Biology of a small RNA virus that infects *Drosophila melanogaster*

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ISBN: 978-91-7601-593-3
Electronic version available at: http://umu.diva-portal.org/
Printed by: UmU-tryckservice, Umeå University,
Umeå 2016,

What would be left of our tragedies,
if an insect were to present us his?

Emile Cioran

Contents

List of Articles included in this thesis…… *ii*
Abstract…… *iii*

Chapter 1: *Drosophila melanogaster* and its Viruses…… 1
1.1 *D. melanogaster,* the Model Organism…… 1
1.2 Viral Pathogens of *D. melanogaster*…… 2

Chapter 2: Nora Virus…… 10
2.1 Discovery…… 10
2.2 Prevalence, Persistence and Pathology…… 10
2.3 Replication and Transmission…….. 12
2.4 Classification: Picorna-like Virus…… 13
2.5 Nora Virus Biology…….. 15
2.5.1 General Characteristics……... 15
2.5.2 Genome Organization…… 15
2.5.3 ORF 1-Encoded Protein: VP1 (**Paper II**)……... 16
2.5.4 ORF 2-Encoded Protein: VP2 **(Paper III)**…… 21
2.5.5 ORF 3-Encoded Protein: VP3 (**Paper I**)…… 23
2.5.6 ORF 4-Encoded Protein: VP4…… 25
2.6 Spontaneous Mutagenesis in the Nora Virus Genome……. 26

Chapter 3: Antiviral immunity in *D. melanogaster*…….. 32
3.1 The RNA Interference Pathway……... 32
3.2 The JAK-STAT Pathway…… 33
3.3 The Toll and IMD Pathways…… 34
3.4 Autophagy…… 34
3.5 Conclusions: **Paper IV**…… 35

Appendix: Materials and Methods…… 38
References…… 41
Acknowledgements……... 52

List of Articles

This thesis is based on the following publications and manuscripts.

I **Sajna Anand Sadanandan**, Jens-Ola Ekström, Venkateswara Rao Jonna, Anders Hofer, Dan Hultmark. VP3 is crucial for the stability of Nora virus virions. Virus Research, Volume 223, 2016, Pages 20-27

II Joel T. van Mierlo, Alfred W. Bronkhorst, Gijs J. Overheul, **Sajna A. Sadanandan**, Jens-Ola Ekström, Marco Heestermans, Dan Hultmark, Christophe Antoniewski, Ronald P. van Rij. Convergent evolution of Argonaute-2 slicer antagonism in two distinct insect RNA viruses. PLOS Pathogens, Volume 8, 2012, e1002872

III **Sajna Anand Sadanandan**, Jens-Ola Ekström, Dan Hultmark. The N-terminus of Nora virus VP2 is important for virion assembly. (Manuscript)

IV **Sajna Anand Sadanandan**, Jens-Ola Ekström, Dan Hultmark. *Drosophila melanogaster* transcriptional response to Nora virus infection. (Manuscript).

Abstract

Drosophila melanogaster has been extensively used as a model organism to study diverse facets of biology, including host-pathogen interactions and the basic biology of its pathogens. I have used the fruit fly as a model to study elementary aspects of Nora virus biology, such as the role of the different proteins encoded by the virus genome. Nora virus, an enteric virus transmitted via the fecal-oral route, does not cause any obvious pathology in the fly, although the infection is persistent. Nora virus genome consists of a positive strand RNA that is translated in four open reading frames (ORF). Since sequence homology studies did not yield much information about the different Nora virus proteins, I have used the cDNA clone of the virus to construct mutants to identify the specific function of each protein. My results have shown that,

1) The protein(s) encoded by ORF 1 are crucial for the replication of the virus genome.

2) The C-terminus of the ORF 1-encoded protein (VP1), is an inhibitor to the RNAi pathway.

3) The transmembrane domain in the N-terminus of the ORF2-encoded protein (VP2) is important for the formation of Nora virus virions.

4) The ORF 3-encoded protein (VP3) forms α-helical trimers and this protein is essential for the stability of Nora virus capsid.

I have also performed RNA sequencing to investigate the transcriptional response of *D. melanogaster* in response to Nora virus infection and my results indicate that,

5) The upregulation of genes related to cellular stress and protein synthesis and the donwregulation of basal digestive machinery, together with the induction of *upd3*, implies major gut epithelium damage and subsequent regeneration.

Chapter 1: *Drosophila melanogaster* and its Viruses

1.1 *Drosophila melanogaster*, the Model Organism

Drosophila melanogaster is a species of fruit fly in the *Drosophilidae* family. *D. melanogaster* has been successfully used as a model organism to study various aspects of biology such as genetics, immunity (Mueller et al., 2010; Xu et al., 2012), physiology, development and cancer. Developmental mechanisms, cellular processes and principles of innate immunity are highly conserved between *Drosophila* and vertebrate organisms. In fact, approximately 75% of disease-causing genes in humans have comparable homologues in the fruit fly (Reiter et al., 2001).

Fruit flies are also very easy to maintain, have short generation times and produce several offspring in each generation. The short and very productive life cycle of *D. melanogaster* is what makes it an extremely useful model organism. A female fruit fly can lay between 30-50 eggs per day throughout her lifetime. A fertilized fruit fly embryo develops into a first instar larva in 24 hours. Following this, the larva takes only 48 hours to become a third-instar larva and then a further $2^{1/2}$ to 3 days to become a pupa. The pupa develops into an adult in 3-5 days and the life cycle continues. Hence, within a short period of 10-14 days (depending on temperature conditions), we can obtain several hundreds or thousands of fruit flies. Unlike mosquitoes, wherein a part of their life cycle happens in water making it difficult to use them in laboratory research, it is very simple to maintain large cultures of *Drosophila*. Moreover, because of their small size, it is very easy to sustain millions of flies in the laboratory at any given time and they do not require any special care. Their diet is modest and the only maintenance they require is a temperature controlled environment (25° preferably) and regular food change.

The genome of *Drosophila melanogaster* is compact and has fewer redundant regions as compared to mammals. Hence, it is easier to identify the effect(s) of single mutations in the fruit fly. It is also very easy to modify the genotype of *D. melanogaster* to create mutants or

transgenic flies. There are several resources available from where one can obtain *Drosophila* mutants or strains with RNAi constructs aimed against specific genes. *Drosophila* are further endowed with polytene chromosomes that are oversized and have barcode-like banding patterns of light and dark regions. This facilitates the easy identification of any and all changes made to the genetic make-up of the flies.

1.2 Viral Pathogens of *Drosophila melanogaster*

Insects constitute a major portion of all animals that live on earth and they also happen to be the most diverse group of organisms. This diversity also leads to immense miscellany in the pathogens that infect insects. Most insect viruses have ancient evolutionary relationships, which have spanned millions of years, with their host. Many a time insect viruses are transmitted to vertebrate animals or to plants, causing deadly diseases. This makes the twenty odd diverse groups of known insect viruses interesting from a medical and agricultural point of view, apart from general scientific interest (Huszar and Imler, 2008). Some of the well described single stranded RNA viruses that infect insects belong to the following families, *Birnaviridae* (*Drosophila X virus*), *Togaviridae* (*Sindbis virus*), *Flaviviridae* (*Yellow fever virus*), *Rhabdoviridae* (*Vesicular stomatitis virus*), *Bunyaviridae* (*Rift Valley fever virus*) and *Nodaviridae* (*Flock House virus*). Apart from these well-described virus families, is a large group of unclassified and classified insect viruses that are very similar to the mammalian picornaviruses. These are characterized by small positive-strand RNA genomes enclosed within un-enveloped icosahedral virions. They are grouped under a general category of picorna-like viruses. *Dicistroviridae, Iflaviridae* and *Nodaviridae* are the major picorna-like virus families known to infect insects, while other families within this group are known to be important pathogens of vertebrate animals, fungi, and plants (*Picornaviridae, Caliciviridae, Partiviridae, Potyviridae, etc.)*

The Dipterans are a large order of insects that undergo metamorphosis during their life-cycle. Viruses that infect dipterans have been studied primarily for reasons of medical prominence, because several clinically important viral pathogens use dipteran insects as vectors. It is very interesting to note that although picorna-like viruses are usually studied

for their clinical or agricultural importance, the model used extensively for their characterization is the *Drosophila,* a fruit fly that is of very little medical or agricultural importance.

Most identified natural viral pathogens of *Drosophila* are RNA viruses. In fact, only two DNA viruses (*Insect Iridescent Virus 6* and the *Kallithea Virus*) have so far been observed to infect *Drosophila* (Constantino et al., 2001; Webster et al., 2015). Among the RNA viruses, the best-described viruses are the *Drosophila C virus* (*Dicistroviridae*) and the *Sigma virus* (*Rhabdoviridae*) (Brun, 1980; Jousset et al., 1977). The *Drosophila A virus* and the *Drosophila P Virus*, both picorna-like viruses, are also natural pathogens of *Drosophila* (Brun, 1980). Apart from these, the *Drosophila X virus* and the *Drosophila F virus*, belonging to *Birnaviridae* and *Reoviridae* families respectively, are also capable of infecting *D. melanogaster*. The fruit fly has also been used to model host-pathogen interactions between human arboviruses and their host. The most infectious arboviruses of public health concern fall within the *Alphaviridae*, *Flaviviridae* and *Bunyaviridae* families. Viruses belonging to each of these groups have been studied using *Drosophila* as a model system (Chotkowski et al., 2008; Filone et al., 2010; Hughes et al., 2012). Similarly, the *Cricket paralysis virus* (Plus et al., 1978) and the *Flock House virus* (Dasgupta et al., 1994), which have a much broader host range than viruses specific to *Drosophila* have also been comprehensively studied in the fruit fly. Let us now look closer at some viral pathogens of *Drosophila* wherein I will describe viruses restricted to insects and vertebrate viruses that have been studied using the *Drosophila* model.

1.2.1 Drosophila C Virus (DCV)

The *Drosophila C virus* belongs to the *Cripavirus* genus, which was initially classified as *Picornaviridae* (Jousset et al., 1977). When detailed analysis was performed on the approximately 9 kb positive-strand RNA genome of DCV, it was observed to have two open reading frames (Johnson and Christian, 1998) as opposed to the single reading frame seen in *Picornaviridae* (Johnson and Christian, 1998). It was also seen that the capsid proteins of DCV were encoded at the 3' end of the genome, while in *Picornaviridae* the structural proteins are encoded in

the 5' region (Johnson and Christian, 1998). The observation of these and other differences led to the construction of a new family called the *Dicistroviridae*, under the *Picornavirales* order (Bonning and Miller, 2010).

