosis to their advantage. The evolutionary race between viruses and other organisms has often led viruses to evade the immune systems, with P53 often emerging as a target (65, 110, 111). For example, the human papillomavirus (HPV) protein E7 can block P53-induced apoptosis, leading to immortalization of infected cells, and successful viral infection (112). Similarly, the adenovirus protein E4-ORF3 inhibits P53 expression by promoting heterochromatin formation at the P53 promoter, which ensures that infected cells do not undergo apoptosis, therefore allowing the virus to use the host machinery to its own advantage (111).

In *Drosophila*, induction of apoptosis during the early stages of FHV and CuniNPV infection can limit viral replication (65, 66). Injecting either FHV or CuniNPV into adult flies leads to dP53 and rpr up-regulation. Higher levels of rpr lead to the depletion of DIAP1 and activation of caspases, ultimately leading to cell death (Figure 2) (65, 66). In loss-of-function fly mutants with the transcriptional factor dP53 deleted, which would be expected to inhibit apoptosis, FHV is found in higher abundance compared to WT flies (65). Knocking-down DRONC in the fat body using RNAi, which would also be expected to inhibit apoptosis, lead to increased FHV replication suggesting that induction of apoptosis during the early stages of infection aids in removal of the virus (65).

While the effects of apoptosis on FHV abundance following viral injection have been demonstrated, it is not yet clear whether induction of apoptosis has an impact on survival of the organism. Furthermore, the involvement of apoptosis in protection against oral FHV infection has not yet been determined, therefore it is not clear whether apoptosis-mediated protection is dependent on the route of virus infection.

*Wolbachia*

While host-derived defence mechanisms are important for mediating antiviral protection, symbiotic relationships between hosts and bacteria can be just as important. An example is the interaction between the endosymbiotic bacteria *Wolbachia pipientis* and its *Drosophila* host. *Wolbachia* is a maternally transmitted intracellular endosymbiotic alpha-proteobacteria (family *Rickettsiaceae*) found in approximately 40% of all arthropods and nematodes (113). It was first discovered in 1924
in the mosquito *Culex pipiens*, due to the incompatible crosses it caused (114). *Wolbachia* can cause sexual parasitism through cytoplasmic incompatibility, male killing and feminization of genetic males (115). Cytoplasmic incompatibility has perhaps been the most widely studied *Wolbachia* effect, and leads to non-viable offspring when *Wolbachia* infected males are mated with *Wolbachia* free females, while reciprocal crosses produce viable offspring (116, 117). These sexual parasitism effects lead to quick *Wolbachia* fixation in natural insect populations, leading to large geographical abundance of the bacterium.

*Wolbachia* can form three types of symbiotic relationships in insects: (1) obligate mutualism, in which both organisms require each other for survival. An example of this is the interaction between *Wolbachia* and the wasp *Asobara tabida*, where *Wolbachia* plays and essential role in host reproduction, as removing the bacterium using tetracycline inhibits oogenesis (118). (2) Parasitism, in which one organism benefits at the expense of the other. In both *Drosophila* and mosquitoes, *Wolbachia* can act as a parasitic symbiont due to its life shortening and reproductive parasitism effects it exerts on its host (115, 119, 120). (3) Facultative mutualism, where an organism provides protection against another invading organism. The focus in this thesis will be on *Wolbachia*’s role as a facultative mutualist, due to the protective role it plays in the tripartite *Drosophila-Wolbachia*-virus interaction.

*Wolbachia*-mediated antiviral protection in *Drosophila*

The protective effect of *Wolbachia* was first discovered in the *Drosophila* host, where it was shown to delay mortality induced by infection with several RNA viruses such as DCV, FHV and CrPV, but not against a DNA virus IIV-6 (14, 32). Further studies performed in *Drosophila* found *Wolbachia*-mediated protection not to be confined to the abovementioned viruses, but that protection extends to a diverse range of viruses such as bluetongue and dengue viruses (121, 122). Presence of *Wolbachia* in *Drosophila* injected with DCV or FHV is often associated with reduced viral accumulation and increased lifespan compared to *Wolbachia*-free flies (Figure 3) (14, 32), however that is not always the case, as not all *Drosophila-Wolbachia* associations are protective against virus infection. The *Wolbachia* strains wMelCS, wRi, wAu, wMel and wMelPop, confer protection against DCV-induced mortality following DCV injection into adult flies, while the *Wolbachia* strains wNo and DSH-wHa do not (14, 32, 123, 124). The protective *Drosophila-Wolbachia* associations can be further categorized depending on whether they confer protection against viral accumulation (resistance) or fly survival (tolerance). For example, the *Wolbachia*
strain wRi confers tolerance to DVC infection, however does not have a protective effect on viral accumulation (resistance) (123). Several other studies have shown that differences in resistance and tolerance exist in different Drosophila-Wolbachia associations (14, 125, 126), however it is yet to be determined whether the molecular mechanisms underpinning viral resistance and tolerance are the same.

**Figure 3: Wolbachia-mediated protection in Drosophila.** Schematic diagram representing Wolbachia-mediated protection in Drosophila following injection with DCV (A) or FHV (B). The blue lines represent the survival of flies with Wolbachia, while the purple lines represent Wolbachia-free flies. The x-axis represents time post injection, while the y-axis represents percentage of survival. This schematic diagram represents the protection outcome seen in many experiments.

**Wolbachia-mediated antiviral protection in mosquitoes**

Wolbachia-mediated protection is not confined to Drosophila, as it has been observed in Aedes aegypti and Aedes albopictus mosquitoes artificially infected with Wolbachia, where protection has been observed against important human pathogens such as dengue, chikungunya, West Nile virus and yellow fever virus (124, 127-139). In mosquitoes, Wolbachia protection is characterized by reduced viral accumulation and transmission, but generally does not affect host survival (140). Not all Wolbachia-mosquito associations lead to a protective phenotype, in fact Culex tarsalis mosquitoes artificially infected with wAlbA show higher West Nile virus infection rates compared to Wolbachia-free mosquitoes (141), suggesting that multiple mechanisms may be controlling viral resistance.
Wolbachia protection observed in artificially infected *Ae. aegypti* has provided a basis for utilizing this system in arboviral suppression studies in areas affected by medically important arboviruses (132). High rate of maternal transmission and strong cytoplasmic incompatibility facilitate the establishment of the bacterium within a population, making it a potentially viable option for controlling arboviral transmission. Since Wolbachia was first proposed as an efficient tool against arboviral transmission, several groups have begun programs targeted at releasing Wolbachia-infected mosquitoes in natural populations. The first of such releases occurred in 2011 in Queensland (Australia), where wMel infected *Ae. aegypti* mosquitoes successfully invaded two local *Ae. aegypti* populations (132). Since then, several releases have taken place in Australia, Indonesia, Vietnam, Brazil and most recently Colombia (www.elimatedengue.org).

**Mechanisms of Wolbachia protection**

The mechanism through which Wolbachia mediates resistance and/or tolerance is not yet fully understood, however some mechanisms have been suggested to be important. Evidence exists for the involvement of miRNAs (142, 143), competition for host-derived resources (144) and elevated reactive oxygen species (145, 146) in antiviral protection, however the exact molecular mechanisms through which this pathways act are not yet clearly understood. Immune stimulation in mosquitoes artificially transinfected with Wolbachia has been proposed as a mechanism of Wolbachia protection (147), however no immune stimulation is observed in *Drosophila* naturally infected with Wolbachia (124, 148, 149), casting doubt on the involvement of the immune system on Wolbachia-mediated protection. One thing that all protective Wolbachia strains share is high density within their respective organism, suggesting that high Wolbachia density may serve as a prerequisite for antiviral protection (123, 125-127, 131, 139, 150, 151). Gradually reducing Wolbachia density in both *Drosophila* adults and mosquito cell culture using tetracycline leads to a dose dependent loss of antiviral protection (131, 150), further supporting the importance of density. While the importance of Wolbachia density has been demonstrated in adult flies and mosquitoes, no study to date has determined the importance Wolbachia density in larvae or assessed the effect of Wolbachia on antiviral protection in larvae.

**Impact of Wolbachia-mediated protection on viral dynamics**

One of the most interesting aspects of Wolbachia-mediated protection is the effect Wolbachia has on natural viral dynamics. By interfering with viral replication, Wolbachia can decrease the
transmission of arboviruses such as dengue and chikungunya in its mosquito host (127, 129, 135, 137). Decreasing transmission of arboviruses would likely decrease the number of infected human hosts, which in turn would have a negative impact on circulation of arboviruses in natural populations. A recent study suggested that as much as a fifth of all arthropod species may benefit from Wolbachia-mediated protection (126), therefore the impact of Wolbachia on viral circulation in natural arthropod populations could be significant. The effect of Wolbachia on natural viral dynamics is hard to predict due to a range of factors influencing viral transmission. Modelling studies have identified factors such as reproduction rate, death rate, maturation rate and Wolbachia transmission rate as factors important in controlling dengue transmission dynamics (152-154). While a range of factors influencing viral transmission in the presence of Wolbachia have been identified, the effects of life-stage susceptibility on virus dynamics have not yet been considered.

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 (155-157). Differences in life-stage susceptibility could have an impact on the host’s ability to survive and transmit the infection, which could affect viral dynamics. Studies in insects such as Chrysodeixis chalcites, Lymantria dispar and Lepidoptera Noctuidae have shown that viral susceptibility can vary throughout the life-stages (158-160), raising the question whether Wolbachia protection in Drosophila and mosquitoes could be life-stage dependent. Studies concerned with Wolbachia-mediated protection in both flies and mosquitoes have to date been performed solely on adults without consideration of other developmental stages. Furthermore, comparison between Drosophila and mosquito studies could be confounded by the fact that different routes of infection were used in the studies. Studies performed on mosquitoes utilized ingestion of contaminated blood-meal as a viral delivery method, whereas studies in Drosophila utilized intra-thoracic injections. Viral injections bypass the fly’s natural immune barriers present within the midgut, and can cause a differential immune response compared to virus feeding alone (15). It is yet to be determined whether the molecular mechanisms behind Wolbachia protection are dependent on the route of infection and whether similar molecular mechanisms control protection in Drosophila and mosquitoes. Studies on orally infected Drosophila could facilitate comparisons between flies and mosquitoes, and could be used to further characterize Wolbachia protection mechanisms.
Aims of thesis

Antiviral defence is of great importance for many organisms, as the ability of an organism to mount an antiviral response can affect host survival and virus dispersal. Recent studies showed that the route of pathogen entry can influence immune regulation, which lead to a renewed interest in the effect of a natural route of infection on immune regulation. The aim of this thesis was to utilize the oral route of virus infection to study Drosophila’s antiviral responses in an effort of gaining a greater understanding on immune regulation in natural insect populations.

