Virus Dynamics in Naturally Varroa-Resistant Honeybee Populations

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Cover: Bee with symptomatic *Deformed wing virus* beside a healthy bee
(Photo: B. Locke)

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Abstract

The ectoparasitic mite, *Varroa destructor*, together with its associated viruses is the most significant threat to honeybee (*Apis mellifera*) health world-wide. Since the introduction of varroa to the new host species, the European honey bee (*Apis mellifera)*, it has been responsible for the near complete eradication of wild and feral honeybee populations in Europe and North America. However, a unique honeybee population on the island of Gotland, Sweden, has acquired resistance to the mite through a natural selection process. A recent study also showed that Gotland mite-resistant population might have adapted tolerance and resistance to virus infections. This suggests that virus-host interactions may play a key role in the long-term survival of this population. The aim of this thesis was to investigate the role of the viral and bacterial microbiome in the enhanced survival of the mite-resistant (MR) honeybees on Gotland, to compare the role of virus tolerance and resistance in other naturally selected mite-resistant honeybee populations, similar to the Gotland population, and to unravel individual level virus-host interactions in honeybees.

First, by using a combination of high-throughput sequencing and different bioinformatics tools we found Lake Sinai virus and Apis rhabdovirus-1, including previously known honey bee viruses, in Swedish honey bees. Further molecular studies showed that Gotland MR bees have developed a colony-level resistance to these viruses, and tolerance to *Deformed Wing Virus* (DWV), the virus most commonly associated with mite infestation. Secondly, differences in the bacterial microbiome between MR and mite-susceptible (MS) bees were studied using the 16S rDNA, but the results indicated little differences between MR and MS bees throughout the season. Finally, individual level susceptibility of MR and MS honey bees to oral virus infection was tested for DWV virus and Acute bee paralysis virus (ABPV). The results demonstrate that DWV and ABPV infection dynamics were nearly identical in MR and MS bees, but that bees from the MR honeybee populations had significantly lower mortality rates than bees from the MS population.

In conclusion, the results of this thesis present strong evidence that naturally adapted mite-resistant honeybees have also adapted, through a natural selection process, tolerance and resistance to virus infections at both the colony and individual level. The bacterial microbiome did not appear to play a role in the enhanced survival of Swedish mite-resistant honeybees but more studies are required to investigate potential bacteria-virus interactions on honeybee health. Future work should aim to identify key genomic regions associated with virus resistance and tolerance that can be incorporated into honeybee breeding programs to improve honeybee health.

Keywords: Apis mellifera, *Varroa destructor*, viral metagenomics, deformed wing virus, acute bee paralysis virus, virus-host interactions

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Dedication

To my parents and teachers

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I **Thaduri S**.*, Locke B., Granberg F., de Miranda J. (2018). Temporal changes in the viromes of Swedish Varroa-resistant and Varroa-susceptible honeybee populations. PLoS ONE 13 (12): e0206938

II **Thaduri S**., Marupakula S., Terenius O., Locke B., de Miranda J.R.* (2019). Temporal dynamics in the bacterial and viral metagenomes of Swedish varroa-resistant and non-resistant honeybee populations. (manuscript)

III **Thaduri S**., Stephan J.G., de Miranda J.R., Locke B.* (2019). Disentangling host-parasite-pathogen interactions in a varroa-resistant honeybee population reveals virus tolerance as an independent, naturally adapted survival mechanism. *Scientific Reports* 9: 6221

IV Locke B.*, **Thaduri S**., Stephan J.G., Low M., Blacquière T., Dahle B., Le Conte Y., Neumann P., de Miranda J.R. (2019). Tolerance to experimental virus infections in four distinct varroa-resistant honeybee populations (manuscript)

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The contribution of Srinivas Thaduri to the manuscripts included in this thesis was as follows:

I First author. Planned and performed all of the laboratory work and data management. Conducted most of the data analyses and interpreted most of the results. Produced most of the figures/tables and wrote most of the text, with editorial assistance from the co-authors. Was corresponding author with the publishing journal.

II First author. Designed the study together with supervisors. Planned and performed most of the field work and all of the laboratory work and data management. Conducted most of the data analyses and interpreted most of the results. Produced most of the figures/tables and wrote most of the text, with editorial assistance from the co-authors.

III First author. Primary responsible for the study design. Performed some of the field work and all of the laboratory work and data management. Conducted part of the analyses and interpreted most of the results. Produced some of the figures/tables and wrote most of the text, with editorial assistance from the co-authors.

IV Second author. Primary responsible for the study design. Performed all of the laboratory work and data management. Conducted part of the analyses and interpreted part of the results. Produced some of the figures/tables and wrote part of the text, with editorial assistance from the co-authors.

Abbreviations

16S rDNA	16S subunit of the ribosomal RNA gene
ABPV	Acute bee paralysis virus
AFB	American foulbrood
AmFV	*Apis mellifera* filamentous virus
ARV	Apis rhabdovirus
BeeMLV	Bee Macula-like virus
BQCV	Black queen cell virus
BSRV	Big Sioux river virus
BVX	Bee virus X
BVY	Bee virus Y
CBPV	Chronic bee paralysis virus
CCD	Colony collapse disorder
cDNA	complementary DNA
Cq	Quantification cycle
DNA	Deoxyribonucleic acid
DWV	Deformed wing virus
EFB	European foulbrood
hbs-LAB	honeybee-specific lactic acid bacteria
IAPV	Israeli acute paralysis virus
IMD	Immune deficiency pathway
IRES	Internal ribosomal entry site
IRG	Intergenic region
JAK/STAT	Janus kinase/Signal transducer and activator of transcription
JNK	Jun-N-terminal kinase
KBV	Kashmir bee virus
KV	Kakugo virus
LSV	Lake Sinai virus
MC	Melting curve

MR	Mite-resistant
MS	Mite-susceptible
ORF	Open reading frame
PAMP	Pathogen associated molecular patterns
PRR	Pathogen recognition receptors
RdRP	RNA-dependant RNA polymerase
RNA	Ribonucleic acid
RP49	Ribosomal protein gene 49
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SBPV	Slow bee paralysis virus
SBV	Sacbrood virus
SQ	Starting quantity
UTR	Untranslated region
VDV-1	Varroa destructor virus-1
VP	Viral protein
VPg	Viral protein genome linked
VSH	Varroa sensitive hygiene

1 Introduction

The Western honeybee, *Apis mellifera*, is the most versatile, ubiquitous, and economically important managed pollinator worldwide. The economic value of insect pollination for agricultural crop production has been estimated at € 22 billion in Europe and € 153 billion globally (Gallai *et al.*, 2009). Approximately 35% of global food production depends on the insect pollination of which 90% is performed by managed honeybees (Klein *et al.*, 2007). Many of these crops consist of fruits, nuts, seeds, and vegetables which provide micronutrients essential for human health (Smith *et al.*, 2015; Chaplin-Kramer *et al.*, 2014). In addition to commercial pollination services and products, honeybees play a significant role in sustaining natural plant biodiversity as an ecosystem service provider (Potts *et al.*, 2010).

In recent years, honeybee colony losses have increased in the United States and Europe. These colony losses have a serious negative impact on the apicultural industry as well as on the ecosystem. Even though diverse biotic and abiotic stressors are involved in these colony losses, honeybee pathogens and diseases play a crucial role in these colony losses (Neumann & Carreck, 2010). The ectoparasitic mite, *Varroa destructor*, in combination with its associated viruses, currently considered to be one of the cardinal causes of honeybee colony mortality worldwide. Furthermore, the spread of *Varroa* has also changed the global honeybee viral landscape (Martin *et al.*, 2012). The mite population grows exponentially in the infested colony, leading to increased virus transmission opportunities and viral epidemics, which eventually results in the death of the colony in 2 to 3 years unless active mite population control strategies are implemented. As a consequence, feral and wild honeybee colonies in Europe and North America have been nearly eradicated (Le Conte *et al.*, 2010).

In spite of these deadly effects of the mite on *A. mellifera*, a few unique honeybee populations in North America and Europe have survived without active mite control for more than 20 years (Locke, 2016b; Le Conte *et al.*,

2007; Fries *et al.*, 2006). These populations have naturally acquired mite resistance traits that reduce the mite's reproductive success (Locke, 2016b). One of the most comprehensively studied varroa mite surviving honeybee populations is on the island of Gotland, Sweden. This population also acquired tolerance and resistance, at the colony level, to viruses that are directly transmitted by varroa and other viruses that are not directly transmitted by varroa mites (Locke *et al.*, 2014).

1.1 Honeybees

Honeybees are eusocial, oviparous, holometabolous insects that live in large numbers in a colony. The colony consists of three castes: one reproducing female queen bee, 20,000 – 40,000 female worker bees and 200-300 male drones. Honeybees have a haplodiploid sex determination system; female workers and queens emerge from fertilized diploid eggs whereas male drones arise from unfertilized haploid eggs. Fertilized eggs become either a queen or a worker depending upon whether the resulting larvae are fed with royal jelly or worker jelly by the nurse bees performing brood care (Winston, 1991).

Drone bees have a life span of around six weeks and their primary purpose is to mate with virgin queens. After the copulation, the drone will die and any drones left in autumn are expelled from the colony. The queen bee life span is approximately three years, and she mates once in her lifetime with several drones. The queen stores all the sperm in her spermathecal for her entire lifetime and she lays fertilized eggs at a rate of around 2000 per day (Winston, 1991). Honeybee worker bees in temperate regions are classified as short lived-summer bees or long-lived winter bees. Worker bees emerging in spring and mid-summer have a life span of about six weeks, whereas winter bees can survive up to eight months. Worker bees undergo a behavioral development based on age, termed temporal polyethism. Young, 2-3 week old, worker bees perform different tasks inside the hive which includes brood care, cleaning and building the comb. After three weeks, the adult workers shift to foraging for pollen and nectar outside the hive for the remainder of their life (Winston, 1991).