DCV was first discovered in wild populations of *D. melanogaster* in the 1970s (Jousset et al., 1977). DCV happens to be one of the best studied viruses among all known viral pathogens of *D. melanogaster*. The virus is transmitted horizontally via the fecal-oral route and has very mild effects on the mortality of infected flies (Gomariz-Zilber, 1993). But when injected into adult flies, the virus causes lethality (Jousset et al., 1972) and the cause behind this shift in pathology is not fully understood. DCV infection also induces nutritional stress as a result of intestinal obstruction in *Drosophila* (Chtarbanova et al., 2014). Several transcriptional response studies have been conducted to identify the *Drosophila* genes that are differentially expressed as a result of DCV infection (Dostert et al., 2005; Hedges and Johnson, 2008; Roxström-Lindquist et al., 2004; Sabin et al., 2010) and it is apparent that the innate immune system of the fly plays a major role in the control of DCV infection.

1.2.2 Drosophila P Virus (DPV)

This is an unclassified single-stranded RNA virus, which is placed under the broad category of picorna-like viruses. It was first discovered as a natural pathogen of *Drosophila* by Plus and Duthoit (1969). Although it displays very little pathology when vertically transmitted from mother to offspring, it causes female sterility and a reduction in life span when it is injected into adults (David and Plus, 1971). Pathology observed during P virus infection (by injection) suggests that it causes malfunctioning of the malpighian tubules. Electron microscopy studies of the virus have indicated it to be around 25-30nm in diameter, displaying properties of other picorna-like viruses (Teninges and Plus, 1972).

1.2.3 Drosophila A Virus (DAV)

When DCV, DPV and DAV were initially identified in the 1970s, it was shown that DCV was the most pathogenic and DAV the least pathogenic among the three (Plus et al., 1975a; Plus et al., 1975b). Unclassified as

yet, the *Drosophila A virus* contains an RNA genome in a virion of approximately 30nm diameter. Although originally thought to be a picorna-like virus, studies have indicated that the DAV genome has characteristics previously attributed only to *Birnaviridae* and *Tetraviridae* (Ambrose et al., 2009). The positive strand RNA genome of DAV has two open reading frames, wherein the replicative genes are encoded in ORF 1 and the structural proteins in ORF 2, like it is in *Dicistroviridae* (Ambrose et al., 2009).

1.2.4 Sigma Virus

The host-pathogen relationship between *Sigma virus* and *Drosophila* has been studied since the late 1930s (L'Héritier and Teissier, 1937; L'Héritier, 1957). It was discovered after L'Héritier observed that some fly strains were paralyzed after CO_2 anaesthetization whereas others were quick to recover from the effects of CO_2. Further investigations into this phenomenon showed that this was a result of *Sigma virus* infection. The virus is vertically transmitted by both sexes via the germ cells (Longdon and Jiggins, 2012) and it infects at least six species of diptera, including 5 *Drosophila* species and one *Muscidae* (Longdon et al., 2010; Longdon et al., 2011). The *Sigma virus* genome is composed of a linear, negative-strand RNA that is 12.6 kb long. It is classified under the *Rhabdoviridae* order within the *Mononegavirales* family. *Sigma virus* has been identified in wild populations also, making it a natural viral pathogen of *Drosophila* (Carpenter et al., 2007; Wilfert and Jiggins, 2010). Since the possibility of fruit flies encountering high levels of CO_2 in nature is minimal, it is possible that *Sigma virus* infection in wild populations has very little effect (Longdon et al., 2010). It has also been theorized that infected flies are less fit as compared to uninfected flies under high population density conditions, leading to a loss of *Sigma virus* from the population (Yampolsky et al., 1999). With respect to antiviral immunity, known immune response pathways such as Toll and IMD signaling cascades do not appear to be differentially regulated following *Sigma virus* infection (Carpenter et al., 2009), although these results were in contradiction to the study conducted by Tsai et al. (2008).

The *Vesicular stomatitis virus* (VSV), also a member of the *Rhabdoviridae* family, causes foot and mouth disease like symptoms in

infected humans and cattle (Letchworth et al., 1999). VSV is capable of infecting cells from a wide-array of organisms and it has hence been studied using the *Drosophila* model also (Wyers et al., 1980). Similar to *Sigma virus* infection, VSV infected flies become sensitive to CO_2 but mortality has not been observed. The innate immune system of the flies including the RNAi pathway (Mueller et al., 2010) and autophagy (Shelly et al., 2009) play an important role in the control of VSV titers and pathology.

1.2.5 Drosophila F Virus (DFV)

DFV belongs to the *Reoviridae* family that is characterized by a dsRNA genome. The DFV virion is non-enveloped and approximately 60-70nm in diameter. It is non-pathological and has been identified in laboratory stocks and wild *Drosophila,* indicating that it is a natural pathogen of the fruit fly. No vertical transmission has been observed and the virus is assumed to transmit via contact or ingestion (Brun, 1980). Another member of the *Reoviridae* family, the Bluetongue virus, is capable of infecting *Drosophila*, albeit under experimental conditions (Shaw et al., 2012)

1.2.6 Drosophila X Virus (DXV)

The DXV was first isolated as a contaminant in *Drosophila* cell lines during studies on *Sigma virus*, but it has never been identified as a natural pathogen in wild *D. melanogaster* (Teninges and Richard-Molard, 1979). DXV infected flies display an increased sensitivity to CO_2 and an increase in mortality (Dobos et al., 1979; Teninges and Richard-Molard, 1979), although the mechanisms behind either of these pathologies have not been characterized. The DXV genome is composed of a bi-segmented double stranded RNA and a nucleocapsid that is enclosed in a non-enveloped icosahedral virion. Of the two RNA segments, segment 1 encodes the structural proteins and the protease while the shorter segment 2 encodes the RNA dependent RNA polymerase (Dobos et al., 1979; Nagy and Dobos, 1984). Based on genome characteristics, it is classified as belonging to the *Entomobirnavirus* genus within the *Birnaviridae* family (Delmas, 2001).

1.2.7 Cricket Paralysis Virus (CrPV)

The CrPV is a well-studied *Cripavirus* belonging to the *Dicistroviridae* family (Christian and Scotti, 1994). It is characterized by a single-stranded positive-sense RNA genome with two open reading frames (Christian and Scotti, 1994). The CrPV is not a natural pathogen of *Drosophila* but it has been extensively studied using both *Drosophila* cell lines and flies (Scotti et al., 1996). Having been detected in at least five different orders of insects it has one of the widest host-ranges observed among insect viruses (Scotti et al., 1981). In *Drosophila*, CrPV infection leads to cytopathic effects and mortality (Scotti et al., 1996) due to a near-complete cessation of host-protein synthesis (Garrey et al., 2010; Wilson et al., 2000). The virus also has a strong defense against the RNAi pathway because it encodes a suppressor of silencing in the 5' end of its genome (Nayak et al., 2010).

1.2.8 Flock House Virus (FHV)

The FHV has a linear, positive-sense, bi-partite RNA genome wherein RNA1 is 3.1 kb long and RNA2 is 1.4 kb long (Dasgupta et al., 1994; Schneemann and Marshall, 1998; Schneemann et al., 1998). RNA1 encodes the RdRp while RNA2 encodes the structural proteins of the virus (Dasgupta et al., 1994; Schneemann et al., 1998). It is a prototype member of the *Nodaviridae* family, under the *Alphanodavirus* genus (Scotti et al., 1983). FHV is not a natural pathogen of *Drosophila*, but it is known to infect a very wide array of hosts, even crossing the kingdom barrier and infecting plants (Dasgupta et al., 2007; Price et al., 1996; Selling et al., 1990). FHV has been comprehensively studied using *Drosophila* cell lines and adult flies, wherein it causes lethality only when injected (Dasgupta et al., 1994; Johnson and Ball, 1999). FHV also produces a 0.4 kb long sub-genomic RNA3 which encodes the suppressor-of-silencing protein B2 to contest the RNAi pathway (Galiana-Arnoux et al., 2006; Li et al., 2002). However, cells infected with FHV combat the spread of infection through apoptosis, which would then block the production of virus particles (Liu et al., 2013).

1.2.9 Insect Iridescent Virus 6 (IIV-6)

Named after the characteristic iridescence observed in infected insects, the *Insect iridescent virus 6* belongs to the genus *Iridovirus* placed under the family *Iridoviridae*. This is a large virus approximately 185 nm in diameter, encapsidating a large double-stranded DNA genome of 212,482 bp. The virus encodes an estimated 211 open reading frames with several redundant elements in the DNA sequence (Eaton et al., 2007; Jakob et al., 2001). IIV-6 has a fairly large host range (Jakob et al., 2002) and it was the first DNA virus shown to be capable of replication in Drosophila cells (Constantino et al., 2001). Injecting the virus into adult *Drosophila* results in death (Teixeira et al., 2008). Since dsRNA is produced as a replication intermediate during IIV-6 life cycle, it is recognized and targeted by the RNA silencing machinery (Bronkhorst et al., 2012).

1.2.10 Kallithea Virus

More than 20 novel *Drosophila* viruses were identified in the study conducted by (Webster et al., 2015). Out of all the viruses identified, there was only one DNA virus, the Kallithea virus, which was closely related to the *Nudiviruses*. They also observed that the RNAi pathway targets this virus and that it encodes an miRNA. However, the prevalence of Kallithea virus is modest at best, since it was detected in less that 1% of *D. simulans* and around 5% of *D. melanogaster* tested (Webster et al., 2015). A later study also conducted by Webster et al. (2016) showed the presence of a third DNA virus in *D. obscura* and *D. immigrans*. This virus was closely related (98% sequence identity) to the *A. vulgare* DNA iridescent virus.

1.2.11 Other viruses

Sindbis virus (SINV) is the prototype member of the alphaviruses and it has been significantly studied using the *Drosophila* model. It is a mosquito borne virus that causes arthritis and the Pogosta disease (Ockelbo disease) in humans (Sane et al., 2010). The receptor used by SINV to enter a host cell was identified in *Drosophila* and the ortholog of the same receptor in vertebrates is used by SINV for entry (Rose et al., 2011; Strauss et al., 1994). Although SINV infection does not cause

lethality or cytopathic effects in *Drosophila* the infection once established is persistent (Mudiganti et al., 2010). The immune response pathways of *Drosophila* appear to have an important role in the control of infection, as flies with non-functional immune responses experience increased virus titers and mortality (Avadhanula et al., 2009).

