Nora virus as a model to study persistent infections in *Drosophila melanogaster*

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Cover: Atomic force microscopy micrograph of the Nora virus
Whenever we reach a summit, higher ones appear ahead of us.

Antoun Saade

To Ragheed...

إلى رغيد...
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II. Jens-Ola Ekström*, **Mazen S. Habayeb** and Dan Hultmark. *Drosophila* Nora virus capsid proteins differ from those of other picorna-like viruses. (manuscript)


IV. **Mazen S. Habayeb**, Jens-Ola Ekström and Dan Hultmark. Nora virus persistent infections are not affected by the RNAi machinery. (manuscript)

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Abstract

*Drosophila melanogaster* has been widely used as a model organism to study the immune responses against bacteria, fungi, parasites and viruses. Here, I present a *D. melanogaster* virus as a model to study persistent virus infections. I have discovered and characterized the Nora virus, a small picorna-like RNA virus able to persistently infect *D. melanogaster*. The Nora virus genome encodes four open reading frames; a feature not present in other picorna-like viruses. The Nora virus is not closely related to any other virus family, but rather is the first virus in a new family of picorna-like viruses. The major replicative proteins of this virus are encoded in the second open reading frame and the capsid proteins are encoded in the fourth open reading frame. The sequence of the capsid proteins are not obviously related to any other previously described protein. By looking at expressed sequence tags (EST) projects, we identified an EST sequence from the parasitic wasp *Nasonia* which appears to encode proteins that have sequence similarity to the Nora virus capsid proteins. I have shown that the Nora virus persists in the fly intestine however I did not observe serious pathological effects in the infected flies. The virus is shed through feces and the transmission occurs horizontally via the ingestion of virus-contaminated food. Moreover, I observed variability in the viral titers among single flies of the same infected stock. Some flies are able to clear the Nora virus but not others and this phenomenon seems to be titer-dependent. Surprisingly, none of the known *Drosophila* antiviral responses play a role against the Nora virus. In conclusion, my work shows that studying the Nora virus interaction with the *Drosophila* immune system can lead to new findings on viral persistence mechanisms of RNA viruses and of *Drosophila* viral innate immunity.
Abbreviations

Janus kinase (Jak)
Signal Transducers and Activators of Transcription protein (Stat)
Drosophila C virus (DCV)
Internal ribosome entry site (IRES)
Drosophila P virus (DPV)
Drosophila A virus (DAV)
Drosophila X virus (DXV)
Sigma virus (SIGMAV)
Sacbrood virus (SBV)
Virus–induced RNA1 (vir-1)
Small interfering RNA (siRNA)
microRNA (miRNA)
Deformed wing virus (DWV)
Cricket paralysis virus (CrPV)
Solenopsis invicta virus 2 (SINV-2)
RNA interference (RNAi)
Piwi-associated interfering RNA (piRNA)
Open reading frame (ORF)
Quantitative reverse transcription-PCR (QRT-PCR)
RNA-dependent RNA polymerase (RdRp)
Introduction

*Drosophila melanogaster* is a tiny insect that has a size of approximately 3 mm. It nourishes itself by feeding on yeast growing in decaying fruits and hence the name, fruit fly. In nature, *Drosophila* faces various environmental insults, one of them being the large amounts of viruses, bacteria, fungi and other parasites present in the nourishing rotting fruits. This environment puts high demands on *Drosophila* to cope with all the invading pathogens it encounters during its life time. The ability of *D. melanogaster* to survive these situations reflects the strength of the insect’s immune system. Viruses are among the most ancient and abundant parasites on the biosphere (Breitbart & Rohwer, 2005) and they have been in contact with insects probably as long as insects have existed. Some viruses infect the insect but get defeated by the immune system, others are able to defeat the insect and cause lethality, whereas some are able to persist in the insect and have a continuous ability to infect it without causing lethality. This persistence phenomenon probably reflects coevolution between the virus and the *Drosophila* immune system resulting in the maintenance of the equilibrium and survival of both. I have taken advantage of this wonderful insect to study the persistence phenomenon. This thesis describes the discovery and the characterization of the persistent Nora virus and its interaction with its host, the insect *Drosophila melanogaster*.

*Drosophila* as a model organism

In order to advance our knowledge in biology, one should conduct experiments with an aim of revealing new findings. Performing experiments and testing a hypothesis is a difficult task without having a well defined system which can be relied upon. *Drosophila melanogaster* has been used as model organism to address various biological aspects since the beginning of the 20th century. These studies have led to ground-breaking findings that have shaped today’s understanding of biology (Rubin & Lewis, 2000). Some advantages of using this model organism are the ease with which one can rear them in the laboratory, the very short generation time of about 14 days at 25°C and the ability to produce hundreds of offspring from few pairs of flies. In addition, the sequencing of the whole genome, the advanced molecular and genetic tools available and the availability of large collection of mutant stocks has led to the understanding of complex biological processes in areas of genetics,
development and immune defense. Some of these discoveries provided the basis for understanding similar process in mammals. With its rich history and its current molecular and genetic advances, this organism will remain to be a valuable source for future progress in biology.
**Picornavirus**