1.2 Honeybee pathology

Similar to any other living organism, honeybees are susceptible to a diverse range of pathogens and diseases. The honeybee nest cavity maintains a relatively constant temperature and humidity which provides an ideal environment for parasites and pathogens. Furthermore, thousands of

individuals in a colony living close together, and contacting via trophallaxis or casual contact, provide various opportunities for pathogen transmission. The honeybee pathosphere consist of pathogens like bacteria, viruses, fungi, and microsporidia and parasites such as varroa mites and tropilaelaps mites.

1.2.1 Varroa destructor

The ectoparasitic mite, *Varroa destructor,* is currently considered the major threat to honeybee health and the apiculture industry around the world (Boecking & Genersch, 2008). The original host of *V. destructor* is the eastern honeybee *Apis cerana*. Due to the conditions provided by the global trade and transport of honeybees, *Varroa destructor* was successful in crossing the species barrier and infest the western honeybee, *Apis mellifera,* in the first half of the last century (Rosenkranz *et al.*, 2010). Since then, *V. destructor* has spread to infest *Apis mellifera* colonies throughout the world, although it has not yet been found in Australia and few isolated islands (Genersch, 2010; Rosenkranz *et al.*, 2010). Based on mtDNA cytochrome oxidase I (CO-I) gene sequence analyses, several *V. destructor* haplotypes have been found, but only two are able to reproduce in *A. mellifera* colonies: the Korean haplotype that has a near global distribution and the Japanese haplotype that has only been reported in Japan, Thailand, and North and South America, and is considered less virulent than the Korean type (Anderson & Trueman, 2000; de Guzman & Rinderer, 1999).

Figure 1. Varroa mite on an adult bee. (photo: Barbara Locke)

V. destructor is an obligate parasite of honeybees, *i.e.* the mite completes all its life stages within the colony (Figure 1). The mites are brown in color and are flat and oval in shape, measuring 1.1mm x 1.6mm in length. Adult female *Varroa* mites have two distinct life stages: a phoretic phase where mites attach to the adult bees traveling within or between colonies; and a reproductive

phase within a sealed brood cell during honeybee pupal development (Rosenkranz *et al.*, 2010). During the phoretic phase, the female mite adheres between the abdominal segments of the adult bee and feeds on the fat body (Ramsey *et al.*, 2019) and hemolymph (Glinski & Jarosz, 1984) of the bee by piercing the soft intersegmental membrane. In order to find an appropriate brood cell for reproduction, female mites preferentially travel on nurse bees (Kraus, 1993).

The reproductive phase begins when the adult female enter the brood cells of 5th instar larva before cell capping by sensing the chemical volatiles released from the larval cuticle (Le Conte *et al.*, 1989). The female mite lays eggs in the closed cell, and her egg laying is synchronized with the development of bee pupae. The first egg is usually unfertilised and develops into a male since varroa use a haplodiploid mode of reproduction. The mother mite continues to lay eggs and all remaining eggs are fertilized and develop into females. A normally reproducing adult female mite lays 5 and 6 female eggs in worker cell and drone cell, respectively. The mite offspring develop through well-defined developmental stages: protonymph, deutonymph, mobile protochrysalises stages, and immobile deutochrysalises stages. Mating takes place between the adult mites (Rosenkranz *et al.*, 2010). Inside the capped cell, both the adult female and her progeny feed on the fat body (Ramsey *et al.*, 2019) and hemolymph of developing pupae which cause severe nutritional deficiency and physiological alterations in the developing bee (Amdam *et al.*, 2004). All mature female daughter mites emerge with the adult honeybee and subsequently infest nurse bees for transportation to new brood cells (Kuenen & Calderone, 1997).

Pathology

The pathology of *V. destructor* infestation on honeybees is determined by two factors: the feeding activities of the mite and the mite's role as a vector of viruses. The mite feeds on the bee by injuring cuticle of pupae and adults and sucking substantial amounts of hemolymph and fat body (Ramsey *et al.*, 2019). The loss of hemolymph interferes with the pupal development which in turn results in the reduction of size and weight of the hatching bees (De Jong *et al.*, 1982). The parasitized drones lose 11-19% of their body weight based on infestation rate, which eventually results in decreased flight performance and lower ability to mate (Duay *et al.*, 2002). For foragers, it has been observed that they show reduced learning ability and their orientation and homing is impaired (Kralj *et al.*, 2007).

Apart from direct effects caused by *V. destructor* on bee health and performance, there are indirect and devastating effects caused by viruses that

are vectored by the mite. Before the invasion of *V. destructor*, most of the virus infections were covert infections (Boecking & Genersch, 2008; Bailey & Ball, 1991). A recent study, based on the molecular evolutionary tools to track the spread of DWV around the world, has shown a tight link between the global spread of the varroa mite and the parallel spread of deformed wing virus (Wilfert *et al.*, 2016). Earlier studies showed contradictory results of the impact varroa on the bee immune system. Initial studies indicated that varroa parasitism induces immune suppression (Yang & Cox-Foster, 2005). Subsequent studies showed increased expression of immune related genes (Kuster *et al.*, 2014). Recent observations showed that the mite feeding activity, through the removal of either hemolymph or fat body tissue, diminishes the immune response of the bee and results in increasing viral densities (Annoscia *et al.*, 2019; Ramsey *et al.*, 2019). More about these viruses is discussed in detail in the virus section. Despite the lethal effects of varroa on *A. mellifera*, some bee populations have developed resistance to mite infestation which is discussed in more detail in **section 4.5.**

1.2.2 Bacteria

The two formalized bacterial pathogens of honeybees are *Paenibacillus larvae* and *Melissococcus plutonius*, which are the primary causative agents of American foulbrood (AFB) and European foulbrood (EFB) respectively. Both bacteria are pathogenic to honeybee larvae, but not to adult bees (Genersch, 2010).

American foulbrood

American foulbrood is a lethal disease that spreads rapidly within the colony and to other colonies in the apiary due to the usage of contaminated bee equipment and robbing by worker bees (Genersch, 2010; Fries & Camazine, 2001). American foulbrood is a notifiable disease in many countries and strict measures are regulated by corresponding authorities (Vanengelsdorp & Meixner, 2010). The tough endospores are the only infectious forms of this organism and they are only infectious to bee larvae. After the ingestion of bacterial spores by the larvae, the spores germinate and massively proliferate in the mid-gut which eventually leads to the invasion of tissues and death of the larvae (Yue *et al.*, 2008). In the AFB infected colony, clinical symptoms include a scattered and irregular pattern of brood due to healthy capped brood and infected uncapped brood. The caps of the dead brood appear darker than those of healthy brood and are often sunken and punctured. The infected larvae gradually turn into brownish semi fluid-like mass (ropy mass) that finally dries

into a hard scale. Since the spores are resistant to high temperatures and disinfectants, the ideal method of AFB control is the destruction of infected colonies. However, antibiotics are widely used outside Europe to treat AFB disease but antibiotics cannot eradicate AFB completely as they only have a bacteriostatic function. Moreover, antibiotics are not effective for bacterial spores which are the primary mode of transmission of AFB disease (Genersch, 2010).

European foulbrood

In contrast to American foulbrood, European foulbrood affects younger unsealed brood and the diseased larvae die when they are four to five days old. The diseased larvae, unlike normal larvae, are twisted around the walls or stretched out in the cell and the color of the larvae changes from pale white color to brown and finally to grayish black (Forsgren, 2010). Larvae are infected by ingesting food contaminated with *M. plutonius* and the infected bacteria proliferate in larval midgut. The pathogenesis of EFB is still obscure, but several reports suggest that the pathogen competes with larvae for food which results in the death of the larvae by starvation. The control of EFB includes use of antibiotic oxytetracycline hydrochloride and shook swarm method: destroying the infected combs and shaking the bees onto clean foundation and boxes (Forsgren, 2010; Genersch, 2010).

Honeybee microbiome

In animals, the gut microbiome supports the host in detoxifying harmful molecules, providing essential nutrients, protecting against invading pathogens and parasites and developing and shaping the immune system (Eckburg *et al.*, 2005). Based upon 16S ribosomal RNA gene sequence studies, the gut microbiome of the *A. mellifera* adult worker honeybees consists predominantly of a distinctive set of nine bacterial species. The core gut microbiome consists of *Snodgrassella alvi*, *Gilliamella apicola*, two species of *Lactobacillus*, and a *Bifidobacterium* species, and these species can be found in every adult worker bee worldwide. The other less numerous, and also less prevalent, bacteria are the *Bartonella apis*, *Apibacter adventoris*, *Frischella perrara* and *Acetobacteraceae* (Regan *et al.*, 2018; Engel *et al.*, 2016; Kwong & Moran, 2016). Gut bacteria transmission between colony members is mediated through the oral-faecal route, oral trophallactic interactions, contact with hive material, social interaction with hive mates, and the consumption of stored pollen or bee bread (Kwong & Moran, 2016; Powell *et al.*, 2014).

