To a first approximation, all animals are insects.

Robert May

GHENT UNIVERSITY

FACULTY OF BIOSCIENCE ENGINEERING

RNAi efficiency in insects: dsRNA uptake mechanisms and viral suppressors of RNAi

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The author's own artwork depicting the main subjects of this thesis: the RNAi process, the bumblebee and the Colorado potato beetle.

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LIST OF ABBREVIATIONS

AA	amino acid
ABPV	*Acute bee paralysis virus*
ACN	acetonitrile
Ago	Argonaute
A. mellifera	*Apis mellifera*
AMP	antimicrobial peptides
ANOVA	analysis of variance
bp	base pairs
BQCV	*Black queen cell virus*
B. terrestris	*Bombus terrestris*
CBPV	*Chronic bee paralysis virus*
cDNA	complementary DNA
chc	clathrin heavy chain
CDS	coding DNA sequence
Ce	*Caenorhabditis elegans*
C. elegans	*Caenorhabditis elegans*
CPB	Colorado potato beetle
CPZ	chlorpromazine hydrochloride
CrPV	*Cricket paralysis virus*
CV	coefficient of variation on the normalized relative quantities
Dcr	Dicer
D. melanogaster	*Drosophila melanogaster*
dpi	days post infection
dsRNA	double-stranded RNA
dsX	double-stranded RNA targeting gene X
DWV	*Deformed wing virus*
EPPO	European and Mediterranean Plant Protection Organization
ESI-MS	electrospray ionization mass spectrometry
FDR	false discovery rate
$HDMS^E$	high definition mass spectrometry
GFP	green fluorescent protein
GO	gene ontology
IAPV	*Israeli acute paralysis virus*
IRES	internal ribosome entr site
KBV	*Kashmir bee virus*
LC_{50}	lethal concentration which corresponds with 50% mortality
Ld	*Leptinotarsa decemlineata*
L. decemlineata	*Leptinotarsa decemlineata*

LD_{50}	lethal dose which corresponds with 50% mortality
lncRNA	long non-coding RNA
M	geNorm M value
miRNA	microRNA
MMTS	methyl methanethiosulfonate
mRNA	messenger RNA
n	number of individuals
ncRNA	non-coding RNA
nt	nucleotides
ORF	open reading frame
p	probability
PAMP	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PCA	principal component analysis
PCR	polymerase chain reaction
piRNA	piwi-interacting RNA
ppb	parts per billion
ppia	*peptidylprolyl isomerase a*
ppm	parts per million
PRR	pattern recognition receptor
RISC	RNA-induced silencing complex
RNAi	RNA interference
rpl23	60S ribosomal protein L23
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
RT-qPCR	reverse transcription quantitative polymerase chain reaction
SBPV	*Slow bee paralysis virus*
SBV	*Sacbrood virus*
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
S. gregaria	*Schistocerca gregaria*
Sid	systemic RNA interference-deficient
Sil	systemic RNA interference-deficient -1-like
siRNA	small interfering RNA
Tc	*Tribolium castaneum*
T. castaneum	*Tribolium castaneum*
TCEP	tris(2-carboxyethyl)phosphine
TEABC	triethylammonium bicarbonate
TEM	transmission electron microscope
ubi	*polyubiquitin B*
UPLC	ultra performance liquid chromatography
UTR	untranslated region

VDV-1	*Varroa destructor virus 1*
vha	vacuolar H+ ATPase
vha16	16 kDa subunit of the vacuolar H+ ATPase
vha68	68 kDa subunit of the vacuolar H+ ATPase
VPg	virus genome-linked protein
VSR	viral suppressor of RNAi
w/v	weight/volume

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SCOPE

RNA interference (RNAi) has been heralded as promising in entomological studies; in both research, as a gene knockdown tool, and for practical applications such as pest control and pollinator therapeutics. Unfortunately, the optimism of the early days has been tempered because of large variability in RNAi efficiency and efficacy over the different insect species. Some insects, such as most beetles, show a great RNA efficiency, even without optimization, whereas other insects, such as most lepidopteran species, do not.

Various reasons for the variability in RNAi efficiency in insects can be identified. We have selected two processes to focus on, each within an appropriate model system. The first factor is situated at the beginning of the RNAi process at whole body level: the uptake mechanisms for double stranded RNA (dsRNA), the trigger of RNAi. DsRNA uptake from the midgut lumen is imperative for many RNAi applications as feeding is the most practical way of dsRNA delivery. We chose to examine this in the Colorado potato beetle, which has an exceptionally efficient RNAi response. The second factor is located downstream of this process. After the dsRNA enters the cell, the core RNAi pathway exerts its function. Viruses present in the cytoplasm, whether chronic infections or not, may influence this pathway in various ways, for example by encoding viral suppressors of RNAi (VSRs). For this factor, the bumblebee was chosen as test environment, as there is considerable knowledge available on viral infections and antiviral immunity in bees.

In **Chapter I,** a brief overview of the current knowledge on RNAi in entomology is given and the factors that can affect RNAi efficiency are listed. The two selected factors, dsRNA uptake mechanisms and viral suppressors of RNAi, are described in detail. Additionally, the two model systems, the Colorado potato beetle *Leptinotarsa decemlineata* and the bumblebee *Bombus terrestris*, are discussed.

Chapter II is an intermezzo which describes the contributions that were made to the genome annotation projects for both the Colorado potato beetle and the bumblebee. The presence of the RNAi core genes is assessed in both species and some remarkable observations are mentioned.

The first selected factor, dsRNA uptake in the Colorado potato beetle, is the subject of **Chapter III**. From literature, two pathways are known likely to be involved: Sid-1 transmembrane proteins and clathrin-dependent endocytosis. First, various genes, representative for these pathways, were identified. An RNAi-of-RNAi setup was devised to test the involvement of these genes. Additionally, the efficacy of one of the identified genes as a target for RNAi-based crop control was evaluated.

To examine whether any of the known bumblebee viruses expresses a functional suppressor of RNAi, we have first attempted to identify a virus with a known suppressor, that can infect bumblebees. A likely candidate was *Cricket paralysis virus*, belonging to the same family as some bumblebee viruses. In **Chapter IV**, its infectious potential through both injection and feeding was evaluated using mortality scoring, RT-PCR and RT-qPCR. A tag-based negative strand-specific detection system was devised to confirm replication. As the results obtained here could have implications for wild bees, also natural infection-mimicking experiments and a limited prevalence screening were undertaken.

Chapter V then examines whether a functional suppressor of RNAi is present in *Israeli acute paralysis virus* (IAPV), a well-known virus of bees, using a two-fold approach. In the IAPV genome, two regions were found which could encode for a small protein. Using high definition mass spectrometry, an attempt was made to confirm the presence of these viral proteins in the ovaries. The second approach made use of a functional RNAi assay, in which the effect of IAPV presence on the RNAi efficiency was assessed in different bumblebee tissues. Based on the information gained in Chapter IV, *Cricket paralysis virus* was used as a control.

In Chapter V, an interesting contradiction was found where the ovaries have similar virus titers as the fat body, but show a profoundly different RNAi response. Also, it is known that IAPV presence can have sublethal effects on the reproduction potential of the bumblebee. Therefore, in **Chapter VI,** the dataset obtained in chapter V was further examined to gain insight in what is occurring in the ovaries during IAPV infection. Differentially expressed proteins were identified and examined using Gene Ontology information.

Finally, in **Chapter VII***,* we describe how the knowledge gained in this thesis fits within the broader scope of RNAi in insects. First, the implications of the results on dsRNA uptake and its links with the systemic properties of RNAi are evaluated, both for the Colorado potato beetle and other insects. The second part focuses on the various ways in which viral infections can influence the RNAi system of the host, especially in the context of eusociality and multivirus/multihost infections. For both factors, future perspectives, both on fundamental knowledge and on practical applications, are presented.

CHAPTER I:
INTRODUCTION

1.1 RNA interference

The central dogma of molecular biology states that genetic information is coded in DNA, which is transcribed into messenger RNA, which is in turn translated to form effector molecules, the proteins. These processes are elegantly summarized in the statement: “DNA makes RNA and RNA makes protein” (Crick 1970). The advent of genome sequencing revealed that, even though the majority of the genome (70-90%) is transcribed, only 1-2% is protein coding (Birney et al. 2007). More and more evidence is emerging that RNA is not merely a messenger, but that non-coding RNAs (ncRNAs) can also act as an effector, resulting in a much more complex genetic programming than previously anticipated (excellently reviewed in Morris and Mattick 2014).

1.1.1 *Classes of non-coding RNAs and RNA interference*

It is unclear to what extent the many ncRNAs, detected using RNA sequencing, are truly functional, but so far multiple classes of ncRNAs with often regulatory biological functions have been identified. They are divided into two categories, small and long ncRNAs, depending on their size, with 200 nucleotides (nt) forming the boundary (Kashi et al. 2016). Some types of ncRNAS are present over all kingdoms, while others are unique to taxonomic groups. An overview of the classes of ncRNAs, identified in the human genome, is given in Figure 1.

Several classes of small ncRNAS are indispensable for normal cell activity in eukaryotes: the ribosomal (rRNA) and transfer RNA (tRNA), necessary for protein translation, and the small nuclear RNA (snRNA) and small nucleolar RNA (snoRNA), involved in RNA biogenesis and maturation. Other classes are grouped together under the term RNA interference (RNAi), based on the fact that they exert their regulatory role through double stranded RNA (dsRNA) effectors and interfere with gene expression at the RNA level. The small RNAs linked to RNAi are the small interfering RNAs (siRNAs), microRNAs (miRNAs) and piwi-interacting RNAs (piRNAs), all of which are dsRNA molecules, 20-35 nt in length. Various other classes have been identified, often involved in developmental regulation (reviewed in Farazi et al. 2008).

The situation for the long ncRNAS (lncRNAs) is more complex because of the large diversity in biogenesis, structure and function. They have been shown to be involved in epigenomic modification of chromatin, regulation of gene expression, mRNA transcription and translation, etc. (Kashi et al. 2016). Several links have been made between aberrant expression of lncRNAs and diseases in humans (Esteller 2011). Also in insects, reports are emerging of an abundance of (functional) lncRNAs (Brown et al. 2014; Jayakodi et al. 2015; Jenkins et al. 2015; Wu et al. 2016).

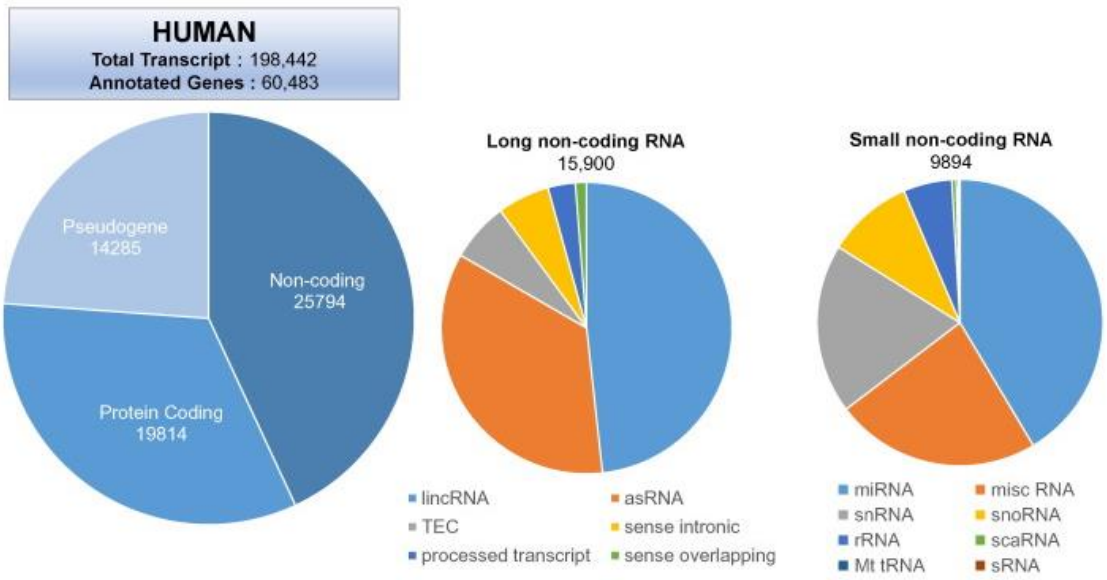

Figure 1. Current annotation for the human genome illustrating the complexity and abundance of non-coding RNAs.
Loci are divided in three different categories – protein coding, pseudogene and non-coding – according to version 22 of the GENCODE release (October 2014 freeze, GRCh38). Non-coding genes are further classified into long and small non-coding RNAs: lincRNA, long intergenic non-coding RNA; TEC, to be experimentally confirmed transcript; asRNA, antisense RNA; miRNA, microRNA; snRNA, small nuclear RNA; rRNA, ribosomal RNA; Mt. tRNA, tRNA located in the mitochondria genome; misc. RNA, miscellaneous other RNA; snoRNA, small nucleolar RNA; scaRNA, small Cajal body-specific RNA; sRNA, small non-coding RNA (adapted from Kashi et al. 2016).

1.1.2 *Biological role of RNAi in insects*

In the phenomenon of RNAi, gene expression is inhibited in a sequence-specific matter, often referred to as the 'silencing' of a gene. It was discovered in the nematode *Caenorhabditis (C.) elegans* (Fire et al. 1998) and its functionality has been shown to be conserved in a wide range of eukaryotic organisms, from unicellular protists, over plants and fungi, to animals (Fire 2007). As stated before, there are three types; siRNA-mediated, miRNA-mediated and piRNA-mediated RNAi, with as common denominator the interposition of a dsRNA molecule. The three classes can be roughly separated by the way in which they inhibit gene expression and their biological roles, but they are also intertwined by shared pathway components and biological functions.

In vertebrates, antiviral immunity is based on the interferon response, which modulates various functions of the immune system (reduced translation in infected and neighboring cells, increased antigen presentation on specialized immune cells, etc.) after virus recognition. Invertebrates and plants do not possess an adaptive immune system, here antiviral immunity is achieved by siRNA-mediated silencing (Li et al. 2002; Keene et al. 2004; van Rij et al. 2006). During viral replication,

dsRNA intermediates are produced which are recognized and trigger the RNAi pathway to form siRNAs, resulting in the destruction of the viral genomes.

Next to its role in the antiviral immune response, which is a response to exogenous dsRNA, the siRNA pathway also plays a role in gene regulation and maintaining genomic integrity, through the use of endogenous siRNAs (endo-siRNAs) (Czech et al. 2008; Piatek and Werner 2014). These latter two functions are also covered by the other two classes, the miRNAs and piRNAs. The first miRNA was discovered in *C. elegans* (Lee et al. 1993; Wightman et al. 1993) and since then many more have been discovered (1100 confirmed in humans (Friedlander et al. 2014), 256 in the *Drosophila (D.) melanogaster* miRBase (Kozomara and Griffiths-Jones 2011) and 130 in the bumblebee *Bombus terrestris* (Sadd et al. 2015)). The majority of miRNAs are transcribed from intergenic regions and form a hairpin dsRNA structure due to internal nucleotide complementarity. After processing, a mature miRNA is obtained, which can target multiple mRNAs and as such, regulate whole sets of genes (Friedman et al. 2009). PiRNAs are likewise involved in gene regulation, but seem to be limited to germline cells and thus are important for development. All three types of small ncRNAs are also thought to maintain genomic integrity by protecting against transposable elements (reviewed in Malone and Hannon 2009).

In this work we will be focusing on the siRNA system because of its biological role in antiviral immunity and its applications in pest control and insect therapeutics. These applications are based on the fact that the siRNA pathway reacts to exogenous dsRNA, and thus in vitro designed dsRNA can be used to manipulated gene expression or viral titers.

1.1.3 *The siRNA pathway*

A schematic overview of the siRNA pathway is given in Figure 2. The trigger of this pathway is long dsRNA, which is generated in the cell as an endo-siRNA or as an intermediate of viral replication, or taken up by the cell from its environment. This dsRNA is recognized by the endonuclease Dicer-2 (Dcr-2) and processed into 21-25 nt long fragments (length is fixed within a species) having a two-nucleotide overhang at the 3' end, called siRNAs (Bernstein et al. 2001). Aided by the cofactor R2D2 (Liu et al. 2003), these fragments are then transferred onto the RNA-induced silencing complex (RISC), a protein complex with Argonaute-2 (Ago-2) as its main functional component (Meister et al. 2004). Subsequently, the RISC is activated by removing the passenger strand so that the guide strand, carrying the sequence complementary to the target, becomes accessible. Then, this single-stranded RNA (still encompassed in the RISC complex) can locate target

messenger RNA (mRNA) in the cytoplasm by complementary base pairing and bring about its cleavage, after which the mRNA fragments are degraded (Hammond et al. 2000).

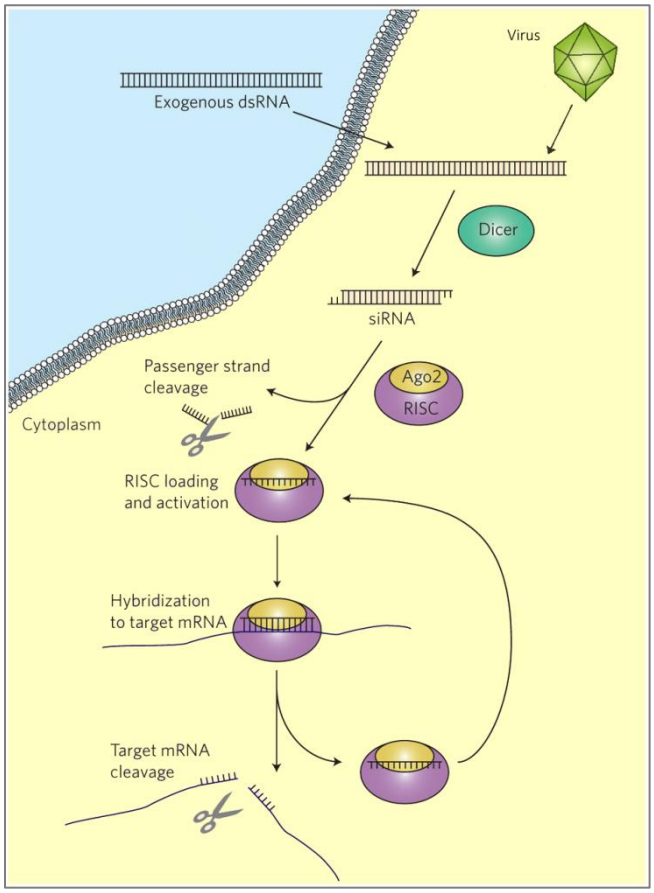

Figure 2. The RNA interference pathway.
Double-stranded RNA (dsRNA), introduced exogenously or produced as an endo-siRNA or intermediary of viral replication, is being cleaved by Dicer-2 into siRNAs. These associate with Argonaute-2 (Ago2) and guide the RISC complex to complementary (messenger) RNA, which is subsequently cleaved and degraded (adapted from Kanasty et al. 2013).

1.1.4 *Environmental / systemic RNAi*

The aforementioned pathway describes the siRNA pathway at a cellular level, but in most organisms RNAi is not restricted to one cell, which greatly contributes to its effectiveness. On one hand, exogenous dsRNA can be incorporated into the cytoplasm by taking up dsRNA from the environment, a feature named environmental RNAi (Whangbo and Hunter 2008). On the other hand, the RNAi signal can be spread throughout the body, eliciting an RNAi response in tissues that had no direct contact with the original dsRNA (Jose and Hunter 2007). The signal molecule of this process, named systemic RNAi, is thought to be the long dsRNA or a fragment of it. This hypothesis is based on the fact that, after feeding dsRNA to the Colorado potato beetle, dsRNA fragments were detected throughout the body, with a size comparable to that of the original

dsRNA and significantly longer than the 21-25 nt fragments expected for siRNAs (Ivashuta et al. 2015). This feature is essential in antiviral immunity in insects and plants as peripheral host tissues can be immuno-primed awaiting the spread of the virus throughout the body (Saleh et al. 2009; Karlikow et al. 2014).

1.1.5 *Applications of RNAi in entomology*

One of the most important characteristics of RNAi is its sequence specificity, which allows for precise targeting of certain genes. The various applications of RNAi in insect studies can be divided into three major categories:

- RNAi as a research tool: Because of its specificity and easy application, RNAi is a useful tool in functional genomics. Silencing a gene of interest reduces the corresponding protein levels, resulting in a disturbed function. Effects can be evaluated by mortality scoring, observing behavioral/phenotypic/metabolic differences, immunochemical staining, RT-qPCR analysis of affected genes, western-blotting to follow protein expression, etc.
- RNAi as a crop protection strategy: Targeting particular essential genes can lead to (sub)lethal effects in insects as their protein levels are (sometimes greatly) reduced. In applications, the dsRNA would have to be applied through transgenic plants or by spraying dsRNA or dsRNA-producing inactivated bacteria or viruses onto the crops, and hence taken up through the digestive system of the insect (Baum et al. 2007; Mao et al. 2007).
- RNAi as a therapeutic: Viral pathogens pose a substantial threat to beneficial insects such as bees which provide invaluable pollination services. DsRNA targeting viral sequences can be administered through the food to domesticated honeybees and bumblebees and help them resist a viral attack (Maori et al. 2009; Hunter et al. 2010; Piot et al. 2015).

1.1.6 *Problematic RNAi efficiency*

RNAi is a promising tool in many research and product development areas, but its widespread use is hindered by the large variability that is seen across and within insect species. In general, RNAi is very efficient in Coleoptera and Orthoptera, but is less so for some, but not all, hymenopteran, dipteran, hemipteran and lepidopteran species. Various explanations have been suggested for the observed differences between different experimental setups within one species and between species:

- Factors inherent to the experimental setup: life stage, siRNA or dsRNA, dsRNA length, delivery method, dose, target gene (region, protein turnover,…), evaluation timepoint, examined tissue, etc.
- Factors inherent to the insect species: dsRNA degradation in the saliva/midgut (Yue and Genersch 2005; Christiaens et al. 2014; Wynant et al. 2014c), dsRNA sequestering in the hemolymph (Wynant et al. 2014a), different (efficiencies of the) dsRNA uptake mechanisms (Chapter III), absence/presence of RNAi core genes (Chapter II), absence/presence of systemic and/or environmental RNAi, expression levels of RNAi core genes, etc.
- Factors inherent to the insect individual: nutritional status, immunological status, viral infections triggering enhanced systemic properties (Saleh et al. 2009), virus-produced suppressors of RNAi (Chapter V), etc.

In this work, two factors (fully underlined) were examined for their ability to affect RNAi efficiency. They were selected because they represent two different levels of influence. DsRNA uptake mechanisms might explain why RNAi works well in some species, but not in others. On the contrary, viral infections exert their influence on an individual/colony/strain level and might explain why some successful RNAi experiments cannot be repeated in other research groups. These two factors will be discussed in detail in the following sections because of their importance for this work. Also some other factors will be mentioned throughout this thesis: the first dotted underlined factor was partly covered through genome annotation collaborations, the second will make a short appearance when examining the virus-produced suppressors of RNAi because of their connectedness.

1.2 dsRNA uptake mechanisms

In most entomological RNAi applications, the dsRNA is administered extracellularly: to the gut lumen after feeding, to the hemocoel after micro-injection or to the growth medium in the case of cell lines. Therefore, it is necessary that the cells are able to take up the dsRNA from the environment by themselves. The first dsRNA uptake mechanism was described in the nematode *C. elegans* where four *systemic RNAi-deficient* (*sid*) genes were found, named *sid-1*, *sid-2*, *sid-3* and *sid-5* (Winston et al. 2002; Hinas et al. 2012; Jose et al. 2012). In insects only homologs for *sid-1* were found, and not in all insects, as the model insect *D. melanogaster* does not have any. However, in this insect, receptor-mediated clathrin-dependent endocytosis has been found responsible for dsRNA uptake (Saleh et al. 2006; Ulvila et al. 2006). Since those discoveries, experiments examining dsRNA uptake mechanisms have been performed in many insects, providing seemingly contradictory results about the role of these two pathways. This topic will be further discussed in Chapter III.

1.2.1 *Sid-1-like transmembrane proteins*

The Sid-1-like proteins are 11 transmembrane domain-containing channel proteins (Figure 3A) that, in insects, are present in all examined tissues during all developmental stages (Tian et al. 2009; Bansal and Michel 2013). In *C. elegans,* this protein is responsible for dsRNA import into cells (Winston et al. 2002), but not necessary for the export of the systemic silencing signal (Jose et al. 2009). In the nematode's apical intestinal membrane, dsRNA uptake seems to occur through a collaboration between Sid-1 and Sid-2, whereas in the other tissues Sid-2 is absent and Sid-1 functions independently (Winston et al. 2007; McEwan et al. 2012). In insects the number of *sid*-1 homologs, often called *sid-1-like* (*sil*) genes, is variable among different species. An overview of the number of homologs and what is known about their involvement is given in Table 4 in Chapter III.

1.2.2 *Receptor-mediated clathrin-dependent endocytosis*

Receptor-mediated clathrin-dependent endocytosis is a targeted form of endocytosis, initiated when extracellular macromolecules are recognized by a receptor. Aided by the adaptor complex AP2, this recognition recruits clathrin triskelia to the receptor. The term triskelion derives from the fact that a clathrin molecule consist of 3 branches, each containing a heavy chain and a light chain, which allow different clathrin molecules to assemble into a cage-like structure around the budding vesicle. After invagination of the vesicle, about 50-100 nm in size, it is pinched of the plasma membrane by dynamin. In the cytoplasm, the vesicle, containing multiple receptors and

the bound macromolecules, releases its clathrin triskelia and becomes an endosome. Here, the contents can be released into the cytoplasm, or fuse with the lysosome for digestion of the macromolecules (Figure 3B) (Grant and Sato 2006).

This process has an essential role in neurotransmission, signal transduction and nutrient uptake in eukaryotes. The endocytic pathway is also exploited by toxins, viruses and bacteria to enter the cell (McMahon and Boucrot 2011). It has been discovered to be involved in dsRNA uptake in the *Drosophila* S2 cell line as multiple genes, either connected directly to endocytosis, involved in cellular trafficking of the endocytic vesicles or the release of dsRNA from the endosome, were deemed indispensable for dsRNA uptake. In the same experiments two scavenger receptors of dsRNA, Eater and SR-CI, were identified (Saleh et al. 2006; Ulvila et al. 2006).

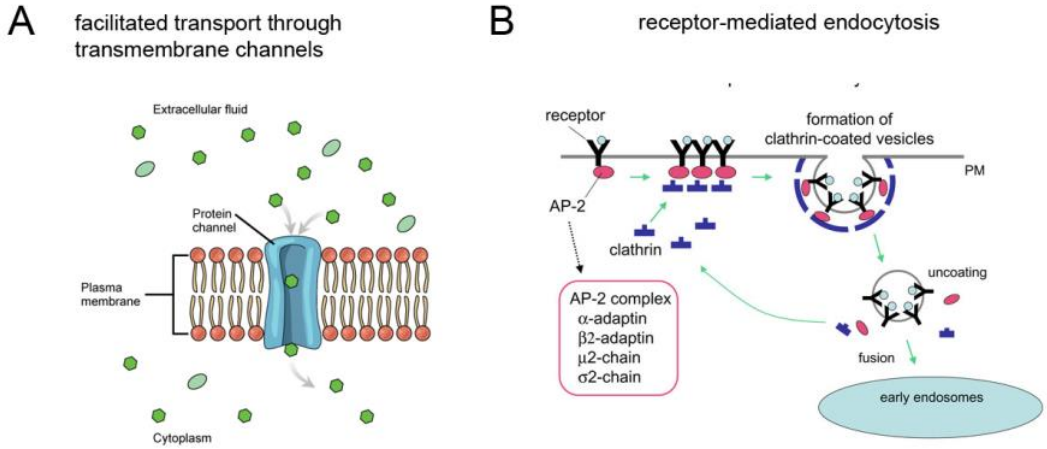

Figure 3. Schematic overview of the two cellular double-stranded RNA (dsRNA) uptake mechanisms discussed in this thesis.
Panel A: the Sid-1(-like) proteins form transmembrane channels through the plasma membrane through which dsRNA can be taken up passively (image by Mariana Ruiz Villareal, released into the public domain). Panel B: dsRNA is recognized by a receptor which recruits clathrin to form an invaginated vesicle coated by clathrin molecules. This vesicle is then released into the cytoplasm (Grant and Sato 2006).

1.3 Viral suppressors of RNAi

To overcome the powerful antiviral response of the RNAi pathway, viruses have evolved a wide range of suppression mechanisms. If they are mediated by virus-encoded proteins, those are grouped under the term viral suppressors of RNAi (VSRs). An interesting feature is that most VSRs retain their suppressor function when expressed in a cross-kingdom system (e.g. insect VSR in plant systems and vice versa (Li et al. 2002; Lakatos et al. 2004; Guo and Lu 2013)), indicating they are targeting conserved parts of the RNAi pathway and do not need any host factors. Indeed, many VSRs exert their function by binding dsRNA and/or siRNAs and sequestering them from Dcr-2 and Ago-2. Despite their small size, many VSRs seem to be multifunctional: they are able to affect the RNAi pathway in distinct ways and even interfere with other antiviral immune systems and hormone signaling of the host (reviewed in Csorba et al. 2015). This mechanistic diversity, coupled with their unrelated sequence and structure, indicates that they have evolved independently within and across kingdoms. In plant viruses, many more VSRs have been identified than in invertebrate viruses, possibly because until now, more effort has been placed in identifying VSRs in plants than in insects (Csorba et al. 2015).

1.3.1 *VRSs in plant viruses*

The RNAi pathway in plants, often referred to as post-transcriptional gene silencing (PTGS) instead of RNAi, is based on the same two protein families as in insects, the Dicers and the Argonautes, but the number of these proteins is different with each protein having slightly different functionalities (reviewed in Seo et al. 2013). Even before the basics of the RNAi pathway were fully understood, viruses encoding small proteins that could interfere with the silencing process in plants had been described.

In the *Tobacco etch potyvirus*, the in-frame P1/HC-Pro protein was found to promote movement of the viral particles within the plant and enhance its virulence by binding duplex siRNAs (Anandalakshmi et al. 1998; Kasschau and Carrington 1998; Lakatos et al. 2006). The 2b protein of various Cucumoviruses originates from a sub-genomic mRNA strand, a smaller viral mRNA strand which is the result of irregular transcription and is not encapsidated into new viral particles. This 2b protein modulates virulence in multiple ways, including small RNA and Argonaute binding (Li et al. 1999; Csorba et al. 2015). At the moment, more than 50 different VSRs are known over many viral genera, some viruses even encoding more than one suppressor protein, prompting the suggestion that a VSR would be found in nearly all plant viruses, if examined sufficiently (Li and Ding 2006; Csorba et al. 2015).

1.3.2 *VSRs in insects*

The first discovery of a VSR, encoded by an animal virus, was the B2 protein of *Flock house virus*, because of shared features with the *Cucumovirus* 2b protein. It is also located in the +1 reading frame of the carboxyterminal region of the RNA-dependent RNA polymerase and translated from a subgenomic mRNA strand (Figure 4A) (Li et al. 2002). It has been shown to bind both siRNAs and longer dsRNAs (Chao et al. 2005) and hinder siRNA biogenesis by associating with the PAZ domain of Dcr-2 (Singh et al. 2009), affecting the RNAi pathway at (at least) two distinct steps. As of yet, 13 VSR proteins have been described in insect or arthropod-borne (arbo) viruses (Table 1). Parallel to plant viruses, relatedness might suggest a VSR at the same genomic location, but does not always guarantee identical functionality. Next to VSR proteins, in the listed arboviruses small subgenomic RNAs (sfRNAs) were found that were able to inhibit Dicer-activity (Schnettler et al. 2012). However, their functionality has only been proven in human systems (their true hosts) and it is unclear whether they are also used to evade the invertebrate antiviral RNAi system during the non-pathogenic infection in insect vectors (Gammon and Mello 2015).

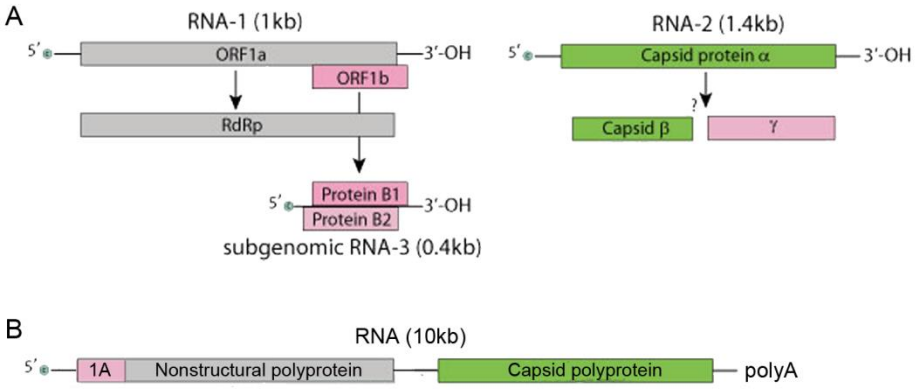

Figure 4. Location of the viral suppressors of RNAi (VSRs) in the genome of *Flock house virus* and *Cricket paralysis virus/Drosophila C virus*.
Panel A: The B2 protein of *Flock house virus* is translated from the subgenomic RNA-3 strand, situated in the +1 reading frame at the 3' end of RNA-1. Panel B: In the *Dicistroviridae*, the 1A protein is located in frame at the 5' end of the nonstructural protein in the single RNA strand.

In light of the scope of this thesis, in the family of the *Dicistroviridae*, the 1A protein has been shown to be a functional suppressor encoded by *Cricket paralysis virus* (Nayak et al. 2010) and *Drosophila C virus* (van Rij et al. 2006). This VSR is located in the normal reading frame, at the 5' end of the nonstructural polyprotein (Figure 4B). Interestingly, although closely related, their 1A sequences show very little sequence similarity and the resulting proteins have a different mode of action. These VSRs are discussed in detail in Chapter V.

Table 1. Known/putative suppressors of RNAi encoded by insect viruses and arboviruses (Bronkhorst and van Rij 2014; Gammon and Mello 2015).

Virus	Family	RNAi suppressor	Genomic location of the VSR	Proposed mechanism of RNAi suppressor	References
RNA viruses					
Flock house virus	*Nodaviridae*	B2	+1 frame, subgenomic mRNA strand	Binding long dsRNA prevents cleavage by Dcr-2; Binding siRNA prevents incorporation into RISC; Dcr-2 binding	(Li et al. 2002; Li et al. 2004; Chao et al. 2005; Aliyari et al. 2008; Singh et al. 2009)
Nodamuravirus	*Nodaviridae*	B2	+1 frame, subgenomic mRNA strand	Binding long dsRNA prevents cleavage by Dcr-2; Binding siRNA prevents incorporation into RISC; Inhibition of Dcr-2 activity	(Li et al. 2004; Sullivan and Ganem 2005; Aliyari et al. 2008)
Wuhan nodavirus	*Nodaviridae*	B2	+1 frame, subgenomic mRNA strand	Binding long dsRNA prevents cleavage by Dcr-2; Binding siRNA prevents incorporation into RISC; Dcr-2 binding	(Qi et al. 2011; Qi et al. 2012)
Mosinovirus	*Nodaviridae*	B2	+1 frame, subgenomic mRNA strand	Binding long dsRNA prevents cleavage by Dcr-2	(Schuster et al. 2014)
Drosophila C virus	*Dicistroviridae*	1A	In frame, stop-go translation product	Binding long dsRNA prevents cleavage by Dcr-2	(van Rij et al. 2006; Nayak et al. 2010)
Cricket paralysis virus	*Dicistroviridae*	1A	In frame, stop-go translation product	Inhibition of Ago-2 slicer (endonuclease) activity	(Wang et al. 2006; Nayak et al. 2010; van Mierlo et al. 2012)
Drosophila X virus	*Birnaviridae*	VP3	In frame	Binding long dsRNA prevents cleavage by Dcr-2; Binding siRNA prevents incorporation into RISC	(Valli et al. 2012; van Cleef et al. 2014)
Culex Y virus	*Birnaviridae*	VP3	In frame	Binding long dsRNA prevents cleavage by Dcr-2; Binding siRNA prevents incorporation into RISC	(van Cleef et al. 2014)

Table 1 (continued).

Virus	Family	RNAi suppressor	Genomic location of the VSR	Proposed mechanism of RNAi suppressor	References
Nora virus	Unassigned	VP1	In frame	Inhibition of Ago-2 slicer (endonuclease) activity	(van Mierlo et al. 2012; van Mierlo et al. 2014)
Dimm Nora-like virus	Unassigned	VP1	In frame	Inhibition of Ago-2 slicer (endonuclease) activity	(van Mierlo et al. 2014)
Dengue virus	*Flaviviridae*	NS4B	In frame	Inhibition of Dcr-2 activity [a]	(Kakumani et al. 2013)
West Nile virus	*Flaviviridae*	sfRNA	Product of incomplete degradation of viral genomic RNA	Inhibition of Dcr-2 activity [a]	(Schnettler et al. 2012)
Dengue virus	*Flaviviridae*	sfRNA	Product of incomplete degradation of viral genomic RNA	Inhibition of Dcr-2 activity [b]	(Schnettler et al. 2012)
DNA viruses					
Heliothis virescens ascovirus-3e	*Ascoviridae*	Orf 27 (RNAse III)	In frame	Degradation of siRNA	(Hussain et al. 2010)
Invertebrate iridescent virus type 6	*Iridoviridae*	340R	In frame	Binding long dsRNA prevents cleavage by Dcr-2; Binding siRNA prevents incorporation into RISC	(Bronkhorst et al. 2014)

[a] Experimental data obtained using human Dicer, inhibition of Dcr-2 in insects is presumed.

[b] Presumed function based on similarity to *West Nile virus* sfRNA and ability to inhibit RNAi in insect cell assays.

1.4 Insects and viruses used in this work

1.4.1 *Insect morphology*

The insect body is divided into three parts: the head, the thorax and the abdomen. The thorax carries six legs and generally two pairs of wings, which might be modified for other functions such as protection, sound production, etc. whereas the abdomen contains most of the insect organs. The internal morphology is usually divided into five systems encompassing various organs as shown in Figure 5. Some typical characteristics (compared to other animal systems) are:

- Insects have an open circulating system with hemolymph as body fluid, responsible for the direct transport of nutrients, salts, hormones, waste products and immune system components between organs and tissues. Fluid movement originates from peristaltic contractions in the dorsal vessel which push the hemolymph through the tube from the abdomen (dorsal vessel = heart) to the head (dorsal vessel = aorta) where it is released near the brain and moves through the body cavity back to the abdomen. Unlike mammalian systems, oxygen is delivered to the tissues by trachea which are connected to the environment by small openings, the spiracula.
- The digestive system is divided into three parts: the fore-, mid- and hindgut. An important feature of the foregut is the crop where the food is temporarily stored before moving to the midgut where most of digestion takes place. The hindgut is the site of water reabsorption. At the transition of midgut and hindgut, Malpighian tubules are attached which absorb waste products from the hemolymph and transfer them to the hindgut.
- The nervous system consists of a brain and two nerve cords along the thorax and abdomen with clusters of nerve cells, the ganglia, in each segment. These ganglia can operate independently from the brain to control activities within that segment.
- The fat body (not depicted) is a loose tissue lining the integument of the abdomen and surrounding other organs. This organ is responsible for energy storage and homeostasis, detoxification processes and the production of hormones and other signaling molecules, including those belonging to several immune response pathways.

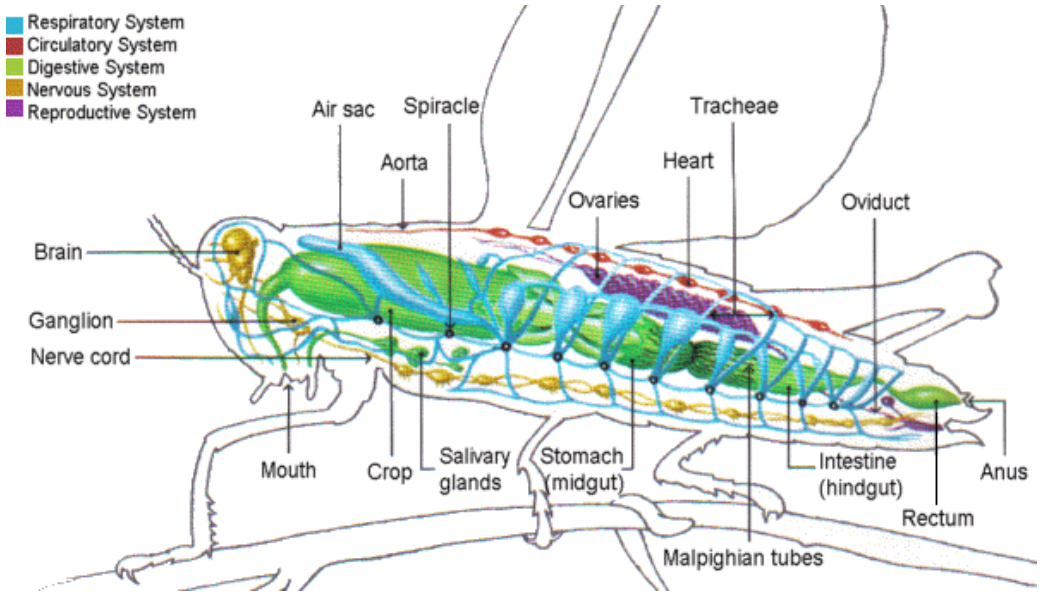

Figure 5. Internal morphology of insects (World Book Inc.).

1.4.2 *The Colorado potato beetle*

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata*, is an important pest of solanaceous vegetables and easily recognizable by black stripes on a yellow-orange background running over the elytra (Boiteau and Le Blanc 1992). It belongs to the holometabolous order of the beetles, which harbors over 350000 described species (Malone and Hannon 2009). The most distinctive feature of this order is the hardening of the forewings into elytra which protect the hindwings when not in flight (Figure 6).

Kingdom	Animalia
Phylum	Arthropoda
Class	Insecta
Order	Coleoptera
Family	Chrysomelidae
Genus	Leptinotarsa
Species	L. decemlineata (Say, 1824)

Figure 6. The Colorado potato beetle (CPB).
Upper left: adult CPB (photograph by Scott Bauer/Agricultural Research Service, USDA). Bottom left: CPB larvae (photograph by Ian Marsman). Right: CPB taxonomy, condensed (ITIS).

1.4.2.1 Life cycle

The life cycle of the CPB can be divided into seven stages: an egg, four larval, a pupal and an adult stage. At the end of spring, adult beetles start to emerge from the soil where they have been overwintering. They migrate to a host plant from the Solanaceae family and, after mating, the females deposit egg masses on the underside of the leaves (Boiteau and Le Blanc 1992). After four to ten days, the eggs hatch into the first larval stage. By subsequent moltings the larval instars complete the four stages, during which they feed vigorously off the host plant's leaves. This takes about two to three weeks. At the end of the fourth stage, the larvae bury themselves in the soil to pupate. After five to ten days, the adults emerge which can either immediately produce a second generation or feed briefly, before entering a diapause in the soil. There can be multiple generations each year depending on the climate (Weber 2003).