The picorna-like superfamily includes viruses that belong to several families, but still share similar characteristics. Literally the term “picornavirus” means “small-RNA virus”, it derives from the Greek word for small (pico) and the abbreviation for the ribonucleic acid (RNA). These viruses have a small (approximately 27-30 nm in diameter) non-enveloped virion (not surrounded by a membrane layer) and a positive single-stranded RNA genome encapsidated in a virion comprised of the structural (capsid) proteins. These viruses utilize their genomic RNA as an exclusive message for a single polypeptide, from which all viral proteins are produced as a result of processing (Ryan & Flint, 1997). Viruses belonging to this superfamily include the families *Picornaviridae* (which infect vertebrates), *Dicistroviridae* (which infect invertebrates), the floating genus *Iflavirus* (which infect invertebrates), *Sequiviridae* and other families (which infect plants). The poliovirus, which belongs to the *Picornaviridae*, was one of the first studied viruses due to its medical importance at that time. Thus, the major progress in the understanding of these viruses comes from research which was done on the poliovirus. The life cycle of these viruses can be summarized into the following stages: (1) binding to the cell receptor, (2) uncoating and genome release in the cytoplasm, (3) protein synthesis, (4) protein processing, (5) RNA replication, (6) virion assembly and (7) virion release (Fig. 1).
Figure 1. Schematic overview of the picornavirus life cycle. (Adapted from Maria Gullberg, University of Kalmar, Sweden).
**Drosophila viruses**

Viruses isolated from *D. melanogaster* have been first reported in the late 1960’s. I will describe these viruses, starting with picorna-like viruses and then covering other classes of viruses. Only three viruses have been characterized as picorna-like: *Drosophila* A virus (DAV), *Drosophila* P virus (DPV) and *Drosophila* C virus (DCV).

**Table 1. Drosophila melanogaster viruses.**

<table>
<thead>
<tr>
<th>Name</th>
<th>Family</th>
<th>Genome</th>
<th>Transmission</th>
<th>Diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCV</td>
<td>Dicistroviridae</td>
<td>ssRNA (+)</td>
<td>Horizontal</td>
<td>25-30</td>
</tr>
<tr>
<td>DPV</td>
<td>Unclassified</td>
<td>ssRNA (+)</td>
<td>Horizontal and Vertical</td>
<td>25-30</td>
</tr>
<tr>
<td>DAV</td>
<td>Unclassified</td>
<td>ssRNA</td>
<td>Horizontal and Vertical</td>
<td>25-30</td>
</tr>
<tr>
<td>DXV</td>
<td>Birnaviridae</td>
<td>dsRNA</td>
<td>Horizontal</td>
<td>60</td>
</tr>
<tr>
<td>DFV</td>
<td>Reoviridae</td>
<td>dsRNA</td>
<td>Horizontal</td>
<td>60-70</td>
</tr>
<tr>
<td>SIGMAV</td>
<td>Rhabdoviridae</td>
<td>ssRNA (-)</td>
<td>Vertical</td>
<td>45-100</td>
</tr>
<tr>
<td>Gypsy</td>
<td>Metaviridae</td>
<td>ssRNA (+)</td>
<td>Horizontal and Vertical</td>
<td>100</td>
</tr>
</tbody>
</table>

*Drosophila C virus* is the best characterized *Drosophila* picorna-like virus, and the only one that has been fully sequenced. Similar to other picorna-like viruses, it is a 30 nm non-enveloped virion with a positive single-stranded RNA genome (Table 1) (Jousset et al., 1977). DCV appeared to resemble the picornaviruses (*Picornaviridae*) until its genome was sequenced and shown to belong to the *Dicistroviridae*, which differs significantly from *Picornaviridae* (Johnson & Christian, 1998). Viruses of the *Picornaviridae* genome consists of a single open reading frame (ORF), with the non-structural proteins (helicase, protease and RNA-dependent RNA polymerase) encoded at the 3′ region and the capsid proteins encoded at the 5′ region of the genome. The 5′ terminus includes an internal ribosome entry site (IRES) that allows the viral RNA genome to bind the host ribosome and initiate translation (Fig. 2). In *Dicistroviridae*, the genome consists of two open reading frames, with the non-
structural proteins encoded at the 5’ end, the capsid proteins at the 3’ end and an intergenic IRES region between the two open reading frames (Fig. 2). DCV is horizontally transmitted, which means that the virus can pass from infected flies or contaminated food to other flies or larvae. Depending on the infection mode, a DCV infection in D. melanogaster can lead to different outcomes. The injection of DCV into the body cavity of D. melanogaster is extremely pathogenic and can cause lethality in as short as three days. DCV can multiply rapidly and spread to different organs, including the fatbody, trachea, visceral muscle and the epithelial sheath surrounding the egg chamber in females (Cherry & Perrimon, 2004; Lautie-Harivel & Thomas-Orillard, 1990; Sabatier et al., 2003). By contrast, DCV ingestion is less pathogenic and results in less mortality. The mortality following DCV ingestion is titer-dependent, given that the mortality rate reduces significantly when the virus is ingested at a low titer (Gomariz-Zilber et al., 1995; Jousset & Plus, 1975). It has been reported that the DCV ingestion by larvae results in minor effects, observed as accelerated larval development and enhanced egg production (Gomariz-Zilber et al., 1995).