The *Rift Valley fever virus* (RIFV) is the most well known *Bunyavirus*, which causes a fatal hemorrhagic fever in humans. Arthropods, such as the mosquitoes, are their main means of transmission and they cause very little pathology in the vector (Boshra et al., 2011). RIFV has been inoculated into adult fruit flies to model the interaction between the virus and an insect host. The virus successfully infects *Drosophila* but causes no mortality, although the infection spreads to several tissues of the flies (Filone et al., 2010). Other bunyaviruses such as the *La Crosse virus* and the *Tahyna virus* are also capable of infecting *Drosophila* (Glaser and Meola, 2010; Hannoun and Echalier, 1971). However, the levels of replication for these viruses in *Drosophila* are very low, suggesting either a strong immune response from the fly or a lack of optimal replication conditions for the viruses.

The *West Nile virus*, *Hepatitis B & C viruses*, *Dengue virus*, *Japanese encephalitis virus*, *Tick-borne encephalitis virus* and the *Yellow fever virus* are some examples of clinically important *Flaviviridae* (Fernandez-Garcia et al., 2009; Suthar et al., 2013). The *Zika virus* (Kuno and Chang, 2007), which has recently been in the news for its alleged association with infantile microcephaly (Mlakar et al., 2016) and its rapid spread through the Americas, is also a member of the *Flaviviridae* family. The *West Nile virus* can infect both *Drosophila* cells and adult flies, although the infection is asymptomatic (Chotkowski et al., 2008; Glaser and Meola, 2010; Rose et al., 2011). Dengue virus infection has also been studied using the *Drosophila* model, wherein an RNAi screen was performed to investigate host factors that are crucial for and against infection (Mukherjee and Hanley, 2010).

Chapter 2: Nora Virus

2.1 Discovery

During the initial stages of its discovery in 1996, Nora virus was identified as a fruit fly RNA transcript that was upregulated upon bacterial infection in a differential display screen. This same sequence was also detected in a *D. melanogaster* cDNA library from the late 1980s. Sequence analysis during the early stages showed only a weak similarity to virus sequences, but in the early 2000s a clear resemblance to the Sacbrood virus RNA polymerase was observed. The fruit fly RNA transcript was then re-branded as a virus. The circumstances of its discovery lead to the idea that it was a stress-induced viral infection. The one undeniable fact was the persistence of this infection, observed in samples from as far back as the late 1980s (Dan Hultmark, personal communication).

Mazen Habayeb initiated investigation on Nora virus in 2004, under the supervision of Prof. Dan Hultmark. One of the first experiments performed was to verify the hypothesis that Nora virus infection was induced during stress. However, this was not the case. There was no induction of Nora virus observed after injecting the animals with bacteria or even during other stress conditions such as starvation or high temperature. Nevertheless, the Hultmark group, where Mazen Habayeb and Jens-Ola Ekström went on to characterize several aspects of Nora virus biology and host-response against Nora virus infection, undertook research on Nora virus. The virus receives its name from the Armenian language where Nora means “new” (Habayeb et al., 2006).

2.2 Prevalence, Persistence and Pathology

Webster et al. (2015) investigated the distribution and evolution of known Drosophila viruses in the wild. Around 3000 wild-caught Drosophila flies were analyzed and total RNA sequencing showed that 0.5% of all reads matched DAV, DCV, Sigma virus and Nora virus. Nora virus was prevalent in around 4% of all *D. melanogaster* and approximately 5% of all *D. simulans* sampled. These samples were

collected from 17 locations around the world and *D. simulans* obtained from Marrakesh (Morocco) showed the highest prevalence of Nora virus (around 30% of all *D. simulans* collected in Marrakesh were infected with Nora virus). Nora virus variants have also been detected in wild-caught *D. immigrans* and *D. subobscura* (van Mierlo et al., 2012). *Haematobia irritans* and *Nasonia vitripennis* are also known hosts of Nora virus, which has also been identified in the transcriptomes of several different dipteran, lepidopteran and hymenopteran insect species (for example, see Table 2 of supplementary information in Paper I included in this thesis). These observations indicate that Nora virus is the prototype member of a large family of insect viruses. However, van Mierlo et al. (2014) observed that Nora virus rarely, if ever, moved between hosts, since they could not detect any new lineages of Nora virus from infected *D. subobscura* and *D. immigrans* that were derived from a mixed population.

Although the prevalence of Nora virus in wild-populations of *Drosophila* appears modest (Webster et al., 2015), the virus is very prevalent in laboratory stocks. Habayeb et al. (2006) documented the prevalence of Nora virus infection in laboratory stocks and it was found to be ubiquitously present in several stocks. Nevertheless, the titer at which the virus is observed in the numerous stocks varies to a large extent. The *Relish*^{E23} (Hedengren et al., 1999) fly stock shows the highest virus titers with each individual fly in the population producing large amounts of the virus (Jens-Ola Ekström, personal communication). On the other hand fly stocks such as the *Oregon R* have mixed populations wherein some flies may harbor high virus titers while others may harbor low virus titers (Habayeb et al., 2009b). Flies within the same stock that harbour high virus titers (10^7 viral RNA/fly) are unable to clear the infection while those that begin with slightly lower virus titers (10^4 viral RNA/fly) appear capable of minimising virus load (Habayeb et al., 2009b).

cDNA libraries made in the 1990s (Habayeb et al., 2006) showed the presence of Nora virus RNA and we can still detect the virus in several of our laboratory stocks. The virus has persisted in materials collected over a span of more than two decades and this is an indication of how persistent it is. Several Drosophila viruses, such as the DCV, DPV, DFV, DAV and the Sigma virus, are known to cause persistent infections

(Brun, 1980; Plus et al., 1975b). Viruses belonging to the *Picornaviridae* family are well known as agents of persistent infections. The poliovirus is a classic example wherein the virus persists in the host for long periods, thus causing the post-polio syndrome (Howard, 2005).

The maintenance of a persistent infection requires a balance between virus replication, host cellular functioning and host immune response. An important reason for the successful persistence of Nora virus could be the non-pathological nature of this infection. Although the virus replicates to produce high titers, the life span, eclosion and fecundity of the flies are not affected (Habayeb et al., 2009a). In fact, no pathology of any sort has been directly observed so far. This might suggest that Nora virus is extremely well adjusted to the *Drosophila* environment and has, through years of co-evolution, learnt to evade the fly immune system.

2.3 Replication and Transmission

Nora virus replicates in the fly intestine and higher virus titers were observed in the gut as compared to other fly organs (Habayeb et al., 2009a). In order to identify the site of infection within the intestine, fly guts were dissected and separated into cardia, midgut, hindgut and malphigian tubules. Total RNA was extracted from the dissected tissue and quantitative RT-PCR was performed to quantify virus titer (Figure 1, unpublished data). This experiment showed that the midgut had the highest virus titers, followed by the cardia, hindgut and then the malphigian tubules. This indicates that the virus primarily replicates in the *Drosophila* midgut. Results from a virus reporter developed by Ekström and Hultmark (2016) also indicate that the virus primarily replicates in the fly midgut.

Since Nora virus replicates in the fly gut, virus titers in the feces of infected flies were examined. The amount of Nora virus detected in the feces was as much as was present in the flies. This then suggested that Nora virus is an enteric virus transmitted via the fecal-oral route (Habayeb et al., 2009a). The virus replicates in the fly gut and is excreted via the feces, through which means it then enters a new host. Nora virus is not vertically transmitted since dechorionating embryos of infected flies and culturing them in fresh fly food can clear the infection.

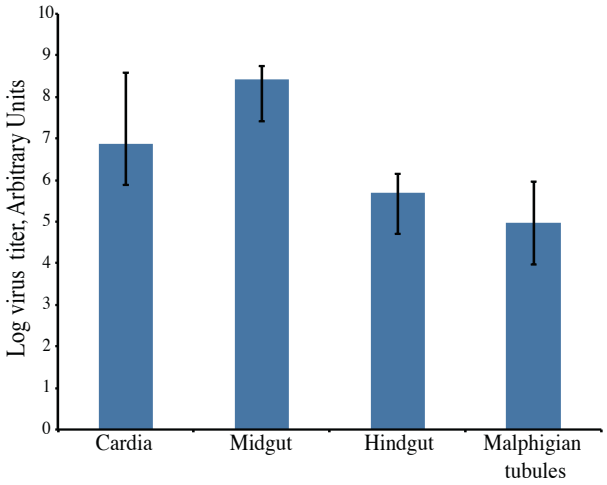

Figure1: Site of Nora virus replication. Quantitative PCR was performed on total RNA from dissected parts of the fruit fly digestive tract. The experiment has been repeated thrice with at least 15-20 flies dissected for each repeat.

2.4 Classification: Picorna-like Virus

Viruses come in a dizzying array of sizes and shapes and have evolved the most ingenious life styles worthy of their "simple" complexity. Among the multitude of characteristics that viruses embody, the fundamental criterion used in the Baltimore virus classification system is the nature of their genetic material. There are viruses that have double-stranded or single-stranded DNA genomes, double or single-stranded RNA genomes, and even reverse-transcribing DNA or RNA genomes. Considering single-stranded RNA viruses alone, they are further classified based on the polarity of the RNA they contain. This difference in polarity has ensued the development of interesting genome organizations and gene expression methods. Upon infection, viruses with positive-sense RNA genomes can directly start translating their proteins. Here, the RNA acts as an mRNA, which is translated by host proteins to produce a single large poly-protein. This poly-protein is post-translationally modified by host or virus proteases to eventually produce the proteins required for completing the virus life cycle. On the other hand, negative-sense RNA viruses need to first produce an "mRNA" before translation can advance. The third variety, called ambisense RNA viruses, are similar to negative-sense RNA viruses, except that they can

translate from the negative strand also. A unique fourth class called retroviruses has a lifecycle that includes a DNA intermediate that has to integrate into the genome of the host prior to viral gene expression.

Positive-strand RNA viruses can be grouped into three categories, picorna-like, alpha-like and flavi-like. Although picorna-like viruses are a large ill-defined group, they are generally said to include 14 families of viruses, apart from several unclassified genera and species (Koonin et al., 2008). Most picorna-like viruses have un-enveloped icosahedral virions of approximately 30nm diameter. A set of well-conserved genes, the RNA-dependent RNA polymerase (RdRp), the protease, a superfamily 3 helicase and a genome-linked protein characterize the RNA genome. The host range of picorna-like viruses is remarkable, considering they have been shown to infect five supergroups of eukaryotes, namely the Unikonta (animals, fungi and Amoebozoa), Plantae, Chromalveolta and the Excavata (Koonin et al., 2008). The *Picornavirales* is the largest order within picorna-like viruses and it contains five families of viruses (*Picornaviridae, Iflaviridae, Dicistroviridae, Marnaviridae* and *Secoviridae*) and several unclassified genera.