Aim 1 To develop a tool able to detect active Drosophila C virus replication and characterize the impact of the route of infection on the outcome of Drosophila C virus infection.

Aim 2 To determine Wolbachia-mediated protection at larval and adult developmental stages following the oral route of virus infection.

Aim 3 To study the effect of apoptosis on Flock House virus infection following the oral and systemic routes of infection.


Chapter 2: Infectivity of Drosophila C virus following oral delivery in *Drosophila* larvae
Abstract

The route of pathogen entry can have a major effect on the ability of the virus to induce a prolific infection, but it can also affect the ability of the host organism to induce an immune response to fight the infection. Transmission of arboviruses that cause serious diseases in humans often begin by an insect ingesting a virus, which then disseminates through the internal organs and tissues and ultimately culminates in viral transmission to a human host. Understanding the effect of a natural route of infection on the host-pathogen interaction may facilitate development of approaches to prevent viral dissemination. *Drosophila* has been a useful model organism for understanding host-virus interactions, however most studies have achieved infection by artificially injecting the virus into the host. Here we develop a single-stranded quantitative PCR able to detect only actively replicating Drosophila C virus (DCV) to study the effect of viral feeding at the early stages of larval development. Exposure of newly hatched larvae to DCV leads to 20% of larvae becoming infected within 12 hours post-contamination, and causes a 14% egg to adult mortality. Using the newly developed tools, the results show for the first time that DCV is able to establish a prolific infection following larval feeding, and suggests that larvae that become infected die before adult eclosion.
Introduction

Exposure of insects to pathogens can occur via different routes, which can have important impacts on the outcome of the infection. Arthropod-borne viruses are commonly transmitted horizontally through oral infection (1), while other insect viruses can be transmitted vertically and/or horizontally (reviewed in 2). Differential immune responses may exist depending on the route of pathogen entry (3, 4), however this is an area that has been largely unexplored. In Drosophila, different routes of viral infection can trigger differential physiological responses, and can influence the host’s adaptation to the pathogen (5).

Drosophila is a strong model for insect host-virus interactions and Drosophila C virus (DCV) is commonly utilised in studies of interactions and host defense mechanisms in Drosophila (6-11). DCV is a member of the Dicistroviridae family of viruses, and is the most well studied Drosophila virus (2, 12). DCV is a non-enveloped, single-stranded positive sense RNA virus with a genome comprising of 9264 nucleotides (13). During active viral replication DCV replicates its genome by synthesizing negative sense RNA, which is used as a template to produce more positive sense RNA (14). The negative strand RNA is not encapsidated in virus particles and can only be detected in cells with actively replicating virus, therefore making it a useful feature for studying viral infection.

While DCV is one of the best-characterized Drosophila viruses, the natural route of infection is poorly characterized (15). Most studies involving DCV have utilized injection, while the natural route of infection has largely been unexplored. One of the benefits of injecting DCV into its host is that the virus induces mortality within 4-6 days post-injection (8, 16), a system that has been widely used to study the innate immune response in Drosophila (17), and the effect of the bacterial endosymbiont Wolbachia on antiviral protection (18).

Injecting the virus bypasses the fly’s natural midgut protection barriers, and can initiate a differential immune response compared to viral feeding alone (4, 5). While systemic DCV infections provide a useful model, the natural route of infection is likely to occur through ingestion of virus-contaminated food (19). Depending on the route of infection and developmental stage, DCV may exhibit different tissue tropism. Following injection of DCV into adult flies the virus spreads to the trachea, fat body, somatic muscles, visceral muscles along the midgut, and the epithelial sheath surrounding the egg chamber (7, 20). In contrast, reports have suggested that
following ingestion of DCV from the first larval instar the virus is confined to the lumen of the digestive tract and the basal part of gut cells (21).

Here we show that larvae fed on DCV-contaminated food do not become persistently infected by the virus, but rather that flies which become infected likely succumb to the infection before adult eclosion. Furthermore we develop a single-stranded quantitative PCR (ssqPCR) able to differentiate between active viral replication and the presence of non-replicating virus, a feature which can be a useful to study DCV infection dynamics.

Materials and Methods

Virus and flies

Plaque purified DCV isolate EB (8, 22) was propagated and purified from Schneider’s Drosophila Line 2 cells (23) and virus titres were determined by 50% tissue culture infectious dose (TCID$_{50}$) as previously described (8, 24). Flies were reared on standard cornmeal media, at a constant temperature of 25°C with a 12-hour light/dark cycle. The rearing media was composed of 8.75 % cornmeal, 1.5 % yeast extract, 7.5 % agar, 0.3 % propionic acid and 0.3 % tegosept solution, while the rest was composed of water. The D. melanogaster line Oregon RC (ORC) was used in the experiments, which has been previously cured of Wolbachia (ORC-T) by tetracycline treatment (25).

RNA extraction, cDNA synthesis and qPCR

RNA was extracted from either larvae or adult flies. The insect samples were frozen and homogenized in Ribozol™ (Amresco) with two 3 mm glass beads using TissueLyser II (Qiagen) for 90 seconds at 30 Hz. The total RNA was precipitated from Ribozol and the samples were treated with DNase (Promega) at 30 minutes at 37°C. 1 μg of total RNA was reverse transcribed using either DCV-tag or rpL32-R primers (Table 2). The primers were used at a total concentration of 500 nM, and were incubated along with RNA at 65°C for 5 min before being set on ice for 1 minute. cDNA was synthesized using SuperScript III™ reverse transcriptase (Invitrogen) at 55°C for 60 min, and then heat inactivated at 95°C for 15 min. For quantitative PCR analysis Platinum SYBR® Green qPCR SuperMix-UDG (Invitrogen) was used as per manufacturer’s instruction using Tag / DCV-R or rpL32-F / rpL32-R primer pairs (Table 2). 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°C 10 seconds, 60°C 10 seconds and 72°C for 20 seconds. This was followed by a standard melt analysis to confirm that only the expected product had been amplified.

**Strand-specific assay**

To generate standard curves for ssqPCR, a 546 bp portion of the DCV\textsubscript{EB} genome (ORF1 from 3788 bp to 4333 bp) (13) was reverse transcribed using the DCV1-rv primer (Table 2) as above. Following reverse transcription, a PCR was performed using Taq polymerase (New England biolabs) and the DCV-F / DCV1-R primer pair (Table 2) was used under the following PCR conditions: 95°C 30 seconds followed by 35 cycles of 95°C 20 seconds, 58°C 30 seconds and 68°C for 40 seconds. The PCR reaction was run on a 1.5 % agarose gel, the DCV fragment was excised from the gel and the DNA was purified using QIAquick gel extraction kit (Qiagen). Subsequently the purified fragment was cloned in both directions into a pGEM\textsuperscript{®}-T \textit{easy} vector (Promega). The viral positive and negative RNA strands were transcribed using T7 RNA polymerase (Megascript) from SacI digested plasmid with the insert oriented in the sense and antisense directions respectively. The RNA transcripts were purified using Ribozol\textsuperscript{TM} (Amresco), and the samples were treated with DNAse as described above. The concentration of the transcripts was determined by spectrophotometry (Epoh, BioTek). The cloned DCV fragment has a molecular mass of 215,895 g mol\textsuperscript{-1}, and one microgram of RNA contains approximately 2.7 x 10\textsuperscript{12} strands of RNA.

**Larval bioassay**

Flies were anaesthetised with CO\textsubscript{2} prior to infection. \textit{Drosophila} flies were injected with 5000 DCV infectious units (IU) using a Nanoject II microinjector (Drummond Scientific) as previously described (8). Flies that died within the first 24 hours were considered to be dead due to the needle injury and removed from the vials. Flies were collected at 4 days post-injection and stored at -20°C until further use. Thirty individual flies were pooled and ground in 600 µl of PBS using two 3 mm glass beads in a TyssueLyser II (Quiagen) at 30 Hz for 60 seconds. The debris was removed by centrifugation at 14,000 RPM for 10 minutes, and the supernatant was filter sterilized to remove bacteria using a Millex GV 22 µm filter (Merck Millipore). Following filtration, the suspension was spread immediately onto bottles containing standard cornmeal media for larval feeding bioassays.
To determine whether flies feeding on DCV from the first larval instar become infected, adult 4-7 day old ORC flies were transferred to vials containing fresh Drosophila food and the following morning 100 eggs were collected, placed on wet sterile filter paper, and transferred into bottles containing extracts of either PBS or DCV injected flies. The hatched larvae were maintained on the treatment media until collection. Samples were collected at larval stages for ssqPCR analysis, or at 4 days post-adult eclosion for survival bioassay analysis.

Larval survival from embryo to adulthood was determined following the larval bioassay to determine if viral feeding at larval stages causes and increase in mortality. Larval mortality was calculated as a percentage of adults post-eclosion compared to the number of eggs before the treatment. Statistical analysis was performed using an unpaired two-tailed Student’s t-test assuming equal variance.

**Time-course of DCV infection**

100 eggs were added to each of four bottles containing DCV-contaminated media. 20 individual larvae were collected at 12, 24, 48 and 72 hours post-contamination, RNA was extracted and the ssqPCR was performed to determine if the larvae became infected.

**Survival bioassays**

Following larval feeding, adults were collected 4 days post-eclosion in order to determine if they became infectious. Five adult flies were pooled, homogenized, and fly extracts were prepared as above. For each treatment, 15 flies were injected with the extracts and daily mortality was scored. Mortality that occurred on the first day post-injection was considered to be due to needle injury. Three replicates of the larval feeding were performed for each group using independent cohorts of flies.

**Results**

**Egg to adult mortality**

To understand the dynamics of DCV infection in larvae, we investigated the effect of viral feeding on egg to adult mortality. Briefly, 300 eggs across 3 different cohorts (100 eggs each) were added to
either phosphate buffered saline (PBS) or DCV-contaminated media and mortality rates were
determined post-adult eclosion. A significantly higher egg to adult mortality (44 %) was observed
in the DCV treatment group compared to the control group (30 %, p < 0.001, Figure 1). The results
indicate exposure to virus as larvae leads to infection and an increase in mortality.

![Graph showing egg to adult mortality following viral challenge per os. Larvae challenged with DCV show a significantly higher mortality compared to the control PBS group. Statistical analysis using an unpaired two-tailed Student’s t-test assuming equal variance indicates that these groups are significantly different (p < 0.001).](image)

**Figure 1. Egg to adult mortality following viral challenge per os.** Larvae challenged with DCV show a significantly higher mortality compared to the control PBS group. Statistical analysis using an unpaired two-tailed Student’s t-test assuming equal variance indicates that these groups are significantly different (p < 0.001).