Genomic and metabolic studies of the bee microbiome indicate that a major portion of the bee microbiota facilitates the fermentation of dietary carbohydrates. These fermentative bacteria include *G. apicola, F. perrara, Lactobacillus Firm-4, Lactobacillus Firm-5 and B. asteroids*. All of these bacteria can metabolize glucose and fructose, the most abundant sugars in nectar, honey, and pollen (Raymann & Moran, 2018; Ellegaard *et al.*, 2015; Engel *et al.*, 2012). The Honeybee microbiome also plays a role in protecting the host against bee pathogens. In infection studies with *E. coli*, bees containing the entire bee community induced the expression of anti-microbial peptides (AMPs) and increased the survivorship compared to microbiota free bees (Kwong *et al.*, 2017). One member of the bee microbiome, *Frischella perrara* causes strong activation of the host immune system where it induces the AMPs and the upregulation of a melanization cascade (Emery *et al.*, 2017). Furthermore, both *in-vitro* and *in-vivo* studies with the honeybee-specific Lactic Acid fermenting Bacteria (hbs-LAB) *Lactobacillus* and *Bifidobacterium* from *A. mellifera* has demonstrated that the hbs-LAB microbiota was successfully able to inhibit the growth of the honeybee bacterial pathogen, *Paenibacillus larvae* which is the causative agent of American foulbrood (Forsgren *et al.*, 2010). Dysbiosis, a disturbance in the gut microbiome, of the honeybee core gut microbiome can increase susceptibility to pathogens. Treatment of bees with the antibiotic tetracycline, severely alters the core gut community composition, decreases the bee survivorship and leads to increased infection by the opportunistic pathogen *Serratia marcescens* (Raymann *et al.*, 2017).

1.2.3 Microsporidia (Nosema *spp.*)

Nosema belongs to the phylum microsporidia and are obligate intracellular spore forming fungal pathogens. The phylum microsporidia have two honeybee-specific pathogens: *Nosema apis* and *Nosema ceranae.* The earlier speculation was that western honeybee, *A. mellifera,* and eastern honeybee, *A. ceranae,* were specifically infected by *N. apis* and *N. ceranae,* respectively (Fries *et al.*, 1996). However, recent studies have shown that *N. ceranae* also infects *A. mellifera* populations around the world (Fries, 2010). The *Nosema* spores act as the infectious agent, which are horizontally transmitted between adult bees via the oral-fecal route by ingestion of spores from the environment. After the ingestion of spores by bees, the spores germinate in the midgut and infect the epithelial cells (Fries, 2010). *N. apis* causes nosemosis disease which mainly characterized by dysentery. In *Apis mellifera*, *N.ceranae* causes major health problems that are characterized by immune suppression, a degradation

of gut epithelial cells and a reduction of bee life span (Antunez *et al.*, 2009; Higes *et al.*, 2008; Paxton *et al.*, 2007). The immunosuppression possibly makes them more vulnerable to other pathogens such as black queen cell virus (BQCV) (Bailey *et al.*, 1983).

1.3 Honeybee viruses

Viruses are likely the most abundant and diverse biological entities on earth (Suttle, 2005), and can be observed wherever life exists. Viruses are non-cellular, opportunistic, and obligate intracellular pathogens. Since viruses are non-cellular, they completely depend on host cell machinery for transcription, translation, and replication. Viruses consist of either RNA or DNA as the genetic material and an outer protein coat, capsid, which encloses the genetic material. Some viruses have an extra envelope covering the capsid and these viruses are known as enveloped viruses (Flint *et al.*, 2015).

The introduction of Next Generation Sequencing (NGS) technologies has revolutionized the discovery of novel viruses. These technologies also enable the identification of viruses present at low titers that do not cause symptoms in the host, allowing future emerging and re-emerging infectious diseases to be detected. Furthermore, these technologies also provide new perspectives about viral biodiversity (van Aerle & Santos, 2017; Shi *et al.*, 2016).

The majority of characterized honeybee infecting viruses have positive-sense single-stranded RNA genomes (+ssRNA), and many belong to the order *Picornavirales*. These consist of common bee viruses in the family *Dicistroviridae* (acute bee paralysis virus (ABPV), Kashmir bee virus (KBR), Israeli acute paralysis virus (IAPV), and black queen cell virus (BQCV)); in the family *Iflaviridae* (Deformed wing virus (DWV), Kakugo virus (KV), Varroa destructor virus-1 (VDV-1), Sacbrood virus (SBV), and Slow bee paralysis virus (SBPV)) (McMenamin & Flenniken, 2018; Brutscher *et al.*, 2016; de Miranda *et al.*, 2010b). Also well characterized are the Lake Sinai viruses, which are in the Sinaivirus genus; Bee Virus X (BVX), Bee Virus Y (BVY); Big Sioux River Virus (BSRV); and Chronic bee paralysis virus (CBPV; unclassified) (McMenamin & Flenniken, 2018; Daughenbaugh *et al.*, 2015; Runckel *et al.*, 2011).

With the availability of high-throughput sequencing studies, several new bee viruses have been identified such as (+)ssRNA viruses include Bee macula-like virus (BeeMLV) in the Tymoviridae family (de Miranda *et al.*, 2015), Apis mellifera flavivirus and Apis mellifera nora virus (Remnant *et al.*, 2017), and Moku virus from Iflaviridae family (Mordecai *et al.*, 2016b). The first *A. mellifera*-infecting negative sense single-stranded RNA viruses (-

ssRNA) were also identified, specifically Apis mellifera Rhabdovirus1 and Apis mellifera Rhabdovirus-2, and Apis mellifera Bunyavirus (Remnant *et al.*, 2017).

Finally, only one honeybee infecting double stranded DNA virus, *Apis mellifera* filamentous virus (AmFV) has been sequenced and characterized (Gauthier *et al.*, 2015).

The most common bee viruses have RNA as genetic material, and they encode and rely on an RNA-dependent RNA polymerase (RdRp) for genome replication. The RdRp enzymes lack proofreading capability, such as found in DNA polymerases, and this leads to high mutation rates (Andino & Domingo, 2015). This results in a population of related mutant viral genotypes around one or more master genotypes, known as a quasi-species. By existing as a diverse swarm of viral genotypes, viruses can achieve a remarkable evolutionary rate, enabling them to overcome different selective pressures (Andino & Domingo, 2015; Lauring & Andino, 2010).

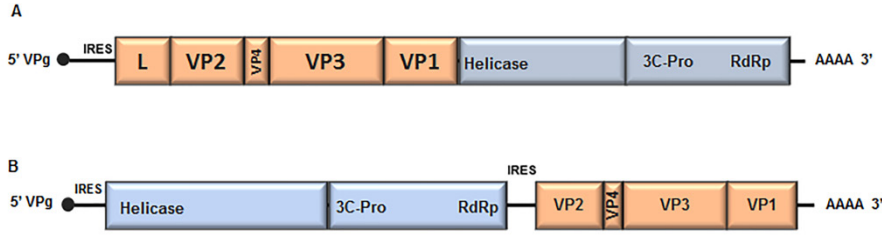

Figure 2. Genome organization of two common honeybee virus families (A) *Iflaviridae* and (B) *Dicistroviridae*.

As indicated above, the majority of bee viruses belong to the order *Picornavirales*, and within in this order, they are further grouped into the two families *Dicistroviridae* and *Iflaviridae*.

1.3.1 Dicistroviridae

The dicistrovirus virion structure consists of an approximately 30 nm icosahedral capsid comprised of 60 copies of three different viral proteins: VP1, VP2, and VP3, and a bicistronic monopartite genome. These are non-enveloped viruses and the host cell entry occurs via clathrin-mediated endocytosis (Bonning & Miller, 2010). The family contains two genera: *Aparavirus*, which includes ABPV, KBV, and IAPV and *Cripavirus,* which includes BQCV. The basic genome organization of *Dicistroviridae* is, as the

name suggests, a single positive strand RNA containing two open reading frames (ORF), separated by an intergenic region (IGR) containing an Internal Ribosome Entry Site (IRES) and flanked by non-translated regions (Figure 2B). The 5' end of the ORF1 also contains an IRES and a 5' CAP covalently linked to a small viral protein (VPg). The larger ORF1 encodes the non-structural proteins: the helicase, protease, and RdRp which are involved in virus replication and protein processing. The shorter ORF 2 is located in the 3' part of the genome and encodes for the four structural proteins VP1 to VP4. The 3' end of the genome has a poly-A tail. The IRES is an important part of the virus genome that helps in regulating viral translation (Bonning & Miller, 2010).

The ABPV-KBV-IAPV complex

Acute bee paralysis virus (ABPV), Kashmir bee virus (KBV), Israeli acute paralysis virus (IAPV) are closely related members of a species complex due to their close genetic relationship, similar route of transmission, the primary host life stage and existing primarily as covert low-titer infections. However, they are extremely virulent when they are injected into pupae or adults, or by feeding with higher doses (10^9 virus particles) per bee (de Miranda *et al.*, 2010a). Observed symptoms for ABPV and IAPV, but not KBV, include rapidly progressing paralysis and trembling, inability to fly and the gradual darkening and loss of hair from the thorax and abdomen, followed by premature death (Maori *et al.*, 2007; Bailey & Milne, 1969). Also reduced mitochondrial function and disturbances in energy related host process are observed in IAPV infections (Chen *et al.*, 2014).

ABPV was discovered in 1963 during transmission studies with chronic bee paralysis virus (Bailey *et al.*, 1963). ABPV has been detected in the brain and hypopharyngeal glands of the adult bees (Bailey & Milne, 1969), and in faeces, indicating several oral transmission routes involving adults, larvae, cannibalized brood, and contaminated food or faeces (Chen *et al.*, 2006). ABPV has also been detected in semen, suggesting vertical transmission (Yue *et al.*, 2006). ABPV has been shown to be vectored by the varroa mite (Ball, 1985), and the arrival of varroa has increased ABPV prevalence. ABPV is also implicated in varroa-associated colony winter mortality (Francis *et al.*, 2013; Berenyi *et al.*, 2006; Bekesi *et al.*, 1999). Based on the modelling study, ABPV can only kill a colony when the large mite populations are present (Martin, 2001). In spite of its acute virulent nature, ABPV is never consistently linked with honeybee colony losses. This could be due to the acutely virulent pathogens transmitted by mites kill the brood in which they reproduce and kill

the adults in which they live, breaking the virus transmission cycle and co-evolution (Schroeder & Martin, 2012).