We do not have enough information about Nora virus biology and hence the virus has not been classified as yet. Sequence homology studies have shown that the genes involved in genome replication are most closely related to the *Dicistroviridae, Picornaviridae* and the *Iflaviridae* (Habayeb et al., 2006). Phylogenetic studies on RdRps from several positive strand RNA viruses, showed the presence of six clades in the RdRp tree. In this study with respect to RdRp relationships, Nora virus placed in a lineage of *Iflaviridae* and insect viruses within the *Comovirus* and *Dicistroviridae* clade. Conversely, comparisons based on the helicase, placed Nora virus within the *Picornaviridae* clade (Koonin et al., 2008). Nora virus exhibits enough sequence similarity with these viruses for us to hypothesize that it is a picorna-like virus, however, the genome organization of Nora virus has never been seen before in this category of viruses. Therefore, it is most likely that Nora virus is the prototype member of a new family of picorna-like viruses (Habayeb et al., 2006).

2.5 Nora Virus Biology

2.5.1 General Characteristics

Nora virus is a non-enveloped, small-RNA virus with an approximate size of 30nm diameter. The monopartite RNA genome is of positive polarity and can be translated directly as an mRNA. Northern blot of the viral RNA gave a single band indicating that sub-genomic RNA is not produced as part of the virus life cycle (Habayeb et al., 2006).

2.5.2 Genome Organization

The positive-sense RNA genome of Nora virus is 12 333 nucleotides long, followed by a poly-A tail (accession number GQ257737) (Ekström et al., 2011). The genome is organized as four open reading frames (Habayeb et al., 2006) (Figure 2) where ORF 1 follows a 478-nucleotide long 5'- untranslated region and the fourth reading frame, ORF 4, is followed by a 394 nucleotide long 3'-untranslated region. The first reading frame overlaps with ORF 2 by 25 nucleotides and ORF 2 overlaps with ORF 3 by 71 nucleotides. On the other hand, there is no overlap between ORF 3 and ORF 4, but an 85 nucleotide long untranslated stretch exists between them. Although frame-shift has been suggested as a possible mechanism of translation for the different reading frames, there is no experimental evidence for or against this.

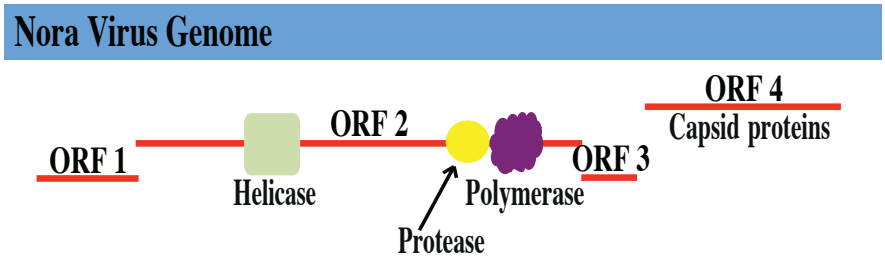

Figure 2: Nora virus genome organization, indicating known genes in the genome.

2.5.3 ORF 1-Encoded Protein: VP1

The functional characterization of VP1 is discussed in **Paper II** included in this thesis.

The VP1 protein is 475 amino acids long with a theoretical molecular mass of 56.3 kDa. Secondary structure prediction of this protein suggests a 53% α-helical structure (Figure 3, unpublished data) with the region between amino acids 177-243 showing the highest probability for an α-helix (red curve in figure 3). This same region also shows a very high probability to form a coiled coil dimer (black curve and purple dotted curve in figure 3). A leucine zipper has also been predicted within this region, wherein leucine residues are located at positions 198, 205, 212 and 219 (LMERVDKLEKMNAALEEENKQL).

The VP1 protein shows no sequence similarity to any previously described virus proteins. However, functional characterization of VP1 indicated that the C-terminus of this protein was involved in the suppression of the RNA interference (RNAi) pathway (Paper II).

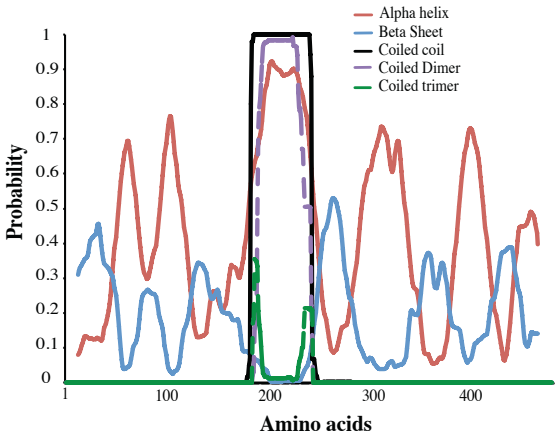

Figure 3: Secondary structure prediction for VP1. Probabilities for α-helix (red), β-strand (blue) and coiled coil (black) according to NetSurfP Secondary structure predictions server and Paircoil2. Probabilities for coiled coil dimer/trimer (purple dotted line/green dotted line) as predicted by Multicoil.

Several species of plants, fungi and insects are known to use the RNAi pathway as an important line of defense against viral infections (Ding and Voinnet, 2007). Double stranded RNAs (dsRNA) are produced as intermediate products during the virus replication cycle and they trigger the RNAi response that cleaves them into small interfering RNAs (siRNA). In insects, the initial recognition and cleavage of dsRNAs is performed by Dicer-2 (Bernstein et al., 2001), a ribonuclease which produces 21-nt siRNAs with 2-nt 3' (Elbashir et al., 2001a; Elbashir et al., 2001b; Zhang et al., 2004). Viral dsRNAs that are cleaved by Dicer-2 are specificity determinants for the RNA-induced silencing complex (RISC). The Argonaute2 protein, which forms a part of the RISC, cleaves the target RNA that is complementary to the cleaved viral dsRNAs (Okamura et al., 2004; Rand et al., 2004).

To counter the RNAi pathway, several plant and insect viruses encode proteins, called viral suppressors of RNAi, which suppress the RNAi pathway (Diaz-Pendon et al., 2007; Ding, 2010; Wang et al., 2006). Certain viruses that encode RNAi suppressor proteins protect their dsRNA by shielding them from Dicer-2. For example, the B2 protein produced by FHV and the 1a protein produced by DCV, both bind to dsRNA, thus protecting it from Dicer-2 activity (Li et al., 2002; van Rij et al., 2006). Certain RNAi suppressor proteins have evolved to target the catalytic activity of Argonaute-2. For example, the 1a protein of the CrPV interacts directly with Argonatute-2 to suppress the gene-silencing pathway (Nayak et al., 2010).

Among *Drosophila* viruses, DCV was the only natural pathogen known to harbor an RNAi suppressor protein, until the results published in Paper II clearly showed that the Nora virus VP1 protein suppresses RNAi activity both *in vivo* and *in vitro*. Although initial investigation performed by Habayeb et al. (2009b) suggested that the RNAi pathway did not affect Nora virus infection, sequencing of 19-29 nucleotide small RNAs from infected w^{1118} *Drosophila* strain proved otherwise. Small RNAs that could be matched to the Nora virus genome were typically 21-nt long and this is the characteristic size of Dicer-2 cleavage products. Thus Nora virus double-stranded RNAs formed as replication intermediates are both recognized and cleaved by Dicer-2 activity.

Once it was established that the RNAi pathway does in fact target Nora virus, the next step was to investigate how the virus manages to evade this attack. An *in vitro* assay (van Cleef et al., 2011) was performed using the four Nora virus ORFs to determine which one of these produced an RNAi suppressor protein. Results from this assay showed that the protein product of ORF 1 was capable of suppressing RNAi activity. N and C-terminus deletion mutants of VP1 further revealed that the C-terminus of this protein mediated the RNAi suppression activity under *in vitro* conditions. The RNAi activity of VP1 could also be detected in *in vivo* assays where it was shown that the protein could not only suppress RNAi activity, but also enhance viral pathogenicity of a recombinant sindbis virus that carried the Nora virus-VP1 protein. In order to characterize the exact mechanism used by the Nora virus-VP1 protein to suppress RNAi, purified VP1 tagged with the maltose binding protein was used to monitor RNAi suppression. These experiments showed that VP1 did not interfere with dsRNA or the Dicer-2 ribonuclease, but it inhibited the RISC complex by disrupting the catalytic activity of Argonaute-2.

In an unpublished experiment (Sajna Anand Sadanandan and Jens-Ola Ekström), we constructed C-terminus mutants of Nora virus (Figure 4) using the cDNA clone of the virus (Ekström et al., 2011). The NV-VP1(Δ401-422) and NV-VP1(Δ401-456) mutants (Figure 4) were microinjected into $Relish^{E23}$ and $Ago2^{414}$ fly strains in order to test the effect of these mutations. We hypothesized that if the C-terminus was involved in suppressing the RNAi pathway, mutant viruses that have a dysfunctional VP1 C-terminus will be unable to successfully replicate in the wild type strain $Relish^{E23}$. On the other hand, these mutants will still be capable of replicating successfully in the $Ago2^{414}$ fly strain that is deficient in the RNAi pathway. We extracted total RNA from the injected animals and performed quantitative RT-PCR to check virus titers. Three other C-terminus substitution mutants, NV-VP1(K366A), NV-VP1(WW375AA) and NV-VP1(W378A) were also constructed using the cDNA clone of the virus and the effect of these mutations was also tested. Our results were unfortunately inconclusive regarding the suppression of RNAi. Virus titer could not be detected for any of these mutants, although the wild type virus gave expected virus titers, indicating that all five mutants were replication-incompetent. This

indicates that genome replication was hampered in all the mutants tested. The assays described in Paper II indicated that the VP1 protein could suppress RNAi, but it is to be noted that the effect of VP1 on RNAi was tested using a plasmid that expressed only VP1 and not any of the other virus proteins. Whereas, in the experiment performed using the Nora virus cDNA clone the entire virus genome is expressed. From this experiment, it is apparent that apart from the RNAi suppression activity, VP1 also plays an important role in the actual replication of the virus genome. However, we must also consider the possibility that the mutations we introduced in the 3' region of ORF 2 may adversely affect the potential frame sift which is important for the translation of the ORF 2 proteins.