1.4.2.2 Economic impact and management strategies

The CPB is native to Mexico and the southwest of the U.S. where it feeds on native Solanacea species. As the total area of potato crops (*Solanum tuberosum*) and intercontinental transport increased greatly during the 19th and 20th century, the beetle adapted to this new host plant (Casagrande 1987) and spread to all climatically favorable parts of Europe and Asia, currently covering about 16 million km^2 (Weber 2003; EPPO 2015). It is the most important insect defoliator of potatoes as the economic injury level has been estimated at one to five larvae per plant, or even less (Senanayakei and Holliday 1990; Nouri-Ganbalani et al. 2010). It is classified as a quarantine pest in most regions, except North America, and a member of EPPO's A2 quarantine list, so measures to limit the spreading of this pest are being undertaken (EPPO 2015).

Once established, a number of strategies can be used in order to keep CPB population densities below the economic injury level. For many decades, chemical insecticides were the preferred method because they proved to be very effective, but soon it became clear that this beetle had an enormous potential to evolve new insecticide resistances (Alyokhin et al. 2008). This incited the search for alternative control methods such as crop rotation, biological control methods or transgenic Cry3A-expressing crops (Newleaf®, Monsanto). However, no biological control method has been found that is effective enough to compensate for its high rearing and handling costs and the Newleaf variety has been removed from the market out of public concern (Grafius and Douches 2008; Alyokhin 2009). Monsanto is developing an RNAi-based topical application targeting CPB, currently in phase II of their Biodirect R&D pipeline (Monsanto 2016).

1.4.3 *The buff-tailed bumblebee*

The buff-tailed bumblebee, *Bombus (B.) terrestris*, belongs to the holometabolous order of the Hymenoptera which comprises over 150000 species (Sharkey 2007). It is considered a primitive eusocial species as its social organization is simpler than that of, for example, honeybees (Cardinal and Danforth 2011). The *B. terrestris* species complex can be distinguished from other bumblebees by two yellow bands of hairs (one at the front of the thorax and one at the front of the abdomen) and white-colored hairs on the final segments of the abdomen as shown in Figure 7 (Prys-Jones and Corbet 2011).

Kingdom	Animalia
Phylum	Arthropoda
Class	Insecta
Order	Hymenoptera
Family	Apidae
Genus	Bombus
Species	*B. terrestris* (Latreille, 1802)

Figure 7. The buff-tailed bumblebee.
Left: adult bumblebee (photograph by Vera Buhl). Right: buff-tailed bumblebee taxonomy, condensed (ITIS).

1.4.3.1 Life cycle

Like most bumblebee species, *B. terrestris* has an annual life cycle. At the end of winter, overwintering queens emerge from the soil and seek out a suitable underground nesting site. A first batch of eggs is being laid within a lump of collected pollen and incubated by the queen by sitting on top of the pollen lump. There are four larval stages which are continuously fed by the queen with pollen and nectar and kept warm, until they pupate and the first workers emerge. These workers then take over foraging duties and the queen continues to lay worker-producing eggs. When the colony grows sufficiently large, the nest switches to the production of haploid males and daughter-queens. These queens build up fat reserves by foraging, during which they are approached by males. After mating, the new queens begin the search for a hibernation site (Goulson 2009).

1.4.3.2 Economic importance and protection strategies

B. terrestris is common in most of Europe, North Africa and the western part of Asia, where it provides an invaluable pollinator service (Goulson 2009). Since the 1990's this species is also reared commercially to be used for the pollination of tomatoes, zucchinis and other greenhouse crops and in fruit orchards. This way, this species has been introduced in South America, New Zealand and East Asia (Velthuis 2002). The transport poses an opportunity for various pathogens, plaguing the bumblebee, such as the fungal *Nosema* species, the protozoan *Crithidia* and *Apicystis* species and various RNA viruses, belonging mainly to the families of the *Dicistroviridae* and *Iflaviridae*, to spread to new wild hosts (Meeus et al. 2011; Murray et al. 2013; Graystock et al. 2014; Goulson et al. 2015).

Since the end of the last century, reports of a declining pollinator abundance and biodiversity have emerged. In Europe, nearly 24% of all bumblebee (*Bombus*) species are threatened with extinction and 48% have a declining population trend, though not *B. terrestris*. The major driver of this deterioration is thought to be the changes in land use and agricultural practices, which have led - and will continue to do so - to a reduction in habitat and food availability (Nieto et al. 2014). Bees, imposed by these continuous and cumulative stress factors, are weakened immunologically, affecting their capacity for countering sudden disturbances, such as pathogen attacks (Goulson et al. 2015). Recently, import prohibitions and improved screening methods have been implemented to prevent pathogen spreading. Additionally, RNAi-based therapeutics, targeting the viral pathogens of honeybees, are being developed which can be used to eradicate viruses from reared honey- and bumblebees and prevent spillover to wild bees (Maori et al. 2009; Monsanto 2016).

1.4.4 *The Dicistroviridae*

The *Dicistroviridae* family, belonging to the order of the *Picornavirales,* together with the other bee-infecting family of the *Iflaviridae*, consists of 15 positive single-stranded RNA virus species infecting arthropods. The virions are icosahedral, non-enveloped and have a diameter of approximately 30 nm (Bonning and Johnson 2010). The phylogenetic relationship between the members of the three genera, Aparaviruses (5 species), Triatoviruses (5 species) and Cripaviruses (4 species), is given in Figure 8 (ICTV 2015).

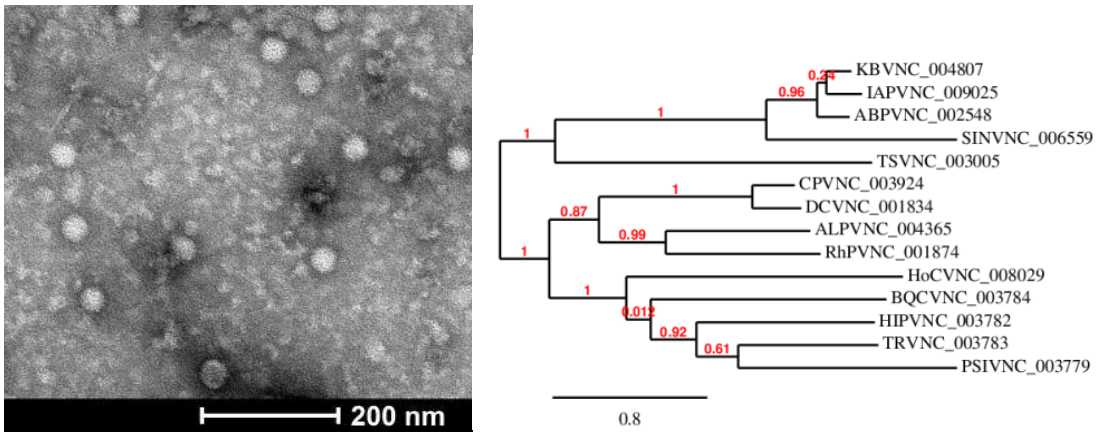

Figure 8. *Dicistroviridae* morphology and phylogenetic organization
Left: *Dicistroviridae* form icosahedral particles of about 30nm. Right: maximum likelihood phylogenetic tree showing the relations between the member species, classified into 3 genera. KBV, *Kashmir bee virus*; IAPV, *Israeli acute paralysis virus*; ABPV, *Acute bee paralysis virus*; SINV, *Solenopsis invicta virus-1*; TSV, *Taura syndrome virus*; CPV, *Cricket paralysis virus*; DCV, *Drosophila C virus*; ALPV, *Aphid lethal paralysis virus*; RhPV, *Rhopalosiphum padi virus*; HoCV, *Homalodisca coagulata virus-1*; BQCV, *Black queen cell virus*; HIPV, *Himetobi P virus*; TRV, *Triatoma virus*; PSIV, *Plautia stali intestine virus* (Echeverría et al. 2015).

1.4.4.1 Genome structure

The single genomic RNA strand (9000-11000 nt) consists of two open reading frames (ORFs), coding for a non-structural and a structural polyprotein, which are post-translationally cleaved into mature proteins (Figure 9) (Garrey et al. 2010). There are two internal ribosome entry site (IRES) elements which enable direct translation of the genomic RNA, one located in the 5' UTR and one in the intergenic region between the two ORFs. A small virus genome-linked protein (VPg) is covalently linked to the 5' end and the 3' end is polyadenylated. The ORF1 encompasses the following coding domains: 1A, 2A, a helicase (HEL), one or more VPg's, a 3C-like protease (PRO) and the RNA-dependent RNA polymerase (RdRp). The capsid proteins are encoded in ORF2 in the following order: VP2, VP4, VP3 and VP1 (Bonning and Johnson 2010).

Figure 9. *Dicistroviridae* genome organization (Welker et al. 2011).

1.4.4.2 Infection, replication cycle and pathology

Dicistroviridae infection commonly results in a chronic infection having sublethal effects such as a reduced longevity and fecundity. But in response to stress, the infection can become acute, leading to paralysis and death (Maori et al. 2007; Chen et al. 2014; Meeus et al. 2014). However, when colony death is observed and is known to be have been caused by a paralysis virus, it is rarely accompanied by evidence of mass paralysis (Maori et al. 2007). One possible explanation is that rapid progression from paralysis to death prevents the accumulation of sufficient live paralytic adults for such effects to be noticed at the colony level (de Miranda et al. 2010). In bumblebees, the viruses are thought to be transmitted horizontally within nests as faeces of infected hosts serve as an infection source. In honeybees, horizontal transmission has been shown to occur also through the sharing of saliva, pollen and honey, during mating and during the sucking of the hemolymph by the *Varroa destructor* mite (Shen et al. 2005; Chen et al. 2006a; Chen et al. 2006b). Vertical transmission has also been suggested for various bee viruses of the *Dicistro-* and *Iflaviridae* (Chen et al. 2006b; Ravoet et al. 2015)

For *Drosophila C* virus, the mechanistically best studied Dicistrovirus, cell entry has been shown to occur through clathrin–dependent endocytosis after recognition by a still-unknown receptor (Cherry and Perrimon 2004). After exiting the endocytic vesicle, the genome is released into the cytoplasm where it is translated by the host cell machinery to produce the non-structural proteins necessary for viral replication and new capsid proteins. Viral particles are assembled from the newly formed RNA genomes and capsid proteins. They can leave the cell early in the infection cycle, or be released when the cell lyses at the end of the infection (Bonning and Johnson 2010).

1.4.4.3 Host defense mechanisms

The first antimicrobial defense systems are the physical barriers formed by the perithropic membrane, the gut and the basal lamina. After the pathogen bypasses these barriers and enters the cell, the insect relies on innate immunity pathways to limit the spread of the virus in the body. After recognition of foreign molecules, called pathogen-associated molecular patterns (PAMPs), by pattern recognition receptors (PRRs), various antimicrobial immune responses are activated (Figure 10). Through a complex cascade, the NF-κB-related Toll and Imd pathways produce antimicrobial peptides (AMPs) which can kill fungi and bacteria (Lemaitre et al. 1997). Additionally, they induce factors which trigger phagocytosis, the production of reactive oxygen species and the melanization (prophenoloxidase) cascades (reviewed in Valanne et al. 2011). A third pathway is the JAK/Stat pathway, which produces factors involved in general stress response, hemocyte proliferation and phagocytosis (reviewed in Agaisse and Perrimon 2004).

As stated in section 1.1.2, the primary antiviral defense system in insects is the RNAi pathway, triggered by the presence of dsRNA replicative intermediates, which act as PAMPs. The receptor is still unknown, but it has been suggested Dcr-2 plays a role in this recognition (Flenniken and Andino 2013). Moreover, it has been shown that also non-specific dsRNA may trigger an antiviral response, possibly because Dcr-2 interacts with other (unknown) immune pathways (Flenniken and Andino 2013; Piot et al. 2015).

In recent years, evidence for the involvement of the other immune pathways in the antiviral response has emerged (reviewed in Merkling and van Rij 2013). The strongest proof comes from the JAK/Stat pathway, where injection with *Drosophila C virus* leads to an upregulation of genes that encode for AMPs and proteins involved in the Jak/STAT pathway in *Drosophila* (Dostert et al. 2005). Additionally, knockdown of various JAK/Stat genes in mosquitoes resulted in increased viral titers (Souza-Neto et al. 2009). For the NF-κB-related pathways the situation is more unclear as multiple studies show conflicting results (overview in Merkling and van Rij 2013). In some cases, viral infection caused AMP production or Toll/Imd-mutants showed enhanced viral infectivity, but not consistently over different cases/experiments. In any case, little is known about the downstream antiviral effectors of these pathways (Merkling and van Rij 2013). Interestingly, there seems to be a link between the JAK/Stat pathway and the RNAi pathway, as dsRNA recognition by Dicer-2 brings about the expression of Vago (Deddouche et al. 2008), which in turn activates the JAK/Stat pathway in mosquitoes (Paradkar et al. 2012).

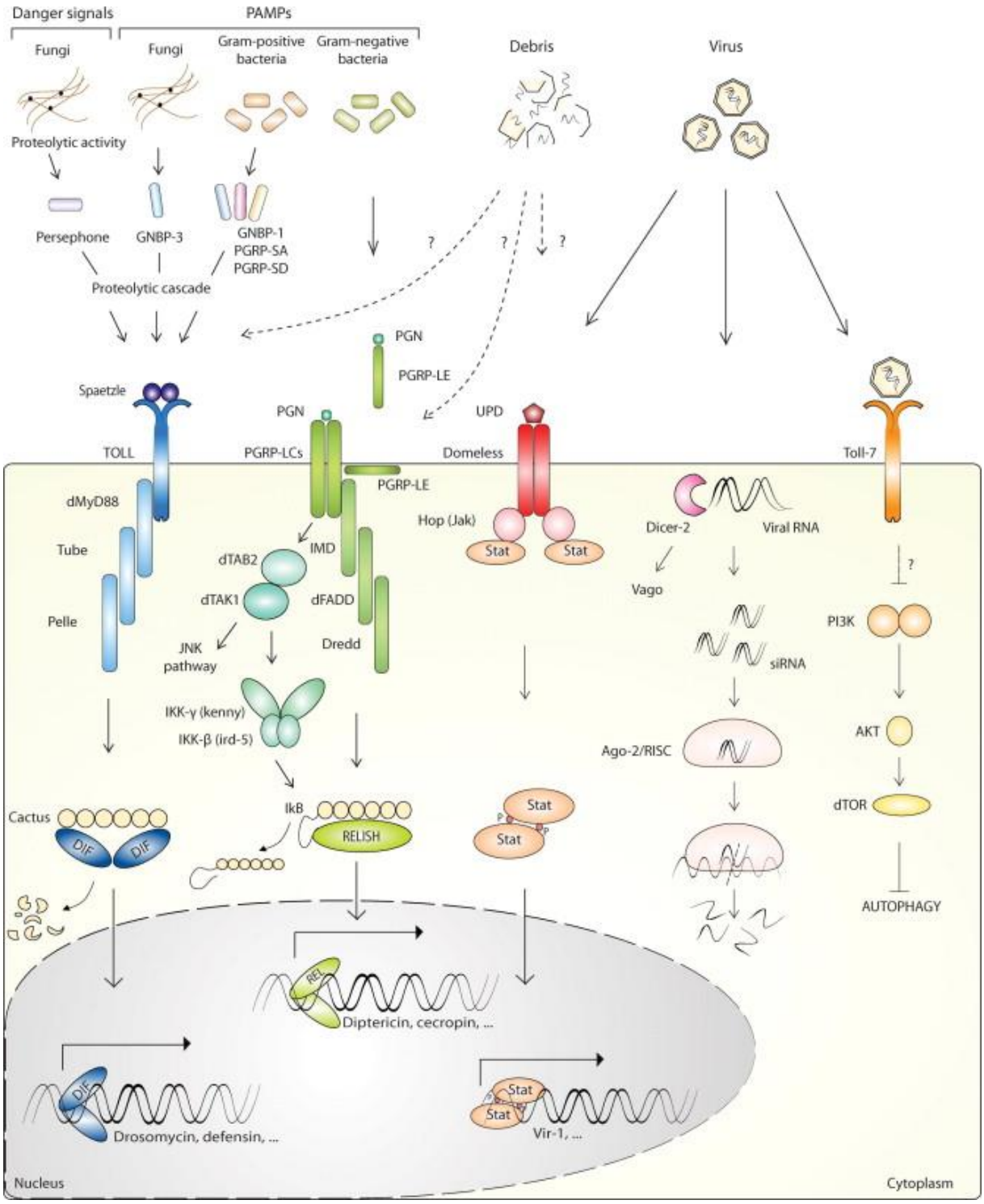

Figure 10. Immune pathways in *Drosophila melanogaster*.
The NF-κB-related Toll and Imd pathways respond to bacterial or fungal infections through a complex cascade to produce antimicrobial peptides such as drosomycin and diptericin. Additionaly, the Jak/Stat pathway responds to stress to produce various immune effectors. Dcr-2 recognizes viral dsRNA which leads to Vago upregulation (cross-link with the JAk/Stat pathway) and a triggering of the RNAi pathway to destroy viral genomic strands (Merkling and van Rij 2013).

1.4.4.4 *Israeli acute paralysis virus* and *Cricket paralysis virus*

For this work, two members of the *Dicistroviridae* are of importance: *Cricket paralysis virus* (CrPV) and *Israeli acute paralysis virus* (IAPV), which is part of the very closely related *Acute bee paralysis virus* (ABPV) - *Kashmir bee virus* (KBV) - IAPV complex (de Miranda et al. 2010). Because of the recent discovery of IAPV and considering many of the primer sets are unable to distinguish between the species, it is likely that some of studies may have misdiagnosed IAPV as KBV or ABPV (Genersch and Aubert 2010). An overview of some important characteristics and how IAPV and CrPV differentiate from each other is given in Table 2.

Table 2 Isolation, genomic sequencing, biophysical properties and biological characteristics of *Israeli acute paralysis virus* and *Cricket paralysis virus* (adapted from Bonning and Johnson 2010).

Virus	Genus	Diameter (nm)	Density (g/ml) [a]	Genome size (nt)	Host range (number of species)	Symptoms	References
IAPV	Apara-virus	27	1.33	9487	Hymenoptera (2) [b]	Paralysis, reduced longevity and fecundity	(Blanchard et al. 2008; Cox-Foster et al. 2007; Maori et al. 2007; Meeus et al. 2014
CrPV	Cripa-virus	27	1.34	9185	Orthoptera (4) Diptera (4) [c] Hemiptera (2) [c] Hymenoptera (2) [c] Lepidoptera (12) [c]	Paralysis, reduced longevity and fecundity	(Reinganum et al. 1970; Reinganum 1975; Scotti 1975; Plus et al. 1978; Manousis and Moore 1987; Anderson and Gibbs 1988; Johnson and Christian 1996; Wilson et al. 2000)

[a] Buoyant density in cesium chloride

[b] KBV has been shown to be naturally occurring in *Apis cerana* (Bailey et al. 1979) and *Vespula germana* (Anderson 1991) bringing the number of hymenopteran hosts to 4.

[c] In natural populations CrPV has only been found in Orthoptera (except for a latent infection in *Apis mellifera*), all other observations concern lab populations or cell lines.

CHAPTER II:

INTERMEZZO: RNAi CORE GENE ANNOTATION IN THE COLORADO POTATO BEETLE AND THE BUMBLEBEE

Parts of this chapter were published in:

Sadd, B.M., Barribeau, S.M., Bloch, G., de Graaf, D.C., Dearden, P., Elsik, C.G., … , Cappelle, K., …, Worley, K.C. (2015) The genomes of two key bumblebee species with primitive eusocial organization. *Genome Biology* 16, 76.

The bumblebee genome consortium generated all sequence data. K. Cappelle annotated a subset of the RNAi core genes, performed additional analyses and wrote the annotation summary.

A Colorado potato beetle genome paper, expected to be published in 2017. Principal authors: Schoville, S.D. and Chen, Y.H.

The CPB genome consortium generated all sequence data. K. Cappelle annotated all RNAi core genes, performed additional analyses and contributed to the annotation summary.

The text of this Chapter was written by K. Cappelle and revised by I. Meeus.

2.1 Introduction

One factor to consider when looking at RNAi efficiency is the presence of the RNAi core genes. Although insects form a huge taxonomical group, little genome information is available. Nearly half of all described metazoan species are insects, and with over 1 million different species they are considered the most diverse group of organisms on Earth (Whiting 2002). Up until recently, only about 70 insect genomes had been sequenced and (partially) annotated, of which 48 belonged to the order of Diptera. In the other orders such as the Coleoptera, comprising over 350000 species, only a few model species had been sequenced. As a consequence, the genetic information available was, in all probability, inadequate when looking at non-model insects. For example, using genetic information from *Tribolium (T.) castaneum*, the coleopteran model insect, to look at processes in the Colorado potato beetle (CPB) might be less relevant than transferring genetic information from the frog to the human situation as there are only about 60000 described vertebrate species (Baillie et al. 2004).

In an attempt to increase our knowledge on genetic diversity in insects, the 5000 Arthropod Genomes Initiative (i5k) was started in 2012 (i5K Consortium 2013). The aim was to 1) organize the sequencing and analysis of the genomes of 5000 arthropod species and 2) grow a community around arthropod genomes that works towards improved sequencing, assembly, annotation, and data management standards. Insect species could be nominated based on various criteria: their ecological role, human impact, conservation needs, intriguing biology, etc. As of yet, 238 insect genomes are available at the NCBI website, and more sequencing and annotation projects are ongoing (i5K Consortium 2016).

As part of the i5k initiative, genome projects were available for both species used in this work. A genome consortium was erected for the bumblebees *Bombus terrestris* (and *Bombus impatiens*) in 2012, nominated because of their invaluable pollination service. Although the honeybee genome was already available at the time (Weinstock et al. 2006), it was deemed necessary to attain the genome of these wild pollinators too. Comparisons could then be made concerning their different ecology, and insights could be gained on the genetics of social behavior (Sadd et al. 2015). The CPB, *Leptinotarsa decemlineata*, was nominated because of its economic value as pest of potato crops. The genome project was started in 2014 and the publication is expected in 2017.

In collaboration with the bumblebee and CPB genome consortia, we searched the genomes of both species for RNAi-related genes, based on a list obtained from the model species *D.*

melanogaster and the closely related *Apis (A.) mellifera* (for Bombus) and *T. castaneum* (for CPB), which both had a sequenced genome available. The RNAi core gene list consisted of the genes coding for proteins belonging to the Dicer and Argonaute families, as well as the dsRNA binding proteins which act as cofactors during the RNAi pathway. For the purpose of this thesis, also the *sid-1-like* genes were included in the analysis. The gene prediction models were examined and corrected when necessary, eventually resulting in a set of annotated core RNAi genes.

2.2 Materials and methods

A list of known insect RNAi core genes was established based on information from *D. melanogaster* (Adams et al. 2000; Saleh et al. 2006), *A. mellifera* (Weinstock et al. 2006) and *T. castaneum* (Richards et al. 2008; Tomoyasu et al. 2008), as well as a CPB transcriptome study (Swevers et al. 2013a) (Supplementary Table 1). The corresponding protein sequences were obtained from the NCBI database and used as queries in the Hymenoptera Genome Database BLAST tool (for Bombus; Elsik et al. 2015) or the i5k BLAST workspace (for CPB; Poelchau et al. 2015) using tblastn searches. From this, the scaffold on which the gene was located, was obtained. The Apollo JBrowse genome browser (Lee et al. 2013), integrated within the Hymenoptera Genome Database (for Bombus) or the i5k workspace (for CPB), provided a platform for gene model verification and editing. The predicted gene models were evaluated by aligning them to the corresponding *D. melanogaster*, *A. mellifera* or *T. castaneum* homologs (both on nucleotide and amino acid level) using Clustal Omega (Sievers et al. 2011) and comparing, among others, intron/exon structure and UTR length. Additionally, multiple RNAseq data sets, incorporated in the JBrowse program, allowed further refinement of the existing gene models. If one gene was divided over multiple scaffolds, attempts were made to construct an assembled gene model.

2.3 Results and discussion

In both *Bombus* species, all expected homologues for genes encoding the core RNAi machinery proteins were found. Amongst these proteins were the two Dicer and two Argonaute proteins that function in miRNA and siRNA pathways, homologues for the dsRNA binding proteins R2D2, Loquacious and Pasha, two additional Argonaute proteins belonging to the piwi-class (piRNA pathway) and the nuclear RNAse III enzyme Drosha, involved in the miRNA pathway (Table 3). In agreement with the results for *A. mellifera*, no third Argonaute protein involved in the RNAi pathway, called *piwi*, was observed.

Also in the CPB, all expected RNAi core genes were found, including R2D2 which had not be found in the CPB transcriptome (Swevers et al. 2013a). However, a striking observation was the apparent duplication of some siRNA core genes. The *dcr-2a* and *dcr-2b* coding DNA sequences (CDSs) showed 60% nucleotide identity to each other and 56% and 54% identity to the *T. castaneum dcr-2* homolog, respectively. Also duplicate genes encoding for Argonaute-2 were found, which were labelled *ago-2a* and *ago-2b.* Unfortunately, the *ago-2b* gene model was incomplete due to gaps in the assembled genome sequence. Therefore, no identities could be calculated. A similar *ago-2* duplication was also uncovered in *T. castaneum*, but in that species there was only one *dcr-2* homolog. It would be interesting to determine whether these duplicates are functionally redundant or whether they have gained different functionalities during evolution, as the duplication might contribute to the RNAi effectiveness in these species (and Coleoptera in general if similar duplications are conserved within the order). It has been shown that all four genes were upregulated after dsRNA exposure, with both *dcr-2* duplicates having a significantly higher, but similar to each other, upregulation compared to the two *ago-2* duplicates (Guo et al. 2015). A screening, which evaluated the involvement of various RNAi genes in the RNAi response in a CPB cell line, revealed a high dependency of the siRNA pathway on both ago-*2* genes and the *dcr-2a* gene, but only a low dependency on *dcr-2b* (Yoon et al. 2016).

Concerning the dsRNA uptake-related Sid-1-like proteins, only one *sid-1-like* homolog was found in *Bombus*, as was also the case in *A. mellifera*. In CPB, two *sid-1-like* homologs were observed. In both cases no homologs for the other SID proteins of *C. elegans* were found. A more detailed overview and discussion of the distribution of the Sid-1-like proteins in the different insect orders is given in Chapter III and Figure 14A.

In general, there are key similarities between the different insect species and orders when looking at the RNAi core genes (Table 3). The Dicer, Argonaute and dsRNA binding protein families are universally present, but their number differs over the insect orders. A more thorough analysis over all sequenced insect genomes would shed more light on whether the duplications in the siRNA pathway of the two examined beetles or the expansion of the miRNA genes in the aphid *A. pisum* are conserved within their respective orders. But it is clear that within orders as large and diverse as for example the Coleoptera, no conclusions can be drawn on the presence of RNAi genes from species that are generally considered closely related, as was suggested in the introduction.

Table 3. Overview of annotated core RNAi genes in some key insect species and the two model species used in this work

	D. melanogaster (Diptera)	*B. mori* (Lepidoptera)	*A.pisum* (Hemiptera)	*A. mellifera* (Hymenoptera)	***B. terrestris* (Hymenoptera)**	*T. castaneum* (Coleoptera)	***L. decemlineata* (Coleoptera)**
RNAse III proteins	*dicer-1* • *dicer-2* • *drosha* •	*dicer-1* • *dicer-2* • *drosha* •	*dicer-1a* • *dicer-1b* • *dicer-2* • *drosha* •	*dicer-1* • *dicer-2* • *drosha* •	***dicer-1*** • ***dicer-2*** • ***drosha*** •	*dicer-1* • *dicer-2* • *drosha* •	***dicer-1*** • ***dicer-2a*** • ***dicer-2b*** • ***drosha*** •
Argonaute proteins	*argonaute-1* • *argonaute-2* • *argonaute-3* • *aubergine* • *piwi* •	*argonaute-1* • *argonaute-2* • *argonaute-3* • *aubergine* •	*argonaute-1a* • *argonaute-1b* • *argonaute-2* • *argonaute-3* • *aubergine* • *piwi* •	*argonaute-1* • *argonaute-2* • *argonaute-3* • *aubergine* •	***argonaute-1*** • ***argonaute-2*** • ***argonaute-3*** • ***aubergine*** •	*argonaute-1* • *argonaute-2a* • *argonaute-2b* • *argonaute-3* • *aubergine* • *piwi* •	***argonaute-1*** • ***argonaute-2a*** • ***argonaute-2b*** • ***argonaute-3*** • ***aubergine/piwi*** •
dsRNA binding proteins	*loquacious* • *R2D2* • *pasha* •	*loquacious* • *R2D2* • *pasha* •	*loquacious-a* • *loquacious-b* * • *R2D2* • *pasha-a* • *pasha-b* • *pasha-c* • *pasha-d* •	*loquacious* • *R2D2* • *pasha* •	***loquacious*** • ***R2D2*** • ***pasha*** •	*loquacious* • *R2D2* • *pasha* •	***loquacious*** • ***R2D2*** • ***pasha*** •
Sid-1-like proteins	/	*sid-1-like 1* *sid-1-like 2* *sid-1-like 3*	*sid-1-like*	*sid-1-like*	***sid-1-like 1***	*sid-1-like A* *sid-1-like B* *sid-1-like C*	***sid-1-like A*** ***sid-1-like C***

* The second *loquacious* homolog in *A. pisum i*s degraded and probably corresponds to a pseudogene.

Green •: siRNA pathway, yellow •: miRNA pathway, red •: piRNA pathway.

Drosophila (D.) melanogaster, Bombyx (B.) mori, Acyrthosiphon (A.) pisum, Apis (A.) mellifera, Bombus (B.) terrestris, Tribolium (T.) castaneum and *Leptinotarsa (L.) decemlineata* (CPB) (Adams et al. 2000; Mita et al. 2004; Weinstock et al. 2006; Richards et al. 2008; The International Aphid Genomics Consortium 2010; Sadd et al. 2015)

Chapter III:

The involvement of clathrin-dependent endocytosis and two Sid-1-like transmembrane proteins in double-stranded RNA uptake in the Colorado potato beetle midgut

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K. Cappelle designed the study and developed the methodology. K. Cappelle performed the assays, aided by C. F. de Oliveira and B. Van Eynde, and analyzed the results. The manuscript was written by K. Cappelle and revised by O. Christiaens and G. Smagghe

3.1 Introduction

The RNAi phenomenon has become a useful research tool in entomology, especially in loss-of-function studies, because of its easy application and high specificity. These same characteristics make RNAi also applicable in crop protection against herbivorous insects. A wide range of target organisms and genes has been described so far, but with a significant variability in RNAi efficiency. The RNAi event comprises two major steps: first, the dsRNA should be taken up by cells and then subsequently processed by the cellular core RNAi machinery, triggering the silencing of the target gene. This makes cellular uptake a key factor and while the core components and their role are already well known in several insects, still many questions remain concerning the dsRNA uptake pathways. Besides the presence of dsRNA degrading enzymes (Christiaens et al. 2014; Wynant et al. 2014c) and/or viral suppressors of RNAi (Li et al. 2002; van Rij et al. 2006; Nayak et al. 2010), another main suspect for explaining the variability in feeding experiments might be the dsRNA uptake efficiency in the insect digestive tract, presumably the midgut (Xiao et al. 2015).

In invertebrates, two different dsRNA uptake systems have been described so far. On the one hand there are the SID transmembrane channel proteins which were discovered in *C. elegans* (Winston et al. 2002). Only for SID-1, homologous genes have been found in many, but not all, insects. For the other transmembrane proteins SID-2 and SID-5 (Hinas et al. 2012), and the tyrosine kinase SID-3 (Jose et al. 2012) no insect homologs have been reported as of yet. These SID proteins are necessary in the systemic RNAi response, for SID-1 and SID-2 it is thought to be through their involvement in dsRNA uptake from the *C. elegans* intestine (Winston et al. 2007). In *D. melanogaster*, no SID-1 homolog is present, but for this species dsRNA uptake by receptor-dependent endocytosis has been demonstrated (Saleh et al. 2006; Ulvila et al. 2006).

In the last few years, articles describing the involvement of one of these pathways in dsRNA uptake have emerged for several insect species, giving valuable information. However, it seems that there is still no consensus about which system is involved (overview in Table 4). The presence of *sid-1* homologs does not necessarily mean that dsRNA uptake is performed by the resulting Sid-1-like proteins. Some experiments indicate a need for *sid-1-like* genes only in feeding setups (Aronstein et al. 2006; Miyata et al. 2014; Li et al. 2015), but this hypothesis is contradicted by injection experiments in *Nilaparvata lugens*, which found a *sid-1-like* gene to be involved. (Xu et al. 2013). It is interesting to note that in two closely related coleopteran insects, *T. castaneum* (Tc) and *Diabrotica virgifera* (Dv), the former has three different *sid-1*-like genes which do not seem to be necessary for dsRNA uptake (Tomoyasu et al. 2008) whereas the latter only has two which are

both confirmed as being involved (Miyata et al. 2014). In most studies, only one pathway has been examined, thus preventing an in-depth evaluation of the distribution of the involvement of these pathways over the different insect families.

This chapter will evaluate the involvement of both pathways in dsRNA uptake in the midgut through the use of an RNAi-of-RNAi reporter system in the Colorado potato beetle (CPB), *Leptinotarsa decemlineata (Ld)*, an important crop pest which shows a robust RNAi response (Baum et al. 2007; Zhu et al. 2011; Zhou et al. 2013; Kong et al. 2014). This reporter system consists of two silencing events: the first, in which a target gene suspected to contribute to the RNAi response is silenced, followed by a second silencing event, in which a reporter gene is targeted. Changes in silencing efficiency of the reporter gene indicate the involvement of the target gene in the RNAi response (Dudley et al. 2002). As a reporter gene, *α-amylase* was chosen as it is expressed mainly in the digestive tract, is silenced easily and has not been associated with any form of cellular uptake (Graveley et al. 2011). To evaluate both pathways, we selected the *sid-1* homologs on the one hand, and the *clathrin heavy chain* (chc) that is playing a role in clathrin-dependent endocytosis on the other hand. Moreover, since it is expected that the vacuolar H^+ ATPase (V-ATPase) has an important role in the release of dsRNA contained in endocytic vesicles (Saleh et al. 2006), two proteins located in functionally different V-ATPase domains were targeted, the subunit A (Vha68) that is located in the peripheral domain (V_1) and the subunit c (Vha16) in the integral domain (V_0) (Beyenbach and Wieczorek 2006). Furthermore, a pharmacological inhibitor of clathrin- dependent endocytosis, chlorpromazine hydrochloride, was administered to see its effect on the RNAi efficiency and to confirm the involvement of the clathrin-dependent endocytosis in the cellular uptake of dsRNA.

Table 4. Overview of reported dsRNA uptake experiments in *C. elegans* and insects.

Organism	Insect order	Environmental RNAi	Systemic RNAi	No. of *sid-1* homologs present	Application method	SID-1 is involved	Endocytosis is involved	Ref.
C. elegans	/	++	++	1	F/I	yes	n.d.	(Winston et al. 2002)
D. melanogaster (S2 cell line)	Diptera	+	+	0	S	no	yes	(Saleh et al. 2006; Ulvila et al. 2006)
B. dorsalis	Diptera	+	+	0	F	no	yes	(Li et al. 2015)
T. castaneum	Coleoptera	+	++	3	I	no	yes	(Tomoyasu et al. 2008); (Xiao et al. 2015)
D. virgifera	Coleoptera	++	++	2	F	yes	n.d.	(Miyata et al. 2014)
L. decemlineata	Coleoptera	++	++	2	F	yes	yes	This work
B. mori	Lepidoptera	-	+	3	I	no	n.d.	(Tomoyasu et al. 2008)
S. gregaria	Orthoptera	-	++	1	I	no	yes	(Wynant et al. 2014b)
L. migratoria	Orthoptera	-	++	1	I	no	n.d.	(Luo et al. 2012)
A. mellifera	Hymenoptera	+	+	1	F	yes	n.d.	(Aronstein et al. 2006)
N. lugens	Hemiptera	-	+	1	I	yes	n.d.	(Xu et al. 2013)

++: present and robust, +: present but not robust, -: not present, F: feeding, I: injection, S: soaking, n.d.: not determined.

Caenorhabditis (C.) elegans, Drosophila (D.) melanogaster, Bactrocera (B.) dorsalis, Tribolium (T.) castaneum, Diabrotica (D.) virgifera, Leptinotarsa (L.) decemlineata (CPB), *Bombyx (B.) mori, Schistocerca (S.) gregaria, Locusta (L.) migratoria, Apis (A.) mellifera* and *Nilaparvata (N.) lugens*.

3.2 Materials and methods

3.2.1 *Insects*

All stages of the CPB were maintained in standard rearing conditions of 25°C, 40% RH and 16:8h light:dark and were fed with fresh potato foliage (*Solanum tuberosum* cv. Bintje). Adult insects were kept in an insect rearing cage, and the eggs were collected manually and transferred to plastic dishes until the hatching of larvae. Neonate first instar larvae were used in the feeding assays and the experiments performed at standard rearing conditions.

3.2.2 *Target and reporter gene selection*

Nucleotide sequences for the target and reporter genes were obtained from a transcriptome database, derived from fourth instar larvae of the CPB, available in-house (Swevers et al. 2013a). Tblastn searches using *T. castaneum* homologs were used to find the corresponding CPB sequences. Primers were designed using Primer 3 (Koressaar and Remm 2007) and are shown in Table 5. The amplified sequences were verified using the LGC Genomics Sanger sequencing service (Berlin, Germany) and submitted to Genbank (*alfa-amylase*: KP273188; *vha16*: KP273189; *vha68*: KP273190; *chc*: KP273191; *silA*: KP273192; *silC*: KP273193). The primers for dsRNA synthesis of the *laccase-2* fragment were found in literature (Yates 2014).

3.2.3 *dsRNA synthesis*

The dsRNA was prepared using the MEGAscript RNAi Kit (Life Technologies, Carlsbad, USA) according to the manufacturer's specifications. The T7-DNA products used as dsRNA template were generated during a PCR reaction (5 cycles using an annealing temperature of 55°C followed by 25 cycles at 60°C) using cDNA of first-instar CPB larvae and the appropriate T7-primers. To correct for potential random effects of dsRNA on the host, a dsRNA sequence targeting green fluorescent protein (GFP) was used as a control. This protein was selected as it has no homologues in insects. For preparation of dsGFP, a linearized plasmid containing the GFP sequence was used as template. The dsRNA was eluted in 50µL hot nuclease-free water and after concentration measurement, stored at -20 °C.

Table 5. List of primers used in Chapter III.

RT-qPCR primers	Sequence (5' – 3')	Length (bp)	dsRNA synthesis primers	Sequence (5' – 3')	Length (bp)
sil-A F	CGAGCGACACGATTGTCATC	130	sil-A_T7 F	TAATACGACTCACTATAGGGCCAGGCCGGATAACTTCGAT	306
sil-A R	ACGGTTTGGTAAGTGCCCTC		sil-A_T7 R	TAATACGACTCACTATAGGGATCTGGAGCATCCGTTTCCG	
sil-C F	TGTCTCCCAGTTGATGATTGCT	122	sil-C_T7 F	TAATACGACTCACTATAGGGTCAAGATGTAACCTTCCGTGG	296
sil-C R	TTGTATCAAAAACAGGGCAACTT		sil-C_T7 R	TAATACGACTCACTATAGGGAGCCCCTATGCAAGACAACG	
chc F	CCTCACTTAGCGTGTGTAGCA	113	chc _T7 F	TAATACGACTCACTATAGGGGCCTCTTGGATGTCGATTGT	275
chc R	CGCCTACGCACCAAATAACG		chc _T7 R	TAATACGACTCACTATAGGGTCTTTGAGGAATCTCTCGGCA	
vha68 F	TGATGGTATCCAACGTCCGCTGA	86	vha68_T7 F	TAATACGACTCACTATAGGGATGGCCACCATCCAGGTAT	407
vha68 R	AGAGTGAAGGCACGTTCACACCC		vha68_T7 R	TAATACGACTCACTATAGGGTTCCTGGTTCTGCCACGTAT	
vha16 F	TGCTGGGGTCAGAGGAATAG	103	vha16_T7 F	TAATACGACTCACTATAGGGGGACCCTTCTTTGGCGTTAT	260
vha16 R	GGCTACAATGAGACCATAAAGACC		vha16_T7 R	TAATACGACTCACTATAGGGGCACCCAAATGTACGAAACC	
α-Amylase F	GGTTTTGCCGGTGTACAGAT	114	α-amylase_T7 F	TAATACGACTCACTATAGGGTTAGGGGGTGAAGCAATCAG	326
α-Amylase R	TAACGACCAGATCCGGAAAC		α-amylase_T7 R	TAATACGACTCACTATAGGGTTCAACGCTCATGATCAAGG	
arf1 F	CGGTGCTGGTAAAACGACAA	135	GFP_T7 F	TAATACGACTCACTATAGGGTACGGCGTGCAGTGCT	495
arf1 R	TGACCTCCCAAATCCCAAAC		GFP_T7 R	TAATACGACTCACTATAGGGTGATCGCGCTTCTCG	
rp18 F	TAGAATCCTCAAAGCAGGTGGCGA	133	Lac2-T7 F	TAATACGACTCACTATAGGGAGCGTCTGGTCTGATTTCGT	442
rp18 R	AGCTGGACCAAAGTGTTTCACTGC		Lac2-T7 R	TAATACGACTCACTATAGGGCAGAGACCCCAACACTGGTT	
Lac2 F	ACCACCCATGTCTTACTTCTGA	105			
Lac2 R	AACTCTCTGGATCTTGACCGGTAT				

sil: *systemic RNA interference deficient-1-like*; chc: *clathrin heavy chain*; vha68: *68 kDa subunit of the vacuolar ATPase*; vha16: *16 kDa subunit of the vacuolar H+ ATPase*; arf1: *ADP-ribosylation factor*; rp18: *ribosomal protein 18*; GFP: *green fluorescent protein*; lac2: l*accase2*.

3.2.4 *Feeding assays*

In order to conduct the RNAi-of-RNAi experiments, a feeding assay for the delivery of dsRNA was developed. At day 0, 1 µL nuclease-free water containing 400 ng dsRNA of the target gene was dropped on a piece of potato leaf of approximately 4 mm^2 and air-dried, after which neonate larvae, starved for 4 hours, were placed onto the leaf pieces. After total consumption of the leaf pieces, the larvae were placed in small insect rearing cages and fed daily with fresh potato leaves. Any larvae that had not completely eaten the leaf piece, were discarded from the experiment. At day 2, the larvae were fed, using the same method, with 400 ng dsRNA targeting the reporter gene. To assess the unaffected RNAi response, two different control groups were necessary, one in which 400 ng of dsGFP was used on both days 0 and 2, the other where 400 ng dsGFP was only used at day 0, but dsRNA against the reporter at day 2. At day 4, the larvae were dissected and the midguts placed into 350 µL of RLT buffer from the RNeasy Mini kit (Qiagen, Hilden, Germany) for RNA extraction. Each treatment consisted of 12 larvae. In the second *chc* experiment, which used the laccase-2 reporter, the time between the first dsRNA and second dsRNA application was reduced from 48 hours to 24 hours. For the chlorpromazine hydrochloride (CPZ; Sigma-Aldrich, St. Louis, USA) treatment a similar setup was used. At day 0, 1 µL CPZ solution (5 µg/µL in dH2O) was fed to the larvae and 6 hours later a second identical dose was applied, together with 400 ng dsRNA against the reporter. At day 2, the larvae were dissected and the midguts were processed as described before.