KBV was first identified from the extracts of the eastern honeybee *A. cerana* originating from the northern part of India, Kashmir (Bailey & Woods, 1977). KBV is prevalent in North America (Cox-Foster *et al.*, 2007; Hung *et al.*, 1996), and New Zeeland (Todd *et al.*, 2007) while rarely reported in Europe (Tentcheva *et al.*, 2004). KBV is serologically, biologically and genetically related to ABPV, although the capsid protein profiles appeared to be slightly different (Allen & Ball, 1995). KBV seems to be the most virulent of all known honeybee viruses as it requires few virus particles to infect both adult bees and pupae by injection. Unlike ABPV, KBV does not exhibit paralysis symptoms but causes death within three days after infection (Bailey *et al.*, 1979). KBV has been found in faeces (Hung *et al.*, 1996), all food sources (brood food, honey, pollen, and royal jelly), and in all larval stages (Shen *et al.*, 2005), which suggests a possibility of horizontal transmission between adult bees to larvae. A vertical transmission route has also been suggested, as KBV RNA was detected in queens (Chen *et al.*, 2005a) and their eggs (Shen *et al.*, 2005), although not in their offspring larvae and adults (Chen *et al.*, 2006). Furthermore, the varroa mite has been shown to vector KBV, and 70% of pupae were infected when mites carrying KBV were transferred to non-infected colonies (Chen *et al.*, 2004).

IAPV was first discovered from dead bees collected from dying honeybee colonies in Israel (Maori *et al.*, 2007), and nomenclature of this virus is predominantly based on that its symptoms that are similar to ABPV. After its discovery in the Middle East, IAPV has now been detected in Australia (Roberts *et al.*, 2017), North America (Cox-Foster *et al.*, 2007), and Europe (Francis *et al.*, 2013; Granberg *et al.*, 2013; Blanchard *et al.*, 2008). The virus has been found in pollen and faeces, and it can be detected in all honeybee developmental stages, as well as in adult workers, queens, and males (drones), suggesting that the virus can be transmitted within and among honeybee colonies by a combination of horizontal and vertical transmission pathways (Chen *et al.*, 2014). The virus can infect all honeybee tissues, but particularly high IAPV titers are found in the gut, nervous system, and hypopharyngeal glands (Chen *et al.*, 2014). The varroa mite has been shown to act as both biological and mechanical vector for IAPV (Di Prisco *et al.*, 2011). The virus has been implicated in Colony Collapse Disorder in the USA (Cox-Foster *et al.*, 2007), and has also been associated with winter colony losses (Blanchard *et al.*, 2008).

Black queen cell virus (BQCV)

Black queen cell virus (BQCV) was first isolated from dead Honeybee queen larvae and prepupae sealed in queen cells, and since the virus was derived from the darkened areas on the walls of queen cells that have infected pupae, the name BQCV was coined. Although BQCV persists asymptomatically in colonies, an overt infection can cause the death of queen pupae and pre-pupae (Bailey & Woods, 1977). The infected queen pupae have a pale yellow color and a tough sac like skin, resembling the pupae infected with sacbrood virus (Chen & Siede, 2007). BQCV is usually found in bees infected with microsporidian parasite *Nosema* (Bailey *et al.*, 1983) and that results in the increased mortality of bees caused by the virus. The virus was also detected in adult worker honeybees (Tentcheva *et al.*, 2004). BQCV is one of the most prevalent honeybee viruses throughout the world (Mondet *et al.*, 2014; Ellis & Munn, 2005; Leat *et al.*, 2000). BQCV was found in pollen and honey, as well as in the gut, which provides evidence for the horizontal transmission of this virus (Chen *et al.*, 2006). The vertical transmission of BQCV from an infected queen to their progeny was also observed (Ravoet *et al.*, 2015b). In a recent study, BQCV was also detected in drone semen sample which indicates the possibility of venereal transmission (Prodelalova *et al.*, 2019).

1.3.2 Iflaviridae

DWV, SBV, SBPV, and the newly discovered Moku virus belong to the genus Iflavirus within the family *Iflaviridae*. Iflavirus virions are non-enveloped, roughly spherical and have icosahedral symmetry with a diameter of 22–30 nm. The genome organization of Iflaviruses consist of a single open reading frame (ORF) flanked by a long 5’ untranslated region (UTR) and a short 3’ UTR and is terminated with a 3’ poly-A tail (Figure 2A). As with mammalian picornaviruses, the capsid proteins (VP1-VP4) are located in the 5’ region of the genome while the non-structural proteins (RdRp, helicase, and protease) involved in virus replication are located in the 3’ region of the genome. The ORF is translated directly into a polyprotein that is subsequently processed by viral encoded 3C-protease to produce functional proteins. The 5’ UTR also contains an IRES which helps in regulating viral translation. The 5’ end of the positive strand RNA virus genome is covalently linked to a protein, VPg, which plays a role in RNA replication, stability, translation, and movement (Valles *et al.*, 2017; de Miranda & Genersch, 2010; Roberts & Groppelli, 2009).

The deformed wing virus (DWV) complex

Deformed wing virus (DWV) is the most comprehensively studied honeybee virus. DWV was first isolated from dead Japanese honeybees (Ball, 1983). DWV infections are associated with the characteristic symptoms of deformed wings, shortened body size and abdomen and discoloration of adult bees which ultimately results in the reduced longevity of adult bees and death of the colony (de Miranda & Genersch, 2010). Nevertheless, these symptoms are more or less linked with varroa-mediated transmission of DWV when the mite feeds on the developing pupae (Mockel *et al.*, 2011). Furthermore, the association of DWV and varroa has a significant role in lowering the winter bee life span and overwintering colony losses in temperate regions (Dainat *et al.*, 2012). However, one study also shows that and association DWV with overwintering colony mortality that was independent of varroa (Highfield *et al.*, 2009). The establishment of a new DWV transmission route, *i.e.* through varroa feeding on developing pupae and adults, has been closely associated with the huge loss of honeybee colonies, and has changed the entire viral landscape of honeybees (Mondet *et al.*, 2014; Ryabov *et al.*, 2014; Martin *et al.*, 2012).

In the absence of varroa, DWV causes covert infections without lethal effects on the colony. DWV has been found in all developmental stages of the bee, including egg, larvae, pupae, and adults (Chen *et al.*, 2005b), and also found in the glandular secretions used to feed larvae and the queen (de Miranda & Genersch, 2010). Apart from vector mediated transmission by varroa, DWV is also transmitted by horizontal transmission, through trophallaxis, faecal-oral transmission, and cannibalized pupae, and through vertical transmission from parent to offspring (Genersch & Aubert, 2010; de Miranda & Fries, 2008). Based on negative strand Reverse Transcription Polymerase Chain Reaction (RT-PCR), DWV has been suggested to replicate in varroa mites collected from deformed bees, but not in mites collected from asymptomatic bees (Gisder *et al.*, 2009).

Kakugo Virus (KV) was isolated from the brains of aggressive worker guard bees (Fujiyuki *et al.*, 2004). The KV genome shows 6% sequence dissimilarity in the RdRp region from the deformed wing virus. This virus was also detected in worker bees in relatively high numbers, suggesting that KV can infect various worker populations in honeybee colonies (Fujiyuki *et al.*, 2006). Varroa Destructor Virus-1 (VDV-1) was initially identified in *Varroa destructor,* and this virus shares 84% nucleotide identity with DWV (Ongus *et al.*, 2004). Subsequently, it was found VDV-1 also replicates in honeybees and the existence of recombination between DWV and VDV-1. Recombinants were reported both in varroa mites and honeybees (Moore *et al.*, 2011; Zioni *et al.*, 2011).

As described earlier, most of the RNA viruses exist as a collection of closely related variants known as a quasi-species. DWV is also a rapidly evolving group closely related variants (de Miranda & Genersch, 2010), and DWV exists as a viral complex comprising at least three master variants—types A, B, and C. According to the ICTV (The International Committee on Taxonomy of Viruses), DWV type A includes original DWV genotype (Lanzi *et al.*, 2006) and Kakugo virus. VDV-1 has now been designated as DWV type B (Mordecai *et al.*, 2016a; Martin *et al.*, 2012). DWV type B has 84% nucleotide identity with DWV type A. Recently, DWV type C has been discovered (Mordecai *et al.*, 2016c) which shares 79.8% and 79.5% nucleotide identity with DWV-A and DWV-B, respectively, and has only been reported in the UK. At the colony level, both DWV-A and DWV-B are linked with significant overwintering colony losses (Natsopoulou *et al.*, 2017; Dainat *et al.*, 2012).

Sacbrood virus (SBV)

Sacbrood virus was first identified in the United States in 1913 (White, 1913). Since its discovery, SBV has been found in every part of the world where beekeeping practices are present (Chen & Siede, 2007). SBV causes a sacbrood disease in honeybee larvae which results in the death of the larva. Larvae infected with SBV fail to pupate, and ecdysial fluid rich with SBV accumulates beneath their unshed skin in the form a sac, after which the disease is named. The color of the infected larvae changes from pearly white to pale yellow, and following after death they dry out making a dark brown gondola–shaped scale. The SBV spreads in the colony through horizontal transmission where nurse bees acquire the virions while removing larvae killed by SBV, and infected nurse bees can then spread the virus by feeding larvae with their glandular secretion and exchanging food with other adult bees (Chen & Siede, 2007).

Even though SBV disease primarily affects honeybee larvae, SBV also infects pupae and adult bees where it induces physiological and behavioral changes. The manifestations include a rapid progression from brood tending to foraging, a strong aversion from eating and collecting pollen and degeneration of hypopharyngeal glands (Bailey & Ball, 1991; Du & Zhang, 1985; Bailey & Fernando, 1972). Earlier studies also detected large amounts of SBV in varroa infested colonies, and the prevalence of SBV in honeybee colonies was found to be positively correlated with the level of varroa infestation (Tentcheva *et al.*, 2004).