**Adult infectivity**

Since natural infections are likely to occur by ingesting contaminated food, we were interested to
know whether the surviving adults emerging from larvae fed on contaminated media were
infectious. In this experiment first instar larvae were exposed to PBS or DCV-contaminated media
until 4 days post-adult eclosion. These flies were defined as the F0 generation. Following
emergence flies were homogenised and extracts were injected into DCV free flies. Homogenates of
flies reared on PBS induced negligible mortality, with over 90% of the flies surviving 15 days post-
 injection (Figure 2a). In contrast, the homogenates of flies reared on DCV-contaminated media
induced 100% mortality within 7 days post-injection (Figure 2a). The data indicates that a high
level of DCV is associated with the F0 population following viral feeding.
Figure 2. Analysis of infection status of flies exposed to DCV as larvae. *Drosophila* were exposed to virus through all larval stages and collected after adult eclosion. Gray dashed lines represent flies exposed to PBS, while black lines represent flies exposed to DCV. All experiments have been performed in triplicates. (a) Homogenates of F0 generation flies exposed to DCV during larval stages are highly pathogenic when injected into DCV free flies. (b) Homogenates from the F1 generation flies from either PBS or DCV groups were not pathogenic when injected into DCV free flies.

To determine whether the virus associated with the F0 flies is passed on to the following generation (F1), the F1 fly extracts were injected into DCV free flies. The homogenates of neither PBS nor DCV groups caused any significant mortality in the injected flies (Figure 2b). The results suggest that the F1 generation carried little or no infectious virus, indicating that DCV was lost between the F0 and F1 generation. Possible interpretations of these results are that: the flies were infected with virus during the F0 generation but that the virus was lost by the time of the assay of the F1 generation; or that the F0 flies were associated with virus through the process of feeding, but that the virus had not infected the flies.

**Single-stranded qPCR assay**

To test whether the flies were infected during the F0 generation and cleared the infection by F1, or if the virus was merely associated with the flies without causing an infection in F0 flies, we developed a ssqPCR. The ssqPCR detects actively replicating virus, by being able to distinguish between positive and negative sense viral RNA. Detecting the negative sense RNA is indicative of actively replicating virus (26, 27). Presence of both the viral positive and negative sense RNA strands during standard qPCR can cause both RNA strands to be detected even if cDNA synthesis is
performed using a strand-specific primer (28). To ensure specific detection we designed a tagged primer which incorporates a tag sequence into cDNA during synthesis from the negative strand template (28-33). The tag sequence added during cDNA synthesis is composed of a 20-nucleotide long tag sequence that carries no sequence homology to either the virus or host organism. The presence of tagged cDNAs can be detected during qPCR, where a tag-specific forward primer, and virus-specific reverse primer are used to amplify the desired cDNA product. In absence of the tag sequence, the presence of the positive strand cDNA reduces the specificity of the ssqPCR (28).

To determine the specificity of the ssqPCR, we performed analysis on positive and negative RNA strands of DCV. When 4x10^6 copies of the negative sense RNA were reverse transcribed using the DCV-tag primer (Table 2) the ssqPCR showed amplification at the 20th cycle, however when 4x10^6 copies of the positive sense RNA were reverse transcribed using the same primer there was no observable amplification by ssqPCR (data not shown). These results indicate that the ssqPCR is able to discern between the positive and negative strands of viral RNA, making it a useful tool for studying DCV replication.

A standard curve was performed to determine the amplification efficiency and detection limits of the assay. The standard curve was generated using ssqPCR by 5-fold serially diluting 1.6 x 10^7 copies of negative sense RNA strand until the detection limit was reached. The standard curve generated by the assay has a slope of -3.446, R^2 of 0.9993 and amplification efficiency of 95.6% (Figure 3a). The lowest dilution the assay could detect reliably was 1 x 10^3 copies of negative sense RNA. The high R^2 and amplification efficiency values suggest that the assay has a high dynamic range of detection (between 1 x 10^3 - 1.6 x 10^7 copies of negative sense RNA) and that using this assay, the standard curve can be used to accurately calculate the absolute number of negative sense RNA strands present within a sample.
Figure 3. (a) Standard curves generated using qPCR on serially diluted negative sense RNA strands. The x-axis represents the Log$_{10}$ of the absolute number of negative RNA strands present before reverse transcription, while the y-axis shows the cycle threshold value (C$_T$), which represents the number of PCR cycles required for the fluorescent signal to cross a specified threshold value. (b) Absolute amount of negative sense RNA strands detected in a homogenized sample of 30 adult flies injected with 5000 IU of DCV compared to adult flies fed with DCV from the first larval instar.

DCV replication detection following viral feeding

Using the ssqPCR we analysed the DCV infection status of the F0 and F1 generation flies. Analysis of positive control flies injected with DCV detected high numbers of negative sense RNA (approx. 2.5 x 10$^5$) 4 days post-injection, in contrast, no amplification was detected in either the F0 or F1 generations (Figure 3b). The ssqPCR data suggests that following eclosion, larvae feeding on DCV-contaminated media do not become persistently infected by the virus.

DCV infection dynamics during larval stages

While there was an increase in egg to adult mortality, 56 % of flies did not succumb to the infection, posing the question whether the surviving larvae were getting infected and recovering, were tolerant to the infection or whether only a small percentage of flies were infected and dying. To address this question, we performed a ssqPCR on individual larvae feeding on DCV infected media for 12, 24, 48 or 72 hours post-larval hatching. At 12 hours post-larval feeding 20 % of larvae showed actively replicating virus, at 24 hours 10 % of larvae showed actively replicating virus, while at 48 and 72 hours our assay did not detect active viral replication (Table 1). These results show that larvae fed on DCV-contaminated media can become infected by the virus. DCV replication is detected only in the first 24 hours post-viral ingestion, suggesting that larvae are susceptible to the virus in the first 24 hours post-embryonic development, and that not all the larvae become infected following viral feeding. While no virus replication was detected past 24 hours post-ingestion, it is possible that viral replication was occurring but at levels below ssqPCR detection.
Discussion

While the mechanism of DCV infection in nature is not entirely understood, it has been reported that DCV does not transmit transovarially, but rather horizontally (19). It has been suggested that DCV can infect its host through direct contact with feces and cadavers (34). In this paper we set out to understand the impact of viral feeding at the early stages of development in *Drosophila*. Following viral feeding we observed an increase in egg to adult mortality, which was associated to the presence of virus. While an increase in mortality was noted, a large proportion of larvae survived to adulthood, posing the question whether the flies reaching adulthood were not infected, recovered from infection, or whether they were tolerant to the virus. DCV is known to be present in natural *Drosophila* populations (35, 36), therefore we hypothesized that flies which survived to adulthood would be infected. However, while homogenates of F0 generation were highly pathogenic (Figure 2a), the pathogenicity was lost in the following F1 generation (Figure 2b). This led to the question of whether the infectivity of the F0 generation was due to a viral infection present within the population, or whether the virus was simply associated with the flies due to direct contact through feeding, without yielding a productive infection. Using a ssqPCR, we failed to detect active viral replication in either F0 or F1 generations, indicating that the infectivity of the F0 population was likely due to virus associated with the flies through feeding rather than a systemic infection.

There are a number of possibilities that may explain why DCV induces mortality at the larval stages but does not persist in adults, these include: 1. not all larvae get exposed to the virus, therefore the flies that are exposed to the virus during the larval stages die before adult eclosion, while larvae that are not exposed to the virus survive to adulthood; 2. larvae are coming in contact with the virus, but a viral threshold exists beyond which larvae become infected and die, while below the threshold the larvae do not become infected or recover quickly from infection; 3. there are differences in susceptibility between larvae. While it is possible that not all larvae get exposed to the virus during feeding, we suggest that this scenario is unlikely, as the viral homogenate is evenly dispersed on the whole feeding surface, and insects are placed on the media at the egg stage, and would therefore have to avoid DCV in the media during feeding. Note that the percentage of egg to adult mortality (14 %) (Figure 1) is similar to the percentage of larvae found to be infected at 12 (20 %) and 24 hours (10 %) post-emergence (Table 1) which suggests that larvae that become infected within the first 24 hours die before adult eclosion. The remaining ~80 % of flies are either not becoming infected or quickly controlling the infection and recovering before 12 hours post-larval feeding, however there is no evidence to support either of these two alternatives. Our results
show that susceptibility occurs at the very early stages post-hatching and indicates that larvae that become infected die before adult emergence.

The reason behind low DCV infectivity through ingestion has not yet been elucidated, as most viral studies have not considered the natural route of infection. Most pathogen-host interactions in nature do not result in a prolific infection (37), which is likely due to the protective gut barrier present in the host organism. Epithelia in the gut function as both physical and chemical barriers to pathogen entry, and following ingestion the pathogen is required to cross the epithelial surface in order to induce a prolific infection (38-41). Expression of AMPs in the gut forms part of the systemic immune response, which alongside reactive oxygen species is used by the fly to fight off bacterial infections (reviewed in 38). The immune response in the gut is not confined to the defense against bacteria, but it can also form a protective barrier against viral infections (4, 42-44). Insects challenged orally by viruses induce or down-regulate the ERK and JAK-STAT pathways, which are required to limit viral infection (42, 43, 45). A recent study showed the importance of the Toll pathway on antiviral immunity following viral feeding in adult Drosophila (4). Adult flies feeding on the highest viral dose show up to 25% mortality. With no significant mortality occurring past 8 days post-infection, it would be interesting to determine whether the remaining 75% of flies become infected and are tolerant to the virus, or whether similarly to larval feeding, only a small percentage of flies become infected.

Differences clearly exist between the route of DCV entry and the effect of the virus on the host. Route of DCV entry effects the adaptation of the host to the virus (5), the induction of the hosts immune system (4), and the ability of the virus to induce a prolific infection. We showed using the ssqPCR that DCV can cause a prolific infection following larval ingestion, however a large number of uninfected larvae suggests that protection mechanisms exist within the host which are either able to prevent the infection from occurring or are able to clear the infection early in the infection cycle, a feature which is not observed following viral injection. Persistent viral infections in nature occur either by a different route of infection, or there are other parameters which we don’t yet understand influencing the establishment of a persistent DCV infection in Drosophila. The ssqPCR described here could be used in future research to study the dynamics of DCV infection, and to determine whether a similar infection dynamics is present in adult flies post-viral feeding. Using the larval model of infection, important immune regulation mechanism could be elucidated, which would be useful for understanding host-pathogen interactions in medically important arboviruses.
References


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Tables

Table 1. Detection of actively replicating DCV in larvae following feeding.