Figure 2. Comparative genomic organization of the Picornaviridae, Iflaviridae and Dicistroviridae. VP1-VP4 encodes the capsid proteins. Non-structural proteins are the helicase (Hel), protease (pro) and RNA-dependent RNA polymerase, or the replicase (Rep). The viruses encode a poly-A-tail (An), a covalently bond protein (VPg) and contain an internal ribosome entry site (IRES).
**Drosophila P virus** (DPV) is another picorna-like virus. It is a 30 nm non-enveloped virion with a single-stranded RNA genome. DPV has not yet been molecularly characterized and is not classified. DPV is endemic in many *D. melanogaster* populations. Naturally infected strains experience little effect and the virus is vertically transmitted from the mother to its offspring. When injected, DPV can reduce the lifespan as well as cause female sterility (Teninges & Plus, 1972).

**Drosophila A virus** (DAV) is the least studied picorna-like virus. It has an RNA genome and a virion of 30 nm in diameter. The virus can be vertically transmitted (Brun & Plus, 1980).

Viruses of other families have also been isolated from *D. melanogaster*. The **Drosophila X virus** which belongs to the *Birnaviridae* family is one of them. As the name of the family reflects, these viruses have a bipartite dsRNA genome (Chung et al., 1996). DXV is non-enveloped and horizontally transmitted. Injection of flies with this virus causes lethality 10-20 days after infection and causes sensitivity to anoxia 5-7 days after infection (Teninges et al., 1979; Zambon et al., 2005). In addition to these viruses, the Sigma virus (SIGMAV) that belongs to the *Rhabdoviridae* family is found in most natural populations of *D. melanogaster*. This family of viruses is enveloped and has a negative single strand RNA genome, which means that it has to be transcribed in infected cells before translation of viral proteins can take place. SIGMAV is vertically transmitted and infected flies are also sensitive to anoxia.

The **Drosophila F virus** (DFV) which belongs to the *Reoviridae* family has been identified in laboratory stocks, natural stocks and tissue culture cell lines of *D. melanogaster*. Its non-enveloped virion has a diameter of 60-70 nm and has a dsRNA genome. DFV is neither pathogenic nor can be vertically transmitted (Brun & Plus, 1980; Plus, 1981; Plus et al., 1975a).

Finally, flies have retrotransposons, some of which are known as endogenous retroviruses. These viruses are mobile genetic elements that replicate by reverse transcription of an RNA intermediate, followed by integration of the resulting DNA into the genome of the host cells. The best characterized endogenous retrovirus in *D. melanogaster* is the *gypsy* element. They are transmitted vertically from mother to the offspring as integrated copies in the genome. The *gypsy* element can be horizontally transmitted, since larvae feeding on extract of *gypsy*
infected flies become infected (Kim et al., 1994). *Gypsy* is repressed by the *flamenco* locus and failure to control it will lead to high rates of transposition that would induce mutability.
Picorna-like viruses in other insects

Many picorna-like viruses that infect other insects have been identified and characterized. Most of these viruses are grouped either in the *Dicistrovirusidae* family or in the *Iflavirus* genus. In this section, I will describe few of these viruses which infect different hosts such as the sacbrood virus, deformed wing virus, cricket paralysis virus and the *Solenopsis invicta* virus 2.

Most of the identified honey bee viruses share the characteristics of picorna-like viruses. The sacbrood virus (SBV) is the causative agent of the sacbrood condition affecting the honey bees (*Apis mellifera*) and resulting in the death of the infected larvae (White, 1917). The name sacbrood originates from the sac-like appearance of the diseased larvae. The sac contains fluid rich with SBV particles accumulating between the body of the infected larvae and its saclike skin (Chen & Siede, 2007). The virus is believed to be transmitted to the larvae by the nurse bees that work on removing the killed larvae. Adults can also get infected with SBV without showing obvious physical signs, but may have decreased life span (Bailey, 1969). The SBV genome has been sequenced and is similar in organization to the *Iflavirus* group (Fig. 2), with the structural genes at the 5’ region and the non-structural genes at the 3’ region (Ghosh et al., 1999).

Another bee virus is the deformed wing virus (DWV). This virus is also a positive–strand RNA virus belonging to the *Iflavirus* group (Lanzi et al., 2006) and pathogenic to both honey bees and bumble bees (Genersch et al., 2006). DWV can cause a persistent infection and is detected in all stages of the honey bees as an inapparent infection (Chen *et al.*, 2005b; Yue & Genersch, 2005). The DWV can be transmitted both horizontally and vertically (Yue & Genersch, 2005; Yue *et al.*, 2007). Horizontal transmission can be divided into two routes: oral transmission by feeding on DWV-contaminated food leading to an inapparent persistent infection or transmission by the injection of the virus through a vector parasite, the mite *Varroa destructor*, leading to the development of adult bees with crippled wing phenotype (hence the name; deformed wing virus) or lethality in larvae (Yue & Genersch, 2005). Interestingly, parasite infestation of honey bees suppresses the immune system, possibly causing an increased DWV titer and the appearance of the deformed wing phenotype following this route of transmission (Yang & Cox-Foster, 2005).
The cricket paralysis virus (CrPV) was isolated in 1970 in the laboratory from early-instar nymphs of crickets that suffered from paralysis in the hind leg, became uncoordinated and died (Reinganum, 1970). CrPV has a wide host range and most importantly it is able to replicate in *D. melanogaster* flies and cell lines (Scotti, 1975). The ability of the virus to infect *D. melanogaster* was helpful in investigating the antiviral immune response in flies (Wang et al., 2006). Sequencing the CrPV genome classified it as a member of the *Dicistroviridae* (Wilson et al., 2000). Studies performed on *D. melanogaster* eggs after dechorionation excluded the vertical transmission possibility and kept horizontal transmission as the likely route for CrPV transmission (Moore & Tinsley, 1982).