Since results from Paper II also showed that only 124 amino acids in the C-terminus of VP1 are important for RNAi suppression activity (figure 4), it may very well be possible that the product of ORF 1 is post-translationally cleaved to produce two or more separate proteins. We could detect one potential 3C protease site between amino acids 70-78, YEKKQNHTF (Figure 4). If this prediction is indeed true, then the N-terminus protein does not include the predicted leucine zipper motif. Since we do not have a method to test the validity of the predicted protease cleavage site at this point, we decided to investigate the role of the leucine zipper motif. We hypothesized that if the N-terminus of VP1 encodes a longer protein (that includes the leucine-zipper motif), this protein may have a function independent of the C-terminus protein. We mutated the predicted leucine-zipper motif, to investigate its role in the virus life cycle (unpublished data, Sajna Anand Sadanandan and Jens-Ola Ekström). A leucine zipper mutant, NV-VP1(Δlz) wherein the four leucines were mutated to glycines (Figure 4), constructed using the cDNA clone was microinjected into *Relish*^{E23} wild type flies. Total RNA was extracted from the flies and quantitative RT-PCR was performed to verify virus titers. We could not detect any virus titer from the leucine zipper mutant, although the flies injected with the wild type virus showed expected virus titers. This suggested that, similar to the C-terminus of VP 1, the predicted leucine-zipper motif is also important for the replication of the virus RNA.

All our results indicate that any and all proteins expressed by ORF1 are important for the actual replication of the virus genome. The protein encoded by the 3'-region appears to have multiple roles as both an RNAi suppressor and as a component of the replication mechanism.

Nora virus VP1 sequence

MINNQTNKKGPQLERVHFGSTQVVGKSTKRRQRGTKLDIEYTVRRNDAPKEQKFLIS
EIFDEKLDKQIKYEKKQNHTFIKPKLNLVIKEEQHITKKVLRGKERAATHAFMKEMV
ESNKIQPSWNVEYEKEIDEVDLFFMKKKTKPFSGFSIKELRDSLIVQSDDKNMAQPT
VMSSIDEIVTPREEISVSAISEQLASLMERVDKLEKMNAALEEENKQLKKEREATIK
SVKKEAKKIKQEKPQIVKKTQHKSLGVNLKITKTKVVGQEQCLEIENTQHKKFVEKP
SMPLKVSKKMTEHQLKKTIRTWYEFDPSKLVQHQKEVLNSVVTNTTFADKVRETGIP
KQKIRYVAKPPAEEKRSIHFYGYKPKGIPNKVWWNWVTTGTAMDAYEKADRYLYHQF
K*REMMIYRNKWVKFSKEFNPYLSKPKMVWEENTWEYEYKTDVPYNFILKWRQLVQTY*
KPNTPIQADWYKISQKQQC

Predicted protease cleavage site, Leucine zipper motif and Viral suppressor of RNAi are indicated.

NV-VP1(Δlz) : Four leucines in the leucine zipper were replaced with glycines

NV-VP1Δ(401-422) : Amino acids 401 to 422 were deleted

NV-VP1Δ(401-456) : Amino acids 401 to 456 were deleted

NV-VP1(K366A) : Lysine in position 366 replaced with Alanine

NV-VP1(WW375AA) : Two tryptophans in positions 375 and 376 were replaced with Alanines

NV-VP1(W378A) : Tryptophan in position 378 was replaced with Alanine

Figure 4: Nora virus VP1 protein sequence. Predicted protease cleavage site (green), leucine zipper motif (red) and the viral suppressor of RNAi (grey) are indicated within the sequence. The different mutants constructed to investigate the function of VP1 are also indicated in the sequence.

2.5.4 ORF 2-Encoded Protein: VP2

The functional characterisation of VP2 is discussed in **Paper III**.

Open reading frame 2 is the largest ORF in the Nora virus genome, producing a 2105 amino acids long peptide. It encodes a replicative cassette similar to picorna-like viruses that includes a helicase, protease and an RNA-dependant RNA polymerase (RdRp) (Figure 2). Both the polymerase and the helicase show similarity to known picorna-like viruses such as the *Picornaviridae* and the *Iflaviridae*. The *Picornavirales* order is characterized by the presence of a well-conserved RNA dependant RNA polymerase (RdRp) and a superfamily 3 helicase. Koonin and Dolja (1993) compared the RdRps and helicases of several viruses that fall within *Picornavirales*. They found that the phylogenetic tree of the RdRp of picorna-like viruses gave six clades. Nora virus falls within the third lineage in Clade 1 (*Comovirus* and *Dicistrovirus*) along with *Iflaviridae* such as the IFV and other insect viruses. However, comparisons based on the helicase, placed Nora virus in the 6th clade along with other *Picornaviruses* (Koonin et al., 2008). But apart from these proteins that have been identified based on *in silico* homology studies, there are large portions of the ORF 2 sequence, where we do not yet know the protein encoded (Figure 5).

As shown in paper III, we performed secondary structure prediction for these uncharacterised regions. The protein encoded by the 5'-region of ORF 2 is estimated to be primarily α-helical. Two hydrophobic regions (amino acids 25-47 and 68-90) were predicted in this protein and these regions showed a high probability for being transmembrane domains (Habayeb et al., 2006). The region between the helicase and protease was also predicted to be predominantly α-helical. However we could not detect any known functional motifs or domains within this region. In order to characterise the function of these unknown proteins I constructed mutants wherein 10 amino acids were deleted from different regions of VP2. These mutants were then tested in $Relish^{E23}$ flies by microinjection. My results showed that out of the three tested mutants, two hindered genome replication and we could not detect any virus titers in injected animals. One of these mutations was placed close to the N-terminus of the predicted helicase and the other was placed in the region

between the Helicase and Protease (Figure 5). The first mutation, which disrupts one of the perdicted transmembrane domains however, did not hamper genome replication. I was able to detect this mutant in the injected animals, but not in the offpsring of the injected animals. This indicated that although genome replication progressed without impediments, the virus was now incapable of transmitting via the fecal oral route. To understand why the virus was not transmission-capable I attempted to purify these mutant virus particles using rate-zonal separation. However, virus particles could not be purified for the VP2 transmembrane mutant. This suggests that although genome replication was occurring in the VP2 transmembrane domain mutant, virus particles were not being assembled.

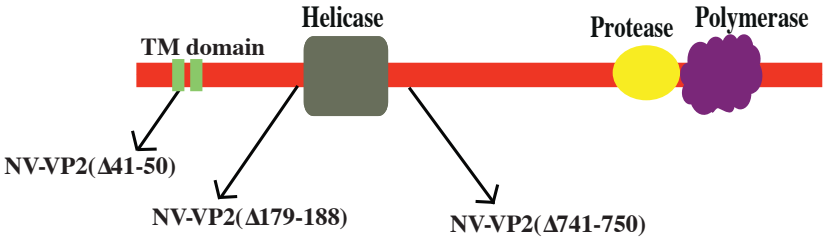

Figure 5: Nora virus ORF 2 image where the helicase, protease and polymerase genes are indicated. The three VP2 mutants tested are also denoted.

As explained earlier, the known genes encoded by ORF 2 are the genes involved in the replication of the genome, but the 5'-region of ORF 2 appears to encode a protein that is not involved in genome replication. The fact that a VP2 N-terminus mutant with a disrupted transmembrane domain can no longer produce virus particles suggests that this protein might be involved in either the translation/maturation of capsid proteins or in the actual assembly of the virus particles. It would be interesting to purify this protein, study its structure and to further dissect how this protein is involved in the production of Nora virus virions.

2.5.5 ORF 3-Encoded Protein: VP3

The functional characterisation of VP3 is discussed in **Paper I**.

The ORF 3-encoded VP3 protein is 281-304 amino acids long, depending on the position of the amino terminal. Considering that there is a 71-nucelotide overlap between ORF 2 and ORF 3, the translation of ORF 3 may occur through a frame shift or via an internal ribosome entry site. This places the theoretical molecular mass of the protein between 31-34 kDa. As shown in paper III, we performed GEMMA (Gas-phase Electrophoretic Mobility Molecular Analyzer) analysis on the purified VP3 protein to verify the molecular mass. GEMMA detects the electrophoretic mobility of particles in air to calculate the molecular mass of the particle. GEMMA results for VP3 showed that the protein in its native form had a propensity to form trimers with an estimated molecular mass of 97 kDa.

Theoretical prediction of the secondary structure of VP3 was first performed by Ekström et al. (2011), where the protein was estimated to be primarily α-helical with a prominent coiled coil motif in approximately the first 200 amino acids. To verify if the predicted structure of VP3 is true, we performed circular dichroism on the purified VP3 protein. Circular dichroism spectra clearly showed that the predicted α-helical nature of VP3 was real and that an estimated 77% of the protein was α-helical. We also performed supplementary *in silico* analysis on the secondary structure of VP3 from not only the *Drosophila* Nora virus, but also from Nora-like virus sequences obtained from other insects. This analysis showed that the predicted coiled coil between amino acids 36-200 was extremely well conserved among all Nora-like viruses, particularly the ones infecting dipteran and lepidopteran insects. Detailed analysis of the coiled coil prediction using different algorithms indicated that this motif was not a single long stretch, but rather split into three short parts. Another interesting observation was that, although the secondary structure of the different Nora-like viruses was remarkably well conserved, their similarity at the sequence level was modest. The amount of similarity between the different Nora-like virus sequences also mirrored the phylogenetic relationship between their hosts. For example, Nora-like virus VP3 sequences from dipterans are more similar to each

other than they are to the lepidopterans and the lepidopteran Nora-like viruses are most similar to each other. When we align only the dipteran Nora-like viruses, the maximum amount of sequence identity is seen in the first 100 amino acids, which covers a portion of the predicted coiled coil. This same region also shows the most sequence identity to lepidopteran Nora-like viruses, indicating that the predicted coiled coil domain plays an important role in the functioning of this protein.

Sequence homology analysis of VP3 does not show any significant relationship to other known virus proteins (Habayeb et al., 2006) and this made it difficult to predict the functional role of VP3 in Nora virus life cycle. When mass spectrometry was performed on the VP4 capsid proteins, small amounts of an ORF 3-encoded protein were also detected (Ekström et al., 2011). This then led to the hypothesis that VP3 was in some manner associated with the structural proteins of Nora virus. I constructed three different Nora virus VP3 mutants using the cDNA clone of Nora virus to investigate its role in the virus life cycle. These mutants were microinjected into *Relish*^{E23} flies followed by total RNA extraction and quantitative RT-PCR. Results from these experiments showed that the predicted coiled coil domain was crucial for the transmission of Nora virus via the fecal-oral route. I then compared the stability of these three mutant virions to wild-type virions under heat and protease treatment conditions. It was then observed that the coiled coil domain of VP3 was essential for the stability of Nora virus virions, while the region downstream of the coiled coil plays a less important role in this function. To the best of our knowledge, Nora virus is the first picorna-like virus shown to have a functionally crucial coiled coil domain in a capsid-associated protein.