<table>
<thead>
<tr>
<th>Time post-infection</th>
<th>Total number of larvae</th>
<th>Number of larvae infected</th>
<th>% larvae infected</th>
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<td>20</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>24 h</td>
<td>20</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>48 h</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>72 h</td>
<td>20</td>
<td>0</td>
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Table 2. Sequence of primers used for reverse transcription (RT), quantitative PCR (qPCR) and cloning.

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<th>Purpose</th>
<th>Reference</th>
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</thead>
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<td>qPCR</td>
<td>This study</td>
</tr>
<tr>
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<td>This study</td>
</tr>
<tr>
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</tr>
<tr>
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<td>cloning</td>
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Chapter 3: *Wolbachia*-mediated antiviral protection in *Drosophila* larvae and adults following oral infection
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*[^11]^-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* *pipiensis*.

*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-*w*Au, DSR-*w*Ri, *w*1118-*w*Mel and the over-replicating and life-shortening *w*MelPop in *w*1118 show a delay in DCV induced mortality when DCV is injected into adult flies, while N7NO-*w*No and DSH-*w*Ha 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}-w^{Mel}, w^{1118}-w^{MelPop}, N7NO-w^No, DSR-w^Ri, Co-w^Au*), 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}-w^{MelPop*} 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 (TCID_{50}) 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’-AAACGCGGTTCATGAGGAGAAGG-3’ (producing a 141 bp PCR product) (49) and *Act5C* 5’-

GACGAAGAAGTTGCTGCTCTGGTT

G-3’ and 5’-TGAGGATACCACGCTTGCTCTGC-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 °C 10 seconds, 52 °C 10 seconds and 72 °C 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 °C 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 $4.4 \times 10^{10}$ and $2 \times 10^{11}$ 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 binominal 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% (Figure 1A, Kaplan-Meier analysis, p < 0.05). We investigated an additional three *Drosophila-Wolbachia* associations DSR-wRi, w1118-wMel, w1118-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 (Figure 1B, D, E, Kaplan-Meier analysis, p < 0.05). 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 (Figure 1C, Kaplan-Meier 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.

Figure 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.

**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 (Figure 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.
Figure 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.
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 w^{1118}-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 (Figure 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) (Figure 2E and Table 1). Unlike the other Drosophila-Wolbachia associations, wMelPop provided complete protection against DCV induced mortality (Figure 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 (Figure 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, $p < 0.05$, Figure 3A, B), while the Wolbachia strain wMelPop shows high densities at both larval and
adult stages (Figure 3A). *Wolbachia* density in the non-protective N7NO-wNo larvae remained at lower densities compared to both wRi and wAu at both developmental stages (Figure 3B), consistent with lack of protection.

**Figure 3.** The density of five 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.
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 (Figure 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 or 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 *w*^{1118}-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 w$^{1118}$-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.
References


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 (-\(w_{ol}\)).
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Chapter 4: The involvement of apoptosis in Flock House virus infection following the oral route of infection
Abstract

Innate immunity plays a crucial role in protection against pathogen invasion in arthropods as they lack adaptive immunity. Apoptosis, a form of programmed cell death, has long been recognized for its importance in tissue reorganization and development, and has recently been accepted as a component contributing to innate immunity. Early induction of apoptosis in *Drosophila* leads to a decrease in accumulation of Flock House virus (FHV) following haemocoelic injection. Because the route of pathogen infection can lead to differential immune regulation and outcome of infection, we investigated whether the antiviral impact of apoptosis is route-of-infection-dependent. Here we show that apoptosis leads to different effects on viral accumulation (resistance) and host survival (tolerance) depending on whether apoptosis is experimentally inhibited or enhanced. We analyzed the response to virus infection of fly mutants with the transcriptional factor dP53 deleted, which would be expected to inhibit apoptosis, and gain-of-function mutants that over-express the pro-apoptotic gene *reaper*, which would be expected to enhance apoptosis. Enhancing or inhibiting apoptosis had no effect on the number of flies that succumb to oral FHV infection (30% in all cases). Inhibition of apoptosis had no effect on FHV accumulation following the oral route of infection, however mortality occurred earlier in dP53 mutants compared to wild type. Conversely, increasing the expression of the pro-apoptotic gene *reaper* in over-expression mutants led to a reduction in FHV accumulation, however did not affect host survival following the oral route of FHV infection. Taken together we propose that apoptosis is likely not involved in the control of primary FHV infection (viral entry and establishment), and that the antiviral effects of apoptosis is tissue-specific.
**Introduction**

Viruses carry a heavy burden on the infected host, in part because they use the host’s intracellular components for their replication. During evolution, organisms have evolved ways of evading pathogen infection through the establishment of the immune system. While mammals have evolved two types of immunity, the innate and adaptive, insects like *Drosophila* lack the adaptive immune system and use the innate immune system as a mechanism of controlling viral infection (reviewed in 1).

Apoptosis has long been suggested to be part of the innate immune system in *Drosophila*, however this is a concept that is only recently becoming accepted. Apoptosis is a controlled mode of cell death, during which cells undergo morphological changes that culminate in cell death and removal (2, 3). Early induction of apoptosis in several organisms can limit viral accumulation through the destruction of the hosts cell machinery necessary for viral replication (4-6).

In *Drosophila*, apoptosis is suppressed by a family of functionally and structurally related proteins called inhibitors of apoptosis (IAPs), with DIAP1 serving as the principal IAP (7). DIAP1 acts as an inhibitor of caspases, that is responsible for a deliberate degradation of the cell (8-10). Following apoptotic stimuli, induction of apoptosis occurs through the transcriptional activation of four genes: *reaper (rpr)*, *hid*, *grim* and *sickle* (RHG genes), which play an essential role in developmentally regulated cell death (11), and are involved in protection against viral invasion (12). Induction of RHG genes often occurs through the activation of the transcriptional factor dP53 (13). Transcriptional activation of the RHG genes leads to a depletion of DIAP1 and activation of the most apical caspase Dronc (14). Once activated, Dronc cleaves and activates executioner caspases, leading to cell death (8-10) (Figure 1).

**Figure 1. Regulation of apoptosis in *Drosophila***. The transcriptional factor dP53 activates the expression of *reaper, hid, grim* and *sickle* genes, which function to promote DIAP1 degradation. Degradation of DIAP1 releases the brakes on apoptosis leading to activation of caspases resulting in cell death.
Until recently, the effect of apoptosis as an antiviral mechanism in *Drosophila* was under question, as infection of cultured *Drosophila* cells with the baculovirus Autographa california multicapsid nucleopolyhedrovirus (AcMNPV) or Flock House virus (FHV) lead to the induction of apoptosis relatively late in the infection cycle (24 h), and blocking apoptosis had no effect on FHV accumulation (15, 16). Unlike cell culture studies, injecting adult *Drosophila* flies with either FHV or Culex nigripalpus Nucleopolyhedrovirus (CuniNPV) leads to a quick induction of apoptosis through the up-regulation of the pro-apoptotic *rpr* and *hid* genes at 1 hour post-injection. The up-regulation of *rpr* and *hid* is followed by the induction of caspases and apoptosis within 2.5 hours post infection (12). Expression of *rpr* and *hid* require the function of dP53 and the irradiation-responsive enhancer region (IRER), as dP53 and IRER loss-of-function mutant flies lose the ability to induce apoptosis and show higher FHV and CuniNPV titers. Suppressing apoptosis by knocking-down the apical caspase Dronc specifically in the fat body results in an increase in AcMNPV and FHV viral titers, suggesting the importance of apoptosis as an antiviral pathway in *Drosophila* (12).

While studies performed in adult flies have shown that systemic viral infections can induce the apoptotic pathway, it is still to be determined whether apoptosis is involved in antiviral protection following the oral route of infection. The importance of the route of pathogen entry on the outcome of infection has been well documented following bacterial infections in *Drosophila*. Injecting bacteria into the hemocoel of *Drosophila* induces a systemic immune response (17-20), while oral infections often lead to a localized immune induction in the gut often limiting the pathogenic effects of the bacterial infection (21-23). There is some evidence that route of infection also leads to differential immune regulation following viral infections, as infections with Drosophila C virus (DCV), FHV, Cricket paralysis virus (CrPV) and Nora virus lead to the induction of the Toll pathway following oral infection but not following a systemic infection (24). Furthermore knocking-down genes essential in Toll pathway regulation has no effect on fly survival or viral accumulation following systemic infection with DCV, FHV, CrPV and Nora viruses, while oral infection with the same viruses results in an increase in the number of infected flies and an increase in viral accumulation (24). Because *Drosophila* is thought to acquire most viruses through ingestion of contaminated media (25), understanding the role of immune pathway regulation in natural *Drosophila* populations will require a better understanding of immune regulation following oral viral infections.

To investigate whether the involvement of apoptosis in antiviral protection is dependent on the route of infection, we analyzed the effect of apoptosis on FHV accumulation and host survival following systemic and oral infection.
Materials and Methods

Virus

Plaque purified FHV (26, 27) was propagated and purified from Schneider’s Drosophila Line 2 cells (28). The virus used for injection experiments was sucrose gradient purified as previously described (26), while the virus used for oral infection underwent only the sucrose cushion purification step.

Fly lines

Flies were reared on standard cornmeal media, at a constant temperature of 25°C with a 12-hour light/dark cycle unless otherwise stated. Drosophila w1118 strain was used as a standard wild type strain (29). The P53 -/- fly line (P53[5A-1-4]), which is homozygous for a 3.3k deletion in the P53 gene was obtained from Bloomington (Indiana University, Bloomington, IN, USA; Stock no. 6815). Over-expression of the rpr and Dronc genes was achieved using the Gal4/UAS system. The driver line (wt+ P[w[+mC]=GAL4-Hsp70.PB}) contains a Gal4 gene under the control of a heat-sensitive Hsp70 promoter and was obtained from Bloomington (Stock no. 1799). Induction of rpr and Dronc was achieved using the responder lines (CyO{UAS-rpr.C}3) and w*;P{UAS-Dronc.FLAG}2. The UAS-Dronc responder line was kindly provided by Dr. Loretta Dorstyn from the University of South Australia, while the UAS-rpr line was provided by Dr. Gary Hime at the University of Melbourne. Crossing the Gal4 driver line with UAS responder lines leads to heterozygous F1 progeny containing both Gal4 and UAS. Transferring Gal4/UAS flies to 30°C leads to the activation of the Gal4 transcriptional factor that contains a DNA-binding domain capable of binding to upstream activation sequence (UAS) allowing transcription of the target gene to occur. Rearing the flies at 18°C suppresses the activation of Gal4, therefore suppressing the transcription of the target gene. Following a cross between the driver and responder lines, the flies were reared at 18°C, and 2-5 day old male Gal4/UAS flies were heat-shocked at 30°C for 2 days and subsequently transferred to 25°C for the remainder of the experiment. All flies used in the experiments were Wolbachia-free.
Western blotting

Western blotting was performed based on a previously described protocol with slight modifications (30). Briefly, 15 flies were ground in PBS and the homogenates electrophoresed through a 10% SDS-PAGE gel. Semi-dry transfer (Biorad) was used to transfer the proteins to polyvinylidene difluoride membranes for 60 minutes at 20V. The membrane was blocked for 1 h (5 % skim milk in PBS containing 0.05 % Tween 20), and incubated with the anti-Dronc antibody at a dilution of 1:500 for 3 h at room temperature, followed by the anti-rabbit (Abcam) antibody for 1 h. The signal was detected by developing the blot with NBT/BCIP substrates (Life technologies) for 10 minutes at room temperature. This was followed by probing the membrane with the AP-conjugated anti-Tubulin (Abcam) antibody at a dilution of 1:100 for 1 h followed by the anti-mouse (Abcam) antibody for 1 h. The blot was developed with NBT/BCIP for 10 minutes.