In order to determine the LD_{50} when silencing *chc*, CPB larvae were fed with 10 different doses of dsRNA targeting *chc* using the same method: 400 ng, 100 ng, 25 ng, 5 ng, 1 ng, 0.75, 0.2 ng, 0.04 ng and 0.008 ng per individual (15 larvae per treatment). Several doses of dsGFP were used as controls: 400 ng, 25 ng, 1 ng and 0.2 ng with each dsChc dose corresponding to a control dose which is either equal or the nearest higher dsGFP dose. Mortality was scored daily for 14 days and statistics were performed using SPSS Statistics 23.0 (Kaplan-Meier survival analysis; log rank test) and SigmaPlot 13 (4 parameter sigmoidal regression fitting).

3.2.5 *RNA extraction, cDNA synthesis and RT-qPCR*

Total RNA was extracted from three pooled midguts (resulting in four biological repeats) using the RNeasy Mini kit and treated with the Turbo DNA-free kit (Life Technologies). For cDNA synthesis, 500 ng of RNA was used in each reaction, performed with SuperScript II Reverse Transcriptase (Life Technologies), according to the manufacturer's specifications.

The RT-qPCR reactions were performed in duplicate in a C1000 Touch Thermal Cycler (Bio-Rad, Hercules, USA) using the GoTaq qPCR Master Mix (Promega, Madison, USA). Each reaction was composed of 1 µL of each primer (10 µM), 10 µL of GoTaq Master Mix and 8 µL of cDNA (diluted 1:40). The amplification conditions were 95°C for 5 min followed by 40 cycles of 95°C for 30 s, and 60°C for 60 s. The reference genes *rp18* and *arf1* were used for normalization of the data (Shi et al. 2013). The amplification efficiency of all primer sets was verified to be between 85 and 115% using ten-fold dilution standard curves. These were repeated for every separate experiment, in parallel with the samples. Additionally, the specificity of each primer set was evaluated using melting curves, ranging from 60°C to 95°C with 0.5°C degree steps. Any samples with a Cq value above 35, a delta Cq between replicates higher than 0.5, or an aberrant melting curve were discarded from the analysis. The results were analyzed using the qbase+ software (Biogazelle, Zwijnaarde, Belgium) and SPSS Statistics 23.0 (IBM Corp 2015).

3.3 Results

3.3.1 *Identification of target and reporter genes*

Two full-length *sid-1-like* (*sil*) coding sequences were found in the CPB midgut transcriptome, *Ld-silA* corresponding to *Tc-silA* (52.3% amino acid (AA) identity) and *Dv-silA* (63.9% AA identity) and *Ld-silC* corresponding to *Tc-silC* (62.9% AA identity) and *Dv-SilC* (66.9% AA identity). Both contain an aminoterminal extracellular domain, which is responsible for most of the variation, followed by 11 transmembrane regions, as was also found for other *sid-1* homologs (Supplementary Figure 1). In addition, the three target genes involved in endocytosis were identified in CPB. For *chc* only a partial sequence was obtained (40% AA coverage and 97% AA identity in respect to Tc-chc), whereas for *vha16* a full-length sequence was attained with an 86.1% AA identity to Tc-Vha16. In the case of *Vha68* a partial sequence covering approximately 67.0% of the *T. castaneum* homolog was found, showing 92.2% AA identity.

As reporter gene, *α-amylase* was chosen as it is not linked to dsRNA uptake and it is expressed in the midgut. A full-length *α-amylase* sequence was found in CPB with a 66.6% AA identity to Tc-α-amylase and 68.8% to Dv-α-amylase. In one case also *laccase-2* was used as a reporter (75% AA identity to Tc-Laccase2) because of a possible link between *α-amylase* expression and food intake.

3.3.2 *Involvement of both sil genes in dsRNA uptake*

As the expression levels of *silA* and *silC* in the dissected midguts remained similar to each other during the experiment (1.55 ± 0.33 and 1.24 ± 0.31, respectively, normalized to the reference genes), identical doses of dsRNA (400 ng) resulted in a silencing of 59% for *silA* and 66% for *silC* after 4 days. Compared to the control in which the RNAi response was unaffected, the silencing of *silA* and *silC* decreased the RNAi efficiency against the reporter gene slightly, though the difference was statistically significant. Rescue of the signal was higher in the case of *silA* (14.4% of the silencing was recovered; p = 0.0002; Figure 11A) than for *silC* where there was 3.4% rescue (p = 0.010; Figure 11B). Increasing the dose of dsRNA against *silC* from 400 ng to 600 ng resulted in a similar *silC* silencing (65%) but the rescue mounted to 8.1% (p = 0.041; Figure 11C). In an attempt to gain more information about the way the two *sil* genes function in relation to each other, the larvae were treated with dsRNA against both *sil* genes simultaneously. A recovery of 14.1% was seen (p = 0.035; Figure 11D).

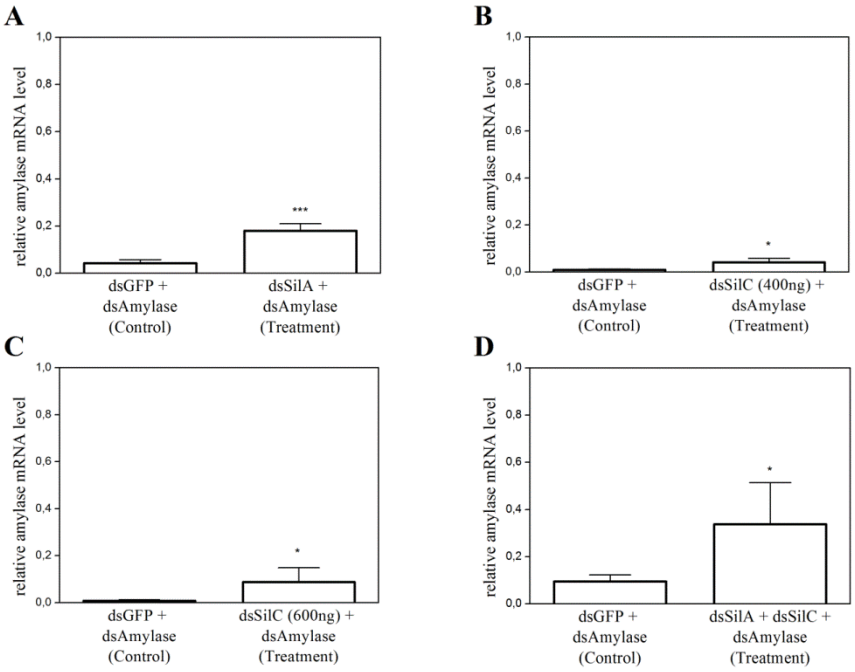


Figure 11. The two *sil* genes in the Colorado potato beetle are involved in dsRNA uptake in the midgut.
L1 larvae were fed dsRNA against the target gene(s) and 2 days later, they were fed 400 ng dsAmylase. The effect of the treatment on the silencing of the reporter was evaluated 4 days after the start of the experiment using RT-qPCR. The columns represent the mean ± SEM, normalized to the *amylase* levels in the dsGFP + dsGFP control (n=12). Statistical analysis was performed using Student's t-test (***: p < 0.001; *: p < 0.05). Treatment with 400 ng dsSilA resulted in a rescue of 14.4% of the silencing effect (A). In contrast, treatment with 400 ng (B) and 600 ng (C) dsSilC brought about 3.4% and 8.1% rescue respectively. Treatment with 400 ng dsSilA and 400 ng dsSilC simultaneously resulted in 14.1% rescue. Standard deviations of the baseline reporter level were: 0.35 (A), 0.15 (B), 0.26 (C) and 0.21 (D).

3.3.3 *Involvement of the endocytic pathway components in dsRNA uptake*

Three different components of the endocytic pathway were considered as target genes: two components of the vacuolar H^+ ATPase complex (*vha68* and *vha16*) and the *clathrin heavy chain* (*chc*). For *vha68* (also known as subunit A), present in the extracellular V1 domain, no recovery of the reporter gene silencing was observed ($p = 0.346$; Figure 12A), even with a silencing of the *vha68* gene of 96.5%. However, for *vha16* (subunit c), in the transmembrane V0 domain, a rescue of 25.4% was detected at a *vha16* silencing of 75% ($p = 0.0016$; Figure 12B).

Unfortunately the effect of silencing *chc* on the RNAi response could not be evaluated using *α-amylase* as a reporter, as disturbed feeding was observed from day 2 onwards, resulting in mortality starting around day 4. Lower doses of dsChc and shorter experiments delayed mortality, but the silencing of the reporter gene could not be assessed reliably as amylase expression levels are linked with food uptake. To further elucidate the involvement of endocytosis, the experiment in which *chc* was silenced was repeated but with a reporter that was not linked to food intake or metabolism in any way, *laccase2*. In this experiment, a chlorpromazine hydrochloride treatment was included and the total experiment duration was reduced, which made it possible to assess the effect of *chc* silencing on dsRNA uptake. For this reporter, silencing was less efficient (60% silencing) and showed more variability. A small rescue ($p = 0.29$) was seen in the case of dsChc treatment (which itself was silenced for 87%) and a larger, near complete rescue ($p = 0.05$) in the case of the chlorpromazine hydrochloride treatment (Figure 12C).

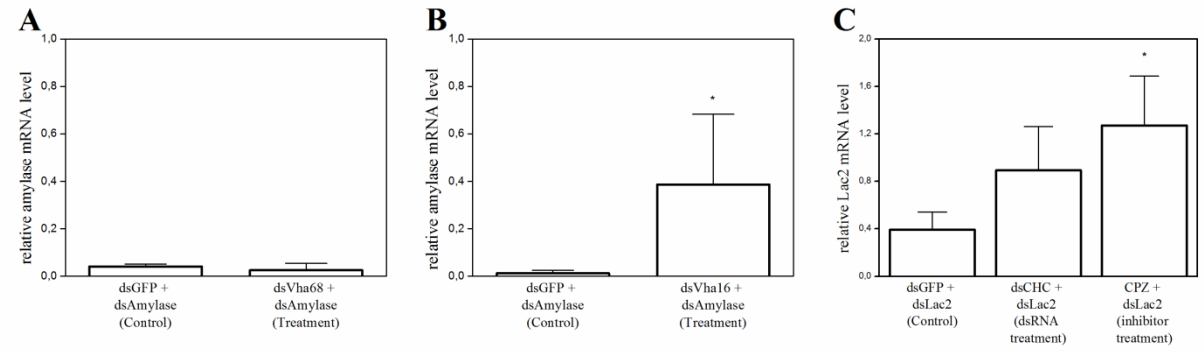


Figure 12. The involvement of two components of the vacuolar H^+ ATPase complex and the clathrin heavy chain in dsRNA uptake in the Colorado potato beetle midgut.
L1 larvae were fed dsRNA against the target gene and 2 days later, they were fed 400 ng dsAmylase (A and B) or 400 ng dsLac2 (C). For the chlorpromazine hydrochloride treatment (CPZ), two doses of 5 µg CPZ were applied, one 6h before dsLac2 treatment, the other simultaneously. The effect of the treatment on the silencing of the reporter was evaluated 4 days after the start of the experiment using RT-qPCR. The columns represent the mean ± SEM, normalized to the amylase levels in the dsGFP + dsGFP control (n=12). Statistical analysis was performed using Student's t-test (A and B) or ANOVA (C) (*: $p < 0.05$) . After treatment with 400 ng dsVha68 (A), no rescue of the silencing effect was observed, whereas treatment with 400 ng

dsVha16 (B) resulted in a rescue of 37.5%. Treatment with 400ng dsChc led to a rescue of 50% and an almost complete rescue was observed in the case of the chlorpromazine hydrochloride treatment (C). Standard deviations of the baseline reporter level were 0.21 (A), 0.23 (B) and 0.11 (C).

3.3.4 *Chc as a target for RNAi-based pest control*

As section 3.3.3 revealed viability impairment caused by silencing of *chc*, different doses of dsChc were tested for their lethal potential. Using Kaplan Meier survival analysis, significant increases in mortality were found for all doses up until 0.2 ng per insect: (400ng: 100% mortality ($p < 0.001$); 100ng: 80% ($p = 0.001$); 25: 100% ($p < 0.001$); 5 ng: 93% ($p < 0.001$); 1 ng: 87% ($p < 0.001$); 0.75: 87% ng ($p < 0.001$); 0.2 ng: 85% ($p = 0.001$); 0.04 ng: 42% ($p > 0.05$) and 0.008 ng: 13% ($p > 0.05$). Using sigmoidal regression fitting (R^2=0.92) the LD_{50} was determined to be 0.053 ng per individual.

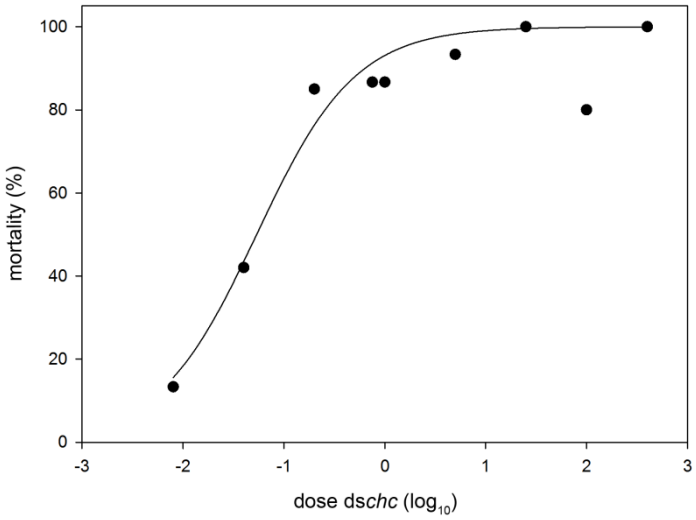


Figure 13. Sigmoidal regression fit for LD_{50} determination of dsRNA targeting *clathrin heavy chain* (dsChc) in Colorado potato beetle larvae.
Mortality after 14 days was plotted for nine different concentrations of dsChc (n=15). For the sake of regression fitting, the doses are log_{10} transformed. A four-parameter sigmoidal curve was used, with constraints at the x-axis infinites of 0 and 100, resulting in the following equation: $y=100/(1+e^{(1,27-x)/0.4885})$ (R^2=0.92). The resulting LD_{50} was 0.053 ng.

3.4 Discussion

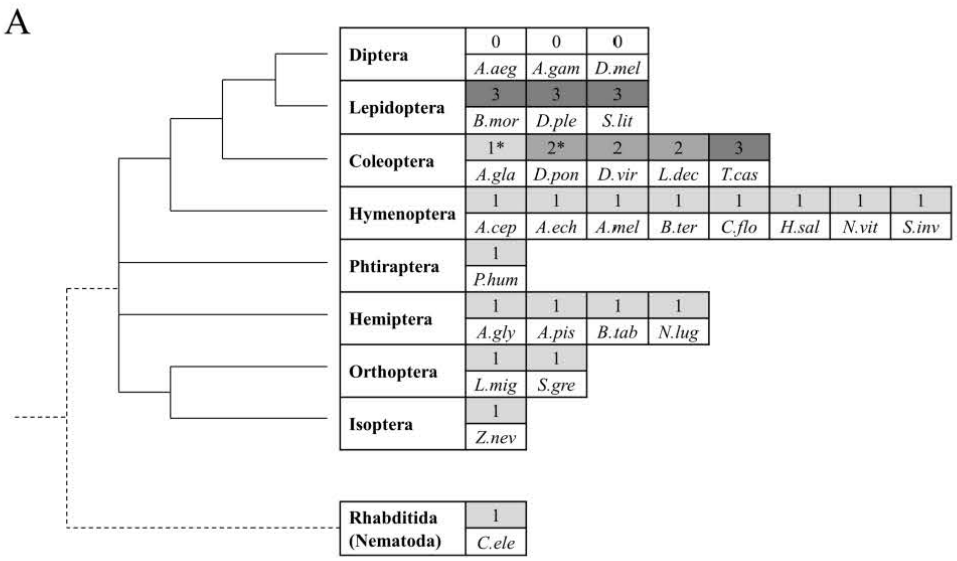
Substantial efforts have been made in order to elucidate all features of the RNAi mechanism. However, the fundamental aspects related to dsRNA uptake in insect tissues continue to be unclear. With the findings of which pathways are involved in the different insect orders, better strategies of delivery could be applied, advancing the applicability of RNAi.

In the CPB, two distinct *sil* genes were identified, as was also reported for *D. virgifera* (Miyata et al. 2014), belonging to the same family of the Chrysomelidae. In contrast, three *sil* genes were found in another coleopteran, *T. castaneum* (family Tenebrionidae). In most other insect orders only one *sid-1* homolog was found. However, in some lepidopteran species, 3 distinct *sid-1-like* genes are present. The distribution of the number of *sid-1-like* genes over the different orders is illustrated in Figure 14A. It is remarkable that all species that deviate from the 1 *sid-1-like* gene formula, namely the Coleoptera, Lepidoptera and Diptera, form a monophyletic clade. This seems to suggest that the presence of multiple *sid-1-like* genes in Coleoptera and Lepidoptera is caused by gene duplication in their common ancestor, and were then lost again in Diptera. We also examined if the *sid-2*, *sid-3* or *sid-5* genes of *C. elegans* showed a similar domain structure to the *sid-1-like* genes, as convergent evolution could possibly explain the presence of multiple *sid-1-like* genes. However, these genes do not contain the typical 11-transmembrane domain barrel structure observed in the *sid-1-like* genes.

Regarding the involvement of the *sil*-genes in dsRNA uptake (Table 4), for Coleoptera it has been demonstrated in *D. virgifera* (Miyata et al. 2014) and now in *L. decemlineata*. However, the *sil*-genes' involvement was discarded in *T. castaneum* (Tomoyasu et al. 2008). This might be connected to the number of *sil* genes present, but it is more likely that other factors are playing here. For example, the dsRNA application method has to be taken into account as testing in *T. castaneum* happened through injection instead of feeding. It is possible that uptake in the midgut occurs through a different pathway than in the hemocoel-surrounding tissues or that the contribution of each pathway to the process of dsRNA uptake depends on the tissues that are examined. The only other study in which a feeding delivery was used to confirm the role of the *sid-1-like* gene was conducted in *A. mellifera*. However, in that case no RNAi-of-RNAi experiment was performed and the authors' conclusion was based on the upregulation of the *sid-1-like* gene after administration of dsRNA, a method which might be insufficient by itself (Aronstein et al. 2006). In the case of injection, there is one study in which a Sid-1-like protein is confirmed as a dsRNA uptake mechanism, namely in *Nilaparvata lugens* (Xu et al. 2013).

In our experiments, silencing *silA* proved to be more effective than *silC*, even to the extent that the rescue corresponding to *silC* silencing is questionable at low doses. Here it is necessary to emphasize that the administration of dsRNA exerts only a partial silencing effect on the target gene. This way, the non-silenced fraction, the other *sil*-gene as well the endocytic pathway are still playing their role in dsRNA uptake. Furthermore, nothing is known on the half-life of the SID-proteins, which could also play an important role. Unfortunately, gene knock-out cannot easily be achieved in CPB. Nevertheless, there is an indication that the Sid-1-like proteins might contribute to dsRNA uptake in the midgut. Because of the low rescues, conclusions about the importance of both Sid-1-like proteins and whether they are functioning independently or not should be made with caution.

It has been suggested that the insect's *sid-1-like* genes show more similarity to *C. elegans*' *chup-1*, previously named *tag-130*, than to *Ce-sid-1 (Tomoyasu et al. 2008)* and that many of the sid1-homologues have been mislabeled (Valdes et al. 2012). This Chup-1 protein is thought to be involved in cholesterol uptake. A phylogenetic tree of all documented *sid-1*(-like*)* and *chup-1* sequences in both insects and nematodes (Figure 14B) indeed shows clustering of the *chup-1* genes within the clade of the insect *sid-1-like* genes whereas the nematode *sid-1* genes constitute a separate clade. When looking at the hydrophobicity plots for some annotated coleopteran Sid-1-like proteins, an 11 transmembrane-profile is visible, similar to the Ce-Sid-1 and Ce-Chup-1 proteins. However, the hydrophobicity score of the 4^{th} and 5^{th} transmembrane region is less pronounced for Chup-1 than for the Sid-1-like proteins (Supplementary Figure 1). It is not unconceivable that in a common ancestor, one protein was responsible for both uptake functions and that in *C. elegans* gene duplicates have evolved in different directions, resulting in Sid-1 and Chup-1, while in insects the protein may have lost the function of cholesterol uptake. Additional experiments, in which the role of the insect Sid-1-like proteins in cholesterol uptake should be evaluated, are needed before we can rule on the loss of function or a potential dual role of these uptake genes. As a conclusion, we can say that there is no clear-cut decision about the evolution of the *sid-1-like* genes in insects yet. However, there is ample evidence concerning the role of Sid-1-like proteins in insects to dismiss the notion that this is a mere case of misidentification. More experiments need to be performed in other to assess a potential dual role of the Sid-1-like proteins in insects.


A
Diptera
0 0 0
A.aeg A.gam D.mel
Lepidoptera
3 3 3
B.mor D.ple S.lit
Coleoptera
1* 2* 2 2 3
A.gla D.pon D.vir L.dec T.cas
Hymenoptera
1 1 1 1 1 1 1 1
A.cep A.ech A.mel B.ter C.flo H.sal N.vit S.inv
Phtiraptera
1
P.hum
Hemiptera
1 1 1 1
A.gly A.pis B.tab N.lug
Orthoptera
1 1
L.mig S.gre
Isoptera
1
Z.nev
Rhabditida (Nematoda)
1
C.ele

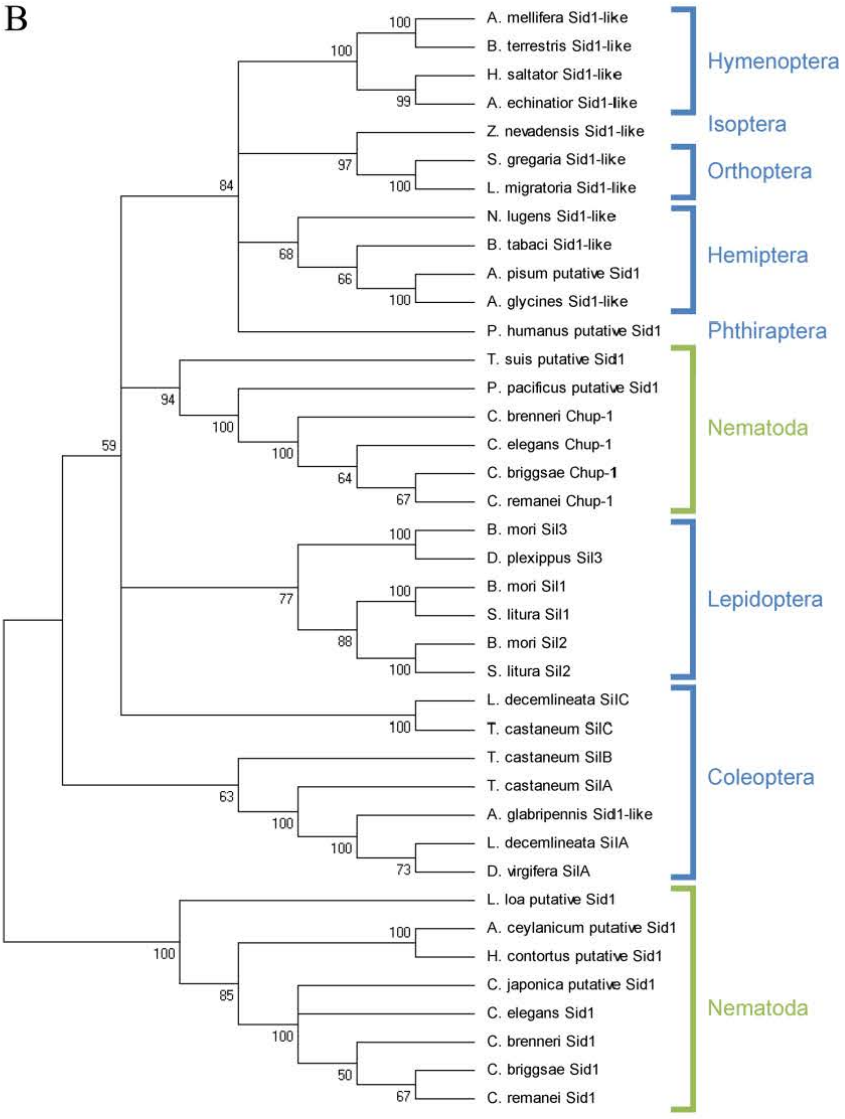

B
A. mellifera Sid1-like
B. terrestris Sid1-like
H. saltator Sid1-like
A. echinatior Sid1-like
Hymenoptera
Z. nevadensis Sid1-like
Isoptera
S. gregaria Sid1-like
L. migratoria Sid1-like
Orthoptera
N. lugens Sid1-like
B. tabaci Sid1-like
A. pisum putative Sid1
A. glycines Sid1-like
Hemiptera
P. humanus putative Sid1
Phthiraptera
T. suis putative Sid1
P. pacificus putative Sid1
C. brenneri Chup-1
C. elegans Chup-1
C. briggsae Chup-1
C. remanei Chup-1
Nematoda
B. mori Sil3
D. plexippus Sil3
B. mori Sil1
S. litura Sil1
B. mori Sil2
S. litura Sil2
Lepidoptera
L. decemlineata SilC
T. castaneum SilC
T. castaneum SilB
T. castaneum SilA
A. glabripennis Sid1-like
L. decemlineata SilA
D. virgifera SilA
Coleoptera
L. loa putative Sid1
A. ceylanicum putative Sid1
H. contortus putative Sid1
C. japonica putative Sid1
C. elegans Sid1
C. brenneri Sid1
C. briggsae Sid1
C. remanei Sid1
Nematoda

Figure 14. Phylogenetic trees showing *sid-1* relationships in insects and nematodes.
A) Phylogenetic tree showing the relationship between the insect orders for which information about *sid-1* homologs is known (basic tree adapted from Ishiwata et al. 2011). Branch length is not indicative for phylogenetic distance. The numbers indicate the number of *sid-1-like* genes reported in this species. *: the number is the result of unconfirmed transcriptome data; it cannot be ruled out that more sid-1 homologs are present. The orders for which more than one or no sid-1-*like* gene have been described, form a monophyletic clade (Coleoptera, Lepidoptera and Diptera).
B) Phylogenetic tree showing the relationship between the different insect and nematode Sid-1(-like) and Chup-1 proteins. The tree was constructed using the Maximum Likelihood method (1000 bootstrap replications) in the MEGA5 software (Tamura et al. 2011) using a subset of sequences which was determined by the MaxAlign webtool (Gouveia-Oliveira et al. 2007). The percentage of trees in which the associated taxa clustered together is shown next to the branches and the tree was condensed with a cut-off value of 50%. Branch lengths are representative for the number of substitutions per site. The nematode Chup-1 sequences cluster together within the insect Sid-1-like proteins whereas the nematode Sid-1 proteins form a separate clade. Sid-1(-like) UniprotKB identifiers: *Aedes aegypti*: /; *Anopheles gambiae*: /; *Drosophila melanogaster*: /; *Bombyx mori*: A9CQK8, A9CQL3, A9CQL1; *Danaus plexippus*: G6D1P5, G6DBM3, G6D1B5; *Spodoptera litura*: V9VIE0, V9VLL3, V9VMZ4; *Anoplophora glabripennis*: V5G0U6; *Dendroctonus ponderosae*: U4U416, U4UCY0; Diabrotica virgifera: Miyata et al. 2014); *Leptinotarsa decemlineata*: KP273192, KP273193 (Genbank accession nrs.); *Tribolium castaneum*: A7YFV8, A7YFW0, A7YFW2; *Atta cephalotes*: W4WKL2; *Acromyrmex echinatior*: F4WVP0; *Apis mellifera*: XP_006565236 (Genbank accession nr.); *Bombus terrestris*: XP_003399893 (Genbank accession nr.); *Camponotus floridanus*: E2AJI6; *Harpegnathos saltator*: E2B9B2; *Nasonia vitripennis*: K7J510; *Solenopsis invicta*: E9I853; *Pediculus humanus subsp. corporis*: E0VXD3; *Aphis glycines*: M1G950; *Acyrthosiphon pisum*: J9K2U4; *Bemisia tabaci*: A0A059TD68; *Nilaparvata lugens*: D9MNS2; *Locusta migratoria*: K9LY69; *Schistocerca gregaria*: X2J861; *Zootermopsis nevadensis*: A0A067R6J9; *Ancylostoma ceylanicum*: A0A016VGN0; *Caenorhabditis brenneri*: G0MS59; *Caenorhabditis briggsae*: A8X6T8; *Caenorhabditis elegans*: Q9GZC8; *Caenorhabditis remanei*: E3LP17; *Loa loa*: E1FKD1; *Haemonchus contortus*: U6NXS9. Chup-1 UniprotKB identifiers: *Caenorhabditis brenneri*: G0NFF0; *Caenorhabditis briggsae*: A8XM97; *Caenorhabditis elegans*: Q9GYF0; *Caenorhabditis remanei*: E3NDW8*; Trichuris suis*: A0A085NGP1; *Pristionchus pacificus*: H3E6H1.

In the case of endocytosis, the conclusion is much more straightforward. We confirmed the role of endocytosis through a substantial rescue when silencing *chc* and a near complete rescue when using a pharmacological inhibitor of endocytosis. As stated before, an RNAi event does not result in a complete inhibition of the process, whereas an inhibitor like chlorpromazine hydrochloride can have a strong effect as it operates on the protein level instead of the mRNA level, a difference that is also visible in our results. It is important to note that the choice of reporter gene needs to be deliberate as the silencing of *chc* can influence metabolic processes like α-amylase production and food uptake in this case. Silencing other components of the endocytic pathway did not cause impaired food intake and the resulting mortality, so it is probably not the endocytic process that is linked with amylase expression, but only its component *chc*, which is involved in other processes besides endocytosis. Mortality after *chc* silencing has also been reported in Schistocerca (S.) gregaria (Wynant et al. 2014b) and *C. elegans* (Saleh et al. 2006). In the former, adults were used, which might be less sensitive to lowered *chc* levels than the larval stages, so that adverse effects

on viability could be postponed long enough to finish the experiment. This lethality could make *chc* an interesting target for RNAi-mediated pest control. Indeed the low LD_{50} value of 0.053 ng dsChc per individual ranks among the lower doses reported in literature for coleopteran species (Table 6). Comparisons between a one-time administration and continuous dsRNA administrations are difficult. However, we have estimated the consumption of leaf material by the larvae to be approximately 0.9 g over the 14 days, which corresponds to a LC_{50} for dsChc of 0.06 bbp, which is comparable to the best targets reported as of yet.

Table 6. Overview of reported LC_{50} values for RNAi-mediated pest control in coleopteran species (adapted from Baum and Roberts 2014).

Species	Target gene	Application method	LC_{50}	Reference
Leptinotarsa decemlineata	*V-ATPase A, V-ATPase E*	Artificial diet (continuously over 12-14 days)	~10 ppb	(Baum et al. 2007)
Diabrotica virgifera virgifera	Multiple (17)	Artificial diet (continuously over 12-14 days)	1-10 ppb	(Baum et al. 2007)
	Snf7	Artificial diet (continuously over 12 days)	4.3 ppb	(Bolognesi et al. 2012)
Diabrotica undecimpunctata howardii	*V-ATPASE A, V-ATPase E, α-tubulin*	Artificial diet (continuously over 12-14 days)	~0.1 ppm	(Baum et al. 2007)
	Snf7	Artificial diet (continuously over 12 days)	1.2 ppb	(Bolognesi et al. 2012)
Phyllotreta striolata [a]	Arginine kinase	Droplets on leaf tissue (every 3 days over 14 days)	0.8 ppb	(Zhao et al. 2008)
Tribolium castaneum	*V-ATPase E*	Artificial diet (continuously over 7 days)	2.5 ppm	(Whyard et al. 2009)
Cylas brunneus	Multiple	Artificial diet (continuously over 5 days)	1-2 ppm	(Christiaens et al. 2016)

[a] This experiment was performed using adult beetles, in all other cases larvae were used.

Silencing the A subunit (*vha68*) in the extracellular domain of the vacuolar H^+ ATPase complex did not trigger any rescue of reporter gene silencing. On the other hand, for subunit c (*vha16*), localized in the integral domain, the rescue was high compared to the rescue noticed for the *sil* genes and comparable to the one observed when *chc* was silenced. A possible explanation for the negative results for Vha68 can be found in the distinct roles of the two subdomains. The integral V0 domain forms the proton-conducting pore, whereas the V1 domain is the site of the ATP hydrolysis, which can dissociate during *Manduca sexta* moulting (Sumner et al. 1995). Also the option that the RNAi-of-RNAi experiment was not sensitive enough should not be dismissed, as silencing the V_1 subunit H in the S2 cell line showed a less pronounced effect on the RNAi

response than Vha16 (Saleh et al. 2006). The association between endocytosis and dsRNA uptake has also been reported in *T. castaneum* (Xiao et al. 2015) and *S. gregaria* (Wynant et al. 2014b) when the dsRNA was applied through injection into the hemocoel, and in the *D. melanogaster* S2 cell line, where a *sid-1-like* gene is absent (Saleh et al. 2006; Ulvila et al. 2006).

We now know that endocytosis is involved and that also the Sid-1-like proteins might play a role in taking up dsRNA from the midgut lumen into the surrounding cells, but concerning the relative contribution of each pathway to the whole dsRNA uptake event, we can only speculate. The larger RNAi rescue that is seen for the endocytosis-related genes (excluding *vha68*) points to a larger involvement of this pathway but the variability of the RNAi response of the reporter genes makes this difficult to assess. Additional experiments, ideally with an improved reporter system, are needed if we want to estimate (and maybe even quantify) the relative contribution of each pathway. Nevertheless, it is indisputable that, when performing dsRNA uptake experiments in other insect species, both pathways need to be examined so that a more complete view on the distribution of the pathways in different insect species and tissues can be obtained. Repeating this set of experiments through both injection and feeding of dsRNA could tell us if the (primary) dsRNA uptake mechanism is different in the insect midgut compared with the hemocoel-surrounding tissues. This could give us a better understanding of the difficulties that are encountered when attempting to provoke an efficient RNAi response through feeding. This could in turn lead to progress toward the use of RNAi for selective insect pest control or/and increasing the resistance of beneficial insects against viral diseases.

CHAPTER IV:

THE PATHOGENICITY OF *CRICKET PARALYSIS VIRUS* IN *BOMBUS TERRESTRIS*

Parts of this chapter were submitted for publication:

Cappelle, K., Meeus, I., Piot, N., Wang, L., and Smagghe, G. The pathogenicity of *Cricket paralysis virus* in the wild pollinator *Bombus terrestris*.

K. Cappelle and I. Meeus conceived and designed the study. K. Cappelle performed the experiments. L. Wang and N. Piot contributed by performing the cell line experiments and collecting the bumblebees from nature, respectively. K. Cappelle analyzed the results. The manuscript was written by K. Cappelle and revised by I. Meeus and G. Smagghe

4.1 Introduction

Transport of domesticated honeybees and reared bumblebees has resulted in a global spread of different parasites and is considered as a substantial threat toward wild bee populations (Meeus et al. 2011; Goulson et al. 2015). Herein viruses serve a particular important role as they have a considerably broad host range (Wightman et al. 1993; Furst et al. 2014; Dolezal et al. 2016), and at least have the potency to inflict damage in numerous species. Viral screenings, host range determination and virulence studies in different bees have therefore become increasingly important. Viruses that are generally included in these screenings in bees mainly belong to two different families: the *Dicistroviridae*, which harbor, among others, *Israeli acute paralysis virus* (IAPV), *Acute bee paralysis virus* and *Black queen cell virus* and the *Iflaviridae*, with *Deformed wing virus*, *Sacbrood virus*, and *Slow bee paralysis* virus being the most important members. The list of viruses detected in pollinators is continuously expanding, due to the increased availability of metagenomic surveys, thereby prompting frequent updates to the list of viruses that are included in the screenings.

A member of the *Dicistroviridae* with an exceptionally broad host range is *Cricket paralysis virus* (CrPV) (overview in Bonning and Johnson 2010). It has been discovered in lab colonies of the Australian field cricket (Reinganum et al. 1970). Since then it has been reported a few times in nature, but most of the information about its host range comes from artificial infections in the lab, where it was found to infect multiple species within the orders of the Diptera, Hemiptera, Hymenoptera, Lepidoptera and Orthoptera (Plus et al. 1978). An interesting observation came from Anderson and Gibbs who found latent CrPV infections when honeybee pupae were injected with buffer solutions and confirmed its presence by serological and ELISA tests (Anderson and Gibbs 1988). Please note that this CrPV identification was based on tests available at the time and thus misidentification due to cross-reactivity with related viruses cannot be excluded.

To this day, this virus continues to be an interesting research subject because of its unconventional translation through two internal ribosome entry sites (IRES) and the presence of a suppressor of RNA interference (VSR), the principal antiviral defense system in insects. This latter feature, coupled with its broad host range, could make this virus a relevant threat to many insects, including pollinator species providing an invaluable ecosystem service. Here we focus on *B. terrestris*, as this wild bumblebee species is a key pollinator in the Palaearctic region. It is also reared commercially and is implicated in the spillover of parasites from reared toward wild bees (Kozomara and Griffiths-Jones 2011; Friedlander et al. 2014). The aim of this work was to evaluate

the pathogenicity of CrPV to *B. terrestris*, both through artificial infections (micro-injection) and infections that are more representative to the natural situation (oral-faecal transmission). Next to an increase in viral titers, replication of a positive-sense single-stranded RNA virus, such as CrPV, can also be confirmed by detecting the negative-sense RNA strand, which is generated during viral replication. In order to achieve this, the cDNA synthesis reaction is performed using a primer, complementary to the negative strand of the virus, instead of an oligodT or random hexamer primer. Strand-specific reverse transcription (RT)-PCR is prone to false positives, caused by self-priming of the positive RNA strand, false priming on the positive strand or an incomplete inactivation of the reverse transcriptase. To avoid this, a non-viral tag can be added to the 5' end of the negative strand-specific primer. In this work, a tag-based primer set was devised for CrPV. IAPV, a closely related and well-studied pathogen of bumblebees, was used to draw some comparisons concerning infectivity and viral loads in different tissues.

4.2 Material and methods

4.2.1 *Bumblebee rearing and infection methods*

All experiments were performed using 5-to-10 day old *B. terrestris* workers, age fixed within each experiment, obtained from Biobest NV (Westerlo, Belgium). Several workers were collected from the colonies and verified to be free of IAPV, *Acute bee paralysis virus* (ABPV), *Kashmir bee virus* (KBV), *Deformed wing virus* (DWV) and *Slow bee paralysis virus* (SBPV) using RT-PCR (Sguazza et al. 2013). One or two workers were collected from each colony and randomly distributed over the microcolonies for the experiments. These microcolonies were placed in an incubator at 30°C, 60% relative humidity and in continuous darkness and fed with sugar water (50 w/v%, BIOGLUC®, Biobest NV) and gamma-irradiated pollen (Soc. Coop. Apihurdes, Pinofranqueado-Cáceres, Spain).

Prior to injections, the bumblebees were sedated on ice in a plastic container for 5 minutes. A volume of 5 µL was used to inject the bumblebees through the abdominal intersegment membrane between the second and third segment using an Femtojet Microinjector (Eppendorf, Hamburg, Germany). For feeding experiments, bumblebees were placed individually in plastic containers in which a small cup with 40 µL feeding solution was placed. This solution consisted of 20 µL sugar water and 20 µL of bumblebee faeces (infected by injection of 10^4 particles per individual). Only bumblebees that had completely consumed the solution were eligible for the experiment.

4.2.2 *Virus production*

The initial CrPV inoculum used for virus production was provided by Dr. Eric Jan (Department of Biochemistry and Molecular Biology, University of British Columbia, Canada) and Dulce Cordeiro dos Santos (Department of Biology, KU Leuven, Belgium). The initial IAPV inoculum was provided by Joachim de Miranda (Department of Ecology, Swedish University of Agricultural Sciences, Sweden).

CrPV was produced within the Schneider-2 (S2) cell line, which was tested to be negative for *Flock house virus*, *D. melanogaster X virus*, *D. melanogaster American Nodavirus*, *D. melanogaster Totivirus* and *D. melanogaster Birnavirus* (Wu et al. 2010). 40 µL of CrPV inoculum (10^6 particles/µl) was used to infect 40.10^6 S2 cells and 15 hours later the resulting viral particles were obtained by repeated freezing and thawing in an ultrafreezer (-70°C) and subsequently applied to 300.10^6 cells. 15 hours later the resulting viral suspension was cleared of cell debris by centrifuging twice: 15' at 800g, followed by 30' at 20000g. The viral suspension was cleaned further by ultracentrifuging 2h, at 4°C, at 100000g in a 15% sucrose gradient. The pellet was collected, resuspended in PBS and tested negative for IAPV, ABPV, KBV, DWV and SBPV using RT-PCR (Sguazza et al. 2013). The suspension was also negative for *Flock house virus*, a typical contaminant of the S2 cell line, encoding a VSR. To evaluate whether other pathogenic entities or toxic compounds in the cell line could have any effect on bumblebees, a control suspension was included where the S2 cells underwent the same procedure, but without CrPV infection.

IAPV was produced by injecting 160 virus-free bumblebees with 500 IAPV particles and waiting three days for the virus to amplify within the body. Then the bumblebees were crushed in 10 mM phosphate buffer (pH 7.0) supplemented with 0.02% diethyl dithiocarbamate. The suspension was centrifuged for 15' at 800g and 4h at 100000g (4°C). The resulting pellet was resuspended in 6 ml demineralized water. Subsequent dilutions were made in phosphate buffered saline (PBS). Contamination of other common bumblebee viruses such as ABPV, SBPV, DWV, KBV, *Chronic bee paralysis virus* (CBPV), *Varroa destructor virus-1* (VDV-1), *Sacbrood virus* (SBV) and *Black queen cell virus* (BQCV), was determined to be less than 0.1% of the IAPV level by RT-qPCR (Niu et al. 2016b) and the stock tested negative for CrPV (determined by RT-PCR).

For both viruses, the concentration of viral particles was estimated by transmission electron microscopy (CODA-CERVA, Brussels, Belgium). Alcian blue-treated grids were deposited on a 15 µl drop of solution for 10 minutes and rinsed two times with water. Afterwards, the grids were stained for 10 seconds on a drop of 2% uranyl acetate (Agar Scientific, Stansted, UK), blotted and

air-dried. The samples were imaged in bright field mode using a Tecnai Spirit TEM (FEI, Hillsboro, USA) with Biotwin lens configuration operating at 120 kV. Five micrographs were recorded per sample using a 4K x 4K CCD camera (Eagle, FEI) at a magnification of 30000 times.

4.2.3 *RNA extraction, cDNA synthesis, RT-PCR, negative-strand-specific RT-PCR and RT-qPCR*

The RNeasy Mini Kit (Qiagen) was used to isolate total RNA according to the manufacturer's instructions, followed by DNAse treatment (Turbo DNA-free kit; Life Technologies). The only alteration from the original protocol was that for larger samples (whole abdomen or whole body), the volume of RLT buffer was increased to 1 ml. 500 ng of RNA was used in each cDNA synthesis reaction, with the SuperScript II Reverse Transcriptase Kit (Life Technologies) using an oligodT primer. The PCR reactions were performed in a Labcycler (SensoQuest, Göttingen, Germany) with the following protocol: 2 min at 94° C, 35 x (30 s at 94°C; 30 s at 56°C; 30 s at 72°C), 3 min at 72°C. 1 µL of cDNA was added to each 25 µL PCR reaction containing 2.5 µL 10 PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 µM primers and 1.25 U Recombinant Taq DNA Polymerase (Life Technologies).