2.5.6 ORF 4-Encoded Proteins: VP4A, VP4B, and VP4C

The Nora virus ORF 4 encodes three proteins, VP4A, VP4B and VP4C, that form the capsid structure of the virions (Figure 2). These proteins were identified through mass spectrometry analysis of the virion since they do not show sequence similarity to any known virus proteins. The organization of Nora virus capsid proteins in the genome is very different from that of other picorna-like viruses (Ekström et al., 2011). Tertiary structure prediction showed that the first and second proteins that are 29 and 28 kDA respectively, form β-strand jelly roll motifs, while the third protein of 45 kDa molecular mass most likely forms an α-helix (Ekström et al., 2011). The observation that Nora virus capsid proteins obtained from fly feces exhibited a molecular mass approximately 1 kDa less than those from flies suggested that the proteins may be post-translationally processed or cleaved as part of a maturation process (Ekström et al., 2011). No known picorna-like viruses have been observed to produce structural proteins with α-helical secondary structure. This, combined with the observation that VP4C is not present in the feces, led to the hypothesis that Nora virus capsids are made of only two proteins, VP4A and VP4B. However, it is also possible that VP4C is still a part of the capsid protein, and despite differences in secondary structures, the three proteins together form a capsid structure similar to other picorna-like viruses.

2.6 Spontaneous Mutagenesis in the Nora Virus Genome

2.6.1 Background

RNA replication in picornaviruses is extremely error-prone because most viral RNA dependent RNA polymerases lack an effective proofreading mechanism. Mutation frequencies in RNA viruses typically range from 10^{-3} to 10^{-6} per site per replication (Holland et al., 1992). This leads to the presence of several quasi-species within a population (Moya et al., 2000; Steinhauer and Holland, 1987). The interaction between the different quasi-species aids in the evolution of that population under varying environmental conditions and stress factors. Such an evolution is however, heavily dependent on replication mechanism, population history and of course selection pressure (Lauring et al., 2013).

The evolution of Nora virus was studied by Webster et al. (2015) and they detected a mutation rate of approximately 4 to 8 x 10^{-4} per nucleotide per year. They also showed that Nora virus proteins are highly conserved with little evidence for positive selection. Three out of four positively selected codons were observed in the structural proteins whereas, the VP1 protein, which has shown rapid host-specialization (van Mierlo et al., 2014), displayed no positive selection.

To study the spontaneous occurrence of mutations in Nora virus cultured under different conditions I performed a long-term experiment. In this study, three different fly stocks, one wild-type, one deficient in the RNAi pathway and one overexpressing a JAK-STAT pathway component were infected with Nora virus. I followed the status of their infection and periodically analyzed the pathogenicity of Nora virus cultured in the two mutant fly stocks. My results indicated that Nora virus was dependent on the over-expression of JAK-STAT and could not survive without it. After 3 years I sequenced the entire virus genome to verify the accumulation of spontaneous mutations. Unfortunately, by this point there was a massive virus contamination in the fly stocks and my sequencing results could provide no information about mutagenesis.

2.6.2 Results and Discussion

The first step in this project was to choose fly stocks where the accumulation of mutations in Nora virus could be followed. Three stocks were chosen, 1) *Relish*^{E23} (Hedengren et al., 1999) where a P-element has been precisely excised from an insertion in the *Relish* gene, thus reverting it to its wild type condition, 2) *AGO2*^{414} (Okamura et al., 2004) that harbors a mutation in the Argonaute-2 protein making it deficient in the RNAi pathway and 3) *Hop*^{Tuml} (Luo et al., 1995), which constitutively over-expresses the JAK-STAT pathway.

The original infection status of these stocks was verified to ensure that they were free of Nora virus infection. Once it was clear that the intended fly stocks were not infected with Nora virus, I then proceeded to infect these flies. Fresh syncytial stage embryos were collected from each of these fly strains and microinjected with the wild type cDNA clone of the virus to establish infection. This ensured that the starting population in each fly stock was homogenous and that any new spontaneously established mutations in the virus populations could be identified. Three replicates called E23-1, E23-2, E23-3, Ago2-1, Ago2-2, Ago2-3 and Tuml-1, Tuml-2 and Tuml-3 were generated for each infected fly stock. These infected flies were bred for 3 generations following which quantitative RT-PCR was performed on total RNA from them to confirm infection status of each replicate of the three infected fly strains. Once virus infection was established in these nine fly populations (Figure 6), the infection was maintained in these stocks for three years such that any changes in the infection status and pathogenicity of the virus could be followed.

Six months after establishing the infection the first pathogenicity assay was performed. In this assay, I took infected male *AGO2*^{414} and *Hop*^{Tuml} flies and transferred them into fresh fly food. These flies were cultured in this fly food for 2 days following which they were removed. The fly food is now contaminated with feces from the infected flies. Uninfected *Relish*^{E23} flies were transferred to the contaminated fly food and cultured for 7 days. The uninfected flies will now be infected because of the contaminated food. Total RNA was extracted from the newly infected *Relish*^{E23} flies and quantitative RT-PCR was performed to verify virus

titers. As a positive control, Nora virus from infected *Relish*^{E23} flies was transferred to uninfected *Relish*^{E23} flies. It was hypothesized that, if for example, the virus cultured in *AGO2*^{414} had accumulated debilitating mutations in the suppressor of RNAi (the C-terminus of the VP1 protein), it would now be incapable of replicating in the *Relish*^{E23} flies that have a functional RNAi system. The first pathogenicity assay however showed that there was no difference in the pathogenicity of the virus irrespective of the fly stock it was cultured in (Figure 7). The virus titers in the original fly stocks were similar to the titers in the newly infected *Relish*^{E23} flies, indicating that there were no significant changes in the master copy of the virus population's genome. So although there may have been mutations in individual virions, these mutations had not altered the genotype of the entire population.

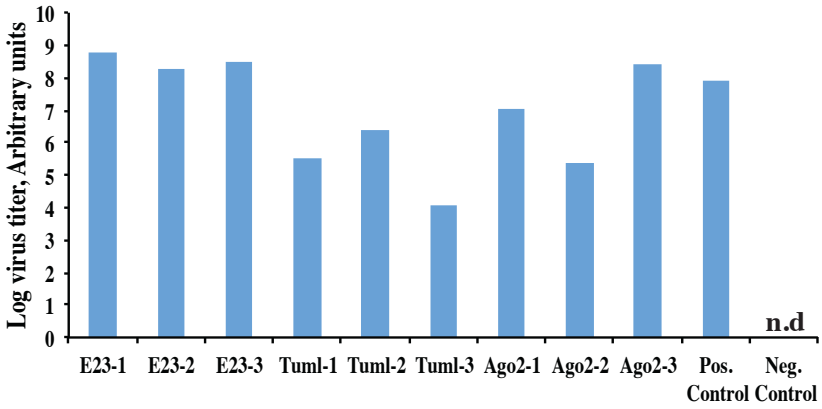

Figure 6: Establishing Nora virus infection in three replicates each of *Relish*^{E23}, *AGO2*^{414} and *Hop*^{Tuml}. RNA from another infected fly stock was used as positive control, while no template was added to the negative control.

The pathogenicity assay was repeated every few months for a period of 3 years to detect any changes in the pathogenicity of Nora virus cultured in *AGO2*^{414} and *Hop*^{Tuml} flies (Figure 7 shows one pathogenicity assay from each year). The first difference that was observed was in the second replicate of *AGO2*^{414}. One year after the infection was established, the Nora virus population from *AGO2*^{414} appeared to have problems in replicating in *Relish*^{E23} flies. Two years after the establishment of infection, all three replicates of both *Hop*^{Tuml} and *AGO2*^{414} exhibited

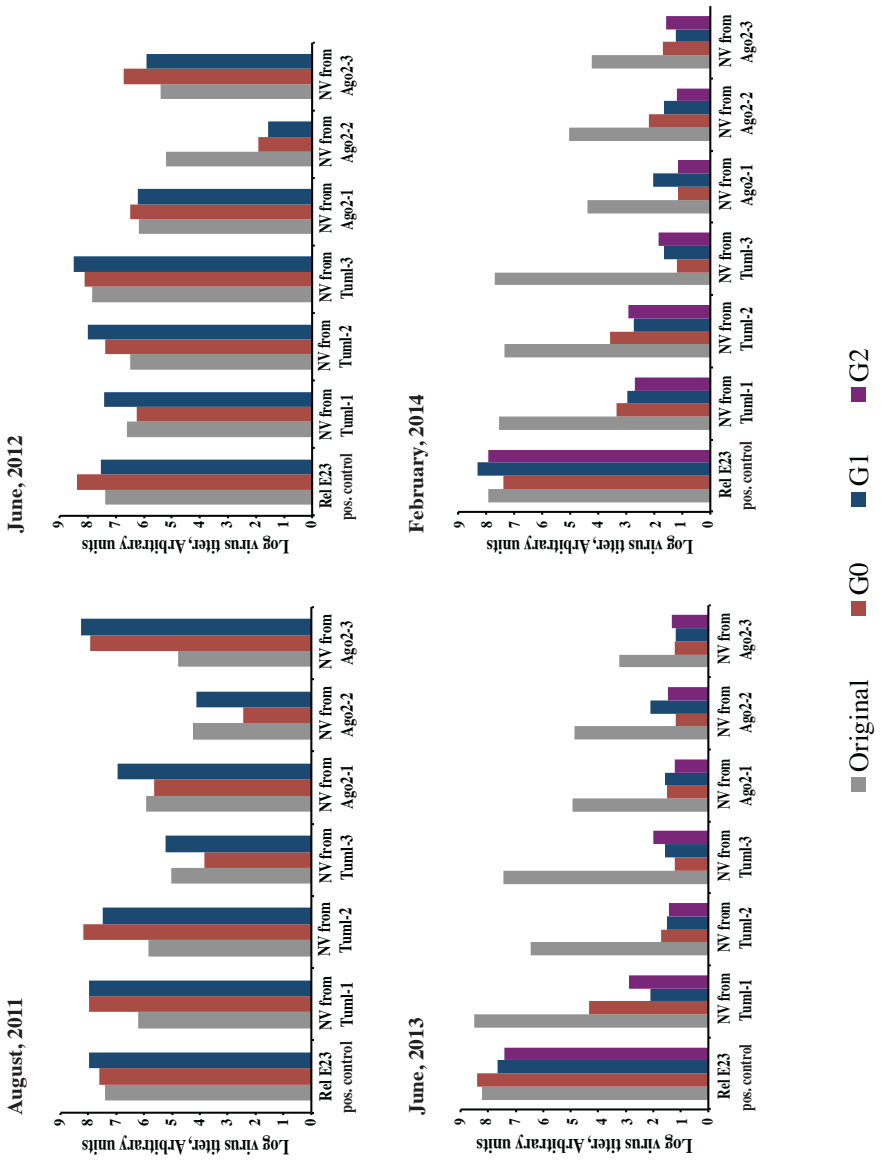

Figure 7: Pathogenicity assay over a period of 4 years. Original (grey bars) is the virus titer in the infected *Hop*^{Tuml} or *AGO2*^{414} flies. G0 (red bars) is the virus titer after transferring the infection from *Hop*^{Tuml} or *AGO2*^{414} flies to *Relish*^{E23} flies. G1 (blue bars) is the virus titer in the offspring of flies used for G0 and G2 (purple bars) is the virus titer in the offspring of flies used for G1.

difficulties in successfully replicating in *Relish*^{E23} flies (Figure 7). This indicates that these populations of Nora virus from *Hop*^{Tuml} were now dependent on the over-expression of the JAK-STAT pathway for their replication. In the case of the *AGO2*^{414} flies, it was speculated that, since the virus cultured in *AGO2*^{414} no longer required a suppressor to the RNAi pathway, it had accumulated mutations in the viral suppressor of RNAi. This version of Nora virus that now had a mutated suppressor protein could not successfully replicate in wild type flies because of the efficient immune response mounted by the RNAi pathway.