The primary polyclonal anti-Tubulin antibody and the secondary anti-mouse and anti-rabbit antibodies were ordered from Abcam (USA), while the polyclonal anti-Dronc antibody was a kind gift from Dr. Loretta Dorstyn from the University of South Australia.

RNA extraction, cDNA synthesis and qPCR

Insect samples were frozen and homogenized in Ribozol™ (Amresco) with two 3 mm glass beads using TissueLyser II (Qiagen) for 90 seconds at 30 Hz. The total RNA was precipitated from Ribozol as per manufacturers instructions and the samples were treated with DNase (Promega) for 30 minutes at 37°C. One microgram of total RNA was reverse transcribed using either FHV-R or rpL32-R primers (Table 1). The primers were used at a final concentration of 100 nM, and annealed to RNA by at heating at 65°C for 5 min before being set on ice for 1 minute. cDNA was synthesized using SuperScript III® reverse transcriptase (Invitrogen) at 55°C for 60 min, and then heat inactivated at 95°C for 15 min. For quantitative PCR analysis Platinum SYBR® Green qPCR SuperMix-UDG (Invitrogen) was used as per manufacturer’s instruction using FHV-F/ FHV-R, Reaper-F / Reaper-R or rpL32-F / rpL32-R primer pairs (Table 1). 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°C 10 seconds, 60°C 10 seconds and 72°C for 20 seconds. This was followed by a standard melt analysis to confirm that only the expected product had been amplified. Two technical replicates were performed for each sample (with a third done where necessary). RT-
qPCR data were analysed using qGENE software (31), and statistical analysis included a two-tailed Student’s t-test to compare differences of the means.

**Oral infection**

Since natural infections are likely to occur by ingesting contaminated food, adult flies were infected by feeding on contaminated media. Oral infection was performed in a plastic vial containing 1.5 x 1.5 cm of sterile filter paper contaminated with 200 µl of a mix containing 75 % of virus extract + PBS and 25 % of dried yeast, at a final virus concentration of $10^{11}$ IU/ml. For the control flies, the virus extract was replaced by PBS. Each vial contained 15 flies exposed to virus for a period of 24 hours. Following the infection period, the flies were set on standard cornmeal media and survival was measured daily. The day on which the flies were set to feed on the viral extract was considered to be day 0. Each experiment was performed 3 times using independent cohorts of flies.

**Injection bioassay**

*Drosophila* were infected with FHV or mock infected with PBS through microinjection into the upper lateral part of the abdomen. Ten anaesthetized 4-7 day old male flies were injected with 250 infectious units (IU) of FHV and maintained on a constant temperature of 25°C and daily mortality was scored. Each experiment was replicated 3 times using independent cohorts of flies. Survival curves were compared using Cox Regression analysis (GraphPad Prism).

**Statistical analysis**

Relative abundance of genes was calculated in qGENE and statistical analysis included the Mann-Whitney t-test to compare differences of the means. Survival curves were compared using Cox Regression analysis (GraphPad Prism). To evaluate whether time-to-death of orally infected flies differed between treatments, Cox Regression analysis was computed on the proportion of flies that succumbed to the infection.
Results

Confirmation of fly mutants

To investigate the role of apoptosis in protection against FHV infection following viral ingestion, we used mutant *Drosophila* flies. Flies containing a deletion in dP53 were confirmed to be homozygous for the deletion using PCR across the deleted region (data not shown). To generate flies with altered levels of apoptosis in additional apoptosis genes, we used the inducible Gal4/UAS system. The F1 progeny containing both Gal4 and UAS-rpr was used to study the effects of rpr over-expression. Because the rpr gene is transcriptionally regulated in *Drosophila* (32-34), RT-qPCR analysis was performed to confirm that rpr induction was successful. Expression analysis of the Gal4-Hsp70/UAS-rpr flies (referred from now on simply as rpr-UP) showed a 4-fold increase in rpr expression at 48 h post heat-shock compared to the same fly line kept at 18°C (Figure 2, t-test, \( p < 0.05 \)), while the WT flies showed no difference in gene regulation at either temperatures (Figure 2, t-test, \( p > 0.05 \)).

![Figure 2. Abundance of reaper mRNA in WT and mutant flies.](image-url)

Relative abundance of the rpr gene as measured by RT-qPCR in wild type \( w^{1118} \) and rpr-UP flies at 18°C and 30°C. Relative expression of rpr was calculated using the host Rpl32 mRNA as a reference. Each bar represents three biological replicates of five individual flies along with the mean ± SD. * represents statistical significant difference in rpr regulation in mutant flies reared at 30°C compared to the same fly line reared at 18°C (\( p < 0.0001 \)).
To induce expression of the caspase Dronc, the Gal4-Hsp70 driver line was crossed to the UAS-Dronc responder line (referred from now on simply as Dronc-UP). Because Dronc is regulated post-transcriptionally (35), Western blot analysis was performed to measure relative protein expression. Western blot analysis was performed on pools of 15 WT and mutant flies before and after heat shock. In WT flies the Drone protein was not detected at either 18°C or 30°C, while Dronc-UP flies showed low protein abundance at 18°C, and an increase in abundance at 30°C (Figure 3), suggesting that the protein was successfully induced.

![Western blot analysis](image)

**Figure 3. Western blot analysis of Dronc protein expression.** Analysis of WT and Dronc-UP mutant flies before heat shock (18°C) and 48 h after heat shock (30°C). The blot shows the abundance of the target protein Dronc (55kDa) and reference protein Tubulin (50 kDa).

**Systemic FHV infection**

To determine whether the effects of apoptosis are dependent on the route of virus infection, we first investigated how knocking out the pro-apoptotic gene dP53 and increasing the expression of the pro-apoptotic genes rpr and Dronc would affect FHV accumulation and host survival following systemic FHV infection. Compared to WT flies, P53 -/- flies injected with FHV had an approximately 7-fold increase in viral accumulation at 24 h post-injection (Figure 4A). Survival bioassays of systemically FHV-infected flies showed that FHV injection caused 100 % mortality of WT flies by day 6, while injecting the same amount of FHV into P53 -/- flies lead to a 100 % mortality by day 5 post-injection (Figure 4B, p < 0.05, Table 2).
Figure 4. Impact of apoptosis on FHV infection by injection. (A, C, E) Relative FHV abundance in adult flies 24 h post FHV injection. Black dots represent FHV abundance in WT flies, while red squares represent FHV abundance in mutant flies. The abundance of FHV RNA1 was calculated using the host Rpl32 mRNA as a normaliser. Each data point represents a single fly, and the graph is fitted with a mean ± SD. Unpaired t-test was used to compare the differences of the means and showed that in all three cases the difference in FHV abundance between WT and mutant flies is significantly different (p < 0.01). (B, D, F) Analysis of mortality of flies injected with FHV. Grey dashed lines represent mock-injected flies (PBS), black lines represent FHV-injected WT flies, while red lines represent FHV-injected mutant flies. Survival of flies is shown from 3 replicates of
10 flies or one replicate of 10 flies for PBS controls. Cox Regression analysis was performed to
determine whether differences in mortality between WT and mutant flies were significant. The data
is representative of 3 biological replicates and the results are shown as means ± SD.

Injecting rpr-UP flies with FHV led to an approximately 14-fold decrease in FHV
accumulation at 24 h post-injection compared to WT flies (Figure 4C, t-test, \( p < 0.05 \)). Survival
bioassay analysis of systemically infected rpr-UP flies showed that the flies succumbed to the
infection significantly later compared to WT flies; FHV injection caused 100 % mortality of WT
flies by day 7, while injecting the same amount of FHV into rpr-UP flies lead to a 100 % mortality
by day 15 post-injection (Figure 4D, \( p < 0.05 \), Table 2).

Injecting Dronc-UP flies with FHV lead to an approximately 11-fold decrease in FHV
accumulation at 24 h post-injection compared to WT flies (Figure 4E, t-test, \( p < 0.05 \)). Scoring
daily mortality of systemically infected Dronc-UP flies showed that the flies succumbed to the
infection significantly later compared to WT flies, as FHV injection caused 100 % mortality of WT
flies by day 7, while injecting the same amount of FHV into Dronc-UP flies lead to a 100 %
mortality by day 13 post-injection (Figure 4F, \( p < 0.05 \), Table 2).

**Oral FHV infection**

To compare the effects of apoptosis between systemically and orally infected flies, we orally
infected P53 −/−, rpr-UP and Dronc-UP flies with FHV and measured the effect it has on FHV
accumulation and host survival following the oral route of FHV infection. Oral infection of P53 −/−
flies lead to no difference in FHV accumulation at 2 or 5 days post-feeding compared to WT flies
(Figure 5A, C). Interestingly, while no difference in viral accumulation was observed, P53 −/− flies
succumbed to the infection significantly earlier compared to WT flies (Figure 5E, \( p < 0.05 \), Table
2). A dichotomy in FHV abundance data was observed where at 5 days post-feeding FHV is either
found at very low or very high abundance. FHV abundance at 5 days post-feeding (Figure 5A)
shows that 70 % of WT and P53 −/− flies contain low levels of FHV while 30 % show at least a 300-
fold higher abundance of virus. Comparison between 2 and 5 days post-feeding (Figure 5A, C)
shows that in 70% of flies, FHV abundance did not change between 2 and 5 days post-feeding,
implying that no FHV replication had occurred in 70% of flies between 2 and 5 days post feeding.
This conclusion is consistent with the survival data, which shows that only 30 % of flies succumbed
to the infection (Figure 5E). Furthermore, no difference in the number of flies succumbing to oral FHV infection was observed between P53 -/- and WT flies.