For negative strand detection, instead of the oligodT primer, a CrPV-specific primer was designed using the primer3 software (Koressaar and Remm 2007) and fused to a tag at the 5' end. Four different tags were selected from literature (Table 7) and tested in various cell lines and bumblebee samples for false positives. The cDNA synthesis protocol was altered slightly, with an increased temperature (50°C instead of 42°C) and shorter reaction time (30 minutes instead of 50 minutes) to avoid mispriming. Residual primers were destroyed by adding 0.5µL Exonuclease I (Thermo Fisher Scientific, Waltham, USA) and incubating for 30 min at 37°C. The enzyme was inactivated at 70°C for 15 min. The cDNA was diluted 1:10 before use in PCR reactions at the conditions described above, but with the tag-primer (not fused to a viral sequence) replacing the F primer.

The RT-qPCR reactions were performed using the same reaction mixture and amplification conditions as described in section 3.2.5, except that here the cDNA was diluted 1:40 or 1:100. As reference genes, *60S ribosomal protein L23* and *ubiquitin* were used for normalization of the data (Table 7; Niu et al. 2014). The same quality control restrictions relating to the Cq values, amplification efficiency and melting curves were used. The results were analyzed using the qbase$^+$ software (Biogazelle) and SPSS Statistics 23.0 (IBM Corp 2015).

Table 7: List of primers used in Chapter IV.

Name	Sequence (5'-3')	Reference
CrPV F	TGTCAACCCGACGCTTACA	This study
CrPV R	TGTATTCCTCTCCCCTCGCA	This study
Tag1-CrPV F	AGCCTGCGCACCGTGGTGTCAACCCGACGCTTACA	(Yue and Genersch 2005)
Tag2-CrPV F	GGCAGTATCGTGAATTCGATGCTGTCAACCCGACGCTTACA	(Plaskon et al. 2009)
Tag3-CrPV F	GGCCGTCATGGTGGCGAATAATGTCAACCCGACGCTTACA	(Vashist et al. 2012)
Tag4-CrPV F	TCGGAATCGCCTAGCTTTGTCAACCCGACGCTTACA	(Celle et al. 2008)
Tag3	GGCCGTCATGGTGGCGAATAA	(Vashist et al. 2012)
IAPV F	CCATGCCTGGCGATTCAC	(de Miranda et al. 2010)
IAPV R	CTGAATAATACTGTGCGTATC	(de Miranda et al. 2010)
Tag3-IAPV F	GGCCGTCATGGTGGCGAATAACCATGCCTGGCGATTCAC	(Vashist et al. 2012)
rpl23_q F	GGGAAAACCTGAACTTAGGAAAA	(Niu et al. 2014)
rpl23_q R	ACCCTTTCATTTCTCCCTTGTTA	(Niu et al. 2014)
ubi_q F	GGTATTTGGATGCCAGTGATTT	(Niu et al. 2014)
ubi_q R	ATGGGCATTTCTACCCCTTTTA	(Niu et al. 2014)

In the tag-fused primers, the tags are underlined. IAPV: *Israeli acute paralysis virus* (EU436443.1); CrPV: *Cricket paralysis virus* (KP974707.1); *rpl23*: *60S ribosomal protein L23* (XM_003400707.2); *ubi*: *polyubiquitin B* (XM_003402262.2).

4.2.4 *CrPV screening in bumblebees collected from nature*

In total, 137 bumblebees (105 *B. pascuorum*, 16 *B. lapidarius* and 16 *B. terrestris* individuals) were collected from 18 study areas (50 ha each) in agricultural landscapes located across the middle and southern parts of the Netherlands. Bees were caught in a 2 m wide transect walk along center of the study areas, stored separately in containers and kept alive until they arrived at the lab. Here, the abdomens were crushed individually in RLT buffer and RNA extraction and cDNA synthesis was performed as described in 4.2.3, except that random hexamer primers were used in this case. For RT-PCR detection, the samples were pooled per five, according to species and sample location.

4.3 Results

4.3.1 *Injection with CrPV causes mortality at high doses*

Three different doses of CrPV (10^6, 10^4 and 10^2 particles per bumblebee) were injected into bumblebee female workers and male drones (only the highest dose for the latter) and mortality was scored (Figure 15). For the highest dose, mortality was nearly 100% for both males and females. At the intermediary dose of 10^4 particles per bee, 50% of the bees survived. RT-PCR detection using oligodT-generated cDNA and the non-tagged CrPV primer set confirmed CrPV presence in the fat body of those bumblebees (3 out of 4) that were still alive at day 14, after being injected with 10^4 particles. For the lower dose and both controls, no mortality was observed. The majority of mortality occurred in the window between 4 days post infection (dpi) and 7 dpi. Uncoordinated movements of the legs and paralysis symptoms were observed in the 24 hours leading up to death. To rule out the possibility that a low contamination of IAPV in the CrPV stock solution was responsible for the paralysis symptoms and mortality, IAPV was confirmed to be absent in the infected bumblebees using PCR.

4.3.1 *Tag-based negative strand RT-PCR detection in a wide host range*

As CrPV is capable of infecting lab populations of many different insect orders, sometimes resulting in chronic asymptomatic infections, distinguishing between positive results caused by the original inoculation dose or by a real infection and thus viral replication is imperative. Therefore, a tag-based system was devised capable of detecting the negative strand of CrPV in many different orders. The four candidate tags were evaluated with the following criteria: 1) no false positives in any of the tested cell lines or bumblebee samples, 2) positive results in bumblebees infected with 10^6 particles 4 days after infection and 3) no positive results for a purified CrPV sample (only containing positive single stranded RNA) .

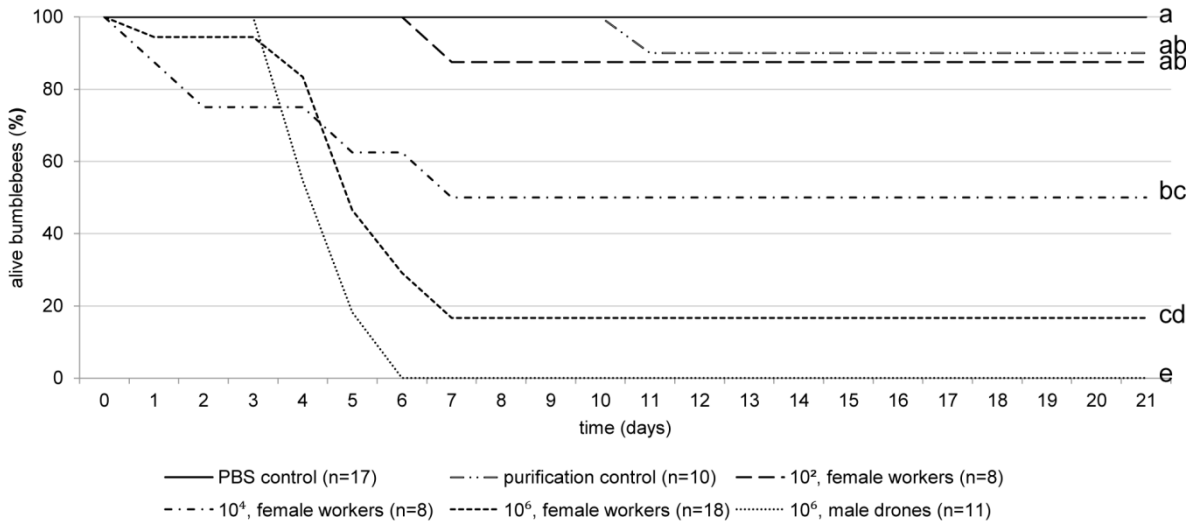


Figure 15. The survival percentage after injection of different doses of CrPV, scored daily.
Statistical differences with the PBS control on an α= 0.05 level (Kaplan-Meier survival analysis, log rank test, in SPSS Statistics 23.0) are denoted by different letters at the end of the graph. Two controls were included, one where PBS was injected and one where a purified suspension from non-infected S2 cells was injected.

As seen in Table 8, only Tag 3 gave a negative result in all the cases, except for the true positive bumblebee sample. Therefore, this tag was used to confirm CrPV replication. The negative result when testing the CrPV stock ensures that no false positive results are generated. This could occur because of primer misbinding or self-starting transcription, due to the positive ssRNA strand folding onto itself, when a large number of viral genome copies is present.

Table 8: PCR results of tag-primer testing in different cell lines and bumblebee samples.

	cf203	gutaw1	Bm5	Hi5	Sf9	S2	cpb	tca	bbee PBS	bbee 10^6	CrPV stock
Tag 1	-	-	-	-	-	+	-	-	-	/	/
Tag 2	-	-	-	-	-	+	-	-	-	/	/
Tag 3	-	-	-	-	-	-	-	-	-	+	-
Tag 4	+	+	+	+	+	+	+	+	+	/	/

+: a band is visible after gel electrophoresis; -: no band is visible; /: not tested. Cf203: FPMI-CF-203 (Sohi et al. 1993); Gutaw1: RP-HzGUT-AW1 (Goodman et al. 2004); Bm5 (Grace 1967) ; Hi5: BTI-Tn-5B1-4 (Granados et al. 1994); Sf9: IPLB-SF-9 (Vaughn et al. 1977); S2: Schneider-2 (Schneider 1972); cpb: BCIRL-Lepd-SL1 (Long et al. 2002); tca: BCIRL-TcA-CLG1 (Goodman et al. 2012); bbee: bumblebee fat body.

4.3.2 *Injection with lower doses of CrPV or feeding with faeces of infected bees results in chronic infections*

Using RT-qPCR analysis an increase in viral titers in the abdomen, 4 days after injection, was confirmed both in the case of 10^4 and 10^6 particles (Figure 16). In the case of 10^4 particles, the infecting dose could not be detected within 40 cycles, but after 4 days, in 4 out of the 7 bumblebees tested, the viral titers were high enough to be detectable. The resulting titers after 4 days were not very high as they fell in the range of the dose of 10^6 particles immediately after injection. However, for 10^6 particles, an over 11,000-fold increase was measured between day 0 and day 4. These results, coupled with the mortality results, suggest an acute infection as a result of the higher infection dose, characterized by a considerably fast viral replication.

The lower dose, however, can have multiple outcomes. As the dose of 10^4 particles per individual did not cause complete mortality, both the normal oligodT-based and the tag-based (for negative strand detection) RT-PCR detection were used to check for CrPV presence and replication in the surviving bumblebees. PCR detection using the regular CrPV primer set confirmed CrPV presence in the fat body (3 out of 4) of bumblebees injected with 10^4 particles that were still alive at day 14. Two of these positive samples showed negative strand amplification using the tag-based assay, confirming viral replication. This indicates that some bees experience a strong acute infection (and die) whereas in others, a chronic infection occurs, with lowered viral titers and replication.

Mimicking a more natural method of infection, bumblebees were also fed with faeces of infected bumblebees. Here, positive results were obtained in fat body (3 of 4) and ovaries (2 of 4), confirming the spread of CrPV in the body. However, the negative strand detection could not confirm replication in these tissues.

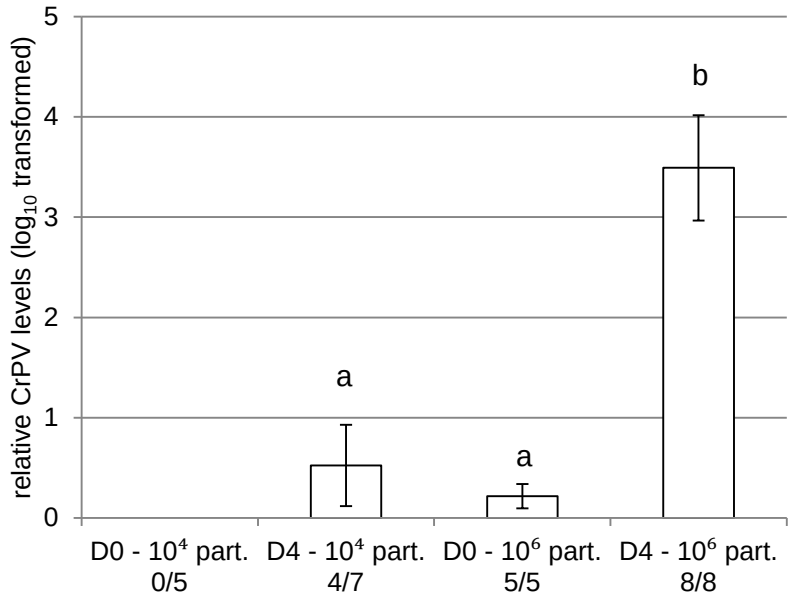


Figure 16 Relative CrPV levels obtained by RT-qPCR, showing an increase in viral titers between day 0 and day 4, for both 10^6 and 10^4 injected particles.
The columns represent the mean ± SEM of the relative virus levels, relative to the normalized level of the reference genes *rpl23* and *ubi*. Statistical differences on an α= 0.05 level are denoted by different letters above the column (independent samples Kruskal-Wallis Test: test stat.=10.7956, df=2, p=0.0045). 10^4 particles could not be detected just after injection, but after 4 days it was detectable in 4 out of 7 bumblebees. For 10^6 particles, the increase was over 11,000-fold.

4.3.3 *Negative strand detection for CrPV and IAPV in different tissues*

A known bumblebee pathogen, closely related to CrPV and causing similar symptoms, is IAPV. The negative strand-specific assay was utilized to assess replication in four different tissues, brain, fat body, midgut and ovaries, and compare it to the viral titers, obtained by RT-qPCR, and this for both viruses. The negative strand of CrPV was detected in all tissues, but not consistently (Figure 17A). Similar band intensities were observed in the fat body and to a lesser extent in the ovaries, whereas in brain and midgut there was only one bright band. In IAPV however, the intensities within one tissue were more equal (Figure 17B). Although the amount of RNA was normalized before cDNA synthesis, one should be careful when using this data for comparisons between tissues and between the different viruses. When comparing with the viral titers, obtained by RT-qPCR on the same samples, no obvious agreement between the two datasets can be seen. In general, for IAPV higher viral titers correspond to a higher replication, except for the ovaries (Figure 17D). For CrPV there are more inconsistencies with relatively high viral titers corresponding with not so bright bands and vice versa (Figure 17C). However, no strong conclusions should be based on this data as the negative strand PCR is an endpoint PCR and some of the bands are saturated so information about their relative intensities is lost.

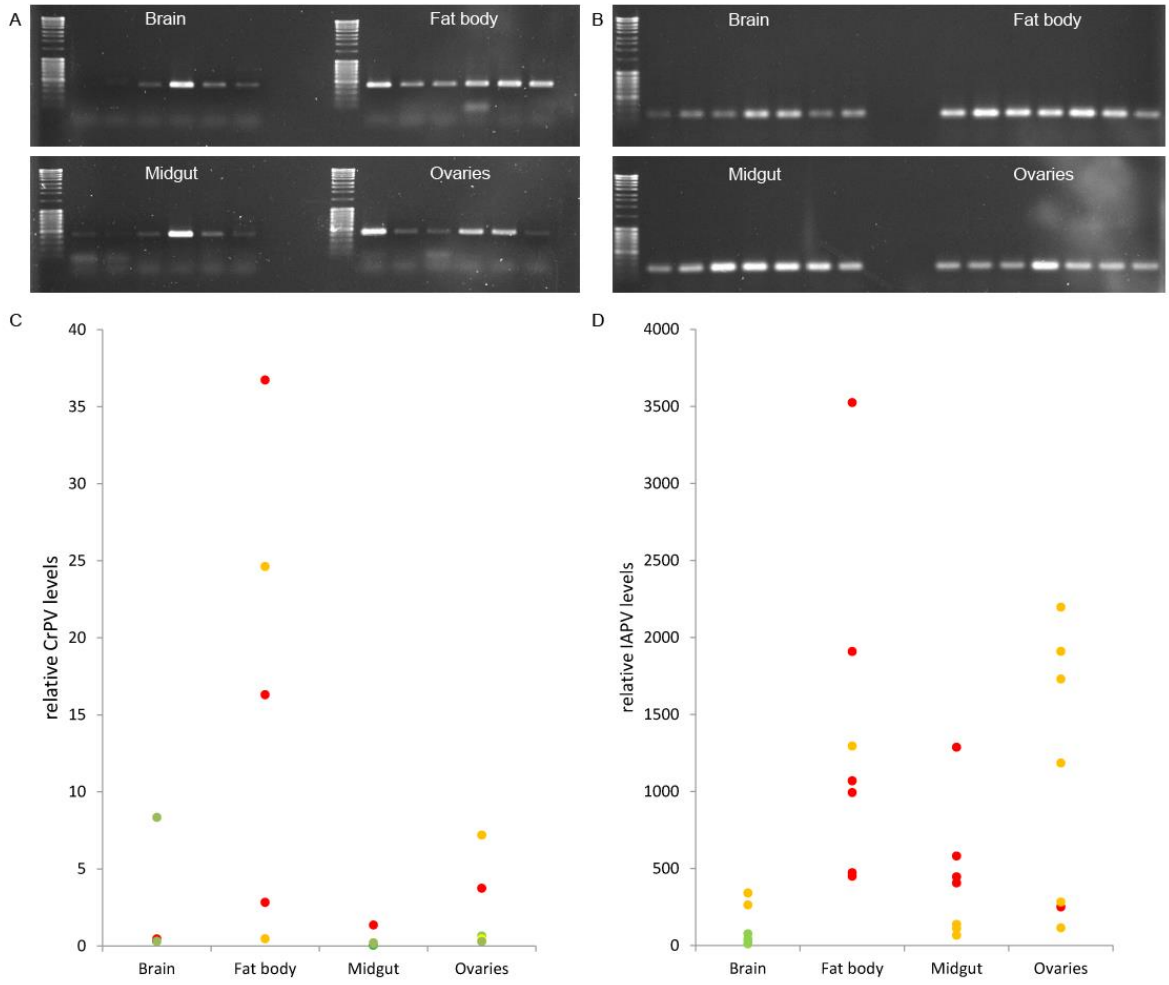


Figure 17. Negative strand detection by tag-based RT-PCR in different tissues (brain, fat body, midgut and ovaries) for CrPV (panel A) and IAPV (panel B).
Equal amounts of RNA were used during cDNA synthesis so, at least, within tissue comparisons are possible. The size of the bands are 406 bpfor CrPV and 224 bp for IAPV. CrPV shows more variability within tissues than IAPV, with similar intensities only in the fat body and to a lesser extent in the ovaries. Panels C and D show the corresponding viral titers obtained by RT-qPCR. Every dot represents a sample and the colors denote the intensities of the bands of the negative-strand specific RT-PCR, with red: a bright, saturated band; yellow: medium intensity band, light green: only a faint band and dark green: no visible band.

4.4 Discussion

From our data, it is clear that CrPV can infect bumblebees in artificial lab conditions using the robust microinjection method of infection. At high doses the symptoms and mortality were similar to those observed for CrPV in crickets (Reinganum et al. 1970) and IAPV in bees (Maori et al. 2007; Wang et al. 2016). Compared to IAPV, the lethal dose by injection was considerably larger (10^4 instead of only a few dozen of particles) and onset of mortality occurred at a slightly later time point (4 dpi instead of 3 dpi) (Niu et al. 2016b). When dropping to lower doses, it seems like some individuals could resist infection, either by preventing the establishment of the infection (negative RT-PCR results) or by keeping the viral titers low enough so there is only limited damage to the host tissues (positive RT-PCR results but no mortality). An important observation was that bumblebees could be infected by feeding with CrPV-containing faeces, suggesting CrPV could be transmitted between bees in natural conditions. However, whether the amount of CrPV particles present in these faeces mimics the CrPV loads, that could be present in nature, remains unexplored.

The less uniform negative strand amplification within the different tissues for CrPV also suggests that a CrPV infection is not established as easily as an IAPV infection. Indeed, chronic infections after IAPV injection do not seem possible when even very low doses of IAPV (~20 particles; Niu et al. 2016b) trigger an acute infection. Virus characteristics, such as their ability to enter the host cell, tissue tropism, production of viral suppressors of RNAi or other virulence factors could determine the replication potential. But this difference could also stem from host factors, concerning both immunity and physiology, allowing the CrPV infection to be kept under control.

The tag-based negative strand detection developed here can be an important tool for distinguishing between positive RT-PCR results caused by the original inoculation dose and those caused by a true infection of CrPV. Moreover, it could also be useful for CrPV detection in samples from nature. Many solitary bees are small and dissection of separate tissues is not practical. Therefore, it is not possible to only examine tissues with no direct access to the environment, such as the fat body. In this case, detection of the negative strand in the whole body would confirm viral infection in these small pollinators. It would also be interesting to do a quantitative comparison of the replication occurring in the different tissues for both IAPV and CrPV, to track infection and replication over the tissues during the course of infection.

This study supports the idea that *B. terrestris* is an experimental host for CrPV and that this virus could be chronically present in nature in bees. As of yet, CrPV has been found as a latent infection once in the honeybee (Anderson and Gibbs 1988) but not in metagenomics surveys in bees (Cox-Foster et al. 2007; Arrese and Soulages 2010; Ghosh et al. 2014). We screened a sample set of bumblebees (N = 137) collected in a geographically (N = 18) limited region (the Netherlands) for CrPV, but have not detected it. A broader screening of bees, especially in areas where CrPV is naturally found in cricket populations, would tell us more about its prevalence. Therefore, we would like recommend that CrPV would be incorporated in general screening programs. The validated tag-based test to assess replication of the virus can readily be used to exclude false positive results.

CHAPTER V:

THE INTERACTION BETWEEN *ISRAELI ACUTE PARALYSIS VIRUS* AND THE RNA INTERFERENCE RESPONSE OF ITS HOST *BOMBUS TERRESTRIS*

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K. Cappelle and I. Meeus conceived and designed the experiments; K. Cappelle performed the experiments and analyzed the data. M. Dhaenens aided in the design, implementation, analysis and reporting of the mass spectrometry part of this study. The manuscript was written by K. Cappelle and revised by M. Dhaenens, I. Meeus and G. Smagghe.

5.1 Introduction

No domain of life is exempt from the threat of viruses (Nasir et al. 2014), which hijack the cellular metabolic pathways to produce the genomic material and proteins needed for their own replication (reviewed in Walsh and Mohr 2011). In order to counter these attacks, organisms have developed various defense mechanisms. As described in Chapter I, in plants, nematodes and arthropods this defense is carried out mainly by the RNAi pathway (Soosaar et al. 2005; Karlikow et al. 2014). This pathway is triggered by dsRNA intermediates that arise during the replication of RNA viruses (van Mierlo et al. 2010; Marques et al. 2013) and results in the destruction of the viral genomic RNA strands, thereby preventing the production of the associated viral proteins (reviewed in Obbard et al. 2009). If the RNAi mechanism acts systemically, a viral sequence-specific signal can be spread to uninfected tissues, resulting in a, at least partially, protected status of these cells (Saleh et al. 2009).

During evolution, viruses have not stood by idly while their hosts developed this RNAi defense mechanism. Viruses, known to encode viral suppressors of RNAi (VSRs), have been found in plants (reviewed in Burgyán and Havelda 2011) and insects (Li et al. 2002; van Rij et al. 2006; Nayak et al. 2010; Schnettler et al. 2012; van Mierlo et al. 2012; van Cleef et al. 2014), the latter were listed in Table 1. These VSRs are often small proteins which exercise their function through various mechanisms such as dsRNA sequestering and Dcr-2 or Ago-2 binding. Within the *Dicistroviridae*, a 166 AA long protein, called 1A, has been proven to be a functional VSR in both the *Drosophila C virus* (van Rij et al. 2006) and the *Cricket paralysis virus* (CrPV) (Nayak et al. 2010). Recently the presence of a similar 1A protein has been suggested in another member of the *Dicistroviridae*, *Israeli acute paralysis virus* (IAPV). The proof of its functionality was based on reduced virus titers after silencing the 1A region, compared to targeting the non-coding 5' IRES region (Chen et al. 2014), indicating the need of 1A for virulence. This method might be insufficient by itself, as 1A is a post-translation product and confounding effects like dsRNA target accessibility were not considered. Another small ORF, tentatively named *orfX* (or *pog*), has been predicted in IAPV and its close relatives but not in CrPV (Firth et al. 2009; Sabath et al. 2009), and the presence of the resulting protein has been confirmed for IAPV in honeybees (Ren et al. 2012) but not for *Solenopsis invicta virus*-1 (Valles and Sabath 2012). As of yet, no functionality has been attributed to this putative 94 AA protein. VSRs can be very potent in the inhibition of the RNAi mechanism and therefore important immunosuppressive virulence factors. The actual virulence of a virus, defined as the relative capacity to cause damage in a host, depends on the balance between the

power of the suppressor and the capacity of the RNAi mechanism of the host to limit the replication of the virus and hence, the production of this inhibitor.

The aim of this study was to investigate to what extent IAPV can influence RNAi efficiency, and to which direction this balance will sway, in the bumblebee *B. terrestris*. This virus is known to pose an important health danger to pollinators such as honeybees and bumblebees (Cox-Foster et al. 2007; Meeus et al. 2014) and is a target for RNAi-based antiviral therapeutics (Maori et al. 2009). As an extra control, CrPV, with its known VSR in *Drosophila*, was included. CrPV has not been reported as a problematic infection of the pollinator community and has a broad host range as it was reported to infect species within the insect orders of Heteroptera, Diptera, Lepidoptera and Hymenoptera, at least in experimental conditions (Plus et al. 1978; Chao et al. 1986; Anderson and Gibbs 1988). Within the concept of virus multi-host dynamics (Manley et al. 2015), the presence of VSRs can severely impact the virulence in different hosts, as the immunosuppressive capacity is dependent on the host immune strategies and their sensitivity towards VSRs. Also within the same host species, VSRs can influence the virulence of co-infecting viruses (Carrillo-Tripp et al. 2016), an important feature now that multi-virus reports in pollinators are emerging (Chen et al. 2004; Wu et al. 2015).

In order to evaluate the effect of IAPV infection on RNAi efficiency in *B. terrestris*, we used a dual approach. First, a proteomic analysis was performed to confirm the translation of the VSR 1A and predicted orfX proteins using the data-independent acquisition method with high definition mass spectrometry ($HDMS^E$). Second, the RNAi efficiency after IAPV and CrPV infection was determined. An assay was developed in which bumblebees were infected with a fixed amount of viral particles and after an incubation period injected with dsRNA targeting *peptidylprolyl isomerase A* (*ppia*), a gene known to remain stable during virus infection (Niu et al. 2014). Silencing levels were evaluated using RT-qPCR, along with expression levels of the RNAi core genes, *dcr-2* and *ago-2*, and the systemic RNAi genes, *ninaC*, *egghead* and *sid-1* (Saleh et al. 2009).

5.2 Material and methods

5.2.1 *Bumblebee rearing and injections*

All experiments were performed using age fixed workers, obtained, reared and verified to be virus-free as described in section 4.2.1 The microinjections were performed as described in the same section, but here a volume of 5 µL was used for all virus solutions (and the appropriate controls), while dsRNA was injected in a volume of 20 µL.

5.2.2 *Virus production*

IAPV was produced within *B. terrestris* pupae as discussed in detail in section 0. Subsequent dilutions of the stock were made in PBS. Contamination of other common bumblebee viruses such as ABPV, KBV, SBPV, CBPV, DWV, VDV-1, SBV and BQCV was determined to be less than 0.1% of the IAPV level by RT-qPCR (Niu et al. 2016b) and the stock was negative for CrPV (determined by RT-PCR). CrPV was produced within the Schneider-2 (S2) cell line as described in 0. The viral suspension tested negative for IAPV, ABPV, KBV, DWV and SBPV using RT-PCR (Sguazza et al. 2013). The concentration of viral particles was estimated by transmission electron microscopy using a standard protocol for negative staining with Alcian blue and uranyl acetate as presented in 0 (CODA-CERVA).

5.2.3 *dsRNA synthesis*

The dsRNA was prepared as described in section 3.2.3, using the appropriate T7-primers (Table 9) and bumblebee cDNA or a GFP fragment-containing plasmid as a template.

5.2.4 *RNA extraction, cDNA synthesis and RT-qPCR*

RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions and treated with the Turbo DNA-free kit (Life Technologies). For cDNA synthesis, 500 ng RNA was used in each reaction, performed with the SuperScript II Reverse Transcriptase Kit (Life Technologies). The RT-qPCR reactions were performed as described in 4.2.3. The results were analyzed using qbase$^+$ software (Biogazelle) and SPSS Statistics 23.0 (IBM Corp 2015). Reference gene stability was evaluated using the geNorm M value and the coefficient of variation on the normalized relative quantities (CV) values generated by the software. The thresholds for the M and CV values were set at 0.5 and 0.2 respectively for within-tissue comparisons and 1 and 0.5 for between-tissues comparisons (Hellemans et al. 2007). An overview of the primers used in this Chapter is given in Table 9. All primers were published (Niu et al. 2014) or designed using the primer3 software (Koressaar and Remm 2007).

5.2.1 *RNAi efficiency experiments, RNAi gene expression levels and pre-infection experiments*

In order to test the effect of viral presence on the RNAi efficiency, an assay was developed, containing three treatments. As a reporter gene the endogenous ppia was chosen as it remains stable in the presence of IAPV (Niu et al. 2014). Bumblebees belonging to the control group, with the purpose of determining the baseline *ppia* level, were injected with 5µL PBS and 24 hours later with 20 µg dsGFP (baseline control group) (You et al. 2010; Niu et al. 2016b).

Table 9. Overview of the primers used in Chapter V.

Primer name	Sequence (5'-3')	Length (bp)	Eff (%)
IAPV_q F	CCATGCCTGGCGATTCAC	203	97-102
IAPV_q R	CTGAATAATACTGTGCGTATC		
CrPV_q F	AAACGCAAAAACAGCGAAAC	110	103-104
CrPV_q R	CACATCAAGCACCAAAGCAT		
rpl23_q F	GGGAAAACCTGAACTTAGGAAAA	143	86-99
rpl23_q R	ACCCTTTCATTTCTCCCTTGTTA		
ubi_q F	GGTATTTGGATGCCAGTGATTT	129	94-96
ubi_q R	ATGGGCATTTCTACCCCTTTTA		
ppia_q F	TCGTAATGGAGTTGAGGAGTGA	132	84-94
ppia_q R	CTTGGCACATGAAGTTTGGAAT		
dcr-2_q F	TGGTCAAAACATCAAGAACAACCA	211	93-97
dcr-2_q R	GATCGGGGCCATACGAACAT		
ago-2_q F	CCGAATGTGGACAATGCTTA	181	95-102
ago-2_q R	AACGGGCAAAGGTGTGATTA		
sid-1_q F	CGAGCCCATCAACGGTAGAA	160	94-107
sid-1_q R	CGAGCCAAATCACAAACGGA		
ninaC_q F	GCGAAACCATCTGGAGGATA	112	91-106
ninaC_q R	ACTCTGTTAGCCGCATCGTT		
egghead_q F	ACCGGAGGACTTAGTTGGAA	122	93-97
egghead_q R	TGCGGAAAGGAAAGAAATGT		
GFP_T7 F	TAATACGACTCACTATAGGGTACGGCGTGCAGTGCT	495	/
GFP_T7 R	TAATACGACTCACTATAGGGTGATCGCGCTTCTCG		
ppia_T7 F	TAATACGACTCACTATAGGGCACTGGTGGAAGGTCCATCT	388	/
ppia_T7 R	TAATACGACTCACTATAGGGAAGGGAAAATGGTGATGATTAGAA		

RT-qPCR primers are denoted by a q in the primer name, dsRNA synthesis primers by a T7. bp: basepairs; Eff.: minimal and maximal amplification efficiencies over the different experiments. IAPV: *Israeli acute paralysis virus* (EU436443.1); CrPV: *Cricket paralysis virus* (KP974707.1); rpl23: *60S ribosomal protein L23* (XM_003400707.2); ubi: *polyubiquitin B* (XM_003402262.2); ppia: *peptidylprolyl isomerase A* (XM_003402218.2); dcr-2: *dicer-2* (XM_012307737.1); ago-2: *argonaute-2* (XM_012312881.1); sid-1: *systemic RNAi deficient 1-like* (XM_012315164.1); ninaC: *neither inactivation nor afterpotential C* (XM_003393094.2); egghead: *beta-1,4-mannosyltransferase egghead* (XM_012321382); GFP: *green fluorescent protein* (M62654.1).

A second group was injected with 5µL PBS and 20 µg dsPPIA and used to assess the RNAi efficiency in absence of the virus (RNAi control group). The virus treatment consisted of a first injection of 500 IAPV or 10^6 CrPV particles, followed by an injection of 20 µg dsPPIA (virus treatment group).

Because of a slower replication of CrPV, the time between virus infection and dsRNA treatment was extended to 48h and not longer to limit the effect of a bumblebee age difference. This way, there is an equal time between the end of the experiment and the onset of death for both viruses. Each group consisted of 7-9 individuals (n). Brain, fat body, midgut and ovaries were dissected 48 hours after dsRNA treatment and stored separately in 350µL RLT buffer. In order to assess the expression levels of the RNAi genes 24 hours after IAPV treatment, a separate experiment was set up (n = 8-10). For the pre-infection experiments, similar timelines were used as in the RNAi efficiency assays. In a first experiment, injection with 500 particles of IAPV was followed by injection of 10^6 particles of CrPV 24h later (n = 9-10), in the other 10^6 particles of CrPV were injected and 48h later, 500 particles of IAPV (n = 11-15). Viral titers were analyzed using RT-qPCR 48h after the second virus infection.

All statistical analyses of RT-qPCR data were performed within SPSS version 23. For experiments that evaluated viral titers, non-parametric methods were used (Friedman/Wilcoxon signed rank tests for dependent samples, Mann Whitney U tests for independent samples). In the functional RNAi assay and the evaluation of the RNAi gene levels, the data was $\log_2$ transformed after which the data satisfied the normality and homoscedasticity assumptions for Analysis of Variance (ANOVA) and t-test testing (Hellemans et al. 2007).

5.2.2 *HDMS^E*

3 days after being injected with 500 IAPV particles, 15 pairs of ovaries were collected from bumblebees. The ovaries were crushed in liquid nitrogen (pooled per three individuals) and two third of the resulting powder was stored at -80°C for mass spectrometry, while one third was dissolved in RLT buffer (RNeasy Mini Kit, Qiagen, Hilden, Germany). Infection was confirmed afterwards by RT-qPCR on the RLT-dissolved sample as described in 5.2.4. The powdered mass spectrometry samples (5 per treatment) were resuspended in 45 µL of 0.5M triethylammonium bicarbonate (TEABC; Sigma-Aldrich), supplemented with sodium dodecyl sulfate (SDS; 0.1 v/v%) and acetonitrile (ACN; 10 v/v%), in the presence of Halt Protease and Phosphatase Inhibitor Cocktail (Perbio Science, Erembodegem, Belgium) and 100 U of benzonase nuclease (Sigma-Aldrich, St. Louis, USA) in a Eppendorf LoBind tube. After sonication on ice, the protein

concentration was determined using the Bradford assay (Bradford 1976). 2.5 µg of protein was reduced in 0.5 M TEABC by adding 1 µL 50 mM tris(2-carboxyethyl)phosphine (TCEP) and incubating for 30 minutes at 60°C, followed by alkylation using 10 mM methyl methanethiosulfonate (MMTS) for 10 minutes at room temperature (RT). Digestion was performed in 1 mM $CaCl_2$ by adding trypsin/lysC (25:1 protein/enzyme ratio; Promega, Madison, USA). The samples were placed overnight at 37°C and evaporated, after which they were resuspended in 0.1% formic acid. 100 ng of each peptide sample was spiked with 25 fmol Hi3 standard and 25 fmol MSPD standard before injection.

The peptides were separated using a nanoscale UPLC system (nanoAcquityUPLC, Waters, Milford, USA) coupled to a Synapt G2-Si mass spectrometer (Waters). Peptides were first trapped in 0.1% formic acid on a 180 µm x 20 mm C18 Trap column. Separation was performed on a HSS C18 1.8 m, 100 m × 250 mm analytical column at a flow rate of 300 nL/min and a temperature of 45°C. As mobile phase A, a 0.1% formic acid with 4% dimethylsulfoxide (DMSO) in water solution was used and 80% ACN containing 0.1% formic acid constituted mobile phase B. Peptides were separated for 60 minutes at 1–40% solvent B and for 1 minute at 40-85% solvent B. 7 min of rinsing (85% solvent B) re-equilibrated the column to the initial conditions. Eluted peptides were analyzed in positive mode ESI-MS using High Definition MS^E ($HDMS^E$) with a collision energy look up table as described in (Distler et al. 2014). The spectral acquisition time of low and elevated energy scans was 0.6 s over an m/z range of 50-2000. [Glu1]-Fibrinopeptide B was used for post-acquisition lock mass correction.

Data analysis of the raw files obtained from the Synapt G2-Si was performed in Progenesis QI (Nonlinear Dynamics) version 2.0. Samples with an alignment score lower than 70% were discarded, resulting in three samples per treatment. Peptides with charges from two to five were retained following data normalization. For peptide identification, the following search criteria were set: trypsin as digestion enzyme, up to one missed cleavage allowed, fixed methylthio cysteine and variable methionine oxidation and deamidation at asparagine and glutamine. The optimal peak picking threshold for protein identification was determined by the PLGS Threshold Inspector. The data was searched by Protein Lynx Global SERVER 3.0.2 (Waters) with peptide tolerance and fragment tolerance set to auto. Protein identifications were obtained by searching a compiled database of UniProtKB/Swiss-Prot entries belonging to IAPV and all *Bombus* species supplemented with the cRAP database (laboratory proteins and dust/contact proteins) and sequences of spiked standard proteins, which was concatenated to a randomized decoy database. The false discovery rate (FDR) for protein identification in PLGS was set to 1% threshold. Only

proteins identified with at least two unique peptides were further considered for relative quantification (normalized to all proteins). For absolute quantification the Hi3 standard was used and only proteins with at least three unique peptides were included.

5.3 Results

5.3.1 *IAPV genome structure and HDMS^E*

An overview of the genome structure of the *Dicistroviridae* IAPV and CrPV is given in Figure 18 and the HDMS^E coverage is denoted by the darkness of the polyprotein sequences' background. The coverage differed between the (poly)proteins; the 64 detected peptides covered 50% of the AA sequence of the structural polyprotein (48 peptides), whereas only 12% of the non-structural protein was detected (16 peptides). Within the orfX protein, none of the 7 predicted peptides (at least 6 AA long) after trypsin digestion were detected. The presence of 1A would be confirmed by detecting the Stop-Go translational cleavage at the NPG▾P site, but the resulting non-tryptic CGDWDSILLLLSGDIEENPG peptide was not observed.

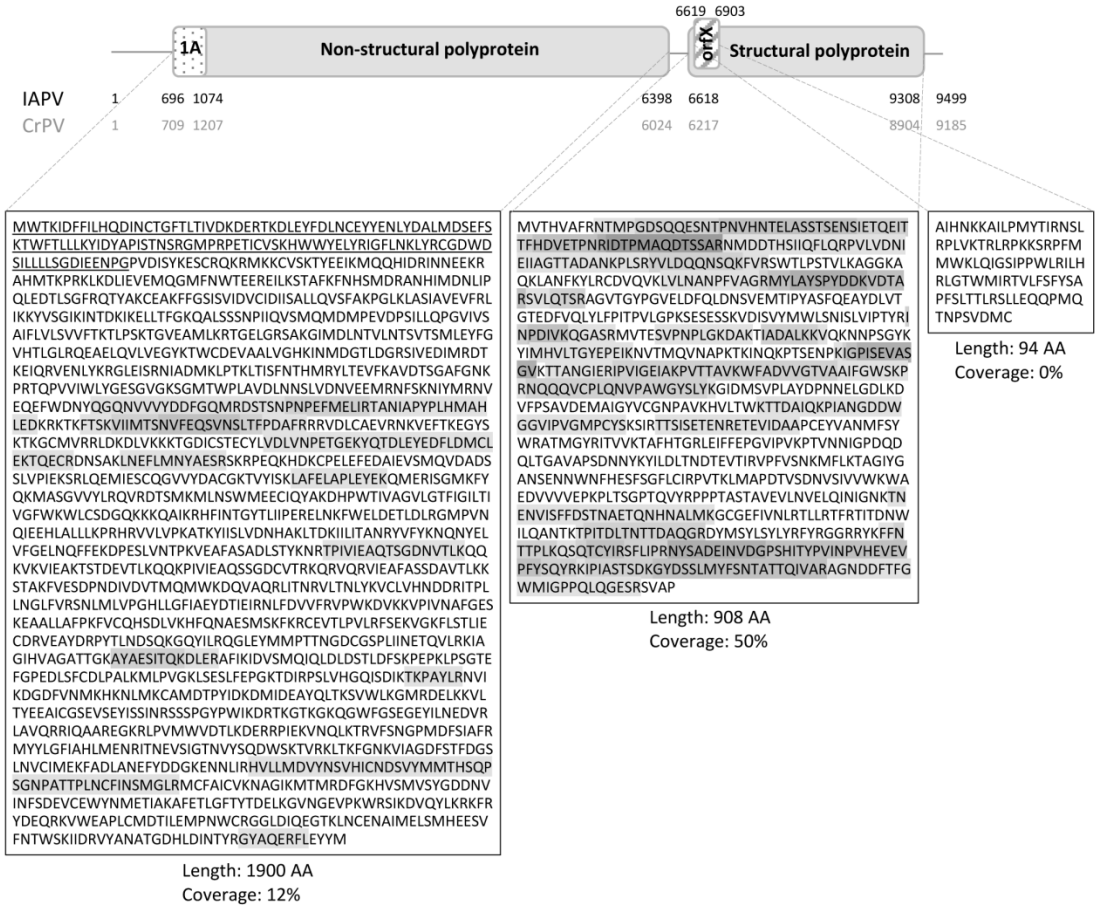

Figure 18. IAPV genome organization and HDMS^E coverage.
The *Dicistroviridae* genome consists of two ORFs, coding for a non-structural and a structural polyprotein. The first stretch of amino acids (AA) in the former polyprotein, upstream from Stop-Go translational cleavage site NPG$^{\blacktriangledown}$P, form the 1A protein (dotted). In IAPV the length of this protein is 126 AA, whereas in CrPV it is 166 AA. In the +1 frame of the 5' end of the second ORF of IAPV the possible orfX (striped) was predicted (not present in CrPV). Start and stop nucleotide positions of the genome, the ORFs and the 1A and orfX CDSs are given in black for IAPV and grey for CrPV. (Poly)protein sequences are given and the location of the 1A protein is underlined. HDMS^E coverage is indicated by the darkness of the AA letter code's background. Polyprotein length and HDMS^E coverage are given underneath the protein sequences.