Recently, a virus infecting the fire ant *Solenopsis invicta* has been identified and characterized. The *Solenopsis invicta* virus 2 (SINV-2) shares some of the characteristics of picorna-like viruses. Its genome is composed of positive single-stranded RNA, with a virion of approximately 30 nm in diameter. However, analysis of the genome revealed 4 major open reading frames with the nonstructural proteins encoded in the fourth open reading frame. Phylogenetic analysis indicates that the SINV-2 is not closely related to the *Picornaviridae*, *Dicistroviridae*, *Iflavirus* and plant small RNA viruses (Valles et al., 2007).
The immune system

The immune system plays the role of defending the host from saprophytic microorganisms and invading pathogens. It is usually divided into innate and adaptive immunity. The innate immune system is found in all animals and plants and is the first to respond to an invading pathogen. It is believed that the innate immune system lacks a memory. This means that repeated invasion with the same pathogen will always lead to a similar response. On the other hand, the adaptive immune system has so far only been found in vertebrates and is the second immune system to act upon a pathogen encounter. During the first pathogen encounter, the adaptive immune response is slow since it first needs to be activated by the innate immunity and requires time for the clonal expansion of an antigen specific receptor. Upon a second encounter of the same pathogen, the adaptive immune system utilizes its memory cells to respond quickly.

Insects are generally thought to lack an adaptive immunity. So, *D. melanogaster* has to depend entirely on its innate immunity to defend itself from the invading pathogens that it faces in its environment. Having the innate immunity as the sole immune system can be viewed either as a disadvantage or an advantage for the scientist. It is a disadvantage because it puts a limit on the use of *D. melanogaster* as a model system to study both the innate and adaptive immune system. However, it can be considered as an advantage since the presence of only the innate immunity facilitates its study without any interference from the adaptive immunity.

**Drosophila viral innate immunity**

In comparison to the well established field of *Drosophila* immunity against bacteria and fungi, the field of viral immunity is still in its infancy. Despite that, studies of viral immunity already proposed few mechanisms for host defense against viruses (Fig. 3).
Figure 3. Schematic representation of the antiviral immune response in *D. melanogaster*. RNAi is induced by the dsRNA intermediate leading to the degradation of viral RNA. The Toll pathway is induced upon a DXV infection and the Jak-Stat pathway upon a DCV infection. This induction will assumingly lead to the translocation of transcription factors into the nucleus, leading to the transcription of antiviral genes.

The RNA interference (RNAi) machinery is identified as one of the antiviral defense mechanisms in *Drosophila*. Transgene silencing was first discovered in plants but its mechanism remained unclear (Matzke et al., 1989; Napoli et al., 1990; van der Krol et al., 1990). The RNAi mechanism was first described in *C. elegans* (Fire et al., 1998), when it was shown that it is based on the ability of the cell to recognize double-stranded RNA (dsRNA) which directs the specific degradation of the corresponding mRNA. Once the dsRNA is recognized in the cytoplasm of the cell, the RNAi machinery processes the dsRNA and uses the cleaved small RNAs to target the corresponding mRNA and initiate its silencing. These small RNAs are divided into three groups: small interfering RNAs (siRNA), microRNAs (miRNA) and the piwi-associated interfering RNAs (piRNA) (Ding & Voinnet, 2007).
In *Drosophila* the dsRNA is processed by two RNase III enzymes, Dicer 1 and Dicer 2, to produce miRNA and siRNA duplexes respectively (Hammond, 2005). Dicer 2 processes viral or other exogenous dsRNA and the resulting siRNAs are directed towards the degradation of the complementary RNA (Fig. 4). In contrast, Dicer 1 processes endogenous mRNA and the resulting miRNAs are directed towards the regulation of the complementary mRNA expression. Finally, the piRNAs are important in the control of retrotransposon transposition and are found in the germline of *Drosophila* (Zamore, 2007). The generality and importance of the antiviral response provided by the RNAi machinery in *Drosophila* is evident by the outcome of several viral infections in loss of function RNAi mutants (Chotkowski et al., 2008; Galiana-Arnoux et al., 2006; van Rij et al., 2006; Wang et al., 2006; Zambon et al., 2006). These mutations lead to increased sensitivity to viral infection and enhanced mortality with higher viral loads in mutant flies. It has also been reported that Dicer-2 after sensing viral RNA in infected cells has a dual function. In addition to its involvement in RNAi, it is also important in the induction of *Vago*, a molecule that participates in the control of viral loads in the fat body (Dedhouch et al., 2008).

Some viruses have evolved to encode suppressors which block the RNAi machinery at different stages. For example, *Drosophila C* virus encodes a suppressor that specifically binds viral dsRNA and inhibits the Dicer-2 mediated processing of dsRNA into siRNA but does not bind short siRNA or disrupt the miRNA pathway (van Rij et al., 2006).

The RNAi machinery is also induced by the uptake of dsRNA via the endocytic pathway into the cell (Saleh et al., 2006). Recently, it was shown that in a challenged fly viral dsRNA is released from infected cells and internalized by uninfected cells leading to a successful systemic antiviral response (Saleh et al., 2009).
**Figure 4.** The mechanism of RNA interference directed towards an exogenous RNA.