Figure 5. Impact of apoptosis on FHV infection by oral feeding. (A, B) Relative FHV abundance in WT and P53 -/- and rpr-UP flies respectively at 2 days post FHV oral feeding. (C, D) Relative FHV abundance in WT and P53 -/- and rpr-UP flies respectively at 5 days post FHV oral feeding. Black dots represent FHV abundance in WT flies, while red squares represent FHV abundance in mutant flies. Relative abundance of FHV was calculated using the host Rpl32 mRNA as a normalizer. Each data point represents an individual fly, and the graph is fitted with a mean ± SD. (E, F) Analysis of mortality of P53 -/- and rpr-UP flies respectively orally infected with FHV. Grey dashed lines represent mock-infected flies (PBS), black lines represent wild-type FHV fed flies, while red lines represent mutant FHV fed flies. Survival of flies is shown from 3 replicates of
10 flies or one replicate of 10 flies for PBS controls. Cox Regression analysis was performed to determine whether differences in mortality between WT and mutant flies were significant. Results are shown as means ± SD.

Orally infecting rpr-UP flies lead to no difference in FHV accumulation at 2 days post oral feeding compared to WT flies (Figure 5B, t-test, p > 0.05), however at 5 days it showed significantly lower FHV abundance compared to WT flies (Figure 5D, t-test, p > 0.05). Interestingly, while a difference in viral accumulation was observed at 5 days post oral feeding, no difference in mortality was observed between rpr-UP and WT flies (Figure 5F, p > 0.05, Table 2). No difference in the total number of flies succumbing to oral FHV infection was observed between rpr-UP and WT flies.

**Discussion**

**P53-mediated protection**

While apoptosis has recently been shown to control virus abundance following systemic FHV infection, the impact of apoptosis on virus-induced mortality and the involvement of apoptosis in antiviral protection following the oral route of infection had not been documented. To compare the effects of apoptosis in controlling virus-induced death following systemic and oral infections, we first determined the importance of the pro-apoptotic gene dP53 in protecting flies from virus-induced mortality and interfering with virus accumulation. Interestingly, the effect of knocking-out dP53 on virus accumulation was route-of-infection dependent, as systemic infection of P53 -/- flies led to an increase in FHV accumulation (Figure 4A), while no such effect was observed in orally infected flies (Figure 5A, C). The observed increase in FHV accumulation following systemic infection of P53 -/- flies is consistent with a previous report (12). While the effect of dP53 on control of pathogen accumulation (resistance) was dependent on the route of infection, the ability of the host to endure infection (tolerance) was not route-of-infection-dependent, as host’s tolerance to virus infection was negatively affected in P53 -/- mutants regardless of the route of virus infection (Figure 4B, 5E). These results suggest that inhibition of apoptosis through dP53-mediated knock-out can affect viral resistance in a route-of-infection specific manner.

**Reaper-mediated protection**

Due to the differences between the two routes of infection following inhibition of apoptosis, we investigated the effect of increasing the expression of the pro-apoptotic gene *rpr* on viral resistance
and tolerance. Up-regulation of rpr in rpr-UP flies showed a similar effect on viral resistance regardless of the route of infection, as in both cases up-regulation of rpr lead to a suppression of FHV accumulation (Figure 4C, 5D). While the effect of rpr on virus accumulation was comparable between the two routes of infection, the effect of rpr up-regulation on virus-induced mortality was route-of-infection dependent. In systemically infected rpr-UP flies a decrease in FHV accumulation was associated with a delay in mortality (Figure 4C, D), while a decrease in FHV accumulation in orally infected flies did not show the same effect, as both the WT and rpr-UP flies showed similar rates of mortality (Figure 5D, F). These results indicate that following the oral route of infection enhancement of apoptosis has an effect on viral resistance, but not tolerance. Because inhibiting or enhancing apoptosis lead to variable effects on resistance and tolerance, it would be interesting to confirm these results by orally infecting Dronc-UP flies with FHV and determine the effect on resistance and tolerance. Unfortunately, Dronc over-expression consistently lead to 70 – 80 % mortality within 24 hours post-feeding regardless of whether the flies were fed on FHV or mock-infected media (Appendix 1).

The results show that the effect of apoptosis in systemically infected flies is consistent with the role of apoptosis as an antiviral mechanism, as induction of apoptosis leads to increased resistance and tolerance following FHV infection (Figure 4C-F), while inhibition of apoptosis leads to a decrease in resistance and tolerance (Figure 4A, B). The same is however not true for oral FHV infections, as inducing/inhibiting apoptosis leads to different effects on resistance/tolerance depending on the route of infection. A distinction is often not made between viral resistance and tolerance, as inhibiting viral replication is often associated with increased survival, however that is not always the case. An example of this is the antiviral protection conferred by the endosymbiotic bacteria Wolbachia in its Drosophila host. In most cases viral tolerance associated with Wolbachia-mediated protection is correlated with a decrease in viral accumulation (resistance), however in some cases Wolbachia can affect tolerance without having an effect on resistance (36).

Here we hypothesize that the differences in resistance and tolerance observed in this study are due to tissue-specific differences in sensitivity to apoptosis. Some support for this hypothesis comes from a comparison between the outcome of infection in Drosophila cell lines and adult flies. In Drosophila cell line DL-1 apoptosis occurs relatively late following FHV infection (36 hours post-infection) and blocking apoptosis has no effect on FHV infection or accumulation (15). Contrary to this observation, FHV injection in adult flies leads to a quick induction in apoptosis (2.5 hours post-injection) and inhibiting apoptosis leads to an increase in viral titer (12). Adding to this, in the current study we show that inhibition/induction of apoptosis in adult flies is dependent on the
route of FHV infection. The apparent differences between the cell lines and adult flies suggest that apoptosis regulation may be tissue-specific, leading to differences in tissue sensitivity to virus infection. Because differences in tissue sensitivity may exist, host virus localization may be dependent on the route of pathogen entry, as the initial site of infection could affect viral competence and the outcome of infection. A study performed in the fall armyworm (*Spodoptera frugiperda*) larvae showed that AcMNPV lacking the apoptosis-inhibiting p35 gene and the neighbouring gene p94 leads to a route-of-infection-specific response to viral accumulation, leading to the hypothesis that the differences in infectivity are due to a tissue-specific response to p35 and/or p94 (37).

One finding that is of particular interest is that inhibiting or enhancing apoptosis has no effect on the number of flies becoming infected with the virus and succumbing to the infection, as both mutant and WT flies showed 30% mortality following oral feeding (Figure 5E, F). Comparing these findings with a recent paper showing that adult mortality following the oral route of FHV infection increased from ~20% in WT flies to ~60% in flies in which the Toll immune pathway has been compromised (24), it is clear that the Toll pathway is involved in determining whether or not a fly will become infected. In contrast, no such effect was observed in dP53 or rpr mutant flies. One interpretation of this is that apoptosis is not involved in the primary FHV infection, but that it may function as an antiviral pathway during secondary infection, however further experiments will be required to test this hypothesis. An alternative explanation is that both dP53 and rpr are not responsible for the activation of apoptosis in the gut, as regulation of apoptosis may be tissue-specific. Understanding FHV progression following the oral route of infection would be invaluable in identifying the tissues that are important in primary FHV infection, however no such studies have been performed to date. Following systemic FHV infection, FHV has been observed in the cardiomyocytes (38), fat body and salivary glands (12), however FHV localization may not be confined to the above-mentioned tissues.

Understanding the observed differences between resistance and tolerance will require elucidation of the mechanisms important in regulating viral resistance and tolerance. Currently, the molecular mechanisms underpinning viral tolerance in *Drosophila* are largely unexplored and poorly characterized. One indication of the mechanism underpinning tolerance during FHV infection comes from a study in *Drosophila* showing that injecting flies possessing mutations in the ATP-sensitive potassium channels with FHV leads to an increase in FHV viral load, increased tissue damage in the heart and earlier mortality compared to WT flies (38). While there is an indication that flies are succumbing to the infection due to the cytopathological effects of FHV in
the cardiomyocytes in the heart, it is not yet clear whether the heart is the only tissue involved in tolerance to virus infection.

Based on current knowledge we suggest that the differences in apoptosis-mediated protection may be due to tissue-specific differences in sensitivity to apoptosis. We furthermore suggest that tissue specific responses to dP53 and rpr likely lead to route-of-infection-dependent differences in FHV localization, leading to the observed differences in viral resistance and tolerance. The mechanisms controlling virus-induced mortality and virus accumulation may be controlled independently from each other through different pathways, and remain to be determined. Future research may be directed at understanding apoptosis-mediated control of virus replication and accumulation, as well as the impact of virus infection on mortality.
References


Tables

Table 1. Primers used in RT-qPCR analysis.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Nucleotide sequence (5’→3’)</th>
<th>Purpose</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaper-F</td>
<td>ACGGGGAAAAACCAATAGTCC</td>
<td>qPCR</td>
<td>(12)</td>
</tr>
<tr>
<td>Reaper-R</td>
<td>TGGCTCTGTGTCCTTGACTG</td>
<td>qPCR</td>
<td>(12)</td>
</tr>
<tr>
<td>FHV-F</td>
<td>CCAGATCACCCTGAAGCTGAAT</td>
<td>qPCR</td>
<td>(12)</td>
</tr>
<tr>
<td>FHV-R</td>
<td>AGGCTGTCAAGCGGATAGA</td>
<td>qPCR</td>
<td>(12)</td>
</tr>
<tr>
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<td>qPCR</td>
<td>(26)</td>
</tr>
<tr>
<td>rpl32-R</td>
<td>AAACGCGTTCTGATGAG</td>
<td>qPCR</td>
<td>(26)</td>
</tr>
</tbody>
</table>

Table 2. Cox-hazard ratio (log-rank) of different mutant *Drosophila* strains orally infected with Flock House virus (FHV) as compared to orally challenged WT flies.