5.3.2 *Virus distribution in bumblebee tissues*

Infections with IAPV and CrPV showed a similar relative distribution over the tissues, with higher viral titers in the fat body and for IAPV also in the ovaria, and lower viral titers in brain and midgut (Figure 19). However, there was a remarkably large variation within the tissues. Interesting to note is that the average viral titer of IAPV in the fat body was around 1400 times the normalized level of the reference genes *rpl23* and *ubi*, whereas for CrPV it was only 16 times, even with an infection dose of only 500 particles of IAPV and 10^6 of CrPV. CrPV replication was confirmed by negative strand detection and showing a 11,000-fold increase in viral titers 4 days after infection compared to the input viral titer minutes after injection (see section 4.3.2).

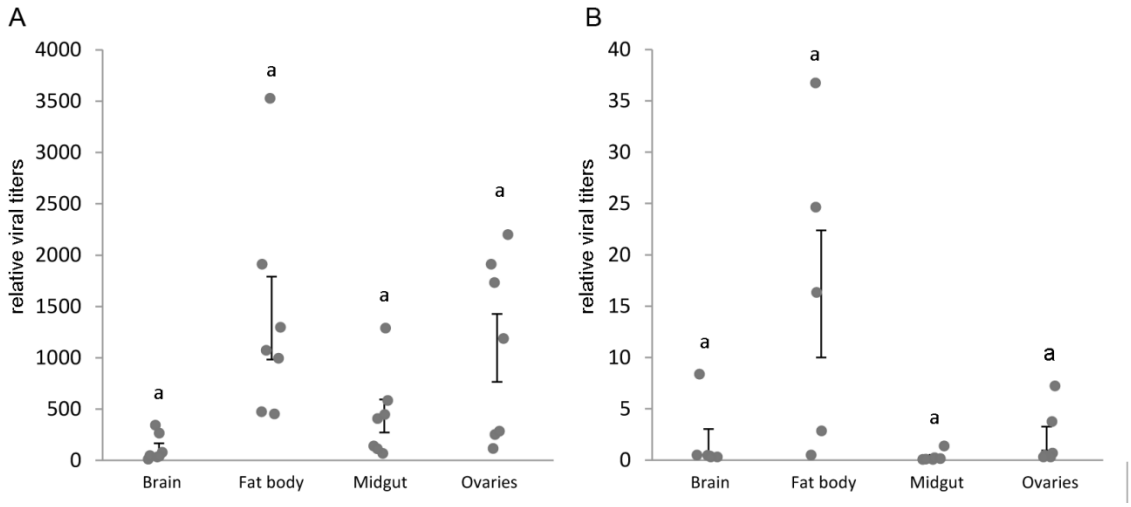

Figure 19. IAPV and CrPV tissue distribution in different bumblebee tissues.
Seven bumblebees were injected with 500 particles of IAPV (panel A) or 10^6 particles of CrPV (panel B)and the viral titers, after 72 hours or 96 hours respectively, were evaluated using RT-qPCR. The dots represent the individual viral titers (n=7), relative to the normalized level of the reference genes *rpl23* and *ubi.* The error bars show the SEM on the mean. Statistical analysis was performed using the non-parametric Friedman rank test as samples from the same individuals are not independent, resulting in significantly different viral titers over the tissues (IAPV: Q-stat.=13.29, p=0.001; CrPV: Q-stat.=10.92, p=0.003). Comparisons between treatments were made using Wilcoxon signed rank tests, none of which differed significantly on an $\alpha = 0.05$ level after Bonferroni correction. In general, there was a considerably large variation within the tissues, but the higher viral titers were found in the fat body and ovaries for IAPV, and fat body for CrPV.

5.3.3 *RNAi efficiency in bumblebee tissues*

In the *ppia*-targeting assays, the RNAi efficiency could be evaluated in both the absence and presence of the viruses. The expression levels of *ppia* in the RNAi control compared to the baseline control measure RNAi efficiency in a virus-free condition, whereas the levels in the virus treatment give an indication on how the virus influences the RNAi efficiency (Figure 20).

In the 'IAPV' experiment, ANOVAs over the three treatments showed significant differences in *ppia* levels in the brain ($F_{2,22}$=7.417, p=0.003), fat body ($F_{2,20}$=29.317, p<0.001) and midgut ($F_{2,22}$=11.218, p<0.001), but not in the ovaries ($F_{2,22}$=0.788, ns). In the following sections the ovaries will not be discussed further. The other tissues will collectively be referred to as 'responsive tissues'. For the 'CrPV' experiment, the *ppia* levels in the fat body were also statistically different ($F_{2,25}$=5.864, p=0.008).

Different silencing efficiencies were observed between the selected bumblebee tissues after 48 hours, in the absence of viruses (comparison between baseline control and RNAi control in Figure 20). For both the 'IAPV' and the 'CrPV' experiment, the expression levels of *ppia* dropped significantly in the fat body (IAPV: p=0.002 (Tukey's HSD); CrPV: p=0.011 (Tukey's HSD)). However, the *ppia* levels dropped by 55% in the 'IAPV' experiment, whereas they only declined by 25% in the 'CrPV' experiment (note that there can be no effect of virus presence in this comparison). In the brain and midgut the *ppia* levels were slightly lowered, but there was too much biological variation in the IAPV experiment to confirm an RNAi event ($F_{2,22}$=7.417, p>0.05 (Tukey's HSD) and $F_{2,22}$=11.218, p>0.05 (Tukey's HSD) respectively).

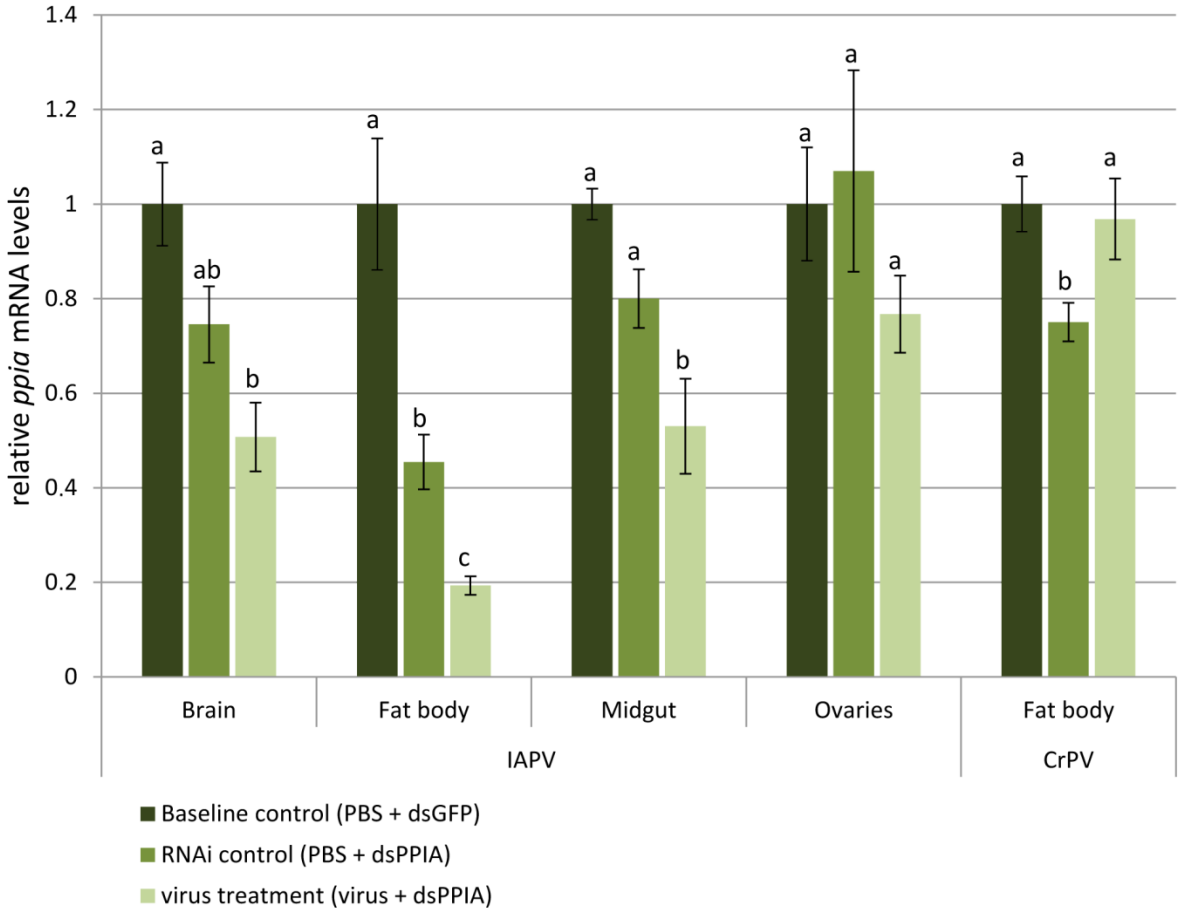

Figure 20. RNAi efficiency in different bumblebee tissues, with and without IAPV or CrPV infection.
Seven to nine bumblebees were treated with 20 µg dsPPIA (or dsGFP in the baseline control). In the virus treatment 500 particles of IAPV or 10^6 particles of CrPV were administered 24 or 48 hours, respectively, beforehand. The effect of the treatment on the silencing of the reporter gene *ppia* was evaluated using RT-qPCR 48 hours after dsRNA application. All data was normalized to the *ppia* levels in the baseline control. Statistical analysis was performed using Analysis of Variance on $\log_2$ transformed data with Tukey's HSD for post-hoc comparisons between the treatments. The columns represent the treatment mean ± SEM (on a linear scale) and statistical differences on an α= 0.05 level are denoted by different letters above the column. Comparing the RNAi control (middle green) to the baseline control (dark green) represents the RNAi efficiency in the different tissues in the absence of viruses, with a significant RNAi event only happening in the fat body. IAPV virus treatment (light green) showed an increased silencing efficiency in brain, fat body and midgut, whereas CrPV treatment resulted in a diminished RNAi efficiency as the expression levels rise to a comparable level as in the baseline control.

5.3.4 *Virus infection alters RNAi efficiency*

When IAPV was administered 24 hours before the dsRNA treatment (virus treatment in Figure 20), a significant silencing effect on the reporter gene *ppia* was noticed in all three tissues of brain ($p=0.002$ (Tukey's HSD)), fat body ($p<0.001$ (Tukey's HSD)) and midgut ($p<0.001$ (Tukey's HSD)) compared to the baseline control, and in the case of the fat body and the midgut also a significant silencing compared to the RNAi control ($p=0.001$ (Tukey's HSD) and $p=0.013$ (Tukey's HSD) respectively).

The *ppia* levels in brain, fat body and midgut were lowered to 51%, 19% and 53% of their original levels, respectively. These results indicate an enhancement of the RNAi effect after IAPV infection in all tissues except for the unresponsive ovaries.

In the case of CrPV only the fat body was analyzed as it was the only tissue showing a significant silencing effect without IAPV presence, which is necessary for confirming RNAi inhibition. The virus was administered to the bumblebees 48 hours before dsRNA treatment (instead of 24 hours for IAPV). This later time-point was chosen because injection of CrPV resulted in slower replication and later onset of mortality of the bees. We have determined the onset of death to occur at 4 dpi and 5 dpi for IAPV and CrPV, respectively (see Niu et al. 2016b and 4.3.1), so this alteration promotes similar virus-host interactions at the moment of dsRNA treatment and RT-qPCR evaluation. 48 hours after dsRNA treatment this resulted in a significant increase in *ppia* levels compared to the RNAi control ($p=0.036$ (Tukey's HSD)). Moreover, no significant silencing could be observed compared to the baseline control ($p>0.05$ (Tukey's HSD)), indicating that the RNAi system might have become impaired after CrPV infection.

5.3.5 *Expression levels of genes involved in RNAi after virus infection*

In order to assess whether the altered RNAi efficiency after virus infection is due to an upregulation of the genes involved in the RNAi pathway, the expression levels of *dcr-2*, *ago-2*, *ninaC*, e*gghead* and *sid-1* were evaluated 24 hours after IAPV infection (the moment the dsRNA is administered; virus injected compared to PBS injected) (Figure 21A) and at the endpoint of the assay (72 hours for IAPV and 96 hours for CrPV; virus treatment compared to RNAi control) (Figure 21B).

First, the RNAi core genes, *dcr-2* and *ago-2*, were assessed in all tissues 24 hours post IAPV infection (Figure 21A). A significant upregulation of *dcr-2* was observed in the brain and midgut ($t_{9.334}=-2.572$, $p=0.029$; $t_{8.497}=-2.275$, $p=0.05$, respectively). A general linear model (GLM) analysis over the responsive tissues showed a significant effect on *dcr-2* over the different tissues ($F_1=5.638$, $p=0.021$). The expression of *ago-2* was increased in midgut ($t_{17}=-4.432$, $p<0.001$) and in the GLM ($F_1=6.356$, $p=0.015$). At the endpoint of the assay (Figure 21B), a significant *dcr-2* upregulation was detected in the fat body ($t_{14} = -2.314$, $p =0.036$) and a similar, but more variable and not significant upregulation in the brain and midgut ($t_{7.68} = -0.996$, $p>0.05$ and $t_{6.90}= -1.165$, $p>0.05$, respectively). The GLM over the responsive tissues showed a significant effect of *dcr-2* ($F_1=5.744$, $p=0.024$). The expression levels of *ago-2* remained unaltered in all tissues.

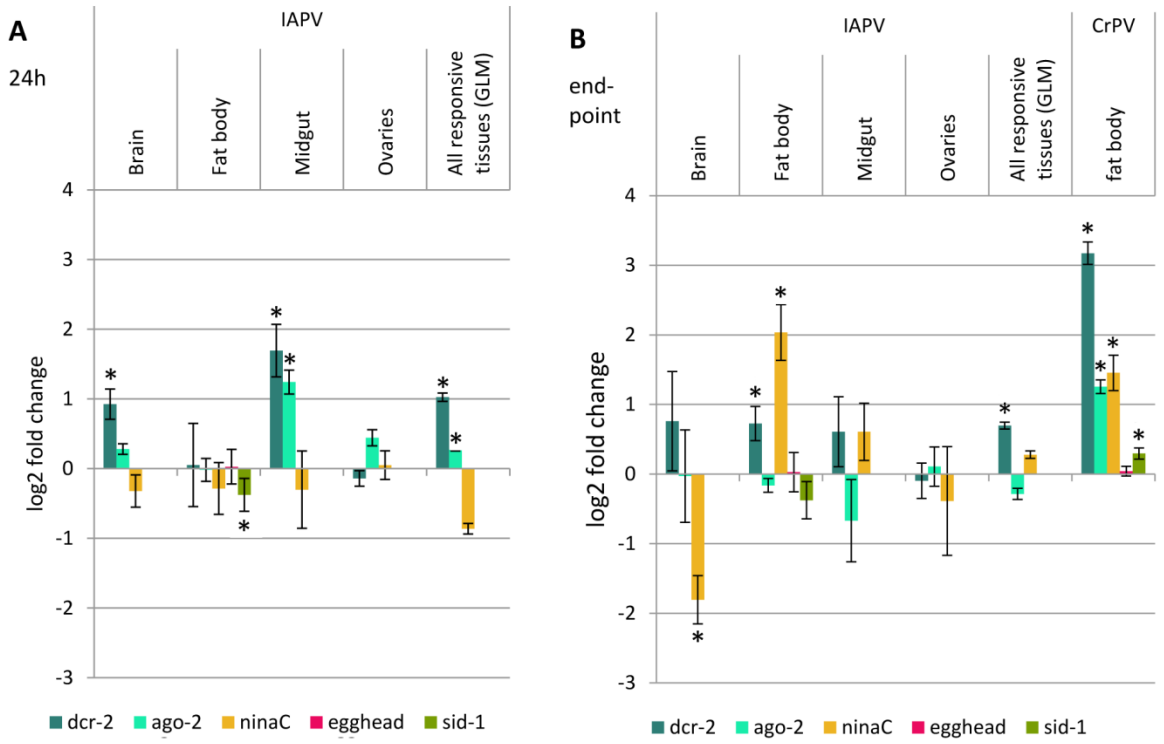

Figure 21. Fold change of selected RNAi genes upon IAPV or CrPV infection.
The expression levels of the RNAi core genes *dcr-2* and *ago-2* and the systemic RNAi genes *ninaC*, *egghead* and *sid-1* were evaluated at the moment of dsRNA application (panel A) and at the endpoint of the RNAi efficiency experiment (panel B) using RT-qPCR. Statistical analysis was performed using the Student's t-test on $\log_2$ transformed data. For the general linear model (GLM) only the responsive tissues, brain, fat body and midgut, were used. The columns represent the mean ± SEM on a $\log_2$ scale, normalized to the control, and statistical differences on an α= 0.05 level are denoted by an asterisk (n=7-9). In the case of IAPV, *dcr-2* showed a significant upregulation in some tissues at both 24 and 72 hours, and also over all responsive tissues. However its fold change is considerably smaller than the 9-fold change of *dcr-2* levels 96 hours after CrPV infection. *Ago-2* expression levels were augmented in the midgut 24 hours after IAPV infection and over all responsive tissues, but no effect was seen at the assay endpoint. For IAPV, the systemic RNAi genes showed occasional alterations in expression levels, however over all responsive tissues the changes were not significant, whereas for CrPV, *ninaC* and *sid-1* are significantly upregulated.

When looking at the systemic RNAi genes *ninaC*, *egghead* and *sid-1* in the fat body, only the former was upregulated (t_{14}= -4.23, p=0.001). Therefore the expression levels of *ninaC* were also determined in the other tissues. However, *ninaC* was significantly downregulated in the brain ($t_{12.10}$= 2.775, p=0.016) and no alteration was observed in the midgut (t_{14}= -1.195, p>0.05) nor in the GLM over all responsive tissues (F_1= 0.715, ns).

For CrPV, there was a much more evident upregulation of the RNAi core genes *dcr-2* and *ago-2* in the fat body after 96 hours (t_{16}=-16.056, p<0.001 and t_{16}=-10.706, p<0.001, respectively), as well as the systemic RNAi genes *ninaC* and *sid-1* (t_{16}=-2.978, p=0.009 and t_{16}=-2.931, p=0.010, respectively). The *dcr-2* upregulation after CrPV infection is over 9-fold, whereas for IAPV over all

responsive tissues not even 2-fold. The expression levels of *egghead* remained unaltered ($t_{10.032}$=-0.195, p>0.05) (Figure 21B).

5.3.6 *IAPV/CrPV levels after CrPV/IAPV pre-infection*

In both cases, there is a clear decrease in viral titers in the fat body compared to the control after pre-infection with the other virus. Pre-infection with CrPV for 48 hours reduced the IAPV levels with 90% (Mann Whitney U = 6, p<0.0001, Figure 22A), whereas pre-infection with IAPV for 24 hours reduced the CrPV levels with almost 99% (Mann Whitney U=2, p=0.0005, Figure 22B). Lower IAPV levels in Figure 22A are due to the evaluation occurring 48 hours post-infection, whereas in Figure 22B they are analyzed 72 hours post-infection.

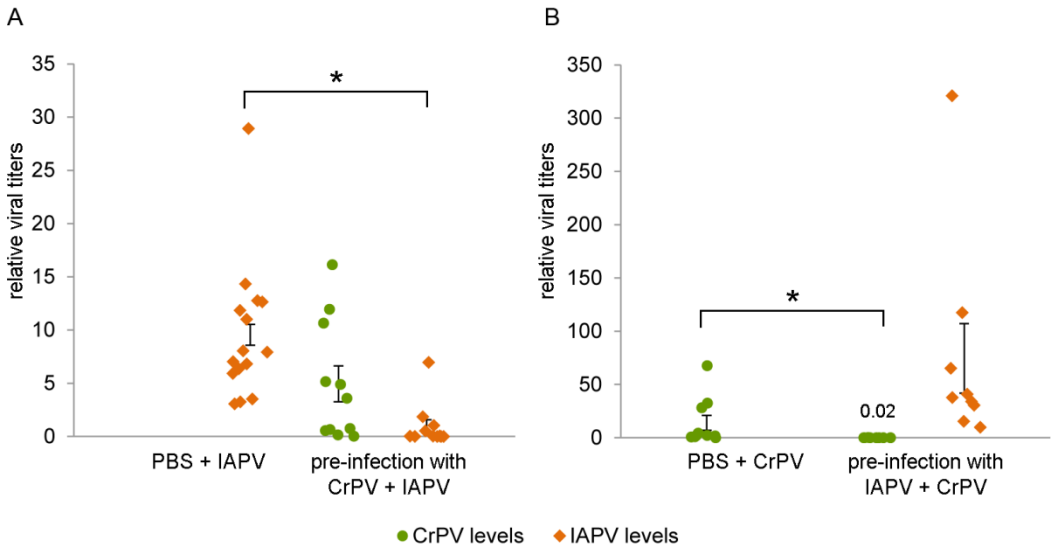

Figure 22. Virus levels of IAPV/CrPV after pre-infection with CrPV/IAPV.
Bumblebees were either pre-infected with 10^6 particles of CrPV and 48 hours later with 500 particles of IAPV (A) or pre-infected with 500 particles of IAPV and 24 hours later with 10^6 particles of CrPV (B). The viral titers were analyzed 48 hours after the second viral infection using RT-qPCR. The dots represent the individual data points relative to the normalized level of the reference genes *rpl23* and *ubi*, and the SEM of the mean is depicted by the error bars (n=10-15). Relevant statistical differences on an α= 0.05 level are denoted by asterisk (Mann-Whitney U test).

5.4 Discussion

Virus presence has been argued to be associated with variability of RNAi efficiency within a species as it can have a dual interaction with the RNAi defense system (Swevers et al. 2013b). On one hand, the presence of viral dsRNA fragments activates the RNAi pathway, on the other hand VSRs can inhibit this powerful antiviral defense system. The RNAi defense potency, in turn, influences viral replication and may determine the survival chances of the host. In this work we examined whether the presence of an immunosuppressive virulence factor of IAPV can tip the scale towards one of these two opposites in the bumblebee.

A first confirmation of the presence of a VSR in IAPV could come on a proteomic level, from the detection of VSR-specific peptides. The ovaries were selected for $HDMS^E$ analysis as they possess several characteristics that maximize the chance of finding small viral proteins. First, the viral titers in the ovaries were considerably high. Second, protein extraction from the fat body, with its similar high viral titers, is challenging, because of the high lipid content. Finally, the ovaries do not contain complex microbiota like the midgut does, which might confound the analysis. The $HDMS^E$ on IAPV-infected ovaries resulted in a coverage that is comparable to a similar setup for IAPV in *A. mellifera* (Michaud et al. 2014) with a higher coverage for the structural polyprotein than the non-structural polyprotein. In order to confirm the presence of the 1A VSR, one specific peptide with the alternative Stop-Go translational cleavage (Wang et al. 2012) needed to be detected. This was not the case, but the lower presence of the corresponding polyprotein or peptide characteristics like the isoelectric point or peptide length could have impeded detection. The same reasons might also explain why no peptides belonging to the possible out-of-frame protein orfX were found, but in this case there are multiple peptides that theoretically should be detectable.

From the $HDMS^E$ result, by itself, it is not possible to confirm with any certainty the absence of these putative VSR proteins in IAPV-infected cells. But, if they are present and functional, they should suppress the RNAi efficiency. The fact that this was not seen during the functional RNAi assay, leads to the conclusion that, even if the 1A or orfX proteins are produced in the cell during IAPV infection, and even if they are functional VSRs, they are not able to inhibit the RNAi system. It could be that their functionality is being outweighed by the RNAi system or that the RNAi system of bumblebees is insensitive towards the VSRs of IAPV.

Not only could the predicted VSR of IAPV not suppress the RNAi efficiency in *B. terrestris*, but also an enhancement of the RNAi activity was observed. The fact that a diminished RNAi effect is seen using the same setup for CrPV ensures the efficacy of the *ppia* assay. An argument could be made

that the difference between the experiments could be explained by the longer incubation of CrPV. We believe it was appropriate to extend the viral pre-infection duration because of the lower virulence of CrPV while a 24 hour age difference between the bumblebees will probably not affect their capacity for RNAi. More important is the question of how this IAPV-induced enhancement of RNAi activity can be explained.

A first explanation could be that virus infection causes the *ppia* levels to drop because of a disturbed cellular machinery. However, we collected ample evidence that this is not the case (Supplementary Figure 2). Another reason could be that the upregulation of the RNAi core genes that is sometimes seen after viral infection in insects (Xu et al. 2012; Galbraith et al. 2015; Niu et al. 2016b) could make this pathway more potent. We noticed a slight (~2-fold) upregulation of *dcr-2* after IAPV infection in some tissues which also showed an increased RNAi efficiency. In contrast, for CrPV a notably larger upregulation (~9-fold) was seen. It seems that this upregulation of *dcr-2* does not determine the outcome of RNAi pathway, possibly because the VSR 1A is acting on Ago-2, a component downstream of Dcr-2 (Nayak et al. 2010). Interestingly, we have previously shown that *dcr-2* silencing does not increase IAPV replication in bumblebees, possibly because of a too robust replication of IAPV (Niu et al. 2016b). Third, host response to IAPV infection could enhance the systemic properties of the RNAi system, facilitating the spread of the silencing signal and resulting in an increased silencing efficiency. This argument seems less adequate as for IAPV, nothing really stood out (except some changes in *ninaC* expression but they are variable over the different tissues). Again for CrPV more significant positive fold changes are noticed (i.e. *ninaC* and *sid-1* in the fat body). The fact that an upregulation of *ninaC* was also seen in the fat body after IAPV treatment might indicate a role of the fat body in triggering a systemic RNAi response, but additional experiments are needed before any conclusions can be drawn on this. The exact mechanism behind the IAPV-induced RNAi efficiency remains inconclusive, possibly one or more genes that drive RNAi efficiency fell out of our selection or a still unknown RNAi signal-spreading mechanism is activated by the presence of IAPV.

The viral influence on the RNAi machinery, a key immune response against viral infections, has great implication for host-virus dynamics. It is important to repeat that IAPV is a known pathogen of various pollinating hymenopterans (Cox-Foster et al. 2007; Meeus et al. 2014), whereas CrPV has been detected once in honeybees (Anderson and Gibbs 1988) and appears to infect bumblebees, but seems to have a wide experimental host range outside of the pollinators (Plus et al. 1978; Chao et al. 1986). The tissue distribution pattern of the two viruses IAPV and CrPV is remarkably similar with both having the highest viral titers in fat body, and considerably large

variation between individuals. In mosquitoes, the fat body has been suggested to be the primary amplifying tissue for the positive ssRNA viruses West Nile Virus (Girard et al. 2004) and Dengue virus (Salazar et al. 2007), where it is thought to be the intermediate station between the primary infection in the midgut and the spreading towards the other tissues. Like others have noted before, this is remarkable as the fat body is also considered a particularly immunocapable tissue (Gillespie et al. 1997) and we have observed this to be a tissue exhibiting a significant RNAi response. Although IAPV induced the antiviral defense system, IAPV reached 100-fold higher viral titers starting from a 2000-fold lower injection dose. This could indicate that RNAi potency is not the primary determining factor in viral infectivity. Cell entry and manipulation of the host cell's protein synthesis or other immune pathways could be more decisive for explaining viral replication dynamics, especially since IAPV has adapted to infect bees, so it could have evolved mechanisms for evading the immune response other than the suppression of RNAi. Also it should be taken into account that the method, by which the viral titers were determined, does not measure viral activity. Therefore, the difference in viral doses administered may not accurately reflect the difference in infectious particles.

Our findings are also interesting in the light of RNAi-therapeutics development and the use of RNAi as a research tool in functional genomics. The RNAi efficiency in the absence of a virus varies between the different tissues examined, with a significant silencing effect only occurring in the fat body and the ovaries which are unresponsive. It is conceivable that the virus could evade the therapeutic in tissues which are insensitive to RNAi, such as the ovaries which showed relatively high viral titers in this study. Variations in RNAi efficiency over insect tissues have also been found in *Anopheles gambiae* with salivary glands that are refractory to conventional RNAi (Boisson et al. 2006) and in many lepidopterans for which RNAi experiments are generally more successful in the hemocoel-surrounding tissues and less in the epidermal tissues (overview in (Terenius et al. 2011)). An insensitivity to RNAi in the ovaria, as observed here, was also seen in *S. gregaria* (Wynant et al. 2012), in the honeybee *A. mellifera* after siRNA injection (Jarosch and Moritz 2011), and attributed to a lack of dsRNA uptake in this tissue in *Locusta migratoria* (Ren et al. 2014). It needs to be noted that the dsRNA was delivered by injection and had direct access to most tissues through the hemolymph, therefore bypassing the midgut barrier. The results also affirm the need for an evaluation on tissue-level instead of whole body-level in similar experiments as both viral titers and RNAi efficiency differ considerably between tissues.

An interesting question emerging from these results is the functionality of the CrPV 1A in bumblebees, a hymenopteran species, which was previously shown to be active in the dipteran

Drosophila (Nayak et al. 2010). Although there is a statistically significant reduction in RNAi efficiency, the fact that the RNAi effect in absence of the virus is not that large prevents a confirmation about its functionality within the bumblebee. As a single bee host is often infected with multiple viruses, VSRs could lead toward competitive or synergistic effects. Pre-infection with a VSR-coding virus, such as CrPV, could facilitate subsequent infection and colonization, while an infection of IAPV would make it more difficult for other viruses to co-infect as the RNAi system becomes more efficient. Carrillo-Tripp et al. showed that pre-treatment with CrPV-1A could induce cytopathogenic effects of *Deformed wing virus*, persistently present in the AmE-711 cell line (Carrillo-Tripp et al. 2016). Our results show a large reduction in viral titers after pre-infection with the other virus. In the case of IAPV pre-infection this could be the result of a combination of an enhanced RNAi machinery and competition for the same host resources. IAPV, under normal circumstances, replicates extremely efficiently in bumblebees. For CrPV, however, this similar reduction of IAPV titers suggests that the VSR functionality, which was dubious in the RNAi assays, is not relevant. The mere presence of CrPV, and its saturation of the translational machinery, limits IAPV replication. It would be interesting to examine how this pre-infection with CrPV influences IAPV virulence in natural infections. As a conclusion we can state that there is a complex interaction between viruses and the RNAi defense mechanism of the insect host. Therefore, VSR functionality cannot be inferred from virus relatedness and needs to be taken into account when looking at virulence and multi-virus/multi-host dynamics.

CHAPTER VI:

PROTEOMIC ANALYSIS OF *ISRAELI ACUTE PARALYSIS VIRUS*-INFECTED OVARIES OF *BOMBUS TERRESTRIS* WORKERS

Parts of this chapter were submitted for publication:

Cappelle, K., Smagghe, G., Dhaenens, M., Deforce, D., and Meeus, I. High definition mass spectrometry reveals proteostatic and metabolic changes after *Israeli acute paralysis virus* infection in *Bombus terrestris* worker ovaries.

K. Cappelle and I. Meeus conceived and designed the experiments; K. Cappelle performed the experiments and analyzed the data. M. Dhaenens aided in the design, implementation, analysis and reporting of the mass spectrometry part of this study. The manuscript was written by K. Cappelle and revised by M. Dhaenens, I. Meeus and G. Smagghe.

6.1 Introduction

Bumblebees can be infected by various bee viruses, mainly belonging to two families: the *Dicistroviridae* and the *Iflaviridae*. The study object of this chapter is IAPV , which typically causes asymptomatic covert infections in bees, which under certain (stress) conditions convert into overt infections, characterized by paralysis followed by death. Although no obvious symptoms are seen with covert infections, they can have an impact on colony survival because of sublethal effects such as a reduced fecundity (Meeus et al. 2014).

Viruses, being obligate parasites, use the cellular machinery of the host to replicate and spread within the host body and to other individuals. The most obvious target for take-over is the translational system of the host, which is used for the production of viral proteins. But also other cellular pathways can be affected by single stranded RNA viruses, such as membrane vesicle trafficking, other (protein) transport systems, lipid biogenesis and various antiviral factors (Colpitts et al. 2011; Vashist et al. 2012; Ryabov et al. 2016). Aside from viral products directly interfering with the cellular machinery and steering it towards enhance viral replication, this replication on itself also has its impact on cell signaling and the metabolic state of the cell because of saturation of the cellular pathways. The extent of these disturbances determines the virulence of a specific virus, but it is counteracted by the host's antiviral immune response.

During viral replication dsRNA intermediates trigger the RNAi defense pathway and upregulation of the RNAi core genes *dcr-2* and *ago-2* after virus infection, and more specifically after IAPV infection in bumblebees, has been reported (Niu et al. 2016b). However, in an exploratory experiment we observed a disparity between relatively high IAPV titers in the ovaries, but a limited antiviral RNAi immune response. In identical experiments in the fat body, there was an upregulation of *dcr-2* and *ago-2*. Aside from RNAi, alternative immune pathways might play a role, therefore a non-targeted proteomic examination of the ovaries after viral infection could be interesting. In this work, we set forward to study how IAPV (direct or indirectly) alters the metabolic and immunological status of the ovaries. For this, a proteomic dataset of IAPV-infected ovaries was compared with non-infected controls using data-independent high definition mass spectrometry ($HDMS^E$). Differentially expressed proteins were identified and analyzed using Gene Ontology (GO) information and possible regulators of the observed changes were further examined using RT-qPCR.

6.2 Material and methods

6.2.1 *Bumblebee rearing and virus production*

The bumblebees were obtained, maintained and injected with 500 particles of IAPV as mentioned in section 5.2.1. The IAPV suspension was produced as described in detail in section 4.2.2.

6.2.2 *HDMS^E experimental setup*

The detailed protocol is described in section 5.2.2. In brief, the ovaries of IAPV-infected bumblebees were crushed in liquid nitrogen (pooled per three individuals) and two third of the resulting powder was stored at -80°C for mass spectrometry, while one third was dissolved in RLT buffer for confirmation of infection. The powdered samples were resuspended in TEABC, supplemented with SDS and ACN, in the presence of Halt Protease and Phosphatase Inhibitor Cocktail and 100 U of benzonase nuclease. After sonication on ice, the protein concentration was determined using the Bradford assay. 2.5 µg of protein was reduced using TCEP and alkylated by adding MMTS. Digestion was performed in 1 mM $CaCl_2$ by adding trypsin/lysC. The samples were evaporated overnight at 37°C and resuspended in 0.1% formic acid. 100 ng of each peptide sample was spiked with 25 fmol Hi3 standard and 25 fmol MSPD standard before injection.

The peptides were separated using a nanoscale UPLC system (nanoAcquityUPLC, Waters, Milford, USA) coupled to a Synapt G2-Si mass spectrometer (Waters) as described in 5.2.2. Eluted peptides were analyzed in positive mode ESI-MS using High Definition MS^E (HDMS^E) with a collision energy look up table as described in (Distler et al. 2014). The spectral acquisition time of low and elevated energy scans was 0.6 s over an m/z range of 50-2000. [Glu1]-Fibrinopeptide B was used for post-acquisition lock mass correction.

6.2.3 *HDMS^E data analysis*

Data analysis of the raw files obtained from the Synapt G2-Si was performed in Progenesis QI (Nonlinear Dynamics) version 2.0. Samples with an alignment score lower than 70% were discarded, resulting in three samples per treatment. Peptides with charges from two to five were retained following data normalization. For peptide identification, the following search criteria were set: trypsin as digestion enzyme, up to one missed cleavage allowed, fixed methylthio cysteine and variable methionine oxidation and deamidation at asparagine and glutamine. The optimal peak picking threshold for protein identification was determined by the PLGS Threshold Inspector. The data was searched by Protein Lynx Global SERVER 3.0.2 (Waters) with peptide tolerance and fragment tolerance set to auto. Protein identifications were obtained by searching

a compiled database of UniProtKB/Swiss-Prot entries belonging to IAPV and all *Bombus* species supplemented with the cRAP database (laboratory proteins and dust/contact proteins) and sequences of spiked standard proteins, which was concatenated to a randomized decoy database. The false discovery rate (FDR) for protein identification in PLGS was set to 1% threshold. Only proteins identified with at least two unique peptides were further considered for relative quantification (normalized to all proteins). For absolute quantification the Hi3 standard was used and only proteins with at least three unique peptides were included.

The list of proteins with differential expression levels (ANOVA p-value<0.05) obtained from Progenesis QI was used for GO enrichment analysis using the Blast2GO software (Conesa et al. 2005). As no GO information was available for any *Bombus* species, the sequences were blasted to a local *A. mellifera* Uniprot database (Weinstock et al. 2006) and subsequently mapped with their GO terms using standard Blast2GO settings. For GO term enrichment analysis Fisher's exact test (as implemented in the Blast2GO software) with an FDR of 0.1 was used using the *A. mellifera* Uniprot database as reference set. GO enriched graphs were produced within the Blast2GO software, the heat map using R (standard parameters; scaling by row) (R Development Core Team 2016) .

6.2.4 *Metabolism and immunity gene expression levels*

Bumblebees (10 per treatment) were injected with 500 particles of IAPV (in 5 µL) or PBS and fat body, ovaries and brain were dissected after 72 hours in RLT buffer. RNA was isolated using the RNeasy Mini Kit (Qiagen) and treated with the Turbo DNA-free kit (Life Technologies). Then, 500 ng of RNA was used in each cDNA synthesis reaction, performed with the SuperScript II Reverse Transcriptase Kit (Life Technologies) using an oligodT primer. All RT-qPCR reactions were carried out as described in section 4.2.3 using the primers listed in Table 10. The results were analyzed using qbase^{+} software (Biogazelle, Zwijnaarde, Belgium) and SPSS Statistics 23.0 (IBM Corp 2015).. Statistical differences were calculated using $\log_2$-transformed data and the Benjamini-Hochberg correction method for multiple comparison at an FDR of 0.05.

Table 10. List of primers used in Chapter VI.

Primer name	Sequence (5' – 3')	Length (bp)	Eff. (%)
IAPV F	CCATGCCTGGCGATTCAC	203	102
IAPV R	CTGAATAATACTGTGCGTATC		
rpl23 F	GGGAAAACCTGAACTTAGGAAAA	143	96
rpl23 R	ACCCTTTCATTTCTCCCTTGTTA		
ubi F	GGTATTTGGATGCCAGTGATTT	129	96
ubi R	ATGGGCATTTCTACCCCTTTTA		
ilp-1 F	CGCTCCTCGACACGGTTAAT	116	92
ilp-1 R	CGGCTCGTTGTAACCTCGAT		
ilp-2 F	GCAAATCGATTATACGCGCTCA	105	106
ilp-2 R	TCCATGTATGTTCCGCTGCT		
ago-2 F	CCGAATGTGGACAATGCTTA	181	90
ago-2 R	AACGGGCAAAGGTGTGATTA		
dcr-2 F	TGGTCAAAACATCAAGAACAACCA	211	90
dcr-2 R	GATCGGGGCCATACGAACAT		

bp: basepairs; Eff.: amplification efficiency; IAPV: *Israeli acute paralysis virus* (EU436443.1); rpl23: *60S ribosomal protein L23* (XM_003400707.2); ubi: *polyubiquitin B* (XM_003402262.2); ilp-1: *insulin-like peptide 1* (XM_012310891.1); ilp-2: *insulin-like peptide 2* (XM_003400730.2); ago-2: *argonaute-2* (XM_012312881.1); dcr-2: *dicer-2* (XM_012307737.1).

6.3 Results

6.3.1 *Disturbed immune response in IAPV-infected ovaries*

Although IAPV levels were statistically different in the fat body and ovaries three days after IAPV infection (general linear model; $F_{1,7}$=13.074, p=0.009), their levels were still comparable (Figure 23A). However, the immune response was considerably divergent. In the fat body there was an upregulation of the key components of the RNAi pathway, *ago-2* and *dcr-2* (t_{16}=-6.241; $p_{BH\ adj.}$<0.001 and $t_{9.5}$=-3.656; $p_{BH\ adj.}$=0.009, respectively) (Figure 23B). In the ovaries none of the selected immune genes was upregulated (Figure 23C). This inactivity of the ovaries was examined further on protein level using HDMS^E.

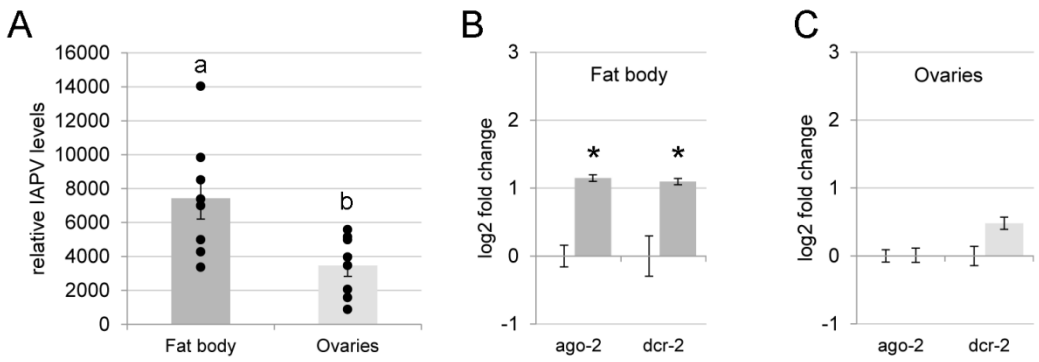

Figure 23. Expression levels of IAPV (panel A) and two core RNAi genes in fat body (panel B) and ovaries (panel C) three days after IAPV infection, evaluated using RT-qPCR.
Statistical analysis was performed as follows: Panel A) a general linear model (GLM) with tissues as fixed and the individual bumblebees as random factors, no statistical difference was found. Columns represent the mean ± SEM, the dots the individual data points, normalized to the reference genes *rpl23* and *ubi*. Panel B and C) Student's t-tests on $\log_2$ transformed data. The columns represent the mean ± SEM, normalized to the control (depicted by their SEM on the left of the corresponding column), and statistical differences on an FDR=0.05 level (Benjamini and Hochberg correction) are denoted by an asterisk (n=8-10).

6.3.2 *HDMS^E overall statistics*

For proteomic analysis, IAPV-infected ovaries three days after infection were used. After run alignment, a total number of 43522 features was obtained, of which 13468 could be assigned to peptide ions (including singly charged, in-source decay ions). These belonged to 1400 distinct quantifiable proteins, 1020 of which had at least two unique peptides, and 701 which had at least three unique peptides. The absolute quantity of the structural polyprotein of IAPV in the infected samples was determined to be about 200 fmol, with absolute quantities of the histone H3 around 3000 fmol. As histone quantities can be used as stable proteomic rulers (2-4% of the total MS signal; Plaskon et al. 2009), the fraction of viral proteins is ensured to be low enough to allow for an accurate normalization over all proteins.

In the ovaries after IAPV infection, 56 proteins were differentially expressed (ANOVA p-value < 0.05): 11 were upregulated, whereas 42 were downregulated. Focusing only on these differentially expressed proteins, principal component analysis (samples as cases and relative protein quantities as variables) shows a clear clustering of the samples and the proteins (Figure 24). An overview of the differentially expressed proteins, their peptide counts, fold change and *A. mellifera* ortholog is given in Supplementary Table 2.