To verify if my hypothesis about the accumulation of mutations was true, it was decided to sequence the entire virus genome. To begin with, a 2300 nucleotides long stretch of the virus genome from each of the three replicates of *Relish*^{E23} and *AGO2*^{414} was sequenced. This region was chosen because it covers the VP1 protein and I wanted to verify my hypothesis that the phenotype change in the pathogenicity of Nora virus cultured in *AGO2*^{414} was due to the accumulation of mutations in the VP1 protein. TOPO-TA cloning was used to clone virus genome fragments from *Relish*^{E23} and *AGO2*^{414}, followed by Sanger sequencing, to sequence the region stretching from 399-2286 nucleotides. These sequences were translated to obtain amino acid sequences and multiple sequence alignment was used to compare the new sequences with the sequence of the cDNA clone. However, the results indicated that the cultures were contaminated because most of the mutations detected were present in both *Relish*^{E23} and *AGO2*^{414}. Only three mutations that were specific to Nora virus cultured in *AGO2*^{414} could be detected. In the first of these unique mutations a glycine at position 34 was substituted with Arginine in all the clones sequenced. In the second and third unique mutations two amino acids, a Cysteine and an Alanine at positions 524 and 527 were substituted with a Valine and an Asparagine respectively. These were the only mutations that were specific to *AGO2*^{414} and were present in more than one clone. The fact that most mutations were present in all the 6 populations of Nora virus sequenced indicated that there has been a contamination in the different fly stocks. They were most likely infected by a different Nora virus strain that was apparently more fit, as its genome was dominant in all the virus populations. The two unique mutations detected may have occurred after this contamination event. Since the amplification and cloning of several fragments of the Nora

virus genome, for each replicate of the virus genome from *Relish*^{E23}, *AGO2*^{414} and *Hop*^{Tuml} is an extremely time and resource consuming process, it was decided that this project be discontinued. Any further results obtained from this project would carry very little significance because of the contamination.

Nevertheless, although the sequencing of Nora virus from *Relish*^{E23} and *AGO2*^{414} and *Hop*^{Tuml}could not be completed, it was interesting to note that there was a change in the pathogenicity of Nora virus cultured in *AGO2*^{414} and *Hop*^{Tuml}. The mutations that occurred in their genomes made it impossible for these virus populations to survive in wild-type conditions.

Chapter 3: Antiviral immunity in *D. melanogaster*

Unlike mammals, insects do not have an adaptive immune system. Their innate immunity has to keep them safe from all the different pathogens that they encounter. This innate immune system is ancient and it is the first line of defense against viral, fungal and bacterial pathogens, as well as parasitoid organisms. *Drosophila* being the excellent model that it is, has been extensively used to study innate immune responses against various pathogens and under various conditions. A number of these pathways are conserved between mammals and *Drosophila* and this has been the focal point of research in immunology using the fruit fly. For example, the discovery of the *Drosophila* Toll receptor (Rosetto et al., 1995) led to the identification of the human Toll-like receptors (Medzhitov et al., 1997). Similarly, the identification of the *Drosophila* peptidoglycan recognition proteins (PGRPs) (Werner et al., 2000) led to the discovery of the human PGRPs (Liu et al., 2001). It is clear that we have gained important insights into the functioning of innate immune responses following bacterial, fungal and viral infection by modeling these infections in the fruit fly.

We know less about antiviral immune responses in flies as compared to anti-bacterial and anti-fungal responses. However it is apparent that flies mount strong immune responses against viral infections. Consensus in the field has been that the RNAi pathway plays the most important role in contesting viruses, but more information is known today about the role of other signaling pathways in antiviral immunity.

3.1 The RNA interference (RNAi) pathway

The RNAi pathway plays an important role in regulating both endogenous and exogenous gene expression. Three different types of RNAi mechanisms have been described in insects, the small interfering RNA (siRNA) pathway, the micro-RNA (miRNA) pathway and the piwi-interacting RNA (piRNA pathway) (Kemp and Imler, 2009). The siRNA pathway recognizes double-stranded RNAs that are formed as intermediary stages in virus genome replication. The recognized double-

stranded RNAs are processed by Dicer-2 to generate virus-derived siRNA that are then loaded onto the RNA induced silencing complex (RISC). The loaded siRNAs act as templates for targeted cleavage by Argonaute-2 (Okamura et al., 2004). Another important component of the siRNa pathway, is Ars2, which is crucial for efficient Dicer-2 mediated processing of dsRNAs (Sabin et al., 2010). The siRNA pathway has been shown to be active against several different viruses (Chotkowski et al., 2008; Sabin et al., 2009; van Rij and Andino, 2006). Apart from its role in the siRNA pathway, Dicer-2 also triggers downstream signaling cascades that can induce the Vago protein. Vago is an antiviral molecule that can restrict viral replication in insects (Deddouche et al., 2008). The induction of Vago via Dicer-2 during DCV infection can negatively control virus titer in the fat body (Dostert et al., 2005; Sabin et al., 2009). It is possible that Dicer-2 induces other genes also, which may play important roles in antiviral immunity.

It is also well documented that viruses often encode a protein that can suppress the RNAi pathway (Li et al., 2002; Nayak et al., 2010; van Mierlo et al., 2012; van Mierlo et al., 2014). Such proteins may interfere with or impede the functioning of Dicer-2, Argonaute-2 or other components of the siRNA pathway.

3.2 The JAK-STAT pathway

In mammals, the JAK-STAT pathway combats viral infections via the interferon response (Dupuis et al., 2003). Although interferon activity has not been observed in insects, the JAK-STAT pathway performs an effective part in insect antiviral immunity also (Dostert et al., 2005; Souza-Neto et al., 2009). JAK-STAT signaling is involved in antiviral immunity against DCV, FHV and CrPV infection in *D. melanogaster*. However, the effect of JAK-STAT appears to be virus-specific since no antiviral response could be detected in the event of SINV, VSV, DXV and IIV-6 infection (Dostert et al., 2005; Kemp et al., 2013). Loss of JAK-STAT pathway leads to a moderate increase in the susceptibility of fruit flies to DCV infection, but an overexpression of JAK-STAT did not induce *vir-1* (Dostert et al., 2005). *Vir-1* or the virus-induced RNA1 is an antiviral gene that has a STAT binding site in its promoter region (Dostert et al., 2005). With respect to Nora virus however, virus

replication appeared to be normal in flies with constitutively activated JAK-STAT signaling (Habayeb et al., 2009b)

3.3 The Toll and IMD pathways

Toll and IMD pathways are both pattern recognition receptor pathways that recognize pathogen-associated molecular patterns. Hence, they are an important part of anti-bacterial and anti-fungal defense. Both pathways result in the induction of different NFκB transcription factors, which in turn trigger the expression of antimicrobial genes.

More recently, the role of Toll pathway in antiviral immunity has been identified, although the exact mechanisms involved are yet to the fully characterized (Costa et al., 2009; Ferreira et al., 2014; Zambon et al., 2005). Toll pathway responses appear to be virus specific, since the pathways or genes triggered by Toll signaling during each virus infection are different. It has been observed that both induction and/or suppression of Toll signaling can restrict both DXV and dengue virus infection in *Drosophila* (Xi et al., 2008; Zambon et al., 2005). But the antiviral activity of Toll pathway is not universal, as DCV infection is apparently not affected by the suppression of Toll signaling (Sabatier et al., 2003). It was shown that the Toll-7 receptor in *Drosophila* induces autophagy as a response to *Vesicular stomatis virus* infection (Nakamoto et al., 2012). This again suggests that Toll signaling has specific antiviral responses. In contrast to DXV, dengue virus and DCV infections inducing the Toll pathway, SINV infection induces the Imd pathway, which is also able to restrict this infection. CrPV infection does not induce the Imd pathway but it is restricted by IMD signaling (Avadhanula et al., 2009; Costa et al., 2009). Antimicrobial peptides such as Dipt8, which are typically produced as a result of Imd pathway induction can restrict SINV infection in flies (Huang et al., 2013).

3.4 Autophagy

Autophagy has been known to play an important role in development, growth and nutrient-usage, cell-death, aging, anti-bacterial and antiviral responses in *Drosophila* (Lamiable et al., 2016; McPhee and Baehrecke, 2009; Yano et al., 2008). Shelly et al. (2009) showed the activation of autophagy by Toll 7 receptor as a response against VSV in *Drosophila*.

Mutant flies with attenuated Toll 7 receptor and autophagy genes were shown to have higher titers of VSV and higher mortality rates (Nakamoto et al., 2012). The process of autophagy results in the degradation of cytoplasmic contents such that the pathogen cannot complete its lifecycle.

3.5 Conclusions: Paper IV

Although considerable progress has been made in unravelling the mechanisms of antiviral immunity, there remain several unanswered questions. In order to identify the different immune response pathways and other factors involved in the responses against Nora virus, we performed RNA sequencing on three populations of *Relish*^{E23} flies infected with Nora virus and compared this to RNA sequencing results from three populations of uninfected *Relish*^{E23} flies.

Our results provided a number of genes that are differentially regulated in response to Nora virus infection, although none of the known antiviral immune responders from the Toll and Imd pathways appear to be activated (except *Drosomycin*, which shows a 4.16 fold activation). On the contrary we have observed that several known Toll and Imd pathway genes are suppressed in our study (*DiptericinB*, *Diptericin*, *CecropinA1*, *Cecropin A2*, *Defensin*, *IM2*, *IM4*). The role of antimicrobial peptides in the defense against bacterial and fungal infections is well-documented but very little is known about the part they play in antiviral immunity. Is the suppression of Toll and Imd pathway genes during Nora virus infection directly influenced by the virus or is this part of an elaborate immune response mounted by the host? The most surprising observation is that none of the known RNAi pathway genes are activated in response to Nora virus infection, although Nora virus is a target of the RNAi pathway (Paper II).