<table>
<thead>
<tr>
<th>Drosophila strain</th>
<th>Infection route</th>
<th>Hazard ratio</th>
<th>95 % C.I.</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>P53 -/-</td>
<td>Injection</td>
<td>2.355</td>
<td>8.408 to 25.42</td>
<td>&lt; 0.0001****</td>
</tr>
<tr>
<td>rpr-UP</td>
<td>Injection</td>
<td>0.2460</td>
<td>0.01316 to 0.06018</td>
<td>&lt; 0.0001****</td>
</tr>
<tr>
<td>Dronc-UP</td>
<td>Injection</td>
<td>0.2588</td>
<td>0.01376 to 0.06120</td>
<td>&lt; 0.0001****</td>
</tr>
<tr>
<td>P53 -/-</td>
<td>Oral</td>
<td>2.549</td>
<td>2.132 to 33.54</td>
<td>0.0309*</td>
</tr>
<tr>
<td>rpr-UP</td>
<td>Oral</td>
<td>1.183</td>
<td>0.3910 to 3.413</td>
<td>0.686</td>
</tr>
</tbody>
</table>
Chapter 5: General Discussion
In nature viral transmissions occur through a variety of routes including aerosol, insects or other animals, sexual contact, direct physical contact and ingestion. Viruses have evolved specialized mechanisms of evading the host immune system, and often target specific tissues, leading to tissue-specific localization within the host. Tissue sensitivity to virus infection is usually not ubiquitous, therefore the route of viral entry can determine the outcome of infection. Distinct routes of infection can lead to different epidemiological effects, as demonstrated for Deformed Wing virus (DWV), a dicistrovirus that infects honeybees. Oral DWV transmission leads to asymptomatic infection, whereas DWV infection following horizontal transmission by the parasitic mite *Varroa* leads to high mortality (1, 2). This is an example in insects where the route of virus infection has a large impact on the severity of infection. Despite the importance of the route of virus infection on viral competence and outcome of infection, this topic is often overlooked.

In this thesis host-virus interaction is addressed in the model system *Drosophila* and focuses on the oral route of virus invasion on intrinsic and extrinsic protective mechanisms. Host-pathogen interactions in *Drosophila* often utilize Drosophila C virus (DCV) as a model, as it is a natural *Drosophila* pathogen and can be extremely pathogenic. DCV infection in natural populations is though to occur through feeding on contaminated media (3), however the exact route of natural infection is yet to be verified experimentally. Laboratory DCV studies have mainly utilized injection as a way of delivering virus to flies, as injecting 5000 infectious units (IU) of DCV into *Drosophila melanogaster* leads to 100% mortality 4 - 6 days post-injection (4). In contrast, allowing *D. melanogaster* flies to feed on $10^{11}$ IU/ml of DCV for 24 h leads to only ~30% mortality by 10 days post-ingestion (Chapter 3) (5). While it is hard to determine how much virus becomes internalized following the oral route of infection, a study performed in *Drosophila melanogaster* found that in a 24 h period flies ingested between 0.5 – 2.5 µl of sucrose + yeast media depending on the sucrose concentration (6). Even if we assume that the flies are ingesting 0.5 µl of media, that would imply that the flies are ingesting approximately $5 \times 10^7$ IU/fly of DCV, an amount which is far greater than through systemic infection. The difference in susceptibility of flies through feeding and injection may be explained by the fact that natural protection mechanisms exist that are bypassed through injection. This effect is not unique to *Drosophila*, as silkworm larvae, for example, require a 1000 to 10,000-fold higher viral titers following oral infection with the cytoplasmic polyhedrosis virus to achieve the same rate of mortality as systemically infected larvae (7).

Following viral ingestion, the virus has to pass through the midgut barrier, which is known to be a strong insect barrier for virus infection (8). In baculoviruses, viral fibroblast growth factors,
matrix metalloproteases and caspases have been shown to be involved in viral dissemination from the lepidopteran midgut (reviewed in 9) however it is yet to be explored whether similar processes are involved in viral dissemination in *Drosophila*. Currently, oral viral infections in *Drosophila* have been largely uncharacterized due to the previous difficulty in developing methods for orally infecting flies. Only recently have methods of orally infecting flies been described, which led to the discovery of the importance of the nutrient responsive ERK pathway in protection against virus infection. The ERK pathway is induced following oral infection with Sindbis virus, vesicular stomatitis virus and DCV. Inhibiting the ERK pathway through the knock-down of Erk leads to increased midgut infection compared to control flies, suggesting the importance of this pathway in protection against virus invasion (10). Other immune pathways are likely to be involved in protection against virus invasion of the gut, however a targeted approach will be required to alter expression of key immune genes specifically in the gut to determine which other pathways are likely to be important.

At the time this thesis was initiated, no reliable way of orally infecting flies existed. Recently, in addition to the methods described in Chapter 2 and 3 of this thesis two different methods of orally infecting *Drosophila* have been described in the literature (5, 10). Experiments performed in the 1980’s used homogenates of flies fed on DCV-contaminated media to inject into DCV-free flies, to show that oral DCV infections lead to persistently infected *Drosophila* populations (3, 11). While these experiments showed that DCV was associated with the flies, the method used is not able to discern between viral infection and viral contamination (inactive virus). Because exposure to contaminated food does not necessarily lead to viral infection, inactive virus associated with the host could be detected using methods that do not analyze an increase in virus titer or new virus replication. To determine whether a persistent DCV infection can be achieved under laboratory conditions, a new method of detecting active viral replication was required. To this end, a single-stranded qPCR (ssqPCR) assay was developed, able to detect only actively replicating DCV by targeting the anti-genomic RNA strand (Chapter 2). The assay showed that oral DCV infection did not lead to persistently infected populations, contrary to what was previously described (3, 11).

While *Drosophila* has been used extensively as a model organism for host-pathogen interaction studies, the research community has largely ignored virus transmission studies, as systemic viral infections often lead to early mortality before virus transmission can occur. Establishing persistent DCV infections, like those found in natural *Drosophila* populations (12), seems to be hard to achieve in the lab, as orally infecting first instar larvae (Chapter 2) or adults
(data not shown) does not lead to a persistently infected population. This suggests that persistent DCV infections in nature either occur by a different route of infection or that there are other parameters that we do not yet understand which influence the establishment of a persistent DCV infection in Drosophila. The ability of FHV to induce persistent infection in Drosophila has not yet been determined, and although FHV is not a natural Drosophila pathogen, it can establish a persistent infection in Drosophila line 1 cell culture (13) suggesting that persistent FHV infections could be possible in adult flies. Establishment of persistently infected fly lines would allow us to study natural viral transmission and could allow dissection of molecular mechanisms underpinning viral infection and dissemination.

The use of the oral route of virus infection in Drosophila is gaining more attention, in fact a recent paper by Ferreira and colleagues studied the regulation of the Toll immune pathway following oral infection with DCV and Flock House virus (FHV). Using a standard qPCR to detect the abundance of DCV genomes, they suggested that DCV is present in all flies and leads to an approximately 30% mortality (5). The infection status of flies was furthermore analyzed using immunofluorescence, and the results showed that DCV was present in approximately 25% of flies at 5 days post-contamination (5), raising the question of whether the remaining 70% of flies were infected. The study furthermore reported a dichotomy in relative DCV genomic copies, where DCV was either present at very low abundance, or at 1000–10,000-fold higher abundance (5). The percentage of flies showing high DCV abundance correlated with the percentage of flies succumbing to the infection. Two interpretations are consistent with this observation: 1) the flies with low DCV genome abundance are infected but able to control viral replication, or 2) the virus genomes detected through standard qPCR come from virus particles which are associated with the flies without cell infection. The immunofluorescence data is important because detection of DCV in the crop, midgut, hindgut, fat body, testis and trachea (5) indicates successful DCV infection, however that was only observed in 25% of all flies. Using immunofluorescence in conjunction with ssqPCR could be useful to determine whether the remaining 70% of flies are infected, as inactive virus associated with the flies would not be detected using this assay. I believe that the newly designed ssqPCR assay could be especially useful for studying oral DCV infections during the early stages of infection when DCV is found in low abundance in both infected and uninfected flies. Using the standard qPCR assay the infected and un-infected flies would be undistinguishable from each other, a problem that could be circumvented using ssqPCR.

While the ssqPCR is a useful method of detecting viral replication, there are some limitations to this approach. While the ssqPCR can detect active infection, a lack of DCV detection
using this assay does not necessarily indicate that an active infection did not occur at some time-point before the assay was performed. This could be overcome by time-course ssqPCR studies, where a DCV replication could be detected even if it is short term. Furthermore, this assay could be used in conjunction with a standard qPCR assay to look for an increase in virus over time. If no negative strand was detected using ssqPCR, and the standard qPCR did not detect an increase in virus over time, it would be unlikely that the fly was infected.

Establishing oral infection in Drosophila was instrumental to begin dissecting host-virus interaction through a natural route of infection. Using the oral route of infection two known antiviral protection mechanisms (Wolbachia and apoptosis) were assessed to determine whether route-of-infection specific differences in protection mechanisms exist. The first protection mechanism that was assessed is the Wolbachia-mediated protection in Drosophila. All experimental evidence supporting the role of Wolbachia in antiviral protection is based on intra-thoracic injections, while Wolbachia protection following the oral route of virus infection remained uncharacterized. From studies performed in mosquitoes, it was clear that Wolbachia can confer protection following the oral route of infection in mosquitoes, however it remained to be determined whether a similar effect could be observed in Drosophila. Using oral infection methods described in Chapter 2, Drosophila larvae and adults were orally infected with DCV to determine whether Wolbachia can protect against oral challenge with DCV. The results showed that Wolbachia protection is not dependent on the route of infection, as protection against mortality was observed following both oral and systemic infections (Chapter 3). Interestingly, Wolbachia protection was dependent on the developmental stage, indicating that protection at adult stages is not always indicative of protection at larval stages. Because as much as a fifth of all arthropod species might benefit from Wolbachia-mediated protection (14), life-stage susceptibility could have a large impact on viral circulation in nature, and it is therefore an interesting and important topic that could be addressed in future studies.

The second antiviral protection mechanism studied in this thesis focused on the role of apoptosis in antiviral protection. Apoptosis is only recently gaining wider acceptance as an antiviral immune mechanism in Drosophila and other organisms, therefore relatively little is known about how regulation of apoptosis affects viral competence. Using both systemic and oral infections I showed that induction/inhibition of apoptosis in Drosophila can have different effects on viral resistance and tolerance depending on the route of infection (Chapter 4). This has important implications, as this is only the second immune pathway in Drosophila that has been shown to have a route of infection-specific effect. Differences in resistance and tolerance observed between...
systemically and orally infected flies are likely due to differences in FHV localization between the two routes of infection, however no studies exist comparing FHV localization in systemically and orally infected flies. While the study in chapter 4 of this thesis suggests that differences in apoptosis-mediated protection exist depending on the route of infection, certain limitations exist in the study. Because any single gene could be involved in multiple pathways, we cannot be sure that knocking out dP53 or enhancing reaper expression, which would be expected to inhibit or enhance apoptosis respectively, would lead to the observed effects solely due to altered regulation of apoptosis. Unfortunately an alternative mutant (Dronc) in which apoptosis was induced did not feed on the infection media, therefore we were unable to confirm the results (Appendix 1). It would be desirable to perform further studies, where inhibition or enhancement of other genes could be used to further understand the importance of apoptosis on antiviral protection. Regardless of the limitation, it is clear that differences between the two routes of infection exist, as systemic FHV infections in flies in which apoptosis was altered were consistent with the role of apoptosis in limiting viral replication, while following oral infection the results were consistent but not compelling. Because of the central role of apoptosis in several processes such as homeostasis and development, altering apoptosis can lead to mortality of the individuals, impeding further studies. One way of overcoming this limitation is by using infectious clones, where pro or anti-apoptotic genes are inserted into the virus before challenging the flies. Infecting mosquitoes with viruses expressing pro-apoptotic genes leads to induction of apoptosis specifically in cells infected with the virus (15), providing a targeted approach of studying the importance of apoptosis in antiviral protection.