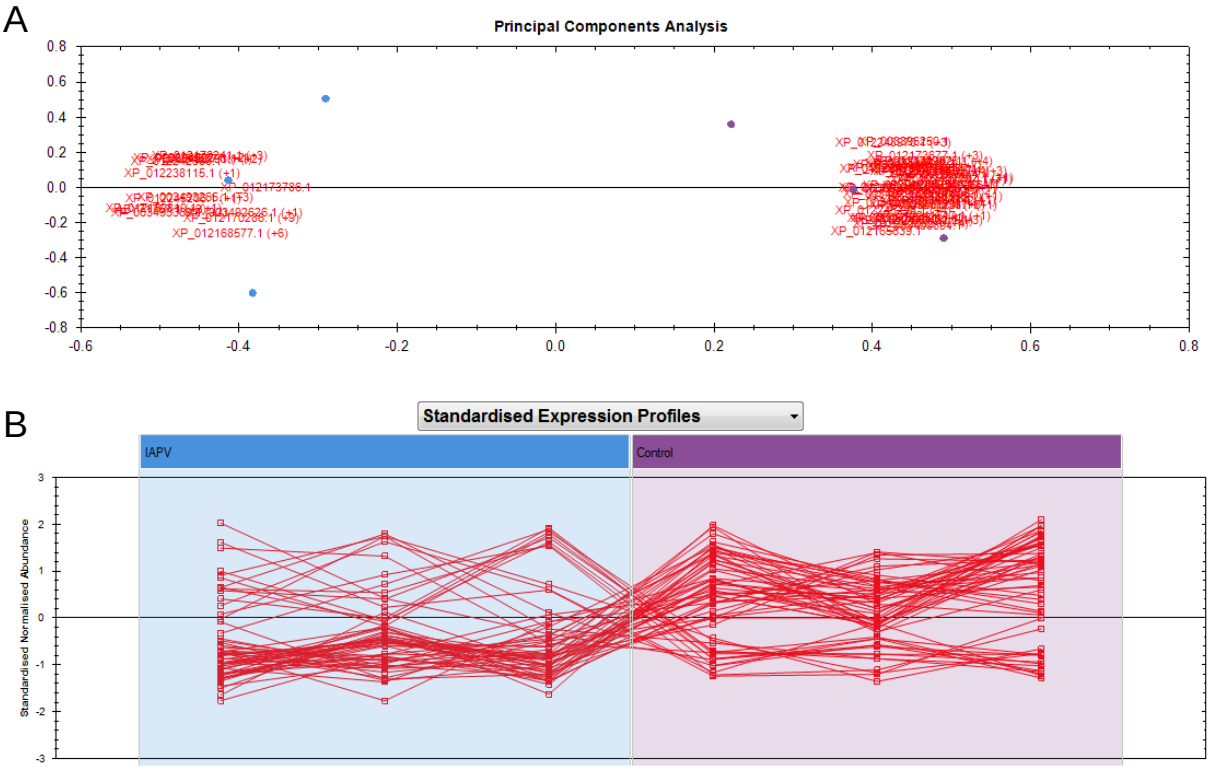

Figure 24. Progenesis QI output for the differentially expressed proteins in IAPV-infected ovaries.
A: Biplot of a principal component analysis on 56 differentially expressed proteins (ANOVA $p < 0.05$) (blue: IAPV-infected samples, purple: control samples, red: differentially expressed). B: The expression profiles of these differentially expressed as depicted by the Progenesis QI software.

6.3.3 *GO enrichment analysis*

Of the 11 upregulated proteins, 10 were successfully blasted onto the *A. mellifera* proteome, and 8 of them could be annotated with GO terms (Supplementary Table 2). GO term analysis (Fisher's exact test, FDR<0.1) revealed two enriched GO terms corresponding to the same two sequences: positive regulation of response to stimulus (GO:0048584; biological process; FDR=0.093) and regulation of response to stress (GO:0080134; biological process; FDR=0.087) (Supplementary Figure 3).

For the downregulated proteins: 36 were blasted to an *A. mellifera* ortholog, and all of them were mapped successfully with their GO terms (Supplementary Table 2). GO term analysis (Fisher's exact test, FDR=0.1) resulted in 30 enriched GO terms over the three GO domains (cellular component, molecular function and biological process; Supplementary Figure 4-6), which could be reduced to 5 most specific terms: ribosome (GO:0005840; cellular component; FDR=0.047), glycolytic process (GO:0006096; biological process; FDR=0.051), glyceraldehyde-3-phosphate dehydrogenase (NAD+) (phosphorylating) activity (GO:0004365; molecular function, FDR=0.047),

nucleotide binding (GO:0000166; molecular function; FDR=0.081) and structural constituent of ribosome (GO:0003735, molecular function, FDR=0.081)

When the differentially expressed were arranged according to function in a heat map (Figure 25), a clear pattern arises: reduced ribosomal constituents, proteasome activity, nucleotide binding (more specifically RNA processing) and metabolic activity. Noteworthy are some enzymes that are directly involved in energy metabolism. Three glycolytic enzymes were downregulated, which catalyze the 2nd (glucosamine-6-phosphate isomerase), 6th (glyceraldehyde-3-phosphate dehydrogenase) and 9th step (enolase) of the pathway. An additional downregulated enzyme is involved in fatty acid beta oxidation: acyl CoA dehydrogenase. A schematic representation of where these enzymes fit in the energy metabolism is given in Figure 26.

6.3.1 *Insulin-like peptide expression levels*

Because of the remarkable downregulation of some proteins involved in energy generation in the ovaries, possible regulators of these systems were further examined using RT-qPCR in both fat body and ovaries. Two *insulin-like peptide* (*ilp*) genes were found in *B. terrestris*, based on their homology to their *A. mellifera* counterparts (*ilp-1*: XM_012310891.1; *ilp-2*: XM_003400730.2). Three days after IAPV infection, a clear upregulation for *ilp-1* and *ilp-2* in the fat body (t_{16}=-5.148; $p_{BH\ adj.}$<0.001 and t_{16}=-3.521; $p_{BH\ adj.}$=0.007, respectively) could be observed, but also a seemingly larger (non-significant) upregulation of *ilp-2* in the ovaries (t_{15}=-2.942; $p_{BH\ adj.}$=0.055) (Figure 27).

Figure 25. Heat map showing the differentially expressed proteins after IAPV infection in the rows and the different treatments in the columns.
The colors denote relative abundance with green meaning highly expressed and red lowly expressed.

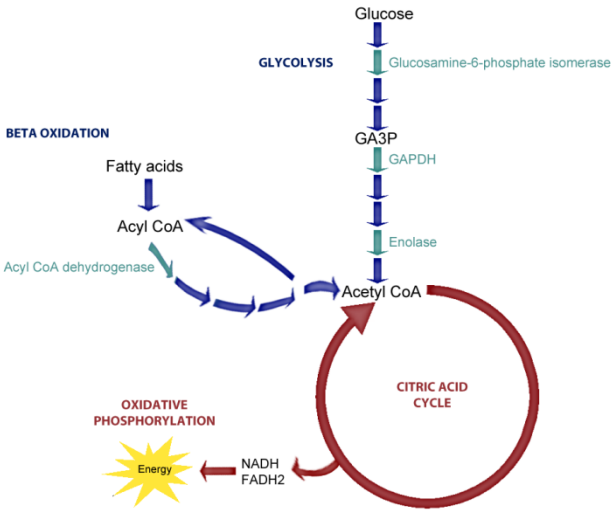

Figure 26. Schematic overview of the energy metabolism pathways.
Glycolysis and fatty acid beta oxidation deliver acetyl coenzyme A to the citric acid cycle, which produces, among others, the electron carriers NADH and $FADH_2$ which are converted into energy. Except for most steps of the glycolysis all reactions occur in the mitochondria of the cells. Enzymes found downregulated in IAPV-infected ovaries are denoted in lighter blue.

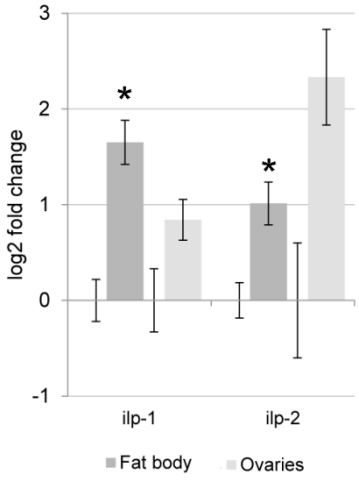

Figure 27. Expression levels of the *insulin-like peptide* genes in fat body (dark grey) and ovaries (light grey) three days after IAPV infection, evaluated using RT-qPCR.
Statistical analysis was performed using the Student's t-test on log_2 transformed data. The columns represent the mean ± SEM, normalized to the control (depicted by their SEM on the left of the corresponding column), and statistical differences on an FDR=0.05 level (Benjamini and Hochberg correction) are denoted by an asterisk (n=8-10).

6.4 Discussion

There are numerous ways to examine the effects that viruses can have on their host, from simple phenotypic evaluations to complex interaction studies between viruses, hosts and their environment. Alterations in the wide range of biological processes in the cell are most often explored at the RNA level, either by transcriptome sequencing or, if available, by using microarrays. Rarely proteomic approaches are used, even though protein levels are a better approximation for protein activity than their intermediary mRNAs. The advent of more powerful and sensitive data-independent acquisition (DIA) mass spectrometry now allows us to assess changes in the proteome in an untargeted manner. This is illustrated here by the quantification of 1400 distinct proteins in bumblebee ovaries, together surfacing a clear pattern of downregulation of general ovarian cell activity upon IAPV infection.

More specifically, the differentially expressed proteins found in this study point towards a dysregulated protein homeostasis. The production of a functional protein comprises of multiple steps: transcription, mRNA splicing and processing, transport to the cytoplasm, translation, protein folding and proteolysis of expendable or erroneous proteins. We observed a downregulation of a set of ribosomal proteins, along with proteins involved in splicing and protein degradation. A disruption of proteostasis was also observed in the head of honeybee pupae after IAPV infection, but with variable outcomes concerning up- and downregulation (Michaud et al. 2014), while in the ova of *Rice Stripe virus*-infected small brown planthoppers, an upregulation of translational processes was seen (Liu et al. 2016). The disruption in proteostasis could be explained in two ways: 1) the virus is trying to hijack the cellular translational system by manipulating certain ribosomal proteins and simultaneously hinder the translation of the host proteins by affecting mRNA maturation or 2) the host is attempting to limit viral protein production by halting its own translation. This latter option is especially alluring, as in most bumblebee workers the ovaries are functionally redundant. Indeed, workers do not contribute to the production of diploid workers or daughter queens (gynes). Only after the colony switch point, when the queens starts the production of drones and gynes, some workers compete with the queen and start to lay eggs developing into drones. It looks that not investing in ovary development, when infected by viruses, is a good strategy to prevent viral spreading. Vertical transmission of IAPV in bumblebees has not been shown yet, but various bee viruses are thought to be transmitted from queens to their eggs in honeybees (Chen et al. 2005; Ravoet et al. 2015). Whether virus infection indeed arrests ovary development in worker bees needs to be confirmed.

Next to protein homeostasis, energy metabolism seems to be affected by IAPV treatment as three glycolysis and one beta oxidation enzyme are downregulated. This would result in less acetyl CoA being generated, leading to reduced ATP production by oxidative phosphorylation. A downregulation of metabolic energy metabolism was also seen in IAPV-infected adult honeybees using microarray analysis (Chen et al. 2014). It is somewhat surprising as in many eukaryotic virus infections the core energy generating pathways are often activated to provide more resources (Celle et al. 2008). This deactivation could represent a strategy of the bumblebee to limit virus replication in a tissue which is redundant, as stated before. Additionally, resources would be conserved and could be put to use in tissues that actively combat the viral invasion, such as the fat body, which is responsible for the production of various humoral immune factors. In the ova of *Rice Stripe virus*-infected small brown planthoppers, a similar downregulation of metabolic processes was observed, along with various proteins involved in mitosis and growth and development (Liu et al. 2016).

The reduced metabolic activity in the ovaries is not a consequence of an overall systemic cessation of essential metabolic and immunogenic systems as a result of IAPV infection. In the fat body, the RT-qPCR data showed a clear activation of the RNAi pathway. Additionally, the ILPs were upregulated in the fat body (and to a lesser extent also in the ovaries). Similar to the situation in mammals, insect ILPs regulate circulating sugar levels and the balance between energy storage and mobilization, but their physiological role may be somewhat different. In some studies, the endpoints of ILP activation are analogous to those seen in mammals, with lowered hemolymph sugar levels and increased carbohydrate storage molecules (Zhang et al. 2009; Morris et al. 2012). In contrast, in *Bombyx mori* the ILP bombyxin facilitates the use of stored energy reserves (Satake et al. 1997). ILPs are part of a complex regulatory network along with, among others, adipokinetic hormones, vitellogenin, the juvenile hormone, USP and EcR (reviewed in Nässel and Vanden Broeck 2016). Also, the number of ILPs is vastly different over different species and the function of these proteins extends further to also include major roles in growth and development in *Drosophila* (Brogiolo et al. 2001; Zhang et al. 2009) and caste differentiation and social behavior in honeybees (Nilsen et al. 2011; Wang et al. 2013). In this study, we used the ILPs as a proxy for metabolic status. It seems that elevated levels of ILP correspond with an immuno-compromised status, as was also seen in the razor clam after bacterial infection (Niu et al. 2016a) and in *Anopheles* after *Plasmodium* infection (Marquez et al. 2011). However, straightforward conclusions cannot be drawn about how exactly the ILPs interact with the energy metabolism as a

result of viral infection. For that, more information is needed on their physiological role and regulation.

A limitation of the proteomic part of this study is the lack of well-annotated genomic and proteomic information for *B. terrestris*. Although the bumblebee genome is published, the NCBI database still mostly consists of computational predictions based on other insect homologs such as *A. mellifera* and *D. melanogaster*. Therefore, it cannot be ruled out that some protein identifications are ambiguous, especially for highly similar proteins or protein domains. Additionally, GO enrichment analysis had to be performed on the *A. mellifera* orthologs and use an *A. mellifera* reference set. It has been shown that the majority of bumblebee genes has orthologs within all bee species, however 118 bumblebee genes did not have a honeybee ortholog (Sadd et al. 2015), so they have fallen out of this analysis. As the reference set is solely used to calculate the percentage of genes belonging to certain GO categories and these bumblebee-specific genes are not enriched in certain groups (except possibly olfactory receptor function (Sadd et al. 2015)), we believe the impact of these shortcomings on our findings is limited. However, it would be advisable not to draw conclusions on the importance of one specific protein; the power of this analysis lies in the global overview of the biological processes. For future reference, the dataset was submitted to ProteomeXChange database for further examination when the genome of *B. terrestris* is better annotated.

Another interesting aspect of this work is the use of proteomic data. Inherent to their biogenesis, protein levels generally lag behind on their corresponding mRNA levels. Moreover, recently more and more information is emerging that there is an extra layer of regulation at the translation steps, with protein levels not necessarily mimicking their corresponding mRNA levels (Lee et al. 1993). As of yet, scant proteomic data, looking at viral infections in insects, is available although proteins are the true effectors of the cellular pathways. Therefore, this work should incite researchers in invertebrate pathology to make use of various novel mass spectrometry approaches having increased differentiating and quantifying power. Additionally, metabolomics studies, in which the end-products of glycolysis, beta oxidation and the citric acid cycle could be assessed, would reveal important information about the impact of viral infections on invertebrate cellular pathways. As a conclusion, this study indicates that IAPV infection causes strong disruptions of some essential cellular processes in the ovaries, such as protein homeostasis and energy metabolism.

CHAPTER VII:

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The large variability in RNAi efficiency, both at species and individual level, has puzzled RNAi-entomologists for almost a decade. In Chapter I we have identified several reasons which might contribute to this variability and selected two to focus on. In this final chapter, some conclusions, interesting remaining questions and future perspectives will be discussed for both factors. Additionally, links with undiscussed factors will be drawn throughout the subsections. For a better comprehension of this chapter, an overview is presented again:

- Factors inherent to the experimental setup: life stage, siRNA or dsRNA, dsRNA length, delivery method, dose, target gene (region, protein turnover,…), evaluation timepoint, examined tissue, etc.
- Factors inherent to the insect species: dsRNA degradation in the saliva/midgut (Yue and Genersch 2005; Christiaens et al. 2014; Wynant et al. 2014c), dsRNA sequestering in the hemolymph (Wynant et al. 2014a), different (efficiencies of the) dsRNA uptake mechanisms (Chapter III), absence/presence of RNAi core genes (Chapter II), absence/presence of systemic and/or environmental RNAi, low expression levels of RNAi core genes, etc.
- Factors inherent to the insect individual: nutritional status, immunological status, viral infections triggering enhanced systemic properties (Saleh et al. 2009), virus-produced suppressors of RNAi (Chapter V), etc.

7.1 DsRNA uptake and transport in insects

In Chapter III, CPB homologs of genes, proven to be involved in dsRNA uptake in *Drosophila* and other insects, were identified. A sensitive RNAi-of-RNAi assay was developed to determine which of these genes were necessary for an optimal RNAi response. Using this assay, we confirmed the involvement of both clathrin-dependent endocytosis, and probably also the *sid-1-like* genes in dsRNA uptake but for these latter two genes the rescue was too small to be completely convincing.

Since these observations were published, a similar assay has been undertaken in a CPB pupal fat body-derived cell line. In this screening, 50 RNAi-related genes, including the dsRNA uptake related genes, were tested. In agreement with our results, several endocytosis-associated genes and the two *sid-1-like* genes were needed for an complete RNAi response (Yoon et al. 2016). Unfortunately, due to the immortalization and continuous passages of cell lines, they may not accurately reflect the natural situation anymore. Additionally, it is impossible to assess the exact role of these pathways in the RNAi response in the whole CPB body.

7.1.1 *What do these results mean for dsRNA uptake in CPB midgut epithelial cells?*

We were the first to report a simultaneous involvement of the two known uptake mechanisms in dsRNA uptake in the insect body (which was later confirmed in vitro). But what exactly happens at the epithelial cell surface of the CPB midgut when dsRNA enters the cell remains unclear. Two questions arise:

1) Do the two different Sid-1-like proteins function independently or not?

2) Do the Sid-1-like proteins and the endocytosis pathway interact with each other to internalize dsRNA?

The various models for dsRNA uptake are depicted in Figure 28. For the nematode *C. elegans*, a model has been suggested where SID-1 and SID-2 interact with each other to take up dsRNA with SID-2 functioning as the receptor at the cell surface, needing at least a part of endocytic pathway, and SID-1 as the channel protein needed for release in the cytoplasm (scenario A; McEwan et al. 2012). These proteins have significant structural differences, e.g. SID-2 has only one transmembrane domain and thus cannot form a channel like SID-1 does. In insects however, the Sid-1-like proteins all share the same structure as the SID-1 protein in *C. elegans* and probably all form channels through the cell membrane. In CPB, two Sid-1-like proteins are present and involved in dsRNA uptake: SilA and SilC. One possibility is that they function independently (scenario B). The extracellular carboxyterminal end of these proteins is likely responsible for the receptor function and as it differs significantly between SilA and SilC, it could have a different affinity for different types of dsRNA or other macromolecules. Another option is that one of the Sid-1-like proteins is present at the epithelial surface and internalizes the dsRNA and that the other plays a role in dsRNA release from vesicles (scenario C), or is involved in dsRNA transport to the neighboring cell layers (scenario D). As the assay evaluated RNAi response in the midgut as a whole, also the underlaying muscle layers were included, so an impairment of this short-distance dsRNA transport would also give a positive result.

With these hypotheses, how can the endocytic dsRNA uptake processes be included? Again, the systems could be completely independent with, based on our results, endocytosis having the largest contribution to the dsRNA uptake process as a whole. The receptor which recognizes dsRNA to trigger endocytosis is not identified yet, but scavenger receptors on the cell surface have been suggested (Ulvila et al. 2006). However, it is also possible that both uptake mechanisms are linked and that the Sid-1-like proteins act as receptors, triggering endocytosis (scenario E). Additionally, it is possible that all dsRNA is first taken up in endocytic vesicles and that it is only in the release of dsRNA from the vesicles that the Sid-1-like proteins enter the picture (scenario F).

However, in this scenario we would expect a much larger decrease in RNAi efficiency after silencing of both *sid-1-like* genes simultaneously, unless they are not essential, only aiding.

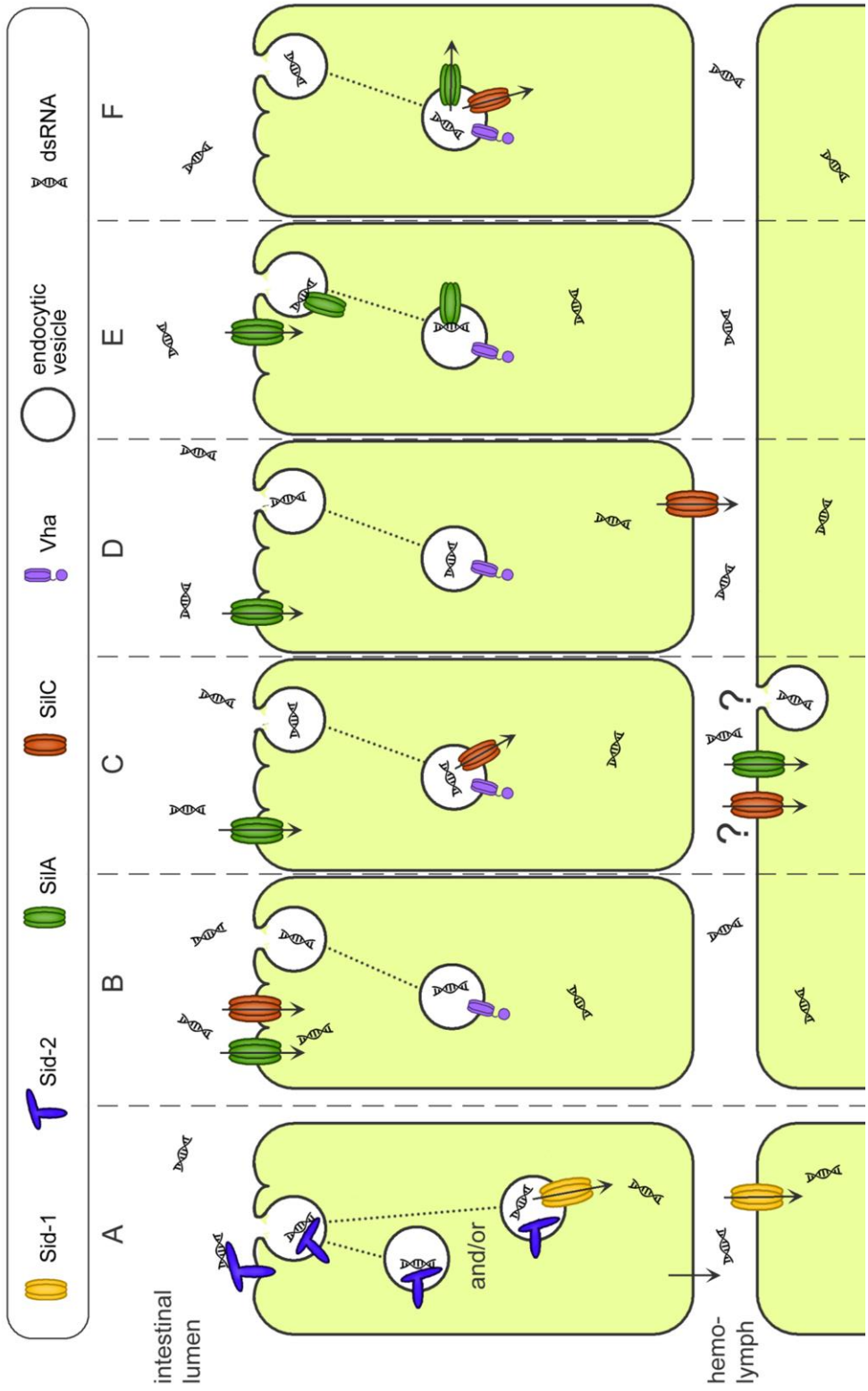

Figure 28. Schematic overview of the different ways dsRNA uptake mechanisms can contribute to dsRNA internalization in the epithelial cells of the Colorado potato beetle.
A: Proposed model of dsRNA uptake in *C.elegans* (adapted from McEwan et al. 2012). B-E: Proposed models of dsRNA uptake, through Sid-1-like (Sil) transmembrane proteins or receptor-mediated clathrin-dependent endocytosis, in the CPB midgut. A combination of multiple models is also possible. B: all uptake pathways act independently from each other. C: One Sil protein takes up dsRNA at the cell surface and the other aids in the release of dsRNA from endocytic vesicles. D: One Sil protein takes up dsRNA at the cell surface and the other is involved in dsRNA export from the cell into the hemolymph. E: One of the Sil proteins acts as the receptor for dsRNA, triggering endocytosis. F: The Sil proteins are not involved in dsRNA uptake at the cell surface, only in the release of dsRNA from the endocytic vesicles. How dsRNA uptake from the hemolymph into peripheral tissues occurs, is not known and depicted here by question marks.

7.1.2 *What do these results mean for RNAi in the CPB body?*

As the assay evaluated RNAi efficiency in the midgut, only uptake from the gut lumen - so environmental RNAi - was at play here. We have tried to also examine other instances of dsRNA uptake by injecting dsRNA against the target genes in the hemocoel and examining the RNAi efficiency in gut and brain. This setup could tell us if those same genes are involved in dsRNA uptake on cell surfaces that are not lining the midgut. Epithelial columnar cells have microvilli and specialized uptake systems at the cell surface facing the lumen and these features are not present in other cell types (Klowden 2007). However, when looking at the two dsRNA uptake systems identified so far, there is no reason to expect that they are not present in other cell types; endocytosis is a universal and indispensable cellular pathway and *sid-1-like* mRNAs have been detected in various tissues (Tian et al. 2009; Bansal and Michel 2013; Wynant et al. 2014b). However, it is certainly possible they function less efficiently in those cells which are not specialized in uptake of macromolecules. Additionally, it is possible that the unknown dsRNA receptor(s) is/are presented more on the epithelial surface of the midgut than on the cell membrane of other cell types.

7.1.3 *What do these results mean for other insect species?*

As discussed in Chapter III, our results, which show an involvement of both pathways, are not in agreement with most other publications on this topic in other insects. Complications arise from the fact that in most studies, only one of the pathways was tested, either because of the author's disinterest or because of technical limitations. Additionally, the experimental design might influence the results. For example, RNAi through feeding does not work (efficiently) in both *S. gregaria* and *T. castaneum*, therefore dsRNA uptake was evaluated only through injection. As mentioned before, dsRNA uptake in hemocoel-surrounding tissues may happen differently than in the midgut epithelium.

To obtain a better understanding of the role of both pathways in dsRNA uptake across all insect orders, further studies are needed in a wide range of insects. Preferably, a cross-species study would be needed, targeting identical gene regions and using the same reporter gene, both through feeding and injection. This would tell us whether the involvement of the uptake pathways can be explained from an evolutionary point of view. In this case, dsRNA uptake would occur similarly in closely related insects, and the reason that this similarity has not been reflected by similar RNAi efficiencies could be because it was obscured by other RNAi-limiting factors. Some evidence for this evolutionary conservation can be found in the distribution of the number of Sid-

1-like proteins over the different insect orders, which makes sense from an evolutionary point of view, as shown in Figure 14.

7.1.4 *Future perspectives on RNAi in insects – fundamental knowledge*

On the subject of RNAi in insects, there is a substantial lack of fundamental knowledge. It is becoming clear that the RNAi pathways are more complex and more interacting than is depicted in most overviews. When applied to the case of CPB, the aforementioned screening of genes essential for the siRNA process included many core genes from the miRNA and piRNA pathways (Yoon et al. 2016). It is possible these pathways are truly interacting, but it could also be that their regulation is overlapping. Additionally, it is still unclear whether duplication of RNAi genes, like for *dcr-2* and *ago-2* in CPB (Chapter II), contributes to a stronger RNAi response.

One of the most alluring remaining questions is the manner in which the RNAi signal is transported in the insect body. From the nematode *C. elegans* it is known that the SID proteins play distinct roles in uptake and transport of the RNAi signal (Feinberg and Hunter 2003; Jose et al. 2009; Hinas et al. 2012; Jose et al. 2012; McEwan et al. 2012), which is thought to be a dsRNA(-derived) molecule (Ivashuta et al. 2015). As discussed in Chapter III, these conclusions cannot simply be transferred to the class of the insects as there are no clear homologs of the different SID proteins. Instead there are multiple Sid-1-like proteins, or none, and another process, receptor-mediated endocytosis, also seems to play an important role. From a fundamental point of view, it is important to identify how exactly this signal is spreading in the insect body. It is likely that after the transport mechanisms are identified, impediments will be found, explaining some of the inconsistencies observed in RNAi experiments.

Also, the identification of the dsRNA receptor would open up possibilities. As stated before, it could explain some of the variability in RNAi efficiency between insect species and tissues. For example, in locusts it was shown that there was no dsRNA uptake in the oocytes and follicle cells in the ovaries and thus no RNAi response (Ren et al. 2014). A similar unresponsiveness of the ovaries to dsRNA was also seen in Chapter V. Whether this impaired uptake is caused by a lack of dsRNA receptors or uptake mechanisms, remains unknown. If identified, expressing the receptor(s) in a cell line would reveal whether they contribute to an efficient RNAi response. This could lead to innovative ways to enhance the RNAi response or even direct dsRNA to specific tissues.

7.1.5 *Future perspectives on RNAi in insects – applications*

Fortunately, the considerable lack of fundamental knowledge on the specifics of the RNAi process has not impeded the development of RNAi-based crop protection applications. Recently the first RNAi-based transgenic crop - corn in which a Cry toxin, glyphosate resistance and RNAi against the western corn rootworm are combined - has been approved by the Canadian Food Inspection Agency (Canadian Food Inspection Agency 2016). Also against other Coleoptera such as the Colorado potato beetle, substantial R&D efforts have been made, both through transgenic crops and sprayable products.

However, filling out the gaps in our fundamental knowledge could help considerably in the development of RNAi-based crop protection in non-beetle species. As stated before, the mechanisms by which the dsRNA is taken up by the cells and spread throughout the body, might contribute to RNAi ineffectiveness. Identifying limiting factors might open up possibilities for an enhanced RNAi response. Delivery molecules could be designed which specifically bind to the dsRNA receptor, or aid in the binding of dsRNA to this receptor. Or, compounds could be administered which trigger or enhance the systemic capabilities. Additionally, this knowledge could tell us more about the possibility of resistance development against RNAi-based crop protection. It is thought that resistance would develop more slowly because long dsRNA encompasses many different siRNAs and multiple target genes can be combined, so that multiple mutations would be required. However, direct alterations of the core RNAi pathway or the dsRNA uptake or systemic properties could lead to an insensitivity of the insect to dsRNA. In the RNAi-based crops, currently being developed, the chance of this type of resistance occurring is diminished by combining the RNAi trait with Cry toxins so that resistance against two very different mechanisms would need to occur for the insect to survive.

7.2 Viral infections influencing RNAi efficiency

Viral infections can have an enormous impact on the RNAi system. A complex network of interactions is at play from the moment the virus infects the individual, both at a cellular and a whole body level. The results discussed in Chapters IV, V and VI shed some light on these convoluted interactions.

7.2.1 *RNAi efficiency in the bumblebee*

In general, *B. terrestris* does not have an efficient RNAi response. High doses of dsRNA are needed, and the results are often disappointing. Where for CPB, the silencing efficiency on mRNA level was consistently over 80% (except for *laccase2*), with 400 ng of dsRNA added, for bumblebees, 50% was the maximum silencing reached after administering 20 µg of dsRNA and this only after experimental design optimization. Of course, the difference in body weight could play a role but when adjusting for this factor the dose amounts to approximately 66 ng dsRNA / mg wet body weight for the bumblebee and 40 ng dsRNA / mg wet body weight for CPB. This minor difference is not likely to be the cause of this huge difference in RNAi efficiency.

Through the work of colleagues, we found no degradation of dsRNA as a result of enzymatic breakdown or instability at non-neutral pH levels in the midgut juice (Vanlede 2014). Additionally, the behavior of dsRNA in the hemolymph was examined by incubating dsRNA-containing hemolymph with proteinase K. This resulted in the expected dsRNA band being visible on an agarose gel, whereas in the control (without proteinase K) it was not. This suggests binding of the dsRNA to a protein present in the hemolymph, impeding its movement through the gel (Snoeck 2015). These proteins have not been identified, but could be similar to the lipophorins in locusts, where they were found to bind to dsRNA in the hemolymph (Wynant et al. 2014a). Whether this binding is responsible for an improved or diminished RNAi response is unclear, but the fact that the RNAi response after injection is very strong in locusts (Wynant et al. 2012), suggests it is not a major impediment. It could even be that these lipophorins are essential for the systemic properties of RNAi.

In literature, various instances in which RNAi has been used to examine gene function in bees, can be found. In bumblebees, in several publications by the same research group 20 µg of the target gene was injected, which is similar to the dose used in this thesis (Kim et al. 2009; Hu et al. 2010a; Hu et al. 2010b; You et al. 2010; Kim et al. 2011), but one publication managed to achieve an RNAi response using only 1 µg (Deshwal and Mallon 2014). In honeybee research, RNAi is used to examine, among others, caste differentiation, foraging behavior and growth and development.

The injected doses in adults and larvae range between 5 µg and 30 µg (Amdam et al. 2003; Wang et al. 2010; Chan et al. 2011; Wang et al. 2013; Li et al. 2016), but in embryo's and specific tissues, such as brain lobes, much lower doses can be used (Beye et al. 2002; Farooqui et al. 2003; Mussig et al. 2010; Mustard et al. 2010). In feeding assays, the concentrations used range between 100 µg/ml and 500 µg/ml (Patel et al. 2007; Nunes and Simoes 2009; Mutti et al. 2011a; Mutti et al. 2011b).

Within bumblebee RNAi experiments, a considerable variability was observed, depending on the experimental design. The most important factors were found to be the target gene, sampling time point and examined tissue. In general, the largest drop in mRNA levels was obtained 48 hours after dsRNA administration. It also proved to be important to look at separate tissues instead of the whole body. As the bumblebee is a fairly large insect, a uniform RNA extraction over different samples is difficult to achieve. Moreover, the results may be distorted by a low RNAi response in unresponsive tissues such as the ovaries or a delayed response in peripheral tissues such as the brain. The highest RNAi efficiency was observed in the fat body, perhaps because of its direct contact with the hemolymph in which the dsRNA was injected. The ovaries seemed to be insensitive to RNAi, possibly because of barriers that the ovaries form to block out dsRNA (Ren et al. 2014). Similar to the way in which the dsRNA uptake mechanisms were evaluated in the CPB midgut in Chapter III, it would be interesting to look at the way in which dsRNA enters the ovaries and how efficiently this process occurs compared to other tissues. Unfortunately, this would require a similar optimization of the setup as was described for the evaluation of dsRNA spreading mechanisms throughout the body, and this proved to be difficult.

7.2.2 *RNAi enhancement as a result of viral infection*

In this work, we set forward to identify a suppressor of RNAi in an important bee pathogen, IAPV. The suspects we had identified, 1A (a known suppressor in some closely related viruses) and orfX (an in silico predicted gene with no confirmed translation nor functionality), were not found using HDMS^{E}. However, the most compelling argument for the lack of suppressor functionality in IAPV came from the RNAi assay. Where a reduced RNAi efficiency was expected, a significant enhancement of the silencing effect was observed. The cause(s) of this enhancement, however, are still unknown.

The most obvious explanation is the activation and upregulation of the RNAi pathway after recognition of IAPV or IAPV-derived proteins or siRNAs. Indeed, a small upregulation of the RNAi core genes was detected, but this upregulation is rather small compared to the upregulation

caused by another virus, CrPV, which presence did not cause an RNAi enhancement. However, in this case, it is possible that an enhancement is occurring, but counteracted by a viral suppressor of RNAi. If this is true, maybe the two-fold upregulation of, for example, *dcr-2* after IAPV infection is enough to cause an increased RNAi efficiency. Here it is also important to realize that, although increased mRNA levels generally lead to heightened proteins levels, the degree of increase may be different and it may be delayed. A second, albeit hypothetical explanation, could be that the virus alters the way in which the RNAi-related proteins work, making them more efficient. Another enticing possibility is that the IAPV infection puts the insect body into an 'virus aware' state with enhanced dsRNA transport capabilities, an improved systemic status, etc. As many of the biological markers to study these factors have not been elucidated yet fully (see 7.1.4), we were unable to uncover the exact cause of the improved RNAi efficiency.

7.2.3 *VSR activity*

Although various VSRs have been identified in plant and insect viruses, little is known about their consequences outside of the lab. Many hypotheses have been expressed concerning the concept of optimal virulence, with virulence defined as the capacity of the virus to damage the host. On the one hand, it is beneficial for the virus to limit its virulence to secure transmission. On the other hand, a certain degree of virulence is needed to ensure that the virus is not outcompeted by other pathogens and that sufficiently high titers are reached to be able to infect other individuals. Some viruses are characterized by a high virulence and fast transmission between individuals, while others have evolved to a perfect pathogen-host co-existence. In this case, the virus is present latently (at low numbers) and chronically (no viral clearance) without damaging the host, hiding throughout the body or in certain tissues. In situations where the viral titers are low, it is easier for the RNAi system to combat the virus (and maybe even clear it from the body). Therefore, it would be beneficial for latent/chronic viruses to diminish the RNAi efficiency, possibly through the use of a VSR.

In the aforementioned cases, only the presence of one viral species was considered. However, when expanding this reasoning to the situation where multiple viral species are present in the same host, as is often the case in bees (Chen et al. 2004; Wu et al. 2015), other factors need to be considered. Here, the selective advantage of the VSR for a virus to evolve to a latent or chronic infection may be lost. The VSR of slow replicating virus A, attenuating RNAi to ensure virus A is not eradicated, can result in massive proliferation of a co-infecting virus B as the bee is immune-compromised. As observed in Chapter V for the combination IAPV/CrPV, there is a strong effect of competition for resources during viral co-infections, so the presence of a VSR could be

unfavorable to the encoding virus. Moreover, one could hypothesize that a fast-replicating virus such as IAPV, which by itself is potent enough to overcome the RNAi defense mechanism, would not produce a functional suppressor to ensure its own competitiveness towards other bee viruses.

VSR activity can also have interesting repercussions for virus adaptation to new hosts. As a confirmation of the usability of the functional RNAi assay in IAPV, CrPV was included in the testing. A minor reduction in RNAi efficiency was observed, but because the control did not have a convincing RNAi effect (because of the aforementioned variability in bumblebees), it cannot be concluded with 100% certainty the 1A suppressor of CrPV is functional in bumblebees. Broad host range functionality of the 1A suppressor would not be surprising as, cross kingdom functionality has already been proven for some of the plant and insect VSRs (Li et al. 2002; Lakatos et al. 2004; Guo and Lu 2013). New host adaptation is commonly characterized by a lower infectivity than in the original host, as a result of unfamiliar barriers, giving the host time to combat the virus (Parrish et al. 2008). However, once the infection is established in a new host, the virulence is generally higher than in the original host, because of a lack of co-evolution. If the immune system would be impaired, it could give the virus enough time to break through the barriers, increasing the chances of a successful infection. It is possible that a broad-spectrum VSR grants a virus the possibility of infecting a broad range of hosts, but this remains mere speculation.

7.2.4 *Other viral products affecting RNAi efficiency*

Besides proteins, viruses can also encode miRNAs, which can influence their own or the host's gene expression. Many of such miRNAs have been found in human DNA viruses (Kincaid and Sullivan 2012), but in insects only a few of these virus-encoded miRNAs have been reported and those were all dsDNA viruses (Hussain et al. 2008; Singh et al. 2010; Wu et al. 2011; Zhu et al. 2013). As transcription of DNA viruses occurs within the nucleus, miRNAs can be processed by the cellular miRNA machinery, which resides there. The functions of these insect virus-encoded miRNAs are the autoregulation of late stage viral replication, to avoid too strong effects on host survival, and the switch to latent infection (Wu et al. 2011; Zhu et al. 2013). The one known instance where the host is affected is the *Bombyx mori nuclear polyhedrosis virus*, which impedes host miRNA export from the nucleus (Singh et al. 2012). Although there are some hypotheses why RNA viruses would not encode miRNAs (destruction of their own genome, no miRNA processing in the cytoplasm), there is evidence that these limitations could be bypassed, suggesting that RNA virus-encoded miRNAs probably will be found eventually (Kincaid and Sullivan 2012; Asgari 2015). Aside from miRNAs, other ncRNAs, able to influence viral replication or the host machinery, could

arise from the viral RNA genome. For instance, the sfRNAs that originate during Dengue virus infection and inhibit Dicer-2 functionality (Hussain et al. 2010), are sometimes classified as miRNA-like.

This raised the question whether similar ncRNAs, either miRNAs or longer subgenomic RNAs, could be encoded by IAPV. Therefore, a small RNA dataset of IAPV-infected bumblebees was examined for virus-derived miRNAs, but no potential candidates were found. However, a set of host miRNA upregulated after IAPV infection was identified (Niu 2015). Further studies will elucidate to what extent these miRNAs affect viral replication, host immune defense or other host pathways.

7.2.5 *How host factors can influence RNAi efficiency*

There are various ways in which the host can alter RNAi efficiency during viral infection, both directly and indirectly. The first one is the triggering and upregulation of the RNAi machinery that is typically seen after virus infection (Lan et al. 2016; Niu et al. 2016b). But the RNAi efficiency can also be diminished: 1) when large amount of viral siRNAs saturate the siRNA pathway, 2) when viral RNA and proteins saturate the cellular pathways and therefore interfere with the production of RNAi-related proteins or 3) when viral proteins manipulate host metabolic or immunogenic pathways to their advantage, which might interfere with the RNAi response. Finally, when infection has evolved far enough to significantly compromise the health status of the host, the RNAi machinery will be shut down, together with most other cellular pathways because of a lack of resources.

It is known that dicistroviruses can have a massive impact on the cellular pathways of the host, especially the translation machinery. For CrPV it has been shown that the translation of host proteins is inhibited by over 80% 4 hours post infection in the *Drosophila* S2 cell line. This corresponded to a massive increase of viral proteins. This shift from host product translation to viral product translation is attributed to a dissociation and inactivation of various eukaryotic translation initiation factors (eIFs), which are not needed for translation from the viral 5' UTR or intergenic IRES, thus limiting only host translation (Garrey et al. 2010). In this scenario, probably very little RNAi core proteins would be produced. It is important to note that this is an artificial and very robust infection in a cell line, and that it may not be completely representative of the in vivo situation where 1) the initial infection dose would probably be significantly lower, 2) cells which are so massively overrun by the virus would enter the apoptosis process, protecting the

neighboring cells/tissues. In the meantime, these other cells/tissues could have been triggered by the systemic RNAi signal and primed for an upcoming infection.

Examining a proteomic dataset of IAPV-infected ovaries using GO analysis, we observed a clear disruption of essential metabolic and proteostatic processes in the ovaries, while there was no complete disruption of the essential processes that would typically be seen in diseased and dying insects. One major implication is a lack of energy production, in the form of ATP, in this tissue. *Drosophila* Dicer-2 has been shown to function in an ATP-dependent manner, a feature which is thought to be conserved in insects (Liu et al. 2003; Welker et al. 2011; Ghosh et al. 2014). Therefore, a lack of ATP generation could result in a diminished dicing capacity, greatly affecting the RNAi efficiency.