We have also observed a 6.83 fold activation of *upd3*, which indicates an upregulation of the JAK-STAT pathway. Results from the pathogenicity assay (Figure 7), which we performed to identify the spontaneous occurrence of mutations in the Nora virus genome, had also indicated a link between Nora virus infection and the JAK-STAT pathway. Those results suggested that Nora virus was dependant on the over-expression

of JAK-STAT signaling for its replication. Could the upregulation of *upd3* that we have observed in the RNA sequencing be connected to this and could the virus have a direct role to play in this activation? However, JAK-STAT activation can also control regenerative activity in the gut epithelium, by the activation of EGFR signaling (Buchon et al., 2010), and hence, the upregulation of *upd3* might indicate damage to gut epithelium and subsequent regeneration. Interestingly, the *Tep4* gene, which is also a target of JAK-STAT (Agaisse et al., 2003) signaling, is also found to be upregulated.

Three members of the *CHKov1* and *CHKov2* (ref(3)D) are also upregulated in response to Nora virus infection. The *CHKov* genes are involved in conferring resistance against *Sigma virus* infection in *D. melanogaster* (Magwire et al., 2011), although the mechanisms behind this resistance are not clear. It has been suggested that these genes provide resistance by affecting choline metabolism and certain rhabdoviruses have been shown to use acetylcholine receptors for entry into host cells (Lentz et al., 1982).

Several heat shock proteins are also activated in response to Nora virus infection. Although the role of stress response proteins (such as the apoptotic genes, heat shock proteins, transporter proteins, etc.) against viral infections are not fully undertsood, they have been slated to play important roles in antiviral immunity (Kim and Oglesbee, 2012; Merkling et al., 2015). Their part in the defense against Nora virus remains to be answered.

As these and several other questions are left unanswered, the only unblemished ”fact” is that the host-pathogen interaction between *Drosophila melanogaster* and Nora virus is very complex. A major theme among most differentially regulated genes in this study is the deregulation of metabolic processes. Several genes which are related to cellular stress management, protein synthesis and cellular regeneration are upregulated while metabolism genes are downregulated. Considering that Nora virus replicates successfully in the fruit fly midgut without causing any visible pathology in its host, we believe that there could be an acute phase of the infection, wherein the gut is damaged, followed by subsequent regeneration of gut epthelium and metabolic activities. Such

complex interaction also indicates that the relationship between Nora virus and its host is ancient and that they have coevolved mechanisms involving both host immune responses and viral counter-responses.

Viruses infect all organisms without prejudice and every organism that has had to confront viruses has developed intricate mechanisms to contest these infections. Insects combat viral infection using their innate immune systems, since unlike mammals they do not have an adaptive immune response. Although the defence against viruses in insects and mammals depend on very different mechanisms, the underlying signaling cascades are very similar. Several pathways and factors discovered in insects have since been found to play major roles in our biology as well. A stronger understanding of the factors that are involved in resisting viral infections in insects will aid us, in undertsanding the basic aspects of antiviral defense, utilising this knowledge to combat vector-borne viral infections and in comprehending our own immune responses.

Appendix: Materials and Methods

4.1 Dissecting Fly Midgut

To identify the location of Nora virus replication fruit fly midguts were dissected as shown by (Tauc et al., 2014). The dissected tissue was washed three times in phosphate buffered saline to remove any adherent virus particles.

4.2 *In silico* Analysis

The NetsurfP 1.1 server (Petersen et al., 2009) accessed via the CBS prediction servers (http://www.cbs.dtu.dk/services) was used for the secondary structure prediction of VP1.

Paircoil 2 (McDonnell et al., 2006) and Multicoil (Wolf et al., 1997), accessed at the MIT Computer Science and Artificial Intelligence Laboratory (http://groups.csail.mit.edu/paircoil2.html and (http://groups.csail.mit.edu/cb/multicoil/cgi-bin/multicoil.cgi) was used for the prediction of the coiled coil motif and its oligomeric nature in VP1.

The percentage of α-helix in the VP1 protein was estimated using PROFsec (Rost, 1996), accessed via the PredictProtein server (Rost et al., 2004) at https://www.predictprotein.org/.

Leucine zipper prediction was performed using Prosite (Bairoch et al., 1997), also accessed via the PredictProtein server (Rost et al., 2004).

3C protease cleavage sites were predicted using the NetPicoRNA 1.0 server (Blom et al., 1996) accessed via the CBS prediction servers (http://www.cbs.dtu.dk/services).

4.3 Constructing VP1 Mutants

The VP1 mutants described in this chapter were constructed with the cDNA clone of Nora virus (Ekström et al., 2011) using standard molecular cloning techniques. The primers used are listed in Table 1.

Table 1: Primers used for the construction of VP1 mutants

Mutant constructs	Primers
NV-VP1(Δlz)	**Forward**: GAACAACTGGCATCTGGGATGGAGAGAGTTGA CAAAGGCAGAAGATGAATGCTGCTGGGGAAGAAGAAAA CAAGCAGGGAAAGAAGGAGAGAGA **Reverse**: TCTCTCTCCTTCTTTCCCTGCTTGTTTTCTTCTTCC CCAGCAGCATTCATCTTCTCGCCTTTGTCAACTCTCTCCA TCCCAGATGCCAGTTGTTC
NV-VP1 (Δ401-422)	**Forward**: GAAAAAGCTGACCGTTATCTGTATCATCAATTT AAACGAAAACCGAAAATGGTGTGGGAAGAGAATACAT GGG **Reverse**: CCCATGTATTCTCTTCCCACACCATTTTCGGTTTT CGTTTAAATTGATGATACAGATAA CGGTCAGCTTTTTC
NV-VP1 (Δ401-456)	**Forward**: GAAAAAGCTGACCGTTATCTGTATCATCAATTT AAACGAAAACCTAACACACCAAT CCAGGCTGATTGGT AT **Reverse**: ATACCAATCAGCCTGGATTGGTGTGTTAGGTTTT CGTTTAAATTGATGATACAGATAACGGTCAGCTTTTTC
NV-VP1 (K366A)	**Forward**: ATTAAGGTCACCACTGGCACAGCTATGGACGCT TATGAAAAAGCTGACCGTTATCTGTATCATCAATTTAAA CGAAAACCGAAAATGGTGTGGGAAGAGA **Reverse**: AATTAACCGCGGCCGCCTTAAGAATGCTACTTA TCATCGTAATTTTT
NV-VP1 (WW375AA)	**Forward**: ATTAAGGTCACCACTGGCACAGCTATGGACGCT TATGAAAAAGCTGACCGTTATCTGTATCATCAATTTAAA CGAAAACCTAACACACCAATCCAGGCTG **Reverse**: AATTAACCGCGGCCGCCTTAAGAATGCTACTTA TCATCGTAATTTTT
NV-VP1 (W378A)	**Forward**: ATTAAGGTCACCACTGGCACAGCTATGGACGCT TATGAAAAAGCTGACCGTTATCTGTATCATCAATTTAAA CGAATCTCGCAGAAACAACAATGTTAA **Reverse**: AATTAACCGCGGCCGCCTTAAGAATGCTACTTA TCATCGTAATTTTT

2.7.4 Establishing Wildtype and Mutant Nora Virus Infection in Flies

Relish^{E23} (Hedengren et al., 1999), *AGO2* (Okamura et al., 2004) or *Hop*^{Tuml} (Luo et al., 1995) fly stocks were used for the different experiments. These flies were reared at 25°C on standard mashed-potato fly food. Prior to microinjection syncytial stage embryos were collected from these flies on apple-juice agar plates. The collected embryos were dechorionated by rolling them gently on double-sided sticky tape. The constructed clones or the wildtype cDNA clone of Nora virus were microinjected into the dechorionated embryos (Spradling, 1986). Approximately 24 hours after microinjection, the larvae that developed from the injected embryos were collected and transferred to fly food supplemented with 0.5mM $CuSO_4$. The $CuSO_4$ activates the metallothionein promoter and thus initiates transcription of the cDNA clone. The larvae were cultured in this fly food until they reached adult stage following which they were transferred to regular fly food.

2.7.5 Quantitative RT-PCR

Quantitative RT-PCR was performed on total RNA extracted using the Aurum Total RNA Mini kit (BioRad). The I-cycle iQ Thermal Cycler (BioRad) was used for one-step quantitative real-time PCR using the probe detected system (Heid et al., 1996). The following primers were used, forward primer: 5'-TTTCACTTTACTGTTGGTCTCC-3', reverse Primer: 5'-ATTCCATTTGTGACTGATTTTATTTC-3' and Taqman probe: 5'-FAM-AGAGTTAGTGGACAAGTTAGAGACTGGCAT-TAMRA-3' (Ekström et al., 2011).

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Acknowledgements

It took all these people to get me here.

Nina Ma'am, thank you for taking such good care of that constantly sick 3 year old.
Roshini**,** you inspired me to read. Thank you!
Kalaichelvi Ma'am**,** you are undoubtedly the best teacher I ever had. Thank you!
Shruthi, Som and Mani, It's a comfort to know that you guys will always be a part of my life (despite everything I've done :P)! Thank you!
Dan Hultmark*,* thank you for this opportunity and for the incredible patience you have shown! I will strive to some day have the knowledge and wisdom that you have.
Jens-Ola*,* this thesis and I are indebted to you. Thank you!
Caroline, my first friend in Umeå, thank you for being the amazing person that you are!
Jan Larsson, thank you for always being kind.
Niklas Arnberg, your inputs saved my work. Thank you!
Hairu, you've been my sounding-board for five long years! Thank you and all the best!
Past and present members of the Hultmark lab**,** Thank you for all the thought-provoking discussions.
All the wonderful friends I've made in Umeå *(you know who you are!),* Thank you for the friday- and non-friday-fikas, corridor-chatter, our li'l mallu get-togethers, Diwali parties, dumb-charades, card games, music, dance and the food! You have all certainly helped make Umeå feel like home ☺

Ammachi, Atha, Akkachi, Machan and Appu*,* I can never thank you enough for welcoming me whole-heartedly into your family. But this is a tiny gesture of my gratitude. Thank you!

Achan and Amma*,* I wouldn't be here without your love. Thanks for believing in me and letting me choose my path. Thanks for making me believe that you will always have my back.

Sinosh, I will not demean what WE have by thanking you. You know the rest.