1.3.3 Recently identified bee viruses

With the advent high throughput sequencing, many novel honeybee viruses are discovered, but here I will only discuss the viruses which are part of my thesis.

Lake Sinai virus (LSV)

Lake Sinai virus was first discovered in the United States in 2009, where two main variants (LSV1 and LSV2) were described (Runckel *et al.*, 2011). Soon after the discovery in the USA, LSV was also detected in honeybee colonies from Spain, Belgium, Sweden and Australia (Remnant *et al.*, 2017; Granberg *et al.*, 2013; Ravoet *et al.*, 2013). LSV group have remarkable diversity, and at present seven LSV variants have been found, although most of these only through partial sequences. LSV-1 and LSV-2 have strong similarities in virion organization, seasonal prevalence, absence of overt symptoms with Bee virus Y and Bee virus X respectively (de Miranda *et al.*, 2013). LSV1 and LSV2 are also detected in the *Varroa destructor* mite, although no negative strand LSV was detected (Ravoet *et al.*, 2015a). LSV infection has been linked in Colony Collapse Disorder (CCD), and a significant positive correlation between LSV and the weakening of the colonies was also found (Daughenbaugh *et al.*, 2015; Cornman *et al.*, 2012).

Apis rhabdovirus (ARV)

Majority of the honeybee viruses have positive strand single-stranded RNA genomes. However Remnant *et al* were the first to characterize two negative-sense strand viruses, Apis rhabdovirus-1 (ARV-1) and Apis rhabdovirus-2 (ARV-2), in *A. mellifera* colonies from three locations: TheNetherlands, South Africa, and the South Pacific (Remnant *et al.*, 2017). Rhabdoviruses are enveloped viruses with a single-stranded negative strand RNA genome, whose particles are 100–430 nm long and 45–100 nm in diameter, and infect a broad range of species including plants and animals, including insects, and are mostly transmitted by arthropod vectors (Longdon *et al.*, 2015). ARV-1 was also detected in honeybee populations from North America, Europe, Middle East, Africa, and South Pacific, suggesting that it has a near global distribution (Levin *et al.*, 2017; Remnant *et al.*, 2017). The replicative form of the ARV-1 genome, *i.e.* the complementary positive strand RNA, was also found in A. *mellifera* and *V. destructor* mites which implies the active replication of ARV-1 in mites and honeybees (Levin *et al.*, 2017).

1.4 Modes of transmission and virulence

For honeybees, pathogens are transmitted either horizontally or vertically (Figure 3). Horizontal transmission involves the movement of pathogens either between honeybee colonies or between individuals within the colony. Horizontal transmission occurs within the colony through various routes, such as oral exchange (trophallaxis), contaminated food sources, contaminated wax, honey, nectar, comb parts, open wounds or by vector-mediated transmission. Furthermore, the horizontal transmission also involves the transmission by cannibalism of dead infected individuals and the faecal-oral route through the ingestion of contaminated faeces. Horizontal inter-colony transmission occurs through robbing, drifting or during foraging. Vertical transmission occurs through reproduction, at the colony level, from mother colonies to swarms. At the individual level, from infected queens to eggs. The venereal transmission also occurs from infected drones to queens, while mating (Amiri *et al.*, 2016; Yañez *et al.*, 2012; de Miranda & Fries, 2008; Chen *et al.*, 2006; Fries & Camazine, 2001).

Virus infections can be categorized into overt and covert infections. Overt infections show obvious disease symptoms and high levels of pathogen burden. Overt infections can be further divided to acute and chronic infections. The characteristics of acute infections are the manifestation of clear symptoms and high pathogen burden in a short time. The overt acute infection can kill the host in a short period of time. Chronic infections characterized by the long lasting production of viral particles over the lifetime of the host, with clear disease symptoms. In conclusion, both acute and chronic infections can induce significant negative impact on host fitness with clear symptoms, with the main difference in the duration and intensity of infection (de Miranda & Genersch, 2010).

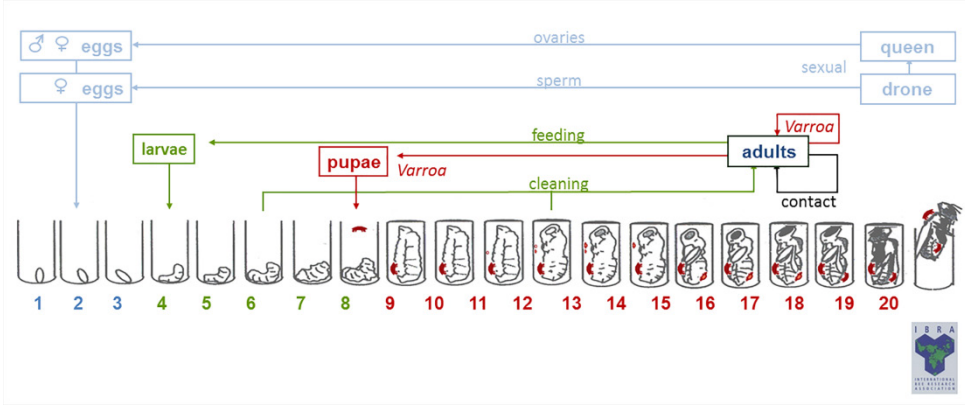

Figure 3. Diagram describing the different possible transmission routes for honey bee viruses (de Miranda *et al.*, 2013).

Covert infections are defined by the absence of obvious disease symptoms in the presence of virus particles, but that may still re-emerge to cause overt infections. Covert infections can be categorized into latent and persistent infections. In latent infections, the virus genomes are either integrated with host DNA or exist as an extrachromosomal episome. Virus particle production is severely reduced. Latent infections are usually seen in retroviruses and no true latency has been demonstrated yet for honeybee viruses. Persistent infection characterized by the production of low viral particles in the infected cell and no destruction of the host cell. In order to establish a persistent infection, the virus has to evade the host immune response. This is possible only if the virus successfully evades the host immune response and regulates gene expression for stable persistence. However, the disturbance of normal homeostasis host may result in a re-emergence of covert infections which have a negative effect on host fitness. These outbreaks could be induced by various factors such as environment, nutrition, colony conditions or stress (de Miranda & Genersch, 2010; Ribière *et al.*, 2002).

The ability of a pathogen to exploit the individual host bee and subsequently the colony depends in its virulence. Virulence is defined as the degree of the disease that a pathogen can cause, which is assumed to increase the host damage or mortality and is generally also positively correlated with the pathogen reproduction rate (de Miranda & Genersch, 2010). According to the trade-off model, virulence evolution in pathogens is governed by a trade-off between pathogen transmission and pathogen virulence. An excessively high pathogen reproduction rate (*i.e.* high virulence) results in premature host mortality and thus insufficient virus progeny to infect a new host. On the contrary, too low a pathogen reproduction rate (*i.e.* low virulence) may have little impact on the host's fitness but the pathogen losses the opportunities to

infect a new host. Coming to the regulation of virulence by the mode of transmission: less virulent pathogens, which have little impact on host fitness, prefer to establish a vertical transmission relationship with the host because the fitness and transmission of the pathogen directly depend on host survival and longevity. However, highly virulent pathogens, which kill host quickly, favor a horizontal transmission relationship with the host since it provides higher opportunities for transmission to a new host, and host death may even enhance the transmission (Fries & Camazine, 2001; Lipsitch *et al.*, 1996).

There is often a disparity between individual and colony level virulence with several honeybee pathogens. Pathogens that are virulent at the individual level may not be virulent at the colony level and *vice versa*. For example, sacbrood virus and the fungal disease chalkbrood are highly virulent at the individual level but not at the colony level (Fries & Camazine, 2001). On the contrary, DWV is by itself not virulent at the individual level but in association with Varroa can be virulent at the colony level. Furthermore, it is this low virulence at the individual level that accounts for DWV being the main virus associated with varroa infestations, because more virulent viruses such as ABPV and KBV kill the brood too quickly for varroa to complete its development on the bee pupae and transmit the virus to new hosts (de Miranda & Genersch, 2010; Sumpter & Martin, 2004; Martin, 2001). Therefore, it is very important to study honeybee and pathogen interactions at both the individual and colony level.

1.5 Honeybee immune system

Similar to mammals, honeybees are vulnerable to the broad spectrum of parasites and pathogens. Mammals have developed and interconnected defense mechanisms, known as innate and acquired immunity. The acquired immune system is mediated by B lymphocytes and T lymphocytes (Parkin & Cohen, 2001). Honeybees, as with all insects, lack this acquired immune system. However, honeybees have a colony level (social immunity) and individual defense (innate immunity) mechanisms to combat pathogens.

1.5.1 Social immunity

Social immunity results from the collective action of individual group members within the colony to fight against the risk of disease transmission. This cooperative defense consists of behavioral, physiological, and organizational adaptations to prevent entrance, establishment and spread of the disease causing agents (Cremer *et al.*, 2007). The best studied examples of

social immunity in honeybees include hygienic behavior and grooming behavior. Hygienic behavior is the ability of worker bees to detect and remove the dead or diseased larvae from among the healthy brood (Boecking & Spivak, 1999; Spivak, 1996). A specific form of hygienic behavior involves detection and removal of brood infested by *Varroa* and has been termed Varroa-sensitive hygienic (VSH) behaviour (Harris, 2007; Ibrahim & Spivak, 2006). Grooming behavior is part of a behavioral defense in bees where bees groom themselves and other nestmates that eventually results in capturing and damaging parasitic mites (Rosenkranz *et al.*, 2010; Peng *et al.*, 1987). Additionally, bees collect plant resins (propolis) that have antimicrobial properties and use them to cement nest cavities which helps in reducing bacteria, viruses and other microorganisms (Kujumgiev *et al.*, 1999).