7.2.6 *Viral infections in social insects*

As described above, viral infections could explain the RNAi variability between individuals. These interactions apply to insects in general, but it is interesting to look at the way (bumble)bee behavior affects the possibility of these interactions occurring. Are there reasons to believe that (bumble)bees carry more viruses than other insects and does this result in different immunity characteristics? For starters, the eusociality of bumblebees and honeybees, characterized by high population densities and low genetic variability, promotes pathogen transmission. Moreover, the unique way in which these pollinators collect resources might contribute to virus spreading. As every flower is visited by many different bees, and every bee visits multiple flowers there is a considerable opportunity for virus transmission between different bee species and even genera. It has been speculated this is the reason why many of the bee viruses have a considerable host range spanning multiple genera. Additionally, it was shown that bees (including solitary bees) have fewer immune genes than other insects, and that this reduction was not the result of sociality (Barribeau et al. 2015). This could make them more susceptible to pathogens, but from an evolutionary point-of-view it would be logical that there are some factors counteracting this. In agreement with the last conclusion, if viruses are more common in social species, one would expect a particularly strong RNAi response in these insects, which does not seem to be the case.

An interesting concept to be considered here is what constitutes an immunological entity; the individual bee or the colony? Analogies have been drawn between individual and social immunity as pathogens have to cross similar type of barriers. The defense mechanisms of both the individual and the colony start with border defenses to prevent pathogen intake, followed by body defenses that prevent the establishment and spread of the pathogen between the body's

cells or the social insect workers. Lastly, germline defenses are employed to inhibit infection of the reproductive tissue or the reproductive individuals in colonies (Cremer and Sixt 2009). Moreover, social immunization has been observed in a few cases, in which contact with pathogen-exposed individuals promotes reduced susceptibility in their nest mates to the same pathogen (reviewed in Masri and Cremer 2014). Whether it would be possible for bee individuals to signal their infected state and trigger enhanced RNAi capabilities in their nest mates, remains wild speculation.

As a consequence of the ambiguity regarding the immunological entity, adaptions that do not seem to make sense from an evolutionary perspective on the individual level, may have been favorited because they lead to an advantage for the colony. In this work, a downregulation of various metabolic and proteostatic processes was observed in the ovaries of bumblebee workers. As this tissue is potentially redundant in the context of colony success, it is possible it is 'switched off' to optimize resource mobilization to combat the virus and limit the spread of virus within the colony.

7.2.7 *Future perspectives on viral infections in bees – fundamental knowledge*

One of the major limitations in virus research in bees is the lack of a continuous cell line which can be infected by the bee viruses. For a while, a honeybee cell line was available in which bee viruses could proliferate (Goblirsch et al. 2013; Carrillo-Tripp et al. 2016), but it proved very difficult to maintain and has now been lost (own experience and personal communication with prof. B. Bonning and prof. Kurtti). Ideally, the cell line would originate from bee tissues, but sometimes viruses can also infect cell lines derived from non-natural hosts. As many of the intracellular pathways, needed for viral replication, are universal, we speculate that virus particle uptake into the cell could be the major limiting factor here. As a consequence, the use of more intrusive virus application methods, such as transfection reagents, electroporation, etc. could enhance the intracellular uptake of bee viruses in non-susceptible cell lines.

Using cell lines can sometimes be preferable to in vivo experiments as the scale reduction allows for large screenings and many of the confounding factors (hormonal regulation, signaling, accessibility, …) are absent. Although they do not always reflect the natural condition, and not every research question can be tackled using them, they are useful in a number of situations. For starters, virus production using a cell line is more practical than in vivo, like the bumblebee pupae used in this study. If the cell line can be kept virus free, the chance of viral contaminants is much

smaller. Additionally, the clean-up of the viral stock requires less effort. Second, cell lines are ideal environments to tackle some fundamental questions in bee virology.

For example, many of the known VSRs and their mode of action have been identified in cell lines as it is relatively straightforward to express the VSR in a virus-free cell line and evaluate its effect on the RNAi machinery (van Rij et al. 2006; Singh et al. 2009; Nayak et al. 2010; Schnettler et al. 2012). During this doctoral dissertation we have attempted to express the orfX of KBV, a bee virus closely related to IAPV, in both the S2 (Schneider 1972) and Hi5 (Granados et al. 1994) cell line. This orfX was predicted computationally, based on a surprisingly lack of stop codons in an out-of-frame segment (Firth et al. 2009; Sabath et al. 2009). Unfortunately, no corresponding protein was detected. This indicates that this protein cannot be translated and/or folded correctly (and thus does not exist) or that specific host factors are needed for proper translation and/or folding, in which case a bee-derived cell line would be necessary.

One of the most intriguing remaining questions is the recognition of the viral pathogen, the dicistrovirus, by the bee host. Here two processes need to be looked at: 1) recognition of viral coat proteins (or regions thereof) by the extracellular domain of a receptor, triggering uptake of the viral particle and 2) the recognition of the viral pathogen-associated molecular patterns (PAMPs) by the immune system. It also possible that these two processes are combined into one event. Random mutagenesis of a dicistrovirus and evaluating the infection potential in the cell line would reveal which part of the virus is recognized by the receptor. Doing this for multiple dicistroviruses would give an indication on whether they could bind on the same host receptor or not. A similar setup could be used to determine which part of the virus is recognized by the immune system, but it could also be that the intracellular recognition of dsRNA intermediaries by Dicer-2 is the only PAMP recognition event and that this causes other antiviral immune pathways to be triggered (Paradkar et al. 2012).

7.2.8 *Future perspectives on viral infections in bees – ecological aspects and applications*

At the moment, dsRNA-dependent products are being developed to help honeybees combat typical bee viruses (Maori et al. 2009; Monsanto 2016). These are based on a continuous administration of dsRNA, targeting the viral mRNA, to the hive. Once a bee is infected, there is little chance of clearing the infection from the body, so it is more a preventive than a curative measure. But there are other principles that antiviral therapeutics in bees could be based on. First, if the virus encodes a VSR, compounds could be administered that block VSR functionality, for example by blocking the catalytic/binding site, without impeding the RNAi pathway. Other

options are based on the fact that the genes involved in immune pathways are not expressed constitutively, but are activated once there is a pathogenic threat (Kemp et al. 2013; Johnston et al. 2014). So in moments when there is a genuine threat to the hives (disease symptoms in the hive, outbreaks in the vicinity, …), the immune pathways could be triggered artificially. This would require a minimal extra investment of resources by the bee, but the health benefits could be major. On the one hand, compounds which trigger the aforementioned 'virus-aware' state, with enhanced systemic RNAi properties, could be administered. This could give the bee an advantage because the time between the first recognition of the virus and the spread of the systemic RNAi signal to peripheral tissues would be reduced. This way, the RNAi defense system would be up and running at the time of virus entry in those distant tissues. On the other hand, the way in which the bee recognizes the virus could be used to develop a therapeutic. If the PAMP is a region of a coat protein, it could be produced in vitro and given to the bees, so that the immune pathways are triggered, even without the virus being present yet. However, it seems like, at least for the RNAi pathway, the trigger is the dsRNA by itself (Garbutt and Reynolds 2012; Lozano et al. 2012; de Faria et al. 2013) and then we need to look at dsRNA-based applications again. A final option is the use of the social immunization trigger as a therapeutic, to place the whole colony into a 'virus-aware' state. However, before all these possible applications could ever be developed, much more fundamental knowledge is needed; both about viral recognition in bees and systemic RNAi spreading in insects, so for now, they remain purely hypothetical.

From the above sections it is clear that the interactions between virus and host, and in particular the RNAi mechanisms of the latter, are complicated. But, they become even more complex when adding various ecological aspects. It is believed that the bee decline that has been observed since the 1950's is caused by a combination of factors such as a change in land use and agricultural practices, resulting in a lack of resources, which in turn makes the bees more vulnerable to other stressors such as pathogen attacks, pesticides, etc. (Goulson et al. 2015) In the last years, there have been indications that pesticide exposure results in an increased susceptibility of bees to virus attacks (Di Prisco et al. 2013). Chemical components in pesticides may directly affect bee health, weakening its defense mechanisms against pathogen attacks. But there could also be indirect, synergistic effects. One possibility that should be assessed here is the way in which chemical components in pesticides might affect RNAi efficiency.

As viruses are an inherent part of all ecosystems and generally are not a major concern in many other insect species in nature, the question arises whether developing therapeutics for virus control is the most useful and effective way to protect the health of domesticated bees. Would it not be better to limit the various stressors which make the bees more vulnerable to pathogen attacks, for example by ensuring adequate floral resources, limiting the use of noxious pesticides, limiting transport etc.? As is often the case, it seems like combination of various strategies might be the way to go. Also for wild bees, it is important to look at viral research in a way that takes into account the wide range of interactions between host, virus and environment when developing new practices aimed at improving wild bee health.

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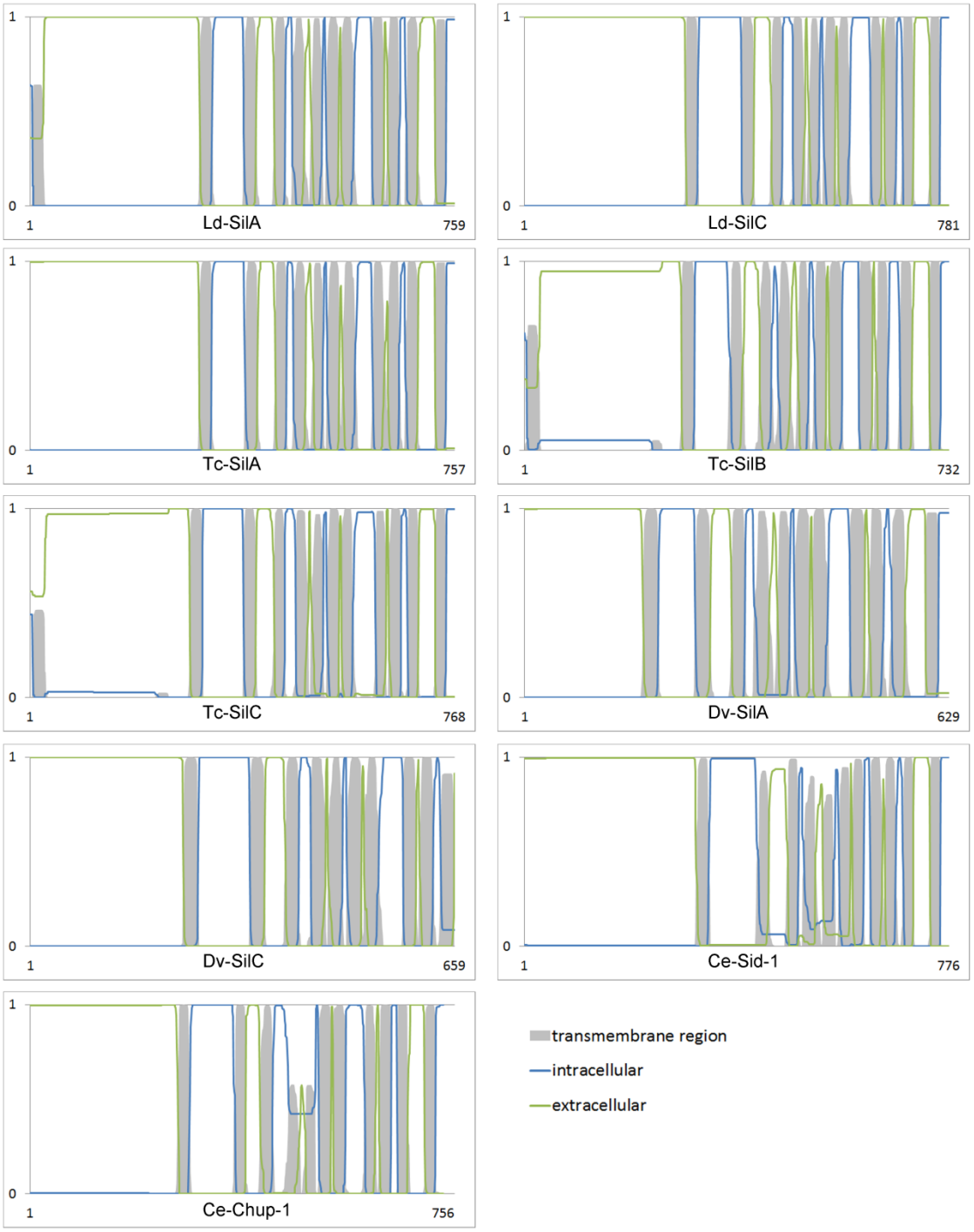
SUPPLEMENTARY DATA

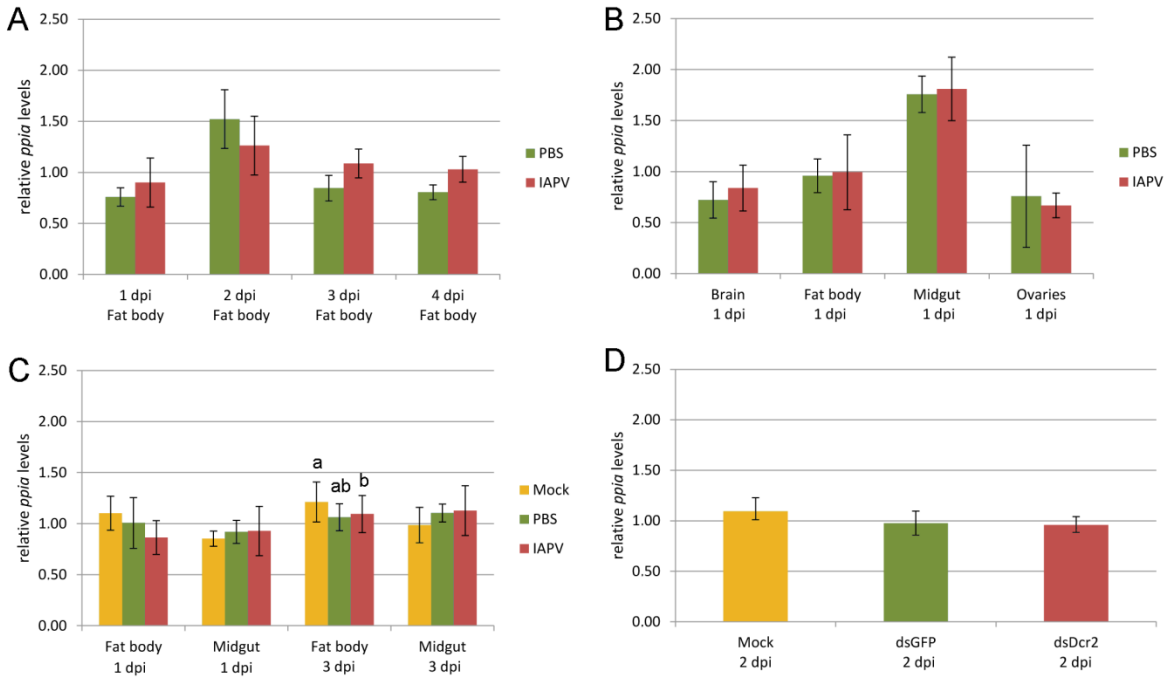
Supplementary Table 1. List of the accession numbers used as queries during RNAi core gene annotation.

	D. melanogaster	*A. mellifera*	*B. terrestris*	*T. castaneum*	*L. decemlineata*
dicer-1	NP_524453.1	NP_001116485.2	XP_003401955.2	XP_008199045	/
dicer-2	NP_523778.2	XP_016773223.1	XP_012163127.1	EEZ99277	AKQ00041.1; AKQ00042.1
drosha	NP_477436.1	XP_016766928.1	XP_003394274.1	KYB24989	/
argonaute-1	NP_725341.1	XP_006571833.1	XP_012170889.1	EFA09197.2	/
argonaute-2	NP_648775.1	XP_395048.4	XP_012168271.1	EFA11590.1; EFA04626	AKQ00044.1; AKQ00045.1
argonaute-3	NP_001036627.2	XP_016771437.1	XP_012170834.1	EFA02921.1	/
aubergine	NP_476734.1	NP_001159378.1	XP_012171701.1	EFA07425.1	/
piwi	NP_476875.1				
loquacious	NP_723813.1	/	XP_003398995.1	EFA09556.1	/
r2D2	NP_609152.1	XP_006560091.1	XP_003395928.1	EFA05903.1	/
pasha	NP_651879.1	XP_006559675.1	XP_003397039.1	EFA05197.1	/
sid-1-like	N/A	XP_006565236.1	XP_012170554.1	ABU63672.1; ABU63673.1; ABU63674.1	ALG36906.1; ALG36907.1

N/A: this gene is not present/found in this species. /: the gene was found, but no genbank identifier is available as of yet. *Drosophila (D.) melanogaster, Apis (A.) mellifera, Bombus (B.) terrestris, Tribolium (T.) castaneum and Leptinotarsa (L.) decemlineata.*



Supplementary Figure 1. Hydrophobicity plots for the coleopteran Sid-1-like proteins, as well as Sid-1 and Chup-1 of *C. elegans*.
The plots were calculated using the TMHMM transmembrane prediction tool (Krogh et al. 2001). The y-axis represents the chance that an amino acid belongs to a certain category: transmembrane region (grey), the extracellular part of the protein (green) or the intracellular part of the protein (blue). All proteins show a similar distribution of 11 transmembrane regions over the whole sequence. The grey sections at half height in the N-terminus of Ld-SilA, Tc-SilB and Tc-SilC are probably signal peptides, which are often hydrophobic in nature.



Supplementary Figure 2. Supplementary experiments illustrating the fact that *ppia* levels remain stable after virus infection or dsRNA treatment.

All bumblebees were injected with 500 particles of IAPV (in 5 µL), 5 µL of PBS, 20 µg of dsRNA or not injected (i.e. mock treatment). The effect of the treatment on the expression of the reporter gene *ppia* was evaluated using RT-qPCR, normalized to *rpl23* levels. Statistical analysis was performed using Student's t-test (panel A and B) or Analysis of Variance (Tukey's HSD post-hoc comparisons; panel C and D) on $\log_2$ transformed data. The columns represent the treatment mean ± SD and statistical differences on an $\alpha = 0.05$ level are denoted by different letters. All other appropriate comparisons are not statistically different. The stability of *ppia* during the course of an IAPV infection is shown in panel A, whereas panel B proves stability over the different tissues. Panel C shows the reproducibility of these results, with a mock infection as extra control and panel D depicts the stability of this gene after non-targeting dsRNA treatment. In one instance there is a minor downregulation of *ppia* 3 days after IAPV infection, but only compared to the mock treatment, so this is probably caused stress after injecting, supplemented with biological variation.

Supplementary Table 2: Overview of the differentially expressed proteins in IAPV-infected ovaries.

B. terrestris genbank identifier	*Bombus* protein name	*A. mellifera* Uniprot identifier	Peptides	Unique peptides	Average fold change	Gene Ontology terms
Upregulated						
gi|350423051|ref|XP_003493369.1|	PREDICTED: collagen alpha-1(I) chain-like [Bi]	A0A087ZZX4	5	3	1,55	GO:0030246
gi|815915550|ref|XP_012242986.1|	PREDICTED: protein transport protein Sec31A [Bi]	A0A088APX8	9	2	6,27	GO:0008021;GO:0005795;GO:0005681;GO:0008380;GO:0090114;GO:0005198;GO:0030127;GO:0045089;GO:0050829;GO:0070971
gi|808134731|ref|XP_012169810.1|	PREDICTED: AP-3 complex subunit beta-2 isoform X1 [Bt]	A0A088A8Q2	5	2	2,25	GO:0030117; GO:0016192; GO:0016020; GO:0030123; GO:0006886; GO:0015031; GO:0006810
gi|350422731|ref|XP_003493265.1|	PREDICTED: cuticle protein 16.5, isoform B-like isoform X1 [Bi]	/	3	3	6,98	/
gi|808117774|ref|XP_012176241.1|	PREDICTED: protein vav isoform X1 [Bt]	A0A088A5G2	3	2	4,76	GO:0046872; GO:0035556; GO:0005089; GO:0035023; GO:0043547; GO:0005622
gi|808135807|ref|XP_012170286.1|	PREDICTED: mitogen-activated protein kinase-binding protein 1 isoform X5 [Bt]	A0A088ARN1	7	4	1,51	GO:0005515;GO:0005622;GO:0007256
gi|808131900|ref|XP_012168577.1|	PREDICTED: putative RNA-binding protein Luc7-like 1 isoform X2 [Bt]	A0A088A1W6	2	2	2,69	GO:0006376; GO:0003729; GO:0005685
gi|340720185|ref|XP_003398522.1|	PREDICTED: 2-oxoglutarate dehydrogenase, mitochondrial isoform X4 [Bt]	A0A088AHH8	10	2	2,78	GO:0016624; GO:0055114; GO:0030976; GO:0008152; GO:0006099; GO:0004591
gi|808145082|ref|XP_012173786.1|	PREDICTED: cytochrome c oxidase subunit 5B, mitochondrial-like [Bt]	A0A088A8C7	13	7	1,36	GO:1902600; GO:0005740; GO:0004129
gi|350420790|ref|XP_003492626.1|	PREDICTED: protein canopy 4 [Bi]	A0A088AAP3	5	4	1,26	/
gi|815920548|ref|XP_012245206.1|	PREDICTED: uncharacterized protein LOC100742071 isoform X2 [Bi]	A0A087ZZ36	6	2	2,93	/
Downregulated						
gi|340708965|ref|XP_003393087.1|	PREDICTED: nuclear RNA export factor 1-like [Bt]	A0A088A7U4	5	3	0,55	GO:0051028;GO:0000166;GO:0003723;GO:0005737;GO:0006406;GO:0006810;GO:0005634;GO:0005622
gi|340709020|ref|XP_003393114.1|	PREDICTED: histone H1A, sperm-like [Bt]	A0A088A825	19	5	0,59	GO:0003677;GO:0005694;GO:0006334;GO:0000786;GO:0005634
gi|340710106|ref|XP_003393637.1|	PREDICTED: 60S ribosomal protein L11 [Bt]	A0A088ABB1	7	3	0,76	GO:0003735;GO:0005549;GO:0030529;GO:0007608;GO:0016020;GO:0050911;GO:0005840;GO:0006412;GO:0004984; GO:0005622
gi|340715501|ref|XP_003396250.1|	PREDICTED: U2 small nuclear ribonucleoprotein auxiliary factor 35 kDa subunit-related protein 1 [Bt]	A0A088A3L1	5	3	0,64	GO:0000166;GO:0003723;GO:0046872;GO:0005634;GO:0003676
gi|340718889|ref|XP_003397895.1|	PREDICTED: probable ATP-dependent RNA helicase DDX43 [Bt]	A0A087ZUZ1	12	4	0,67	GO:0008152;GO:0000166;GO:0003723;GO:0004386;GO:0016787;GO:0005524;GO:0003676
gi|340719892|ref|XP_003398378.1|	PREDICTED: 60S acidic ribosomal protein P0 [Bt]	A0A087ZPA5	24	18	0,70	GO:0030529;GO:0042254;GO:0005840;GO:0005622

Table continues on the next page. [Bt]: *Bombus terrestris*, [Bi]: *Bombus impatiens*.

Supplementary Table 2: Overview of the differentially expressed proteins after IAPV infection in the ovaries (continued)

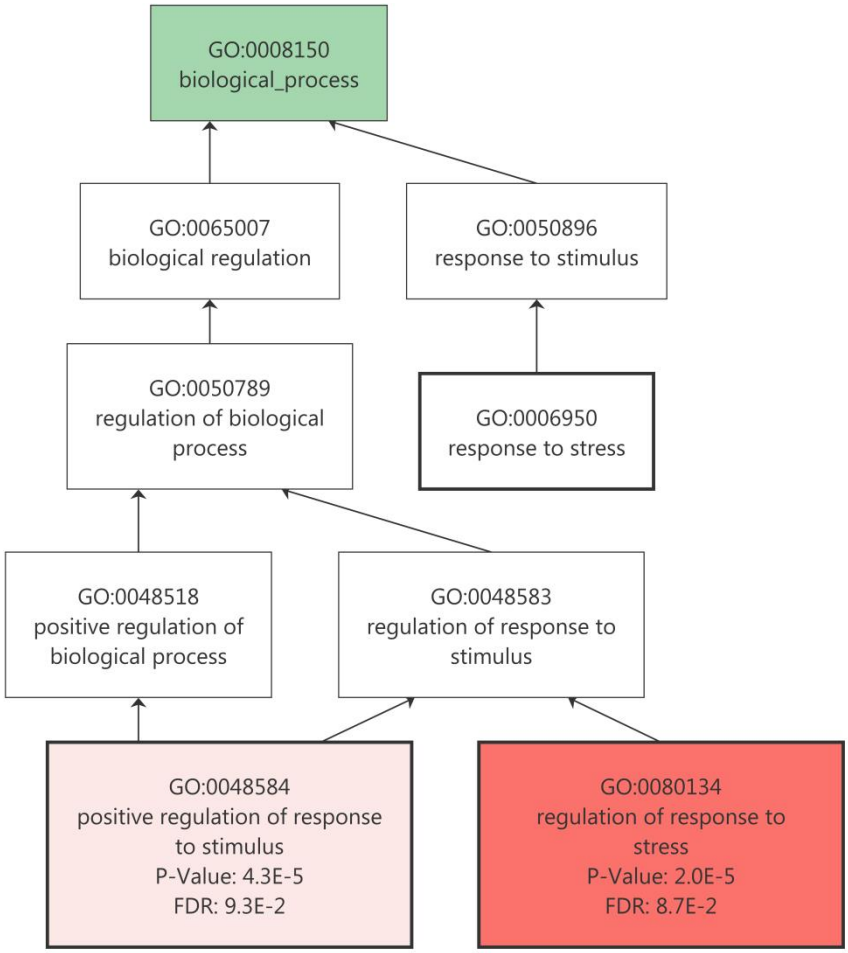
gi|340721477|ref|XP_003399146.1|	PREDICTED: protein kinase C and casein kinase substrate in neurons protein 2 isoform X3 [Bt]	A0A088AC20	13	5	0,56	/
gi|340722499|ref|XP_003399642.1|	PREDICTED: peroxisomal biogenesis factor 19 [Bt]	A0E087ZNK5	6	3	0,75	GO:0005777
gi|340724486|ref|XP_003400613.1|	PREDICTED: tyrosine--tRNA ligase, cytoplasmic-like [Bt]	A0A088AMC1	19	2	0,54	GO:0006418;GO:0000166;GO:0003723;GO:0005737;GO:0004812;GO:0006437;GO:0016874;GO:0000049;GO:0006412;GO:0004831;GO:0005524
gi|340724650|ref|XP_003400694.1|	PREDICTED: 60S ribosomal protein L3 [Bt]	A0A087ZXE4	20	4	0,70	GO:0003735;GO:0030529;GO:0005840;GO:0006412;GO:0005622
gi|340725754|ref|XP_003401231.1|	PREDICTED: dihydropteridine reductase [Bt]	A0A088AGA6	9	8	0,63	GO:0008152;GO:0016491;GO:0055114
gi|340726192|ref|XP_003401445.1|	PREDICTED: Carbonic anhydrase 2-like [Bt]	A0A088ATP0	4	2	0,60	/
gi|340728711|ref|XP_003402661.1|	PREDICTED: glucosidase 2 subunit beta [Bt]	A0A088AUN3	8	3	0,44	GO:0006491;GO:0005509
gi|350402647|ref|XP_003486555.1|	PREDICTED: high mobility group protein DSP1-like [Bi]	A0A088A8Y3	14	8	0,82	/
gi|350407034|ref|XP_003487962.1|	PREDICTED: methylglutaconyl-CoA hydratase, mitochondrial [Bi]	A0A088A241	14	10	0,80	GO:0008152;GO:0003824
gi|350411979|ref|XP_003489506.1|	PREDICTED: ubiquitin-like modifier-activating enzyme 1 [Bi]	A0A088AVS9	20	13	0,30	GO:0000166;GO:0008641;GO:0006464;GO:0016874;GO:0005524
gi|350417890|ref|XP_003491630.1|	PREDICTED: 6-phosphogluconolactonase [Bi]	A0A088A588	12	10	0,66	GO:0006098;GO:0017057;GO:0005975
gi|350419579|ref|XP_003492232.1|	PREDICTED: splicing factor 3A subunit 1 [Bi]	A0A088AG06	4	3	0,68	GO:0003723;GO:0006396
gi|350425330|ref|XP_003494087.1|	PREDICTED: proteasome subunit beta type-6 [Bi]	A0A088AKA7	8	7	0,66	GO:0005839;GO:0004298;GO:0051603
gi|808116847|ref|XP_012175848.1|	PREDICTED: 60S ribosomal protein L5 [Bt]	A0A088AL09	23	12	0,73	GO:0008097;GO:0003735;GO:0005840;GO:0006412;GO:0005622
gi|808125036|ref|XP_012165634.1|	PREDICTED: probable medium-chain specific acyl-CoA dehydrogenase, mitochondrial [Bt]	A0A087ZTI9	26	2	0,57	GO:0008152;GO:0016627;GO:0016491;GO:0050660;GO:0055114;GO:0003995
gi|808125048|ref|XP_012165639.1|	PREDICTED: Zinc finger protein on ecdysone puffs [Bt]	A0A087ZTJ1	29	10	0,53	GO:0046872
gi|808128063|ref|XP_012166937.1|	PREDICTED: 60S ribosomal protein L14 isoform X2 [Bt]	A0A088ANK4	11	9	0,63	GO:0003735;GO:0005840;GO:0006412;GO:0005622
gi|808132994|ref|XP_012169040.1|	PREDICTED: transcription elongation factor S-II-like [Bt]	A0A088A1K6	9	7	0,73	GO:0003677;GO:0008270;GO:0006351;GO:0006355;GO:0032784;GO:0005634;GO:0006357;GO:0003676
gi|808135622|ref|XP_012170202.1|	PREDICTED: HIV tat-specific factor 1 homolog [Bt]	A0A088AKE1	5	5	0,83	GO:0000166;GO:0003676
gi|808137016|ref|XP_012170810.1|	PREDICTED: enolase [Bt]	A0A088AST9	66	51	0,55	GO:0004634;GO:0000287;GO:0006096;GO:0016829;GO:0000015
gi|808141734|ref|XP_012172367.1|	PREDICTED: aldose reductase-like [Bt]	A0A088AGI8	41	9	0,83	GO:0016491;GO:0055114

Table continues on the next page. [Bt]: *Bombus terrestris*, [Bi]: *Bombus impatiens*.

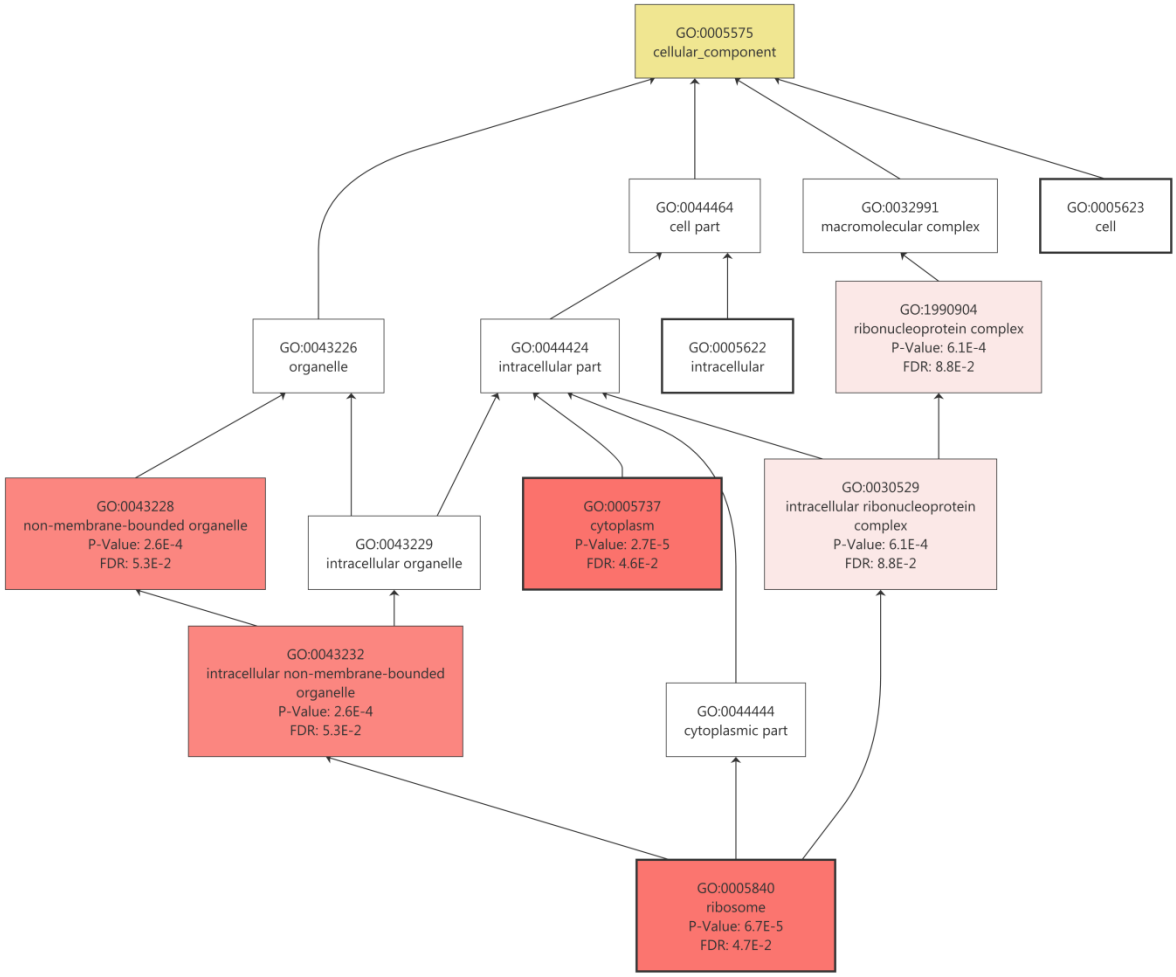
Supplementary Table 2: Overview of the differentially expressed proteins after IAPV infection in the ovaries (continued)

gi|808144131|ref|XP_012173402.1|	PREDICTED: rho GDP-dissociation inhibitor 2 [Bt]	A0A0B4J2M0	8	4	0,57	GO:0005737;GO:0005094;GO:0050790
gi|808144822|ref|XP_012173677.1|	PREDICTED: SAP domain-containing ribonucleoprotein isoform X2 [Bt]	A0A088A896	5	4	0,59	/
gi|815894809|ref|XP_012240082.1|	PREDICTED: catenin alpha [Bi]	A0A088AF89	10	4	0,71	GO:0045296;GO:0051015;GO:0007155;GO:0015629;GO:0005198
gi|815897913|ref|XP_012249675.1|	PREDICTED: protein bric-a-brac 2 isoform X2 [Bi]	O77168	2	2	0,78	GO:0003677
gi|815901918|ref|XP_012237034.1|	PREDICTED: general transcriptional corepressor trfA-like isoform X2 [Bi]	A0A088AFE2	2	2	0,59	/
gi|815904071|ref|XP_012237983.1|	PREDICTED: RNA-binding protein 1-like isoform X3 [Bi]	A0A088AGV6	8	6	0,29	GO:0000166;GO:0003676
gi|815906962|ref|XP_012239252.1|	PREDICTED: golgin subfamily A member 4-like [Bi]	A0A088AAM2	14	2	0,55	/
gi|815908092|ref|XP_012239749.1|	PREDICTED: tubulin alpha-1C chain-like [Bi]	A0A088ANF0	13	4	0,79	GO:0051258;GO:0008152;GO:0005856;GO:0005525;GO:0000166;GO:0005737;GO:0003924;GO:0043234;GO:0005200;GO:0005874;GO:0007017
gi|815909546|ref|XP_012240390.1|	PREDICTED: glyceraldehyde-3-phosphate dehydrogenase 2 [Bi]	A0A088AHC8	47	5	0,73	GO:0016491;GO:0051287;GO:0050661;GO:0006096;GO:0055114;GO:0004365;GO:0016620;GO:0006006
gi|815912412|ref|XP_012241617.1|	PREDICTED: proteasome subunit alpha type-1 [Bi]	A0A088ATC7	14	8	0,40	GO:0005737;GO:0008233;GO:0006508;GO:0005839;GO:0004175;GO:0019773;GO:0004298;GO:0000502;GO:0051603;GO:0016787;GO:0006511;GO:0005634
gi|815913922|ref|XP_012242270.1|	PREDICTED: glucosamine-6-phosphate isomerase [Bi]	A0A087ZPX4	5	3	0,68	GO:0005737;GO:0006044;GO:0004342;GO:0016787;GO:0005975
gi|815919947|ref|XP_012244928.1|	PREDICTED: 26S protease regulatory subunit 10B [Bi]	A0A0B4J2P2	9	7	0,77	GO:0000166;GO:0005737;GO:0030163;GO:0005524;GO:0016787
gi|815924951|ref|XP_012247130.1|	PREDICTED: RNA-binding protein squid-like [Bi]	A0A088AUY1	11	4	0,29	GO:0000166;GO:0003676
gi|815927891|ref|XP_003494397.2|	PREDICTED: cytochrome P450 6a2-like [Bi]	A0A087ZNI0	4	3	0,72	GO:0005506;GO:0016491;GO:0016705;GO:0046872;GO:0020037;GO:0055114;GO:0004497
gi|815929019|ref|XP_012248970.1|	PREDICTED: 40S ribosomal protein S3-like [Bi]	A0A088AV85	13	8	0,74	GO:0003723;GO:0003735;GO:0030529;GO:0015935;GO:0005840;GO:0006412

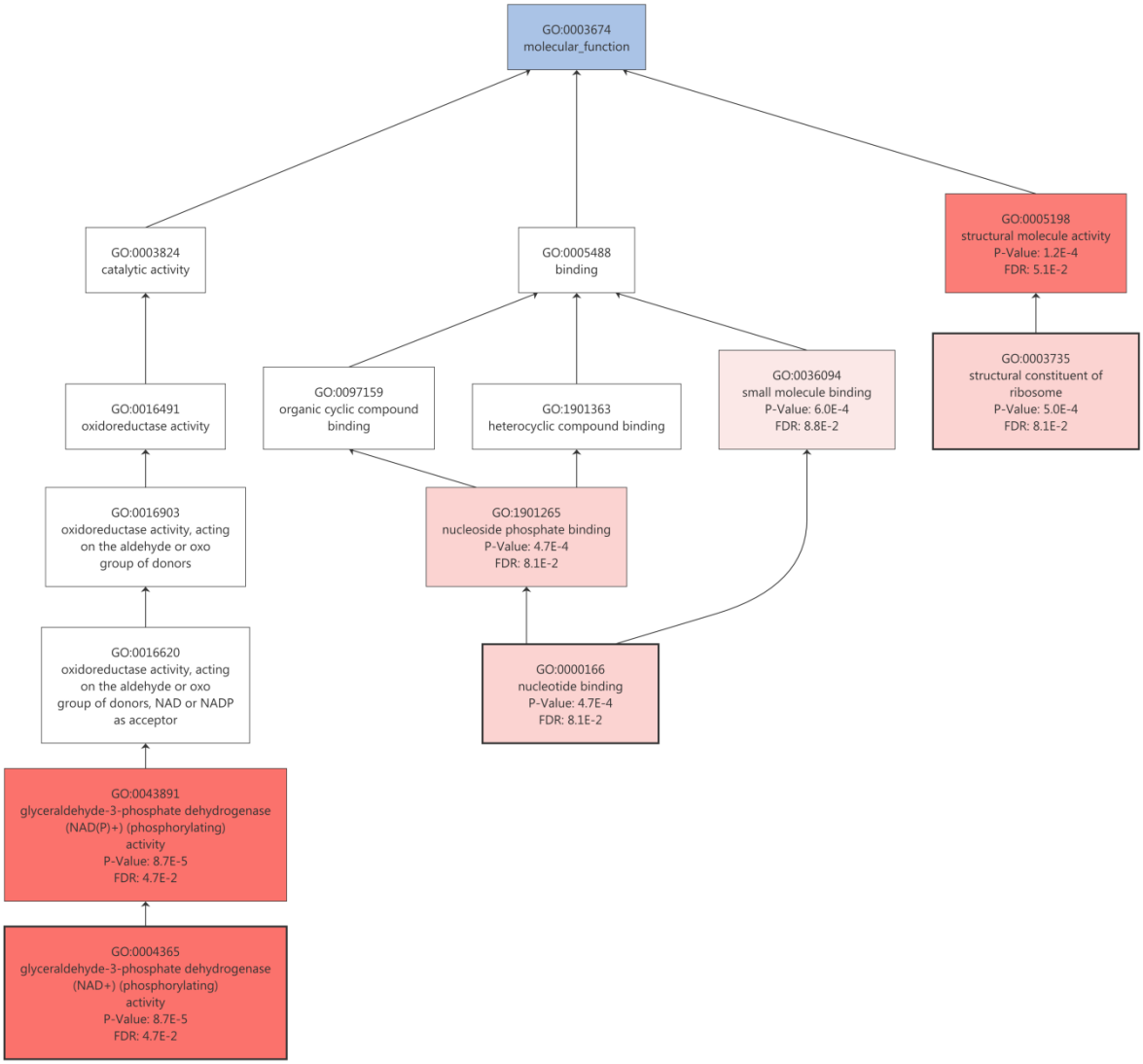
[Bt]: *Bombus terrestris*, [Bi]: *Bombus impatiens.*



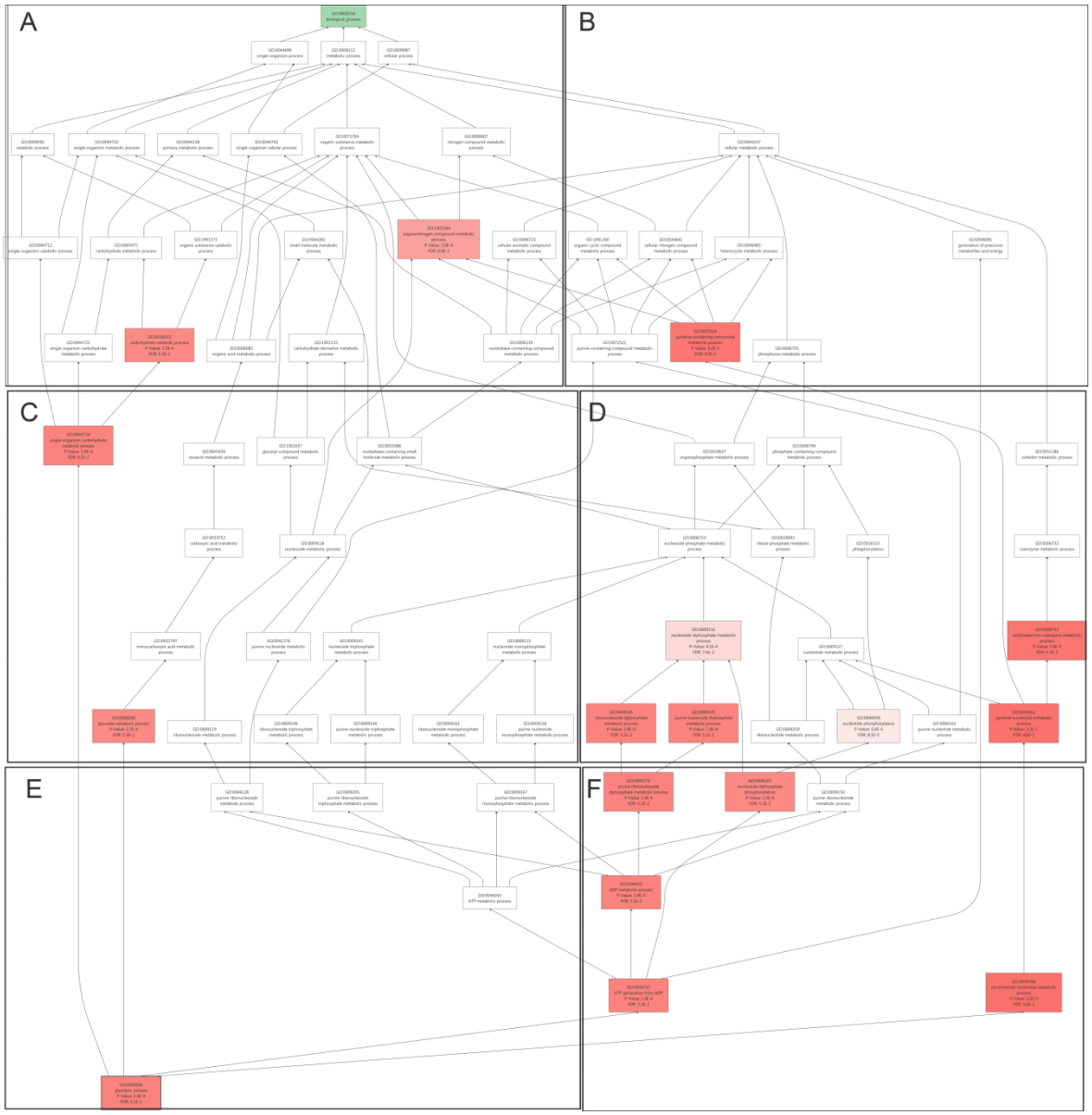
Supplementary Figure 3. Gene Ontology enriched graph as produced by the Blast2GO software for proteins upregulated in the IAPV-infected ovaries.
Significantly enriched GO terms (FDR=0.1) are colored pink or red according to their FDR value. White boxes are not enriched, but show parent-child relationships. In this case, only in the domain of biological processes enriched GO terms were found: GO: 0048584 and GO:0080134.