The Jak-Stat signaling. The Janus kinase (Jak)-Signal Transducers and Activators of Transcription protein (Stat) pathway was found to induce a set of genes following a *Drosophila* C virus (DCV) infection. The majority of these DCV-induced genes are different from genes induced after a bacterial or fungal infection (Dostert et al., 2005). The *virus-induced RNAi* (*vir-1*), one of the highly DCV induced genes contains a STAT binding site in its promoter. Using mutants in the Jak-Stat pathway, it was confirmed that the induction of *vir-1* depends on the Jak-Stat pathway. Loss of the pathway leads to moderate increase in susceptibility to a DCV infection and to increased virus titer. However, overexpression of the Jak-Stat pathway did not lead do the induction of *vir-1* indicating that this pathway is required but not sufficient for the induction of the DCV-inducible genes (Dostert et al., 2005).

The Toll pathway was found to affect the antiviral response in *D. melanogaster* against the *Drosophila* X virus (Zambon et al., 2005). The DXV infection induces expression of antimicrobial peptides known to be activated also by a bacterial or fungal infection. Constitutive expression of single expressed antimicrobial peptide in an immunodeficient background did not decrease the viral loads, suggesting that these peptides do not work as
effector molecules in an antiviral response. A loss of function mutation in the gene Dorsal-related immunity factor (Dif), a transcription factor activated by the Toll pathway, leads to increased viral loads and increased sensitivity to DXV infection (Zambon et al., 2005). These results suggest that the Toll pathway plays a role in inhibiting the replication of DXV in D. melanogaster.

The Jak-Stat and the Toll pathways in D. melanogaster do not seem to have a general role in the antiviral defense in D. melanogaster since these pathways are only activated by specific viruses, and the mechanism of viral resistance is unknown (Huszar & Imler, 2008). In contrast, the RNAi machinery seems to play a general role as an antiviral defense by degrading viral RNA and inhibiting viruses from replicating their genome. The recent finding by Saleh and co-workers (Saleh et al., 2009) provides an evidence for the requirement of the spread of dsRNA to boost the RNAi machinery in order to confer a systemic antiviral immune response in D. melanogaster. Furthermore, the data argue against the categorization of the antiviral innate immune response in Drosophila as an intrinsic cell defense mechanism (RNAi) and an inducible response (Jak-Stat and Toll) (Beutler et al., 2007). The induction of the RNAi machinery at a distant location from the infection site (Saleh et al., 2009) indicates that all antiviral immune responses in Drosophila are inducible.
Persistence

Following a successful infection of the host cells, some viruses are able to evade the immune system and to reside in their host for a long period of time. The infection caused by these viruses is termed a “persistent infection”. Enteroviruses, which belong to the Picornaviridae are known to be able to cause persistent infections in their host (Galbraith et al., 1997). The poliovirus is one of the best studied viruses. It infects via the gastrointestinal tract and infected people may stay asymptomatic. The symptoms appear when the virus reaches the blood and in few cases the central nervous system where it can cause poliomyelitis (inflammation of the grey matter in the spinal cord) that may lead to paralysis (Howard, 2005). In about half of the people who have suffered from poliomyelitis the virus was found to persist in the host for a very long period of time. The persistent infection is believed to contribute to the post-polio syndrome, a condition that is characterized by fatigue, weakness and muscle pain (Howard, 2005). It has also been shown that patients suffering from chronic fatigue syndrome have a significantly higher probability of a persistent enterovirus infection in their stomach compared to control healthy individuals. In addition, the severity of the syndrome correlated with the amount of detected enteroviruses (Chia & Chia, 2008).

Most described honey bee viruses are able to cause a persistent infection. These viruses have been detected in eggs, larvae, adults and queens (Chen & Siede, 2007) and in most cases do not cause any apparent symptoms. More than one inapparent viral infection can occur simultaneously in the same bee (Chen et al., 2005a; Chen et al., 2004) and some of these viruses appear to be inducible. Stressing the bee by injecting different buffers can induce the viruses to replicate to higher titers and to cause apparent symptoms in the bee (Anderson & Gibbs, 1988). The factors that cause this induction are not clear, but it has been shown that varroa mites might induce the replication of an inapparent deformed wing virus by suppressing the immune response of the infested bees (Yang & Cox-Foster, 2005).

*D. melanogaster* persistent viral infections have also been reported. Apart from studies on the transmission (Jousset & Plus, 1975), reports on the *D. melanogaster* persistent viruses are limited to the description of viruses detected in wild-caught and laboratory stocks. DCV, DPV, DFV, DAV and SIGMAV are considered to be persistent viruses as they have been detected
in several laboratory stocks and in wild-caught flies without causing obvious effects (Brun & Plus, 1980; Jousset & Plus, 1975; Plus, 1981; Plus et al., 1975b).

The criteria for defining “persistence” differ between vertebrates and invertebrates. In vertebrates persistence is defined by the ability of the virus to bypass the initial innate immune response and not to be efficiently cleared by the adaptive immune system (Flint et al., 2004). In invertebrates the term “persistence” is often used when viruses are detected in an apparently healthy infected stock and are successfully transmitted to the next generation. It is difficult to have a general criterion for defining “persistence”. The presence of an adaptive and innate immunity in vertebrate as opposed to only an innate immunity in invertebrates is one of the factors that hinder the establishment of a common criterion. Vertebrate viruses have to overcome both the innate and adaptive immune responses whereas invertebrate viruses only have to overcome the innate immune response. Another problem for a general criterion even within the vertebrates and the invertebrates themselves is the difference in the host lifespan. For example the infection by Hepatitis C virus is termed as a persistent infection when viral RNA is detected for more than 6 months (Chemello et al., 1996; Zhong et al., 2006), a time frame that exceeds by far the Drosophila life span. It would be more realistic to establish a customized definition of “persistence” for every virus or group of viruses which infect related hosts.