1.5.2 Innate immunity

At the individual level, honeybee defense mechanisms can be classified into a primary line secondary line of defense. The physical and chemical barriers include the outer cuticle exoskeleton and the peritrophic membranes lining the digestive tract of the individual bee and are considered as the first lines of defense that prevent pathogens from entering the body. They confer a non-specific immunity to honeybees (Chen & Siede, 2007). If a pathogen breaches the first line of defense barriers, honeybees can protect themselves from infection by employing cellular and humoral immune responses which represent the second line of defense (Chen & Siede, 2007).

The cellular immunity is mediated by the hemocytes that come into play the onset of microbial infections. The recognition of microbial pathogens is achieved by germline encoded proteins named Pattern Recognition Receptors (PRRs) that recognize the highly conserved structural motifs on the surface of pathogens, termed Pathogen Associated Molecular Patterns (PAMPs). After the binding of PAMPs to PRRs, a proteolytic cascade is activated that triggers the intracellular humoral pathway that controls antimicrobial peptides (AMP) expression and several other mechanisms such as phagocytosis, nodule formation, encapsulation, and melanization. During phagocytosis, circulating plasmatocytes or granulocytes are activated after the recognition of pathogens by surface receptors, leading to the engulfment and intracellular destruction of invading pathogens. Phagocytosis is the primary response to bacteria, but different bacteria may elicit different immune responses since phagocytosis is a complex and diverse process that requires several sequential signal transduction events (Marmaras & Lampropoulou, 2009). Nodulation is a method of entrapping large doses of bacteria by multicellular hemocyte

aggregates. Encapsulation is the most effective cell mediated immune mechanism works against larger targets such as protozoa or nematodes. After binding to the intruders, hemocytes form a capsule layer around the invader and finally lead to the death of the pathogen inside the capsule (Lemaitre & Hoffmann, 2007).

Insect humoral immune responses involve melanization, and the induction and secretion of antimicrobial peptides by the fat body, which is functionally equivalent to the liver in humans, and by hemocytes (Fallon & Sun, 2001). Melanization has a key role in the defense against a broad range of pathogens, in wound healing, in nodule and capsule formation around the parasites and the production of toxic intermediates that can kill the invading pathogens. Melanization is triggered by the activation of an enzyme, phenoloxidase that catalyzes the oxidation of mono- and diphenols to orthoquinones, which polymerize to melanin. The melanization cascade is triggered by injury or by recognition of PAMPs through PRRs (Marmaras & Lampropoulou, 2009; Lemaitre & Hoffmann, 2007). Antimicrobial peptides (AMPs) are a distinct group of molecules with a variety of antimicrobial properties. The majority of them are cationic or amphipathic peptides that interact with negatively charged lipid membranes and this interaction results in the formation of channels which enables leakage of essential cellular components from the bacteria. Another mode of action of AMPs is that they interact with the DNA, RNA, inner proteins or microbial cell compartments (Danihlík *et al.*, 2015). Four different types of AMPs such as abaecin, apidaecin, hymenoptaecin, and defensin have been identified in the *A.mellifera* hemolymph upon the induction of microbial infections. These peptides appear to be involved in the bee immune response to microbial infections (Danihlík *et al.*, 2015; Casteels & Tempst, 1994; Casteels *et al.*, 1990).

Based on bioinformatic analyses, the honeybee molecular immunity is thought to be mediated through four canonical innate immune pathways, namely the Toll pathway(Toll), the Immune deficiency pathway (Imd), the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway and the Jun-N-terminal Kinase (JNK) pathway (Doublet *et al.*, 2017; Evans *et al.*, 2006). Both Toll and Imd are transmembrane signal transduction proteins, serving as PRRs, that recognize PAMPs. The Toll pathway is activated by fungal and Gram-positive bacterial infections and the Imd pathway is often said to be specific for Gram-negative bacteria. After the activation of the receptors, several intracellular pathways lead to the induction of distinct NF-κB transcription factors, which ultimately result in the activation of immune effector genes, such as AMP encoding, and genes involved in the melanization pathway. Furthermore, Imd signaling also leads to the activation

of components of the JNK signaling pathway (Evans *et al.*, 2006; Hultmark, 2003). In insects, it has been observed that the JAK-STAT pathway mediates an antiviral immune response (Dostert *et al.*, 2005).

Several transcription level studies of honeybees characterized the involvement of immune pathways in the antiviral response. An insect ortholog of mammalian transcription factor NF-κB, dorsal-1A expression has a key role in limiting the DWV infection, and reduced expression of dorsal-1A helped virus multiplication (Nazzi *et al.*, 2012). Newly emerged adult bees orally infected with IAPV exhibited increased expression of Toll pathway members, *e.g.* Toll-6, cactus, and hymenoptaecin (Galbraith *et al.*, 2015). Honeybee larvae infected with either ABPV or ABPV plus *E. coli* did not produce AMPs that suggest that ABPV may suppress the bee immune response (Azzami *et al.*, 2012). Similarly, bees infected with Sindbis virus expressed lower AMPs than mock infected controls (Flenniken & Andino, 2013).

1.5.3 RNA interference (RNAi) pathway

The RNA interference (RNAi) is a post-transcriptional sequence-specific gene silencing mechanism in eukaryotic cells that regulate gene expression. The RNAi pathway is triggered by the detection of exogenous double-stranded RNA (dsRNA), an intermediate generated during RNA virus replication, by an RNase III-like enzyme called Dicer. After the recognition, Dicer cleaves the dsRNA into virus-derived short interfering RNAs (siRNA) of 20-25bp length. These siRNAs are then loaded onto the RNA Induced Silencing Complex (RISC). The RISC complex comprises Argonaute (AGO2), an endoribonuclease and the catalytic component of this multiprotein complex. The RISC complex specifically targets and degrades the viral RNA and thereby restricts the viruses' multiplication (Brutscher & Flenniken, 2015). The virus derived siRNAs and their role in antiviral defense mechanism has been observed in plants, fungi, and invertebrates (Ding, 2010).

The role of RNAi in honeybee antiviral defense has been demonstrated in studies where adult bees or larvae were artificially fed virus specific dsRNA. Adult bees fed with IAPV specific dsRNA resulted in increased bee survival and lower IAPV levels (Maori *et al.*, 2009). Similarly, larvae fed with DWV-specific dsRNA before inoculation with DWV had the lower viral burden and reduced wing deformities than the larvae fed with non-sequence specific dsRNA (i.e. dsRNA-GFP), through survival was not affected (Desai *et al.*, 2012). Deep sequencing analysis of bee samples collected from colony collapse disorder (CCD) colonies revealed abundant siRNA specific for DWV,

KBV, and IAPV (Chejanovsky *et al.*, 2014). This indicates that the honeybees in CCD affected colonies mounted an RNAi mediated antiviral response.

1.5.4 Resistance vs Tolerance

In host-pathogen interactions, the host has developed two broad defense strategies to counter the pathogen: resistance and tolerance. Host resistance is the ability to control the infection by targeting pathogen burden, whereas host tolerance is defined as the ability to reduce the negative impact of an infection on host fitness without affecting the pathogen burden (Raberg *et al.*, 2009).

In social insects like honeybees, it is also important to distinguish between tolerance and resistance at the individual level and at the colony level. A colony containing resistant bees is likely to be resistant at the colony level. The best example is mite-resistant colonies from Gotland and Avignon, which illustrates how varroa resistance (*i.e.* inhibiting mite's reproduction) at the individual level lead to the resistance at the colony level. By contrast, colonies composed of tolerant individuals at least also tolerant at the colony level, but may also be resistant at the colony level, as observed for Nosema-tolerant honeybees from Denmark (Kurze *et al.*, 2016; Hatjina *et al.*, 2014). The colonies selected for Nosema resistance, *i.e.* the low prevalence of Nosema at the colony level, seem to be tolerant at the individual level, *i.e.* they developed high infection intensities but survival was not affected. These Nosema-tolerant honeybees also escape parasitic manipulation of apoptosis, a defense mechanism honeybees use to kill the infected cell. *Nosema ceranae* reduces the host mediated apoptosis mechanism by enhancing the expression of Inhibitor of Apoptosis Protein-2 (*iap-2*) gene. However, the expression of the *iap-2* gene was significantly reduced in Nosema-tolerant bees compared to Nosema-sensitive bees, which suggest that the Nosema-tolerant bees have evolved a mechanism to circumvent the parasitic manipulations (Kurze *et al.*, 2015). The infected tolerant bees probably clear the infection by expelling apoptotic infected cells on defecating flights and reducing the spread of Nosema infection in the colony through the faecal-oral route (Kurze *et al.*, 2015).

1.5.5 Varroa-resistant honeybees

A stable host-parasite relationship exists between the varroa mite and its natural host, the eastern honeybee *Apis cerana*, where the host has developed several strategies to limit the growth of the varroa population. *A. cerana* detects and removes the worker brood that is infested with mites, such that the reproduction of the mites is limited to the drone brood present in the colony

(Rosenkranz *et al.*, 2010; Rath, 1999). The *A. cerana* also has effective grooming and hygienic behavior (Rath, 1999). Moreover, *A. cerana* drone pupae infested with multiple mites are too weak to open their hard cocoon cap themselves and the worker bees intentionally leave the infested drone cells, thus entombing the mites trapped inside the cell that die along with the pupa (Rosenkranz *et al.*, 2010; Peng *et al.*, 1987).