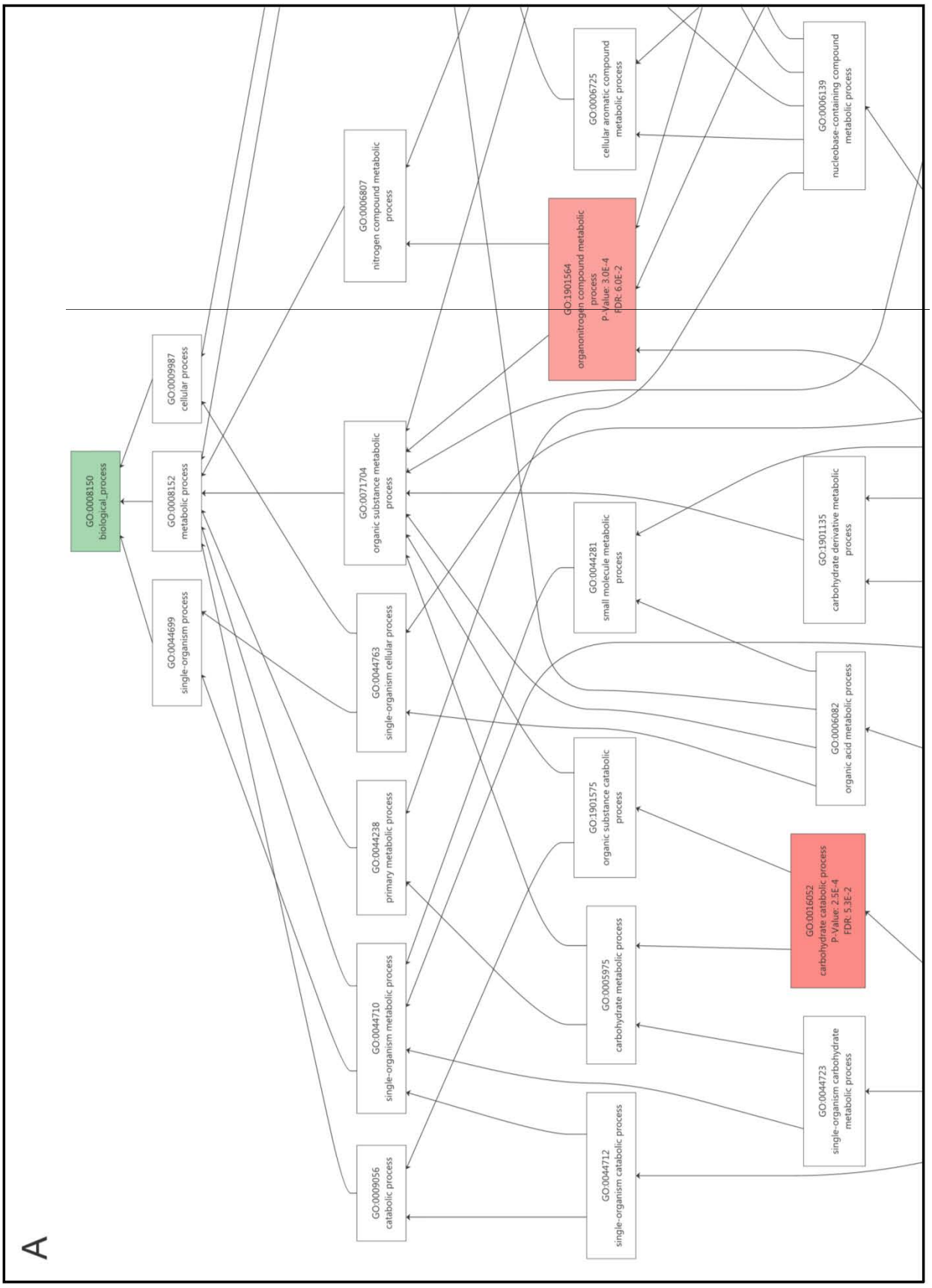
Supplementary Figure 4. Gene Ontology enriched graph showing the cellular component domain, as produced by the Blast2GO software for proteins downregulated in the IAPV-infected ovaries.
Significantly enriched GO terms (FDR=0.1) are colored pink or red according to their FDR value. White boxes are not enriched, but show parent-child relationships.

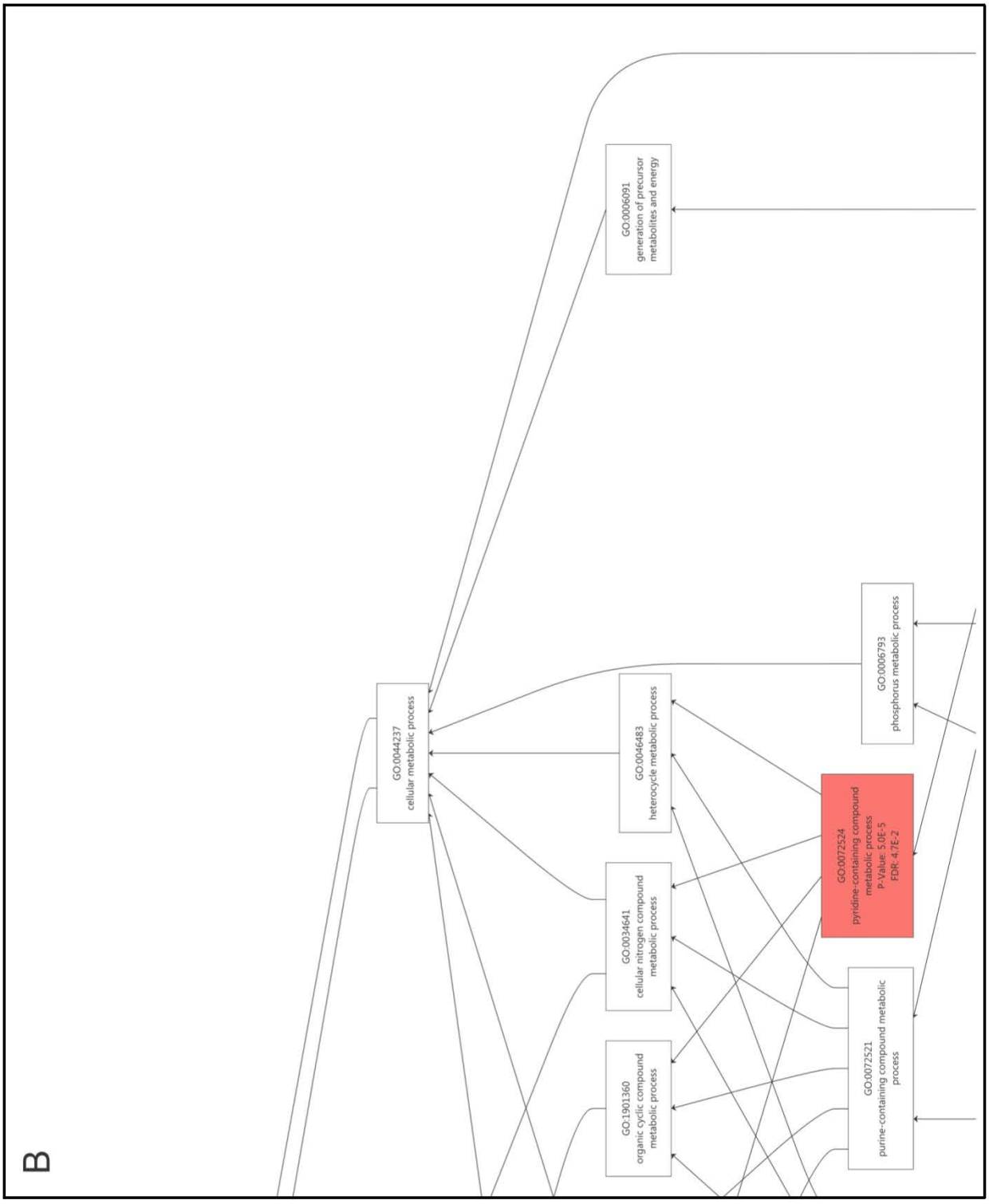


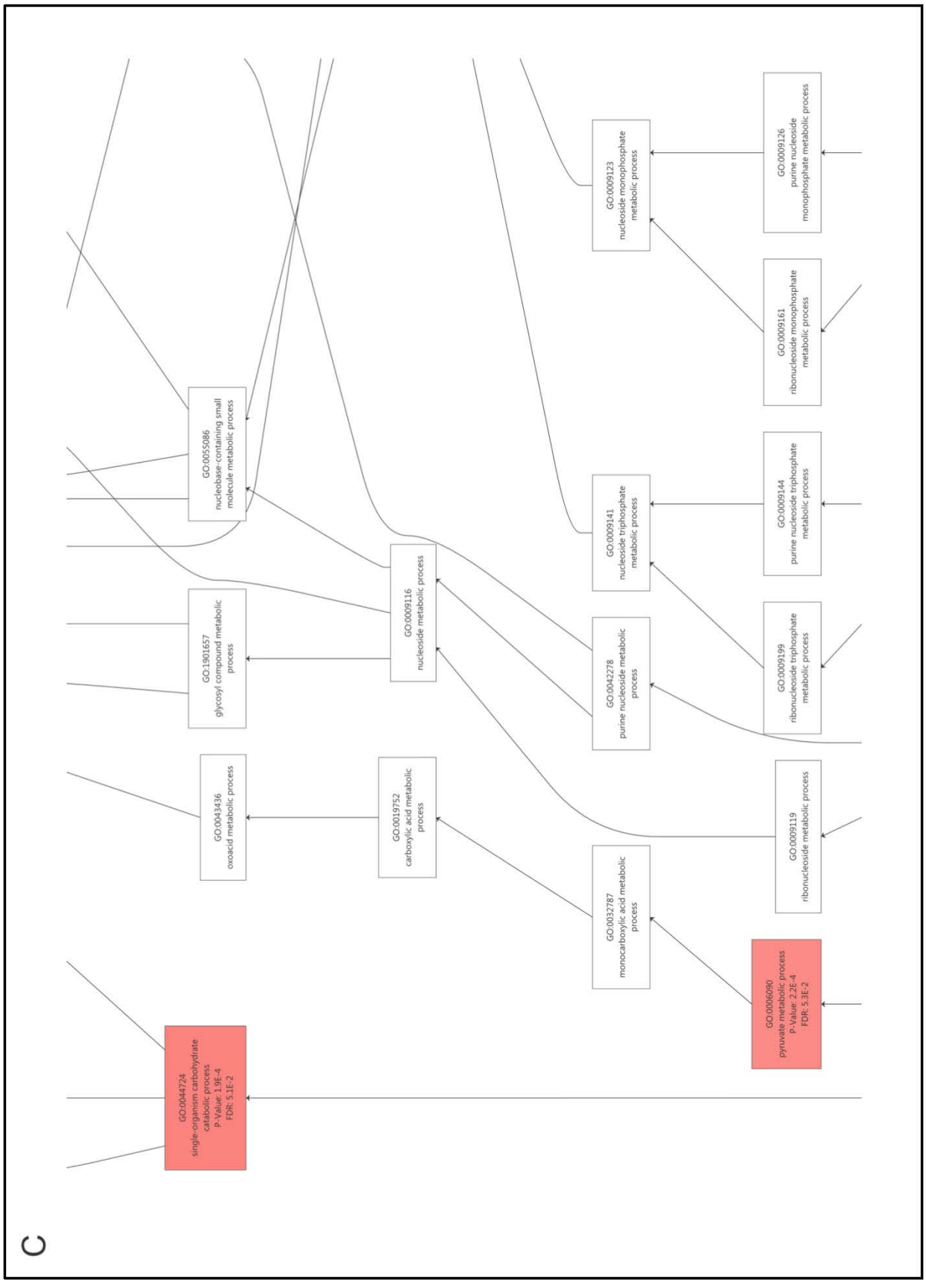
Supplementary Figure 5. Gene Ontology enriched graph showing the molecular function domain, as produced by the Blast2GO software for proteins downregulated in the IAPV-infected ovaries.
Significantly enriched GO terms (FDR=0.1) are colored pink or red according to their FDR value. White boxes are not enriched, but show parent-child relationships.

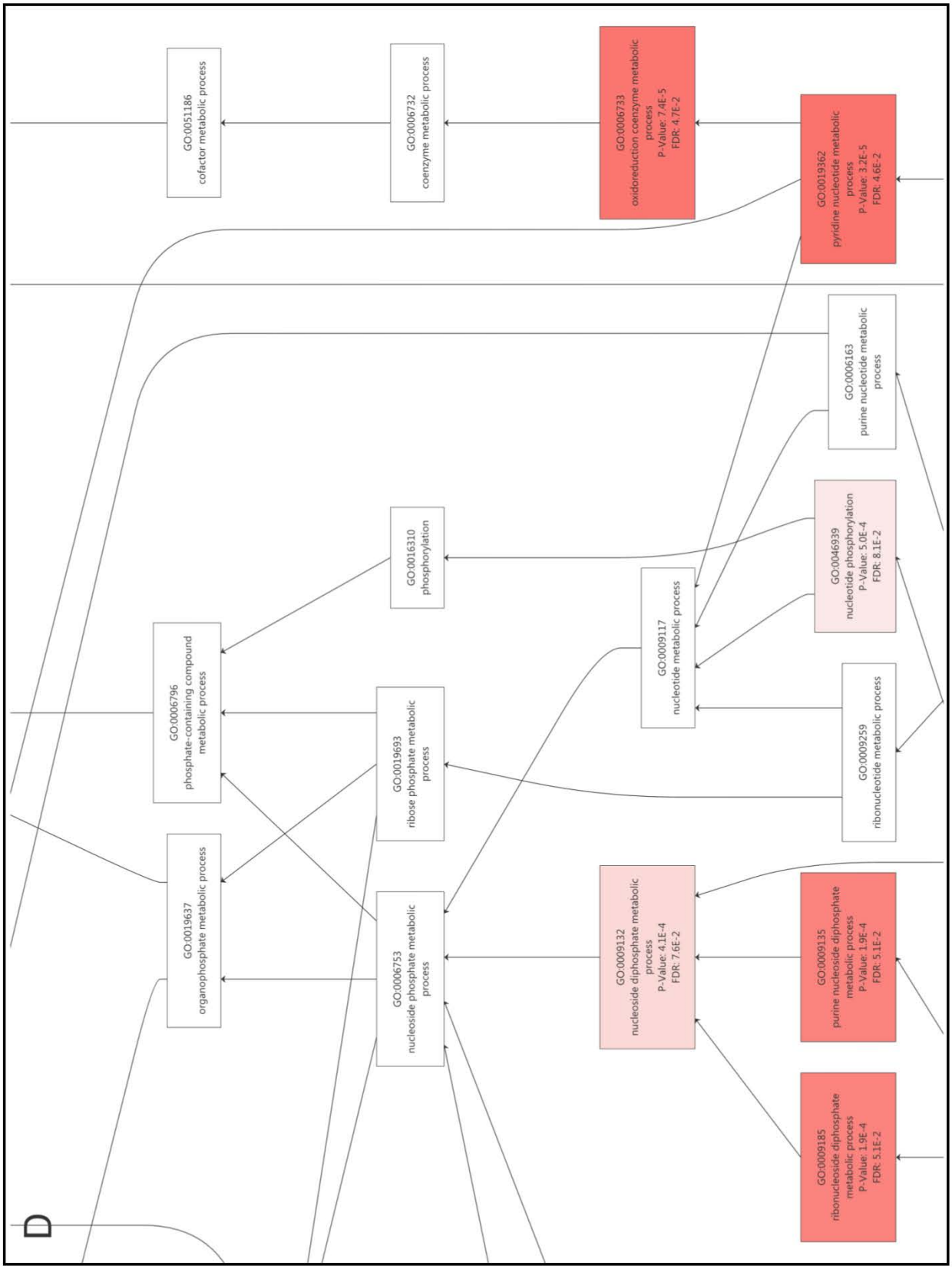


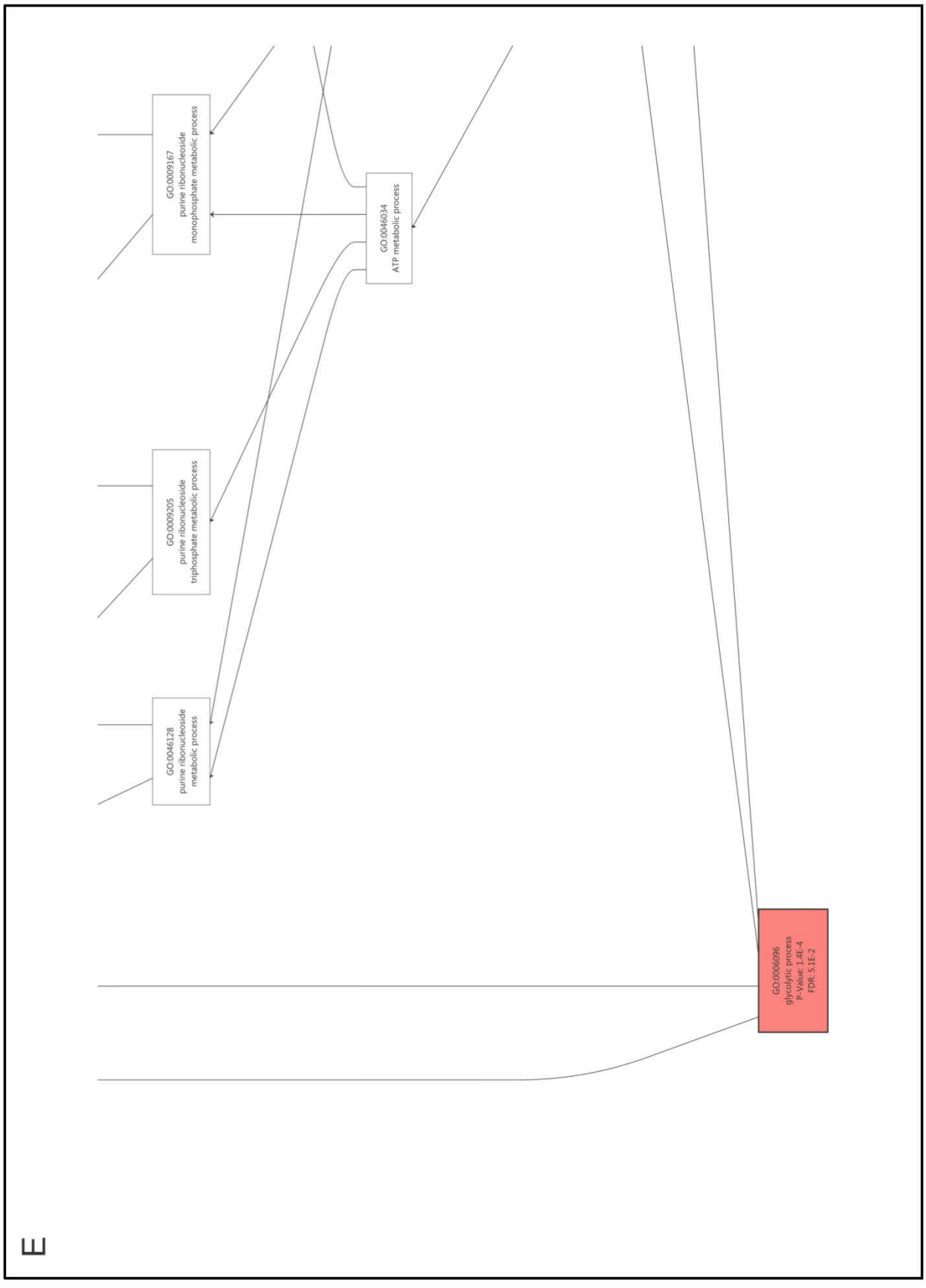
Supplementary Figure 6. Gene Ontology enriched graph showing the biological process domain, as produced by the Blast2GO software for proteins downregulated in the IAPV-infected ovaries.
Significantly enriched GO terms (FDR=0.1) are colored pink or red according to their FDR value. White boxes are not enriched, but show parent-child relationships. Detailed sections of this images are shown on the following pages, as denoted by the different panels.

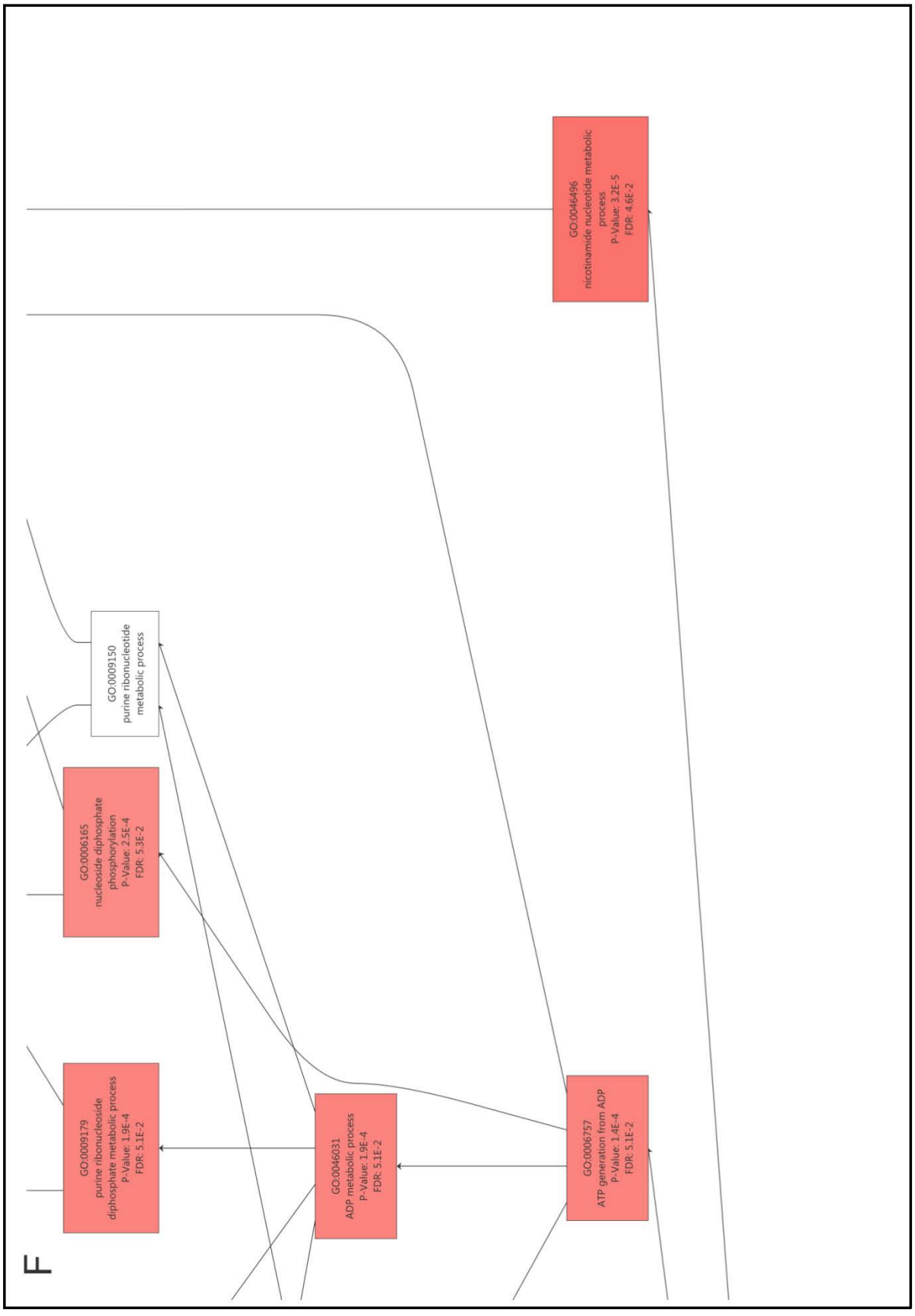

A
GO:0008150
biological_process
GO:0008152
metabolic process
GO:0009987
cellular process
GO:0044699
single-organism process
GO:0071704
organic substance metabolic process
GO:0006807
nitrogen compound metabolic process
GO:1901564
organonitrogen compound metabolic process
P-Value: 3.0E-4
FDR: 6.0E-2
GO:0006725
cellular aromatic compound metabolic process
GO:0006139
nucleobase-containing compound metabolic process
GO:0044763
single-organism cellular process
GO:0044281
small molecule metabolic process
GO:1901135
carbohydrate derivative metabolic process
GO:0044238
primary metabolic process
GO:1901575
organic substance catabolic process
GO:0006082
organic acid metabolic process
GO:0044710
single-organism metabolic process
GO:0005975
carbohydrate metabolic process
GO:0016052
carbohydrate catabolic process
P-Value: 2.5E-4
FDR: 5.3E-2
GO:0009056
catabolic process
GO:0044712
single-organism catabolic process
GO:0044723
single-organism carbohydrate metabolic process


B
GO:0044237
cellular metabolic process
GO:0006091
generation of precursor
metabolites and energy
GO:0046483
heterocycle metabolic process
GO:0006793
phosphorus metabolic process
GO:0034641
cellular nitrogen compound
metabolic process
GO:0072524
pyridine-containing compound
metabolic process
P-Value: 5.0E-5
FDR: 4.7E-2
GO:1901360
organic cyclic compound
metabolic process
GO:0072521
purine-containing compound metabolic
process


C
GO:0044724
single-organism carbohydrate catabolic process
P-Value: 1.9E-4
FDR: 5.1E-2
GO:0006090
pyruvate metabolic process
P-Value: 2.2E-4
FDR: 5.3E-2
GO:0055086
nucleobase-containing small molecule metabolic process
GO:1901657
glycosyl compound metabolic process
GO:0043436
oxoacid metabolic process
GO:0019752
carboxylic acid metabolic process
GO:0009116
nucleoside metabolic process
GO:0032787
monocarboxylic acid metabolic process
GO:0009119
ribonucleoside metabolic process
GO:0042278
purine nucleoside metabolic process
GO:0009141
nucleoside triphosphate metabolic process
GO:0009123
nucleoside monophosphate metabolic process
GO:0009199
ribonucleoside triphosphate metabolic process
GO:0009144
purine nucleoside triphosphate metabolic process
GO:0009161
ribonucleoside monophosphate metabolic process
GO:0009126
purine nucleoside monophosphate metabolic process


D
GO:0051186
cofactor metabolic process
GO:0006732
coenzyme metabolic process
GO:0006733
oxidoreduction coenzyme metabolic process
P-Value: 7.4E-5
FDR: 4.7E-2
GO:0019362
pyridine nucleotide metabolic process
P-Value: 3.2E-5
FDR: 4.6E-2
GO:0006163
purine nucleotide metabolic process
GO:0016310
phosphorylation
GO:0006796
phosphate-containing compound metabolic process
GO:0019693
ribose phosphate metabolic process
GO:0009117
nucleotide metabolic process
GO:0046939
nucleotide phosphorylation
P-Value: 5.0E-4
FDR: 8.1E-2
GO:0019637
organophosphate metabolic process
GO:0006753
nucleoside phosphate metabolic process
GO:0009259
ribonucleotide metabolic process
GO:0009132
nucleoside diphosphate metabolic process
P-Value: 4.1E-4
FDR: 7.6E-2
GO:0009135
purine nucleoside diphosphate metabolic process
P-Value: 1.9E-4
FDR: 5.1E-2
GO:0009185
ribonucleoside diphosphate metabolic process
P-Value: 1.9E-4
FDR: 5.1E-2


E
GO:0009167
purine ribonucleoside
monophosphate metabolic process
GO:0046034
ATP metabolic process
GO:0009205
purine ribonucleoside
triphosphate metabolic process
GO:0046128
purine ribonucleoside
metabolic process
GO:0006096
glycolytic process
P-Value: 1.4E-4
FDR: 5.1E-2


F
GO:0009179
purine ribonucleoside diphosphate metabolic process
P-Value: 1.9E-4
FDR: 5.1E-2
GO:0006165
nucleoside diphosphate phosphorylation
P-Value: 2.5E-4
FDR: 5.3E-2
GO:0009150
purine ribonucleotide metabolic process
GO:0046031
ADP metabolic process
P-Value: 1.9E-4
FDR: 5.1E-2
GO:0006757
ATP generation from ADP
P-Value: 1.4E-4
FDR: 5.1E-2
GO:0046496
nicotinamide nucleotide metabolic process
P-Value: 3.2E-5
FDR: 4.6E-2

SUMMARY

Without a doubt, RNA interference (RNAi) is an invaluable tool in entomology. Its ability to silence genes in a sequence-specific manner, coupled with its easy applicability has led to many applications, both as a research tool and in commercial products (some of them still under development). However, molecular entomologists have been puzzled over the variability in RNAi efficiency that is seen between and among insect species. In this doctoral thesis, we have enumerated factors that could affect RNAi efficiency and have selected two factors to focus on: double-stranded RNA (dsRNA) uptake mechanisms and viral suppressors of RNAi (VSRs). For each factor an appropriate test environment was chosen, for the former the Colorado potato beetle (CPB) and for the latter, the bumblebee *Bombus (B.) terrestris*.

Before studying these complicated processes in detail, the presence of the RNAi core genes was confirmed in both species in a collaboration with, at the time ongoing, genome annotation projects. Most results matched with those obtained in related species, but for CPB an interesting duplication of two core genes was observed.

To examine which pathways contribute to oral dsRNA uptake in the CPB midgut, an assay based on the RNAi-of-RNAi principle was devised. From literature, two pathways were known possibly to be involved, Sid-1-like (Sil)-dependent transmembrane transport and receptor-mediated clathrin-dependent endocytosis. Therefore, representative genes were selected from both pathways. Using the assay, a strong contribution of the *clathrin heavy chain* gene and a smaller one for one of the two selected subunits of *the vacuolar H^+ ATPase* was observed, proving that endocytosis plays a major role. For the two *sid-1-like* genes, *silA* and *SilC*, there was some involvement, but it was less pronounced. To further elucidate the contested association between *sid*-1-*like* genes and dsRNA uptake in insects, these last two genes were examined through phylogenetic and hydrophobicity analysis. Altogether, these results confirmed for the first time the involvement of two pathways in dsRNA uptake in an insect species at the same time. In the general discussion some propositions are made on the various ways these two pathways could interact with each other (or not) and what these results might mean for other insect species. During the course of these experiments, it also became clear that the *clathrin heavy chain* gene had great potential as a target gene for RNAi-based CPB control, with an LC_{50} value that was comparable to the best targets that have been reported so far.

The next part of this work began by identifying a virus with a known VSR, that can infect *B. terrestris*. The most promising candidate was *Cricket paralysis virus* (CrPV) because of its known VSR 1A, its broad host range and its relatedness to some important bee viruses within the family of the *Dicistroviridae*, in particular *Israeli acute paralysis virus* (IAPV). A negative strand-specific tag-based assay was developed to evaluate replication of this virus, both in *B. terrestris* and other insect species. Delivering the viral particles trough micro-injection, complete mortality was observed at the high dose of 10^6 particles per individual, and partial mortality at 10^4 particles per individual. Using RT-PCR and the tag-based assay, a chronic infection was confirmed in the surviving bumblebees. These same techniques were also used to compare virus replication between different tissues, both for CrPV and IAPV. In all tissues, replication was detected, but to a varying degree, and with higher viral titers often, but not always, corresponding with higher levels of replication. Additionally, viral infection was also observed in some bumblebees fed with virus-containing faeces, suggesting that CrPV infections in nature are possible for *B. terrestris*.

In a following step we examined whether IAPV also encodes for a functional VSR, using a two-fold approach. Through a functional RNAi assay, we observed an enhancement of the RNAi system after IAPV infection instead of its suppression, despite only minimal upregulation of the genes involved in RNAi. Moreover, the presence of the candidate small viral proteins could not be confirmed using high definition mass spectrometry. In parallel, when bumblebees were infected with CrPV, with its known VSR, no increase in RNAi efficiency was seen and there may even be indications of suppressor activity. For both viruses, pre-infection with one virus led to decreased titers of the other virus, indicating a major effect of competition. The implications of these results in the context of multi-virus/multi-host are discussed here.

During the VSR experiments for IAPV in *B. terrestris*, some interesting discrepancies were observed. Although both ovaries and fat body had similar viral titers, they showed a remarkably different RNAi response. Therefore, the proteomic dataset of the IAPV-infected ovaries was examined to assess how IAPV alters the host's proteome. Differentially expressed proteins were identified and analyzed using Gene Ontology information. This revealed a number of downregulated processes, with the most notable being proteostasis and energy generation. This attenuated metabolic status of the ovaries was not a consequence of total system failure in the bumblebee, as for instance the fat body was still capable of increasing important immune genes. These observations could be linked with the reduced reproductive potential of bumblebee workers after IAPV infection and raise interesting questions concerning the role of non-essential reproductive tissues in social insects during virus infection.

In a final part, some general conclusions and future perspectives were discussed. This doctoral thesis contributed to the knowledge on RNAi in insects; on the one hand by proving for the first time the involvement of both dsRNA uptake pathways in the same experiment, on the other hand by identifying various direct and indirect ways viral infections can influence the RNAi machinery of their host.

SAMENVATTING

RNA-interferentie is ongetwijfeld een onmisbare techniek in entomologische studies. De manier waarop genen sequentie-specifiek gesilenced kunnen worden, gecombineerd met het gebruiksgemak, heeft geleid tot vele toepassingen, zowel voor onderzoeks- als commerciële doeleinden (waarvan sommige nog in de ontwikkelingsfase). Desalniettemin breken moleculaire entomologen zich al lange tijd het hoofd over de variabiliteit in RNAi-efficiëntie die tussen en binnen insectensoorten gezien wordt. In deze doctoraatsthesis werden factoren opgelijst die de RNAi-efficiëntie kunnen beïnvloeden. We focusten ons op twee factoren: opnamemechanismen van dubbelstrengig RNA (dsRNA) and virale suppressoren van RNAi (VSRs). Voor elke factor werd een gepaste testomgeving gekozen, voor de eerste de Coloradokever (CPB), voor de laatste de hommel *Bombus (B.) terrestris*.

Alvorens deze ingewikkelde processen te bestuderen, werd de aanwezigheid van de belangrijkste RNAi-genen bevestigd in beide soorten. Dit gebeurde in samenwerking met genoomannotatie-projecten die op dat moment lopende waren. De meeste resultaten kwamen overeen met die in sterk verwante soorten, maar voor CPB werd een interessante genduplicatie van twee genen geobserveerd.

Met als doel te onderzoeken welke pathways bijdragen tot de opname van dsRNA in de middendarm van de CPB werd een assay ontwikkeld, gebaseerd op het RNAi-of-RNAi principe. Uit de literatuur was reeds gekend dat twee pathways mogelijk betrokken konden zijn: transmembraantransport doorheen Sid-1-like eiwitten of receptor-gemedieerde clathrine-afhankelijke endocytose. Voor elk van beide pathways werden een aantal representatieve genen gekozen. Met behulp van de assay werd een duidelijke betrokkenheid van *clathrine* en één van de subunits van het *vacuolair H+ ATPase* aangetoond, wat erop wijst dat endocytose een belangrijke rol speelt. Voor de twee *sid-1-like* genen, *SilA* en *SilC*, werd ook een betrokkenheid vastgesteld, maar in mindere mate. Om deze associatie, die vaak in twijfel wordt getrokken, verder uit te klaren werden de *sid-1-like* genen onderworpen aan een fylogenetische en hydrofobiciteitsanalyse. Samengebracht bevestigen al deze resultaten voor het eerst de betrokkenheid van beide pathways tegelijk in dsRNA-opname in een insectensoort. In de algemene discussie werden een aantal mogelijkheden geopperd over de manier waarop deze pathways (al dan niet) met elkaar interageren. Gedurende deze experimenten werd ook duidelijk dat *clathrine* een goede target voor RNAi-gebaseerde bestrijding van CPB zou kunnen zijn, met een LC_{50} waarde die vergelijkbaar was met de beste gerapporteerd tot nu toe.

De volgende sectie van dit werk begon met het identificeren van een virus met een gekende VSR dat *B. terrestris* zou kunnen infecteren. Een veelbelovende kandidaat was het *Cricket paralysis virus* (CrPV) omwille van zijn gekende VSR 1A, zijn brede host range en zijn verwantschap met enkele belangrijke bijvirussen binnen de familie van de *Dicistroviridae*, meer bepaald met het *Israeli acute paralysis virus* (IAPV). Een assay werd ontwikkeld die specifiek de negatieve streng van het virus kon oppikken via een getagde primer om zo de replicatie van het virus te kunnen bevestigen, zowel in *B. terrestris* als andere insectensoorten. Wanneer met behulp van micro-injectie de hommels geïnfecteerd werden, werd een volledige mortaliteit gezien bij de hoge dosis van 10^6 viruspartikels per individu en een gedeeltelijke mortaliteit bij 10^4 partikels per individu. Gebruik makende van de tag-assay en RT-PCR werd een chronische infectie bevestigd in de overlevende hommels. Diezelfde technieken werden ook toegepast om virusreplicatie te vergelijken tussen verschillende weefsels, zowel voor CrPV als IAPV. In alle weefsels werd replicatie gedetecteerd, maar in afwisselende mate. Hierbij kwamen hoge virale titers soms, maar niet altijd, overeen met meer replicatie. Daarnaast werd infectie ook aangetoond in een aantal hommels die gevoed waren met faeces dat virus bevatte, wat suggereert dat CrPV-infecties in de natuur mogelijk zijn bij hommels.

In de volgende stap onderzochten we of IAPV ook codeert voor een functionele VSR via een tweevoudige benadering. Met behulp van een functionele RNAi-assay werd een verbetering van de RNAi-efficiëntie waargenomen in tegenstelling tot de verwachte suppressie, ondanks dat er slechts een beperkte opregulatie van de RNAi-genen was. Daarenboven kon de aanwezigheid van de kandidaat VSRs, kleine virale eiwitten, niet aangetoond worden via hoge definitie massaspectrometrie. Voor beide virussen leidde een pre-infectie met één virus tot een verlaagde titer van het ander virus, wat wijst op een sterk effect van competitie. De implicaties van deze resultaten in de context van multi-virus/multi-host netwerken werden hier ook besproken.

Tijdens deze VSR-experimenten in *B. terrestris* werden enkele interessante onregelmatigheden opgemerkt. Alhoewel de ovaria en het vetweefsel vergelijkbare virale titers vertoonden, werd een verschillende RNAi-respons geobserveerd. Daarom werd de proteoomdataset van IAPV-geïnfecteerde ovaria onderzocht om na te gaan hoe IAPV het proteoom van de gastheer kan veranderen. Differentieel geëxpresseerde eiwitten werden geïdentificeerd en geanalyseerd met behulp van Gene Ontology informatie. Hieruit bleek dat een aantal processen neergereguleerd was, met de meest opvallende proteostase en energieproductie. Deze verzwakte metabole status van de ovaria was niet het resultaat van een algemene stopzetting van de biologische processen in de hommel, aangezien in het vetweefsel nog een aantal belangrijke immuungenen

opgereguleerd waren. Deze resultaten kunnen gelinkt worden met gereduceerde reproductie in de werksters van hommels na IAPV-infectie en roepen vragen op in verband met de rol van niet-essentiële reproductieve weefsels in sociale insecten tijdens virale infecties.

Allerlaatst werden enkele algemene conclusies en toekomstperspectieven besproken. Deze doctoraatsthesis heeft bijgedragen aan de kennis van RNAi in insecten, enerzijds door voor het eerst de betrokkenheid van beide opnamesystemen aan te tonen in éénzelfde experiment, en anderzijds door verschillende directe en indirecte manieren te identificeren, waarop virusinfecties de RNAi-machinerie van de gastheer kunnen beïnvloeden.

CURRICULUM VITAE

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Name:	Kaat Cappelle
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Education

- Doctoral Training Program in Applied Biological Sciences: Cell and Gene Biotechnology
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- Bachelor and Master of Science in Bioscience Engineering: Cell and Gene Biotechnology with great distinction.
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Publication List

- **Cappelle, K.**, Meeus, I., Piot, N., Wang, L., and Smagghe, G. The pathogenicity of *Cricket paralysis virus* in the wild pollinator, *Bombus terrestris*. *In preparation.*
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Presentations

- 49th Annual Meeting of the Society for Invertebrate Pathology (SIP), 24-28 July 2016 in Tours, France.

  Oral presentation: An opposite effect of *Dicistroviridae* on the RNA interference defense mechanism of their host, *Bombus terrestris*.
  Supported by the SIP Virus Division travel award.

- 63rd Annual Meeting of the Entomological Society of America (ESA), 15-18 November 2015 in Minneapolis, USA.

  Oral presentation: Both clathrin-mediated endocytosis and two SID transmembrane proteins are involved in double-stranded RNA uptake in the Colorado potato beetle midgut.
  Supported by the Scientific Research Committee (CWO) of the Faculty of Bioscience Engineering

- COST Action Workshop “RNAi and Health of bees”, 15-18 April 2015 in Ghent, Belgium.

  Oral presentations: 1) RNA interference: Introduction
  2) RNA interference: Experimental design and RNAi efficiency

Tutorships

- Soete, E. (February 2016 – June 2016).

  Opnamemechanismen van dsRNA in coloradokevers en hun targetpotentieel in RNA interferentie-gebaseerde gewasbescherming.
  Bachelor dissertation for Bachelor in Biomedical Laboratory Technology

- Van Eynde, B. (August 2013 – June 2014)

  Role of endocytosis and the SID transmembrane proteins in the uptake of dsRNA in the insect midgut.
  Master dissertation for Master of Science in Bioscience Engineering: Cell and Gene Biotechnology

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