Another problem in the field of persistent viruses is the use of terms to describe a persistent infection. The literature often uses the terms; “inapparent”, “latent”, “persistent” for the same virus infecting the same host. Since terms that are not agreed upon can cause confusion and misunderstanding, I will briefly clarify the way I define these terms. I will use the term “persistence” to describe the phenomenon in which a virus resides in its host for a substantial part of the host life span. “Inapparent” and “latent” can be used to describe the way this persistence phenomenon occurs. Persistence can occur in an inapparent form when the virus is replicating in the host to high or low titers without causing any obvious signs of infection. An inapparent persistent infection can change into an apparent infection that leads to an obvious disease or to death. A latent infection occurs when a virus is in a dormant form and only expressing part of its genome. In this case the virus can become periodically active in acute episodes.
Results and Discussion

My work started from the discovery of an RNA in a differential display-PCR screen aimed to identify upregulated RNAs in the fruit fly following a bacterial challenge. The PCR product from this screen was then used to screen two independent D. melanogaster cDNA libraries which were made in 1990 and 1996. The recovered clones were sequenced from their 5´- and 3´- ends and showed similarity to the RNA polymerase gene of the sacbrood virus which infects honey bees. Furthermore, my first Northern blots during 2004 showed that the same virus was still present in laboratory stocks indicating that this is a persistent virus.

The early indications that injection of bacteria into flies led to the induction of this viral RNA raised the possibility that the Nora virus is stress-induced as observed for some honey bee viruses (Anderson & Gibbs, 1988). However my work did not show an induction of the Nora virus after bacterial injection or other stress regimes such as starvation and high temperature (data not shown). Later I have shown that in Canton S flies (the same strain used which was used for the bacterial injections) a high and a low titer Nora virus infection may occur in two independent vials (Paper I). Moreover, single flies from the same infected stock showed a large variability in the Nora virus titers (Papers III and IV). Thus, the original observation of apparently upregulated viral RNA in flies challenged with bacteria can now be explained as the consequence of randomly occurring high viral titers.

The aim of my project was to characterize the newly isolated virus, examine its interaction with the fly and find possible mechanisms conferring a persistent infection.
To investigate the presence of the virus in the flies, I randomly picked laboratory fly stocks and analyzed them for the presence of the Nora virus. The virus was not detected in most stocks but found at high titers in some stocks. Interestingly, some of the stocks which showed high virus titers came from the same genetic background. This can be explained by the fact that the strain which was used to generate the different mutant stocks was infected with the virus and transmitted the virus to all the generated stocks (Paper I).

To get the full sequence of the virus RNA, I performed 5´ and 3´ rapid amplification of cDNA ends (RACE) based on the sequence revealed in the first clones (Paper I and II). The complete RNA sequence is 12,333 nucleotides followed by a poly-A tail. The genome encodes four open reading frames (ORF), a novelty in comparison to other picorna-like viruses and hence the name Nora, which means “new” in Armenian language. The second open reading frame is the largest and encodes the replicative proteins, whereas the other three remaining open reading frames encode proteins which show no similarity to previously described proteins (Paper I). A phylogenetic analysis based on the most conserved protein regions, the RNA-dependent RNA polymerase (RdRp) and the helicase, suggested that the virus is not closely related to any previously identified picorna-like virus family (Paper I). I further characterized the virus by showing that it consists of a positive-sense single-strand RNA which has no DNA intermediate and a virion of approximately 30 nm in diameter. These features are shared among the picorna-like viruses. A later report described a previously unknown fire ant virus, the *Solenopsis invicta* virus 2 (SINV-2) which also encodes four open reading frames (Valles et al., 2007). However, the organization of the four open reading frames in the SINV-2 is different from that of the Nora virus (Paper I; Valles et al. 2007) and phylogenetic analysis showed that the Nora virus and the SINV-2 are not closely related (Valles et al., 2007).

Based on the lack of sequence conservation, we were not able to find the genes encoding the capsid proteins in the genome of the Nora virus. To identify the capsid proteins, I purified the virus and separated its proteins and further analyzed them by mass spectrometry. The results showed that all protein bands contained sequences from the fourth open reading frame of the Nora virus. While one protein was encoded by the 3´ region of the fourth open reading frame, the two other proteins corresponded to the 5´ region of this open reading frame. We
conducted the same analysis for Nora virus isolated from the feces of flies. In this case, the protein bands encoded by the 5’ region of the fourth open reading frame were still present, whereas the protein band encoded by the 3’ region was absent. This suggests that the protein encoded in the 3’ region of the fourth open reading frame is not a part of the mature viral particle which is found in the feces (Paper II).