The occurrence of natural tolerance to *V. destructor* has also been demonstrated in specific honeybee races of the new host, *A. mellifera*, *e.g.* the Africanized honeybees in South and Central America and *A. mellifera scutellata* in Africa. These bees have developed a stable host-parasite relationship with *V. destructor* and do not require mite control treatments (Locke, 2016b; Correa-Marques *et al.*, 2003; Rosenkranz, 1999). Behavioral traits such as hygienic and grooming behavior are important mite-resistant traits in Africanized honeybees in South America and Africa (Guzmán-Novoa *et al.*, 1999; Rosenkranz, 1999; Corrêa-Marques & De Jong, 1998). The mite reproductive success in Africanized bees in South America has shifted from 50% to over 80%, although the Africanized honeybee population has remained stable without reports of increased mite infestation rates (Locke, 2016b; Carneiro *et al.*, 2007; Rosenkranz, 1999).

1.5.6 Mite resistant population in Europe

Most of the honeybee populations in the tropics are wild and feral population whereas majority of the bees in temperate North America and Europe are managed bees (Moritz *et al.*, 2007). This indicates that the honeybee population in South America and Africa are under constant selective pressure by varroa mites which helps in developing an adaptive resistant mechanism to varroa infestation. Furthermore, the use of chemicals to remove the mites in apiculture practices is a major barrier to developing adaptive strategies (Locke *et al.*, 2012a). However, there are reports of few managed and feral *A. mellifera* Honeybee populations in temperate regions that have survived mite infestations by means of natural selection (Oddie *et al.*, 2017; Le Conte *et al.*, 2007; Fries *et al.*, 2006).

Gotland, Sweden

One of the best-studied mite-resistant populations is located on the island of Gotland, Sweden. As a part of the natural selection experiment, 150 colonies were established on the island Gotland in the Baltic Sea in 1999 (Fries *et al.*, 2003). The colonies were brought from different parts of Sweden, with different genetic backgrounds, and the colonies were equally infested with an

average of 50 mites per colony. The infested colonies were kept without varroa treatment and were free to swarm. During the first three years, more than 80% of the colonies died due to high mite infestation rates that were well over the winter mortality threshold (Fries *et al.*, 2006; Fries *et al.*, 2003). After the initial losses, winter mortality decreased and a small number of honeybee colonies were able to survive and have continued to do so without any varroa treatment (Locke, 2016b; Fries *et al.*, 2006). The mite population is significantly reduced in mite-resistant bees compared to mite susceptible bees and further work has confirmed that low mite population growth rate was a characteristic adaptation of the host, rather than of the parasite (Fries & Bommarco, 2007). The Gotland mite-resistant honeybee population exhibits several mite-resistant traits, such as small colony size and reduced mite reproductive success, with only 50% of the mites producing viable mated daughter mites (Locke *et al.*, 2012a; Locke & Fries, 2011). Reduced mite reproductive success was observed in all colonies with a genetic origin from the Gotland mite-resistant population, which suggest that this trait has a strong genetic component (Locke, 2016a).

Gotland mite-resistant bees are also tolerant and resistant at the colony level to a few honeybee viruses (Locke *et al.*, 2014). Exploring host-virus interactions in this population at the individual and molecular level is a major theme of this thesis.

Avignon, France

Two additional, well-known mite-resistant *A. mellifera* populations are found in Avignon and Le Mans, France. Feral Honeybee colonies and abandoned managed colonies that were not treated for varroa for at least 3 years were established in Avignon (Le Conte *et al.*, 2007). These colonies were allowed to swarm freely and no mite control stratifies were employed. For over seven years, there was no significant difference in the colony mortality between mite-resistant colonies and mite-susceptible colonies. However, mite infestation rates were significantly lower in mite-resistant colonies than in mite-susceptible colonies (Le Conte *et al.*, 2007). One of the best-studied mite-resistant traits in this population is reduced mite reproductive success, with mite reproductive success in the Avignon mite-resistant population reduced by 30% compared to mite-sensitive population (Locke *et al.*, 2012a). Individual level virus tolerance and resistance in this population, compared to the other naturally surviving populations, is the focus of **Chapter IV.**

Østlandet, Norway

A managed Honeybee population in the Østlandet region of Norway has been surviving without varroa treatment for more than seventeen years. A recent study has shown that mite infestation levels were significantly lower in mite-resistant colonies than mite-susceptible colonies. Further, the study also showed mite reproductive success was reduced to 30% in mite-resistant population compared to mite-sensitive population (Oddie *et al.*, 2017). Individual level virus tolerance and resistance in this population, compared to the other naturally surviving populations, is the focus of **Chapter IV**.

Tiengenmeten, The Netherlands

This population was established in 2008 from a mixture of Dutch bees and bees from the naturally adapted Gotland population (Panziera *et al.*, 2017). It has been managed without varroa-control and with additional selection focused on traits such as colony growth rates and the ability to survive winter. The population today is stable without mite control and exhibits mite-resistance traits such as Varroa-Sensitive Hygiene Behaviour (Panziera *et al.*, 2017). Individual level virus tolerance and resistance in this population, compared to the other naturally surviving populations, is the focus of **Chapter IV.**

2 Aims of the thesis

The overall aim of the thesis was to investigate the role of microbial factors in the enhanced survival of mite-resistant (MR) honeybee colonies relative to mite-sensitive (MS) colonies.

The specific aims of the thesis were to:

- Identification of additional microbial factors that differ between MR and MS honeybees
- Confirming the previous observations and to identify microbiome differences between MR and MS honeybees
- Unravel the individual level susceptibility of MR and MS honeybees to oral virus infections

3 Materials and Methods

This section summarizes the methods used to conduct the study in the four chapters of this thesis. A more comprehensive description of the experimental design and methods is given in each individual chapter.

3.1 Honeybee colonies

The honeybee colonies used in the experiments of this thesis come from Gotland (Chapter I-IV), Avignon, (Chapter IV), Oslo (Chapter IV), Tiengemeten (Chapter IV) and are described in the introduction above. The control colonies used in all four studies of this thesis (Chapters I, II, III & IV) were unrelated to the mite-resistant colonies.

Figure 4. Collection of honeybee samples (photo: B.Locke)

3.2 Molecular detection of honeybee viruses (Chapters I – IV)

3.2.1 Nucleic acid extraction

In Chapters I & II, RNA was extracted from pools of 30 adult worker bees sampled from bee colonies at a specific occasion, whereas for Chapters III and IV, RNA was extracted from each experimental time course sample, containing either four larvae or five adult bees. All the samples were placed in a plastic mesh bag and ground to powder using liquid nitrogen and pestle. A primary homogenate was produced by mixing 200 µl/ bee of sterile water to each ground sample. A QiaCube robot (Qiagen) with RNAeasy protocol for plants (Qiagen) was used to extract the RNA from 100 µl of the primary homogenate. The extracted RNA concentration was estimated by NanodDrop, and the purified RNA was stored at -80˚C until further analysis.

In Chapter II, total DNA was extracted by a Qiacube automated extraction robot (Qiagen) from 100 µl of pooled bee homogenate using the DNeasy Blood & Tissue (Qiagen) kit. DNA purity and quantity was determined using a Nano-Drop, and the purified DNA was stored at -80˚C until further analysis.

3.2.2 RT-qPCR

Real-Time quantitative Polymerase chain reactions (RT-qPCR) is a standard technique used to quantify and diagnose honeybee pathogens (de Miranda *et al.*, 2013). This technique was used throughout all the studies. RP49 was added as an internal reference to normalize differences between samples in quality and quantity of RNA. The amount of target virus and RP49 were determined using iScript One Step RT-PCR kit (Bio-Rad) and EvaGreen qPCR kit (in study I) with SYBR Green as the detection chemistry and the Bio-Rad CFX connect thermocycler. For each assay, a ten-fold dilution series of known amounts of each target was also run, along with negative control for absolute quantification. The melting curve analysis and the conversion of RT-qPCR data to estimated copy numbers of each target RNA per bee was done as described previously (de Miranda *et al.*, 2013; Locke *et al.*, 2012b).

3.3 Metagenomic surveys (Chapter I & II)

3.3.1 Library preparation and high-throughput sequencing

In Chapter I & II, total RNA from all the individual MR or MS colonies at each sampling occasion was pooled in equimolar amounts. The pooled RNA was enriched for non-ribosomal RNA and the enriched RNA was used for sequencing. Ion Torrent sequencing libraries were prepared and sequenced at the National Genomics Centre, SciLife Lab, Uppsala, Sweden.

3.3.2 Bioinformatic analyses of RNA sequencing data

A number of bioinformatic tools were used to investigate the raw RNA sequencing data in Chapter I. Sequences were trimmed to remove adapter sequences, and filtered to remove those quality scores lower than Q<20 using PRINSEQ. The metagenomic classification of reads was performed by Kaiju web server, and the taxonomic assignments were visualized using Krona. In order to assemble genome sequences of known and novel viruses, the honeybee host sequences were removed by mapping to the *A.mellifera* genome assembly, Amel_4.5 using the mapping tool Bowtie 2. The unmapped sequences were extracted and assembled *de novo* by using Trinity. The assembled contigs were classified by BLAST querying the NCBI nucleotide (nt) and protein (nr) database using an E-value cut-off of 0.001. Contigs that were classified as viruses were aligned to the respective viral reference genome using CodonCode Aligner 6.02 (CodonCode Corporation) and concatenated, to generate study specific reference genomes for each virus. Then all the individual reads were remapped to back to the various study specific virus reference genomes using Bowtie 2, and these mapped reads were retrieved and counted using Samtools.

3.3.3 16S rDNA amplicon library preparation and bioinformatic analyses

In Chapter II, bacterial 16S rDNA Ion Torrent sequencing libraries were prepared and sequenced by the National Genomics Centre, SciLifeLab, Uppsala, Sweden. The sequencing data were analyzed on the Ion Reporter software (Thermo Fisher Scientific) using a custom designed metagenomics workflow version 5.2. The sequence reads were then aligned against the MicroSEQ 16S rRNA reference database and curated Greengenes database

using Megablast with E value 0.01. The genus and species percentage identity were set at 97% and 99%, respectively.