Regulatory processes in Drosophila might involve both induction and inhibition of apoptosis in a tissue and stage of infection-dependent manner. For example, induction of apoptosis during the early stages of virus infection can in some circumstances slow down viral replication (16), while in other cases induction of apoptosis at the later stages of infection can facilitate viral release and dissemination (17-19). Understanding the regulation of apoptosis following the oral route of FHV infection will likely require additional approaches. Time-course analysis of the regulation of apoptosis following FHV infection could be performed using transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay on whole flies. TUNEL analysis is used to detect apoptosis, and would provide useful information on the spatial and temporal regulation of apoptosis following the oral route of infection. Time course analysis could allow us to determine whether apoptosis is occurring in the midgut following oral challenge, and would allow us to study tissue-specific regulation of apoptosis throughout the infection. Identifying spatial and temporal regulation of apoptosis could allow us to alter gene expression in specific tissues at specified times during
infection, which would allow us to gain a better understanding of the importance of apoptosis in regulating viral infection.

Based on the current literature and the results presented in this thesis, it is becoming increasingly clear that the route of infection can have a large effect on the outcome of virus infection in *Drosophila*. Given the current evidence, I consider that immune response in *Drosophila* should be reassessed using the oral route of virus infection. It is possible that the RNAi, JAK-STAT, Imd and autophagy pathways that been reported to be involved in antiviral protection following systemic infection, are more or less important in virus control following oral infection. Especially interesting is immune regulation in the midgut, as the midgut represents one of the first lines of defense against pathogen invasion. Dissecting the protection mechanisms present within the midgut will be essential in understanding virus invasion.

Information gained from studying *Drosophila* immune regulation will not only be useful in understanding host-pathogen interactions in *Drosophila*, as it could facilitate our understanding of regulation of immunity in other insect systems. Studies on antiviral protection mechanisms in *Drosophila* are only recently beginning to gain greater attention, and a lot of questions remain to be answered. Gaining a better understanding of *Drosophila*-virus interactions will require elucidation of the pathways involved in primary virus infection and the subsequent host-virus interactions leading to viral dissemination and transmission. The information provided in this thesis will hopefully encourage other groups focusing on host-virus interactions to use the natural route of virus infection.
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APPENDIX 1
**Introduction**

This addendum complements the data from chapter 4 of this thesis. Herein are described the additional experiments designed to expand on the results from chapter 4. The aim was to determine whether up-regulation of Dronc would lead to similar effects on FHV abundance and tolerance compared to rpr up-regulation. Furthermore because the difference between systemic and oral infection is in the route of pathogen entry, and because pathogens acquired through oral infection have to pass the midgut barrier to establish a prolific infection, I wanted to determine whether induction of apoptosis in the gut can affect FHV abundance and fly survival, however some unexpected results that are unrelated to virus infection prevented me from collecting usable data.

**Methods**

The methods used in this addendum are described in chapter 4 of this thesis. The fly lines used in these experiments have been described in chapter 4, except for the driver line used to express rpr in the midgut of adult flies. The heat-inducible Myo1A-Gal4, tub-Gal80 driver line was used to drive expression of the rpr gene in the midgut epithelial cells (1) and was provided by Dr. Nic Tapon from the Francis Crick institute (UK).

**Results**

Due to the apparent differences and similarities in apoptosis-related protection between systemic and oral infection, I wanted to study the effect of increasing Dronc expression on FHV protection. Oral feeding of Dronc-UP flies was performed several times without success. Repeated experiments continually lead to approximately 70 – 80% mortality within 24 h post-feeding regardless of whether the flies were fed on mock or FHV contaminated media. Because presence of live yeast in the feeding media could be leading to early mortality, live yeast was replaced with heat-inactivated yeast. Switching between live and heat-inactivated yeast had no effect on mortality, as similar rates of mortality were observed regardless of whether live or heat-inactivated yeast was used (data not shown). Following several attempts, a blue food dye was added to the feeding media to observe whether flies were ingesting the food. The results showed that WT flies were ingesting both mock and FHV- contaminated media, evident by the presence of the blue dye in the abdomen (Figure 1, left). In contrast, no blue dye was visible in Dronc-UP flies in contact with either mock or FHV- contaminated media, suggesting that Dronc-UP flies were not ingesting the media (Figure 1, right).
To determine whether lack of feeding was associated with the composition of the media, the flies were transferred to standard cornmeal media. Rearing Dronc-UP flies on standard cornmeal media did not lead to mortality, suggesting that the composition of the media led to food avoidance.

Due to the inability of orally infecting flies using yeast + PBS media, an alternative method of oral infection was tested. Standard cornmeal media was surface contaminated by spreading a high concentration of purified FHV ($10^{12}$ IU/ml) onto the rearing media. Following contamination, adult WT and Dronc-UP flies were allowed to feed on virus-contaminated media for 24 or 72 h before being transferred onto standard virus-free media. The flies appeared to be feeding on the cornmeal media evident by a lack of mortality, however the flies did not appear to become infected, as no mortality was observed throughout the course of the experiment (by 20 days post-feeding, data not shown). Due to the difficulty associated with orally infecting Dronc-UP flies, this fly line was not used in further experiments.

**Figure 1.** Representative example of WT (left) and Dronc-UP flies (right) following feeding for 4 h on yeast extract containing a blue food die. Presence of the dye in WT flies suggests that the flies were ingesting the media, while lack of blue dye in the abdomen of Dronc-UP flies suggests lack of feeding.

**Midgut rpr induction**

Due to the chemical and physical barriers present within the midgut, and because pathogens have to breach the midgut barrier to establish a prolific infection within the organism, I wanted to test whether inducing rpr expression specifically in the midgut would have an effect on FHV
infectivity. An inducible Gal4 driver line was used under a MyoIA promoter that allows expression specifically in the enterocyte cells (intestinal absorptive cells) present within the midgut. Induction of rpr in the midgut (midgut-rpr-UP) repeatedly lead to approximately 80 – 90 % mortality within 24 h post-feeding regardless of whether the flies were fed on mock or FHV infected media. No further modifications were attempted for this fly line, therefore it is unclear whether the flies were dying due to starvation, or whether morbidity was due to extensive tissue damage present within the midgut. Due to high mortality and inability to perform oral infection experiments, this fly line was excluded from further experiments.

Discussion

Feeding Dronc-UP flies on yeast + PBS media lead to an abnormally high mortality within 24 h post-feeding. A wide range of sensitivity to starvation exist between different Drosophila species, leading to a median survival time between 25 h (in D. sechellia) and 171 h (in D. buzzatii) at 25°C, however the survival time is substantially lower when water is not present as flies become prone to desiccation (2). Because the feeding media is the only source of water present to the flies, mortality observed in Dronc-UP and midgut-rpr-UP flies is most likely associated with a lack of fluid intake rather than starvation. Feeding on the standard cornmeal media but not on the yeast + PBS media suggests that a preference for a food type exists. The same food type preference is not observed in WT flies, suggesting that it is likely due to the mutations present in the Dronc-UP and midgut-rpr-UP flies. The result was somewhat unexpected, as induction of apoptosis has not been previously implicated with food preference.

The reason behind avoidance of yeast + PBS feeding media is unclear, however the genes involved in apoptosis are often involved in other processes such as fly development and homeostasis, ribosome biogenesis, DNA repair and homologous recombination (3-11). Altering the expression of genes involved in the apoptotic pathway likely leads to a range of physiological and behavioural effects, some of which may involve food preference or increased sensitivity to diet-related stress. Designing novel methods of orally infecting flies could overcome this problem, however this has proven to be especially difficult in the past.
The midgut infection barrier

During early stages of oral infection, the virus needs to cross the midgut barrier to establish a systemic infection and spread to the internal tissues. Because the midgut represents both a physical and chemical/immune barrier to pathogen infection (12, 13), it is thought that the interaction between the pathogen and the host in the midgut can determine whether the insect will become systemically infected with the virus. In baculoviruses, viral fibroblast growth factors, matrix metalloproteases and caspases have been involved in viral dissemination from the lepidopteran midgut (reviewed in 13) however it is currently unclear whether similar processes are involved in viral dissemination in *Drosophila*.

Apoptosis in the gut has been implicated as an important mechanism in protecting mosquitoes against arboviral infection. In the refractory mosquito *Culex pipiens*, infection with West Nile virus leads to extensive apoptosis in the midgut, a process which was proposed to limit viral replication in the midgut (14). A recent study showed that infecting *Aedes aegypti* mosquitoes with a Sindbis virus infectious clone expressing the *Drosophila reaper* gene leads to a delay in midgut infection and virus replication (15). A strong selection against *rpr* expression was observed in mosquitoes infected with this virus, which lead to the suggestion that arboviruses have evolved mechanisms to avoid stimulating apoptosis in mosquitoes (15). Contrary to this finding, RNAi silencing of the initiator caspase AeDronc in *Aedes aegypti*, which would expect to inhibit apoptosis, lead to reduced Sindbis virus infection in the midgut, suggesting that some level of apoptosis or caspase activity is required for successful viral replication, which is consistent with the requirement for caspases demonstrated in the baculovirus system (16). Given the data, it is still unclear whether induction and/or inhibition of apoptosis in the gut can serve as a protection mechanism against pathogen infection in insects.

Understanding the role of apoptosis in mediating antiviral protection in the midgut would be facilitated by immune-fluorescence time-course studies focused at understanding progression of FHV infection following the oral route of infection. Construction of FHV infectious clones expressing both pro-apoptotic and anti-apoptotic genes would allow regulation of apoptosis to be confined to the infected cells, which would provide some evidence about the importance of apoptosis following primary FHV infection. Further research is required to better understand the mechanisms involved in the establishment of the midgut protection barrier.
References