At the time of sequencing the Nora virus, no related sequences except for the second open reading frame were deposited in the databases. Recently, EST sequences from the parasitic wasp *Nasonia* were deposited. These sequences showed high similarity to the Nora virus fourth open reading frame and thus so far identify the *Nasonia* virus as the closest relative of the Nora virus. Based on our protein analyses, we hypothesized that the C-terminal protein encoded by the Nora virus fourth open reading frame is not part of the mature virion. This hypothesis is further supported by the fact that the newly identified sequence from the *Nasonia* ESTs has a frameshift in the middle of the equivalent Nora virus fourth open reading frame. The position of this frameshift would allow the translation of proteins that have approximately the sizes of the proteins detected in the Nora virus purification from feces. A difference between the Nora virus and the *Nasonia* virus is the presence of two open reading frames in the region that corresponds to the Nora virus fourth open reading frame. This might give rise to a total of five open reading frames in the newly identified *Nasonia* virus. Complete sequencing of the *Nasonia* virus RNA should be performed to get a better understanding on the differences and similarities.
The persistent high-titer virus infection observed in some fly stocks raised the question about where the virus resides. To answer this question, I dissected flies into three major parts, the head, the abdomen and the thorax. Quantification showed that the highest viral load is found in the abdomen. We subsequently dissected the abdomen into the fatbody, the reproductive tract, the intestine and the remaining carcass. The results clearly showed that most of the viral load present in the abdomen is in the intestine. By electron microscopy, we identified viral particles of a size similar to that of the Nora virus in the lumen of the posterior midgut. Unfortunately, there are still unresolved questions which are of importance to address such as the site of viral replication. A possible reason for not finding the site of viral replication can be due to their presence in a different site than the examined posterior midgut. The demanding task to examine the whole intestine by electron microscopy and the lack of a good antibody prevent us currently from answering this question. Given the fact that most of the viral RNA was found in the intestinal tract, it is likely that the intestine is the site of viral replication. The intestines of infected flies were abnormally fragile during dissection and showed vacuolization in histological sections. It is surprising to observe such morphological changes in these cells without detecting the Nora virus in them. A possible explanation for this observation is that the Nora virus induces morphological changes in cells at distant sites.

Knowing that the virus is concentrated in the intestine, we were interested to see if the virus is able to reach the external environment through the fly feces. The quantity of viral RNA in the feces produced in a five hour period was in fact almost equal to the total viral RNA present in the body of the flies. This result shows that the Nora virus is continuously replicating in the flies and shed with the feces at a high rate.

Since the virus was found in large amounts in the feces it was possible that the transmission of the virus is via an oral-fecal route. My next experiments were designed to find the route of viral transmission. Embryos laid by uninfected flies were allowed to develop into adults on Nora virus-contaminated food. High viral titers were present in the adult flies clearly demonstrating that the virus can be transmitted horizontally via the oral-fecal route. No viral RNA was detected in fly stocks established by dechorionating eggs from infected flies and placing them in fresh uninfected food vials indicating that the Nora virus is not vertically transmitted.
To establish two genotypically identical strains that differ only by the presence or absence of Nora infection, I injected an uninfected Oregon-R stock with virus preparations. The virus titers started to increase after one day and reached a plateau within 7 days. Furthermore, similar viral titers of Nora virus were detected in the offspring of these flies. This demonstrates that it is possible to establish a Nora virus infection by injecting flies with the virus. Using flies which have the same genetic background and only differ in the presence or absence of a Nora virus infection, we were able to study whether the virus has an effect on the fitness of the fly. The survival of infected and uninfected flies was the same for at least the first 50 days. After that we observed a slight difference with infected flies starting to succumb faster than the uninfected flies. Although this might be due to the virus infection, this observation most likely has no significance in nature because flies usually do not live for 50 days as laboratory stocks. However, flies in nature are exposed to various stress factors which are absent in the laboratory setting. Therefore, the small difference in longevity observed in the laboratory might become prominent in nature leading to a significant decrease in the life span of infected flies.

On the other hand, studies have shown that latent viral infections may protect against harmful bacterial infections (Barton et al., 2007). I have not addressed this possibility and therefore, at this stage a beneficial effect of a Nora virus infection cannot be excluded.

I have noticed that the viral titers were variable even between batches of identically treated flies. I thought that this is due to variability in the viral titers of single flies within the same stock. This prompted me to examine the viral titers of the individual flies. Indeed, I found that the viral titers differ by as much as a factor of $10^3$ between single flies from the same vial. This result shows that flies from the same population have different “susceptibility” to the infection. Therefore, in subsequent experiments I analyzed single flies instead of fly batches.
Paper IV

The large variability in the Nora virus titers between individuals of the same infected stock raised the question whether flies are able to clear the virus. To address this question, I conducted experiments by transferring single wild-type flies into clean bottles. This treatment resulted in flies harboring high, medium, low or undetectable titers of viral RNA. Next, I followed the virus titers in the feces of single flies for a period of time. Interestingly, I observed that flies which produced high titers of virus in the beginning of the experiment sustained a high level of virus production during the entire course of the experiment. In contrast, flies that produced less virus at the beginning of the experiment showed a dramatic decrease in viral production during the first four days. Thereafter, the viral production continued to decrease but at a slower rate. A few flies contained the virus at a medium titer, but they tended to either increase or decrease their viral production approaching the high- or low-titer populations. This result showed that the clearance of the Nora virus is threshold-dependent as for a virus to stay at a high titer it has to be over a certain threshold.

To investigate whether the immune system plays a role in controlling the viral titers, we infected flies harboring mutations in genes involved in known D. melanogaster antiviral response pathways. If these genes contribute to the control of the viral titers, I expected to observe either lethality or increased viral titers in mutants compared to wild-type infected flies. I started by infecting mutants in the RNAi pathway which was shown to play an important role during viral infections in D. melanogaster and other organisms (Chotkowski et al., 2008; Galiana-Arnoux et al., 2006; van Rij et al., 2006; Wang et al., 2006; Zambon et al., 2006). Surprisingly, the RNAi mutants did not die from the infection nor had increased viral titers as compared to the wild-type flies and they were still able to clear the virus. We further tested flies mutant in the Toll pathway (Zambon et al., 2005) and observed no difference between mutant and wild-type flies. Finally, we tested a constitutively active mutant of the Jak-Stat pathway (Dostert et al., 2005) assuming that if this pathway plays a role in controlling the viral titers, then constitutively activating the pathway would decrease the viral titers in the infected stocks. The infected Jak-Stat mutant flies gave similar results to the wild-type and the previously tested mutants. Altogether, these data suggest that the observed variability and clearance of the Nora virus are not RNAi-dependent. Additionally, they demonstrate that the Toll pathway is not involved and the Jak-Stat pathway is not sufficient to affect viral clearance.
Since the Toll and the Jak-Stat pathways seem not to be of general importance for the *Drosophila* antiviral defense but rather specific for the DXV and DCV respectively, it was not surprising that these pathways are not essential in the clearance or control of the Nora virus. A more unexpected finding was the absence of lethality or increased viral titers in the RNAi mutants and the ability of these mutants to clear the Nora virus. The inducer of the RNAi machinery is the intermediate dsRNA that is produced during the replication of positive-single strand RNA viruses. The Nora virus is a positive-single strand virus and thus must produce dsRNA as an intermediate during its replication. The high viral loads shed in the feces and the ability of the virus to maintain a high-titer infection in the flies, clearly showed that the Nora virus is replicating in the flies. However, the previous experiment suggested that the Nora virus clearance and replication is not affected by the RNAi machinery. I propose two possible explanations for the insensitivity of the Nora virus to the RNAi machinery. First, the Nora virus encodes a potent inhibitor of the RNAi machinery which renders the virus completely insensitive to the machinery. Therefore, mutating genes in the RNAi machinery will not influence the interaction of the Nora virus with the flies. However, viruses which encode inhibitors of RNAi, such as the *Drosophila* C virus, are nevertheless still affected by the machinery. This becomes evident during an artificial inhibition of the RNAi machinery, which results in increased viral titers and pathogenicity of these viruses (van Rij *et al.*, 2006). The second possibility is that the Nora virus replicates in a compartment where it is not accessible to the RNAi machinery. It is well established that some positive-strand RNA viruses replicate their genome in association with intracellular vesicles (Venter & Schneemann, 2008) and potentially hide the virus replication intermediates from cellular degrading proteins or immune defense.
Concluding Remarks

At the beginning of my project, the field of *Drosophila* picorna-like viruses was limited to the isolation and description of the viruses. Molecular work was done solely with the *Drosophila* C virus which was the only completely sequenced virus (Johnson & Christian, 1998) while the *Drosophila* antiviral immunity work was not yet started. As I progressed with my work, few other laboratories started to investigate the best-described *Drosophila* viruses and their interaction with the immune system. These studies focused on pathogenic viruses causing acute lethality or increased mortality in the infected stocks. In contrast, my work was directed towards the understanding of a virus that causes a persistent asymptomatic infection.

In conclusion, we now have a novel persistent *D. melanogaster* virus that is different from previously described *D. melanogaster* viruses. The virus is highly abundant in the intestine and is transmitted horizontally via the fecal-oral route. There is a high degree of variability in viral titers within the infected fly populations and a threshold titer may determine the persistence of the virus. The mechanism that clears the Nora virus is not provided by the RNAi machinery, the Jak-Stat or the Toll pathways. This basic understanding of the Nora virus establishes the basis for future Nora virus-*Drosophila* interaction studies.

The results from some of the experiments raise more questions than answers. Some of these questions are: What controls the Nora virus titers and keeps the virus in check not to cause lethality? What is the clearance mechanism? How can the Nora virus evade the RNAi machinery? The ultimate goal of a virus is to sustain its existence by replication and spread within its host. The Nora virus-*Drosophila* interaction secures the existence of the virus and its transmission without causing obvious pathological effects on its host; therefore it is an ideal viral-host interaction. As it seems, the control mechanism(s) maintaining this delicate balance are playing an important role, if tipped off may result either in the virus elimination or the host death. The titer-dependent clearance of the Nora virus indicates the presence of host defense mechanisms. Almost certainly the control of the viral loads and the absence of lethality phenotype are not due to any of the known *Drosophila* antiviral mechanisms (RNAi, Jak-Stat and Toll) and it remains to be discovered which components of the immune system play this role. On the other hand, the idea of a virus self-control mechanism should also be considered. Such a control mechanism is not unlikely allowing the presence and spread of the virus without causing lethality. Addressing these questions is important to deeper understand
the Nora virus-\emph{Drosophila} interaction. Future experiments and utilization of newly developed tools, such as the Nora virus cDNA clone will certainly help to answer these questions and may lead to new findings. Hopefully these new findings will contribute to the advancement of the knowledge in the field of the interaction between persistent viruses and innate immunity.
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