3.4 Virus infection experiments (Chapter III & IV)

3.4.1 Virus propagation and infectivity dose optimization

In order to obtain the sufficient DWV and ABPV inocula for oral virus infections experiments, reference DWV-A and ABPV virus stocks were propagated each in fifty white eyed pupae from Varroa free colonies from Åland. Each pupa was injected with 1 microliter of 1/10000 dilution of purified concentrated virus stock. A clarified crude extract was prepared by homogenizing all the 50 pupae in a blender with 10 ml 0.5 M Phosphate Buffer, pH 8.0 (DWV) or 10 ml 0.01 M Phosphate Buffer, pH 7.0 (ABPV), and stored at -80˚C. Before using these crude extracts for virus infection experiments, optimization experiments were conducted to identify the optimum infectious dose for the experiments, considering larval and adult bee mortality rates as optimization criteria. The highest single virus dose that did not cause larvae or adult bee mortality before 96 hour post inoculation was considered as optimum virus infectivity dose. The RT-qPCR analysis of crude extract showed the optimum single inoculation dose for larvae was $1.5\pm \times 10^{8}$ DWV genome equivalents and $5.4\pm \times 10^{7}$ ABPV genome equivalents and for adults $6.0\pm \times 10^{8}$ DWV genome equivalents and $2.1\pm \times 10^{8}$ ABPV genome equivalents.

3.4.2 Experimental design

All infection experiments were performed separately on newly emerged adult bees and on newly hatched larvae. Each infection experiment consisted of one group of DWV inoculated bees, one group of ABPV inoculated bees, and one group of inoculated control bees. The virus inoculation scheme comprised of bees fed with a single infectious dose for a short period of time followed by non-contaminated food, which circumvents the accumulation of virus inoculum. From each infection time course experiment, adult bees and larvae were sampled at different time intervals.

3.4.3 *In-vitro* larval infections

To obtain similar aged larvae, queens from the experimental colonies were confined to a single frame for 24 hrs for egg laying. First instar worker bee

larvae (between 24 – 36 hr old) were grafted with a Chinese grafting tool (Bienenzuchtgeräte, Graze, Weinstadt, Germany) into individual wells of 48 well tissue culture plates, each containing 10 µl of pre-warmed diet (Aupinel *et al.*, 2005). Extra larvae were always grafted to compensate for dead larvae because of the grafting procedure. Larvae were incubated for 24 hrs at 35˚C with a relative humidity of 96% to determine the larval viability. After the 24 hr of incubation, all dead and excess larvae were removed by making sure that one larva per well, 48 living larvae total, were retained for the infection experiment. All the viable larvae were then fed with larval diet, and the group of larvae to be inoculated with the virus were fed with larval diet blended with the optimum single inoculation dose of either DWV or ABPV, as specified above. The larvae were fed daily following the established protocols (Crailsheim *et al.*, 2013) and checked for mortality before feeding. Four larvae from each time point and each infection group were collected in a microcentrifuge tube and stored at -20°C until further analysis.

3.4.4 Adult bee cage infections

The adult bees were hatched on caged frames inside an incubator at 35˚C temperature and 96% relative humidity. For each inoculation group, fifty newly emerged bees from each colony were placed in separate Lyson queen cages. All the caged bees were fed with 2 ml of Bifor, a 66% w/w commercial honeybee sugar solution, over 24 hour period time. Whereas the virus inoculation group were fed with the optimum inoculation dose of either DWV or ABPV (as described above) mixed in 2 ml Bifor. After inoculation, all groups of bees were fed with uncontaminated Bifor for the rest of the time course experiment.

4 Results and Discussion

4.1 Viral metagenomics of Swedish mite-resistant honeybees (Chapter I)

In Chapter I, RNA samples from the MR and MS honeybee analyzed in the original study (Locke *et al.*, 2014) were analyzed using a viral metagenomics approach to characterize the complete virome. The Ion Torrent high throughput sequencing data were analyzed using distinct bioinformatics tools. The metagenome classifier, Kaiju, classified reads as viruses, bacteria or eukaryotic organisms, mostly fungi. The majority of the metagenome in MR bees was assigned to bacteria or fungi (except for the July 2009 MR sample), whereas for MS bees the majority of the metagenome was assigned to viruses. The majority of the viral reads in both the MR and MS honeybees belonged to well-known honeybee-infecting viruses in the *Iflaviridae* (DWV and SBV) and *Dicistroviridae* (BQCV) families. Furthermore, two other known honeybee infecting viruses: Lake Sinai Virus (LSV) with indeterminate classification and Apis rhabdovirus-1 (ARV-1) belonging to the *Rhabdoviridae* family, were also detected in both the MR and MS populations. The full length genomes of all the identified honeybee viruses were generated by *de novo* assembly of sequence reads and further confirmed by Sanger sequencing.

Both LSV and ARV-1 were identified for the first time in Swedish bees. Lake Sinai virus is common in honeybees around the world (Roberts *et al.*, 2017; Granberg *et al.*, 2013; Runckel *et al.*, 2011). In spite of the high prevalence and global distribution of LSV, its pathology remains unknown. LSV infection has been found in association with poor colony health (Daughenbaugh *et al.*, 2015) and Colony Collapse Disorder (CCD) (Cornman *et al.*, 2012). A BLAST search of the LSV found in these Swedish colonies

showed a close resemblance with LSV3, although with only about 90% nucleotide identity to the reference genome. However, the LSV3 isolates from MR and MS population were only about 92% identical to each other. Apis rhabdovirus-1 (ARV-1) is an enveloped negative-sense single strand RNA virus that was recently discovered in honeybees, varroa mites, and in the bumblebee *Bombus impatiens* (Levin *et al.*, 2017; Remnant *et al.*, 2017). A separate Apis rhabdovirus-2 (ARV-2) was also detected in Honeybees, and both ARV-1 and ARV-2 were phylogenetically closest to Farmington virus (FARV), a virus originally isolated from birds (Palacios *et al.*, 2013).

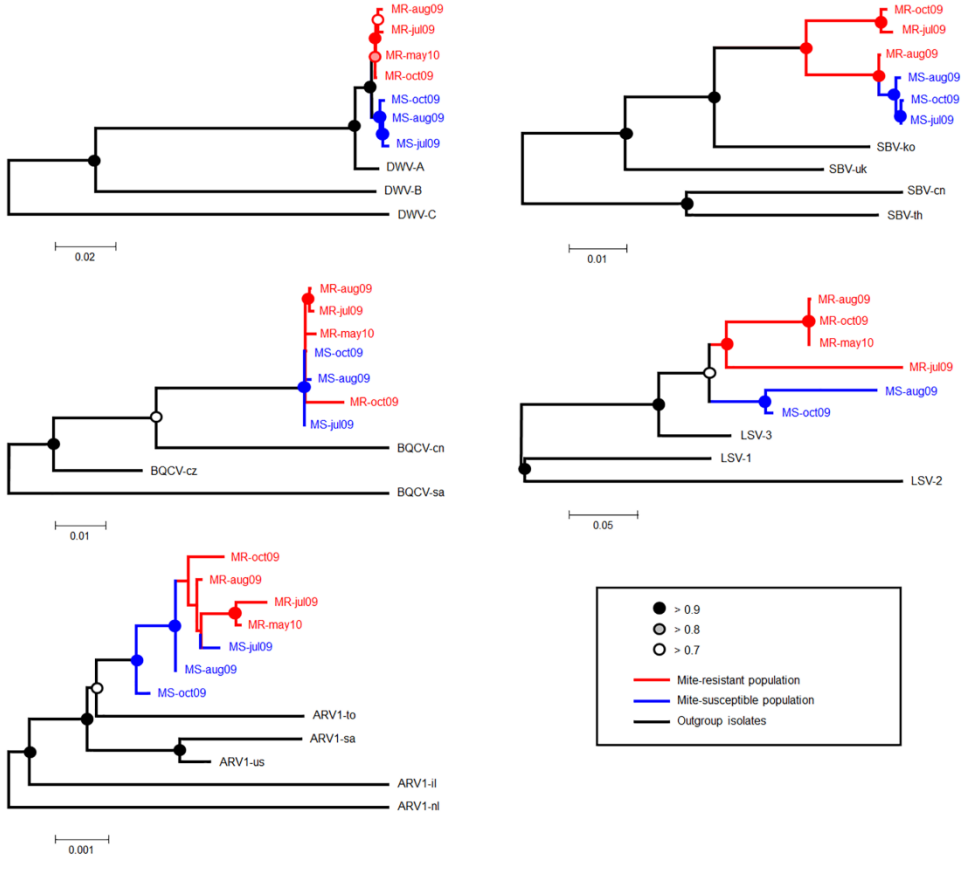

Figure 5. Phylogenetic analyses of identified honeybee virus genome sequences (Chapter I). Reconstruction of the phylogenetic relationships between the consensus virus sequences in the different seasonal samples of the MR (red) and MS (blue) honeybee populations. The reconstructions are obtained through a Maximum Composite Likelihood (MCL) approach, with the most likely reconstruction presented. The open, shaded and closed circles indicate different probability intervals for the partition of the taxa across the node in question, based on 500 bootstrap replicates (Thaduri *et al.*, 2018).

Phylogenetic analyses were conducted on the population level consensus sequences of all the identified bee viruses of the MR and MS honeybee populations. The analysis has shown a consistent separation between the MR and MS seasonal isolates by their population of origin: more clearly so for DWV, SBV and LSV, less well resolved for ARV-1 and BQCV (Figure 5). This separation into population-specific clusters indicates that there might be pre-existing genetic differences between the MR and MS population viruses from when the MR population was created in 1999 (Fries *et al.*, 2003) or could be the result of the selection for survival in the mite-resistant population, perhaps resulting in reduced virus virulence, which would be expressed as increased tolerance or resistance of the MR population to virus infections (Locke *et al.*, 2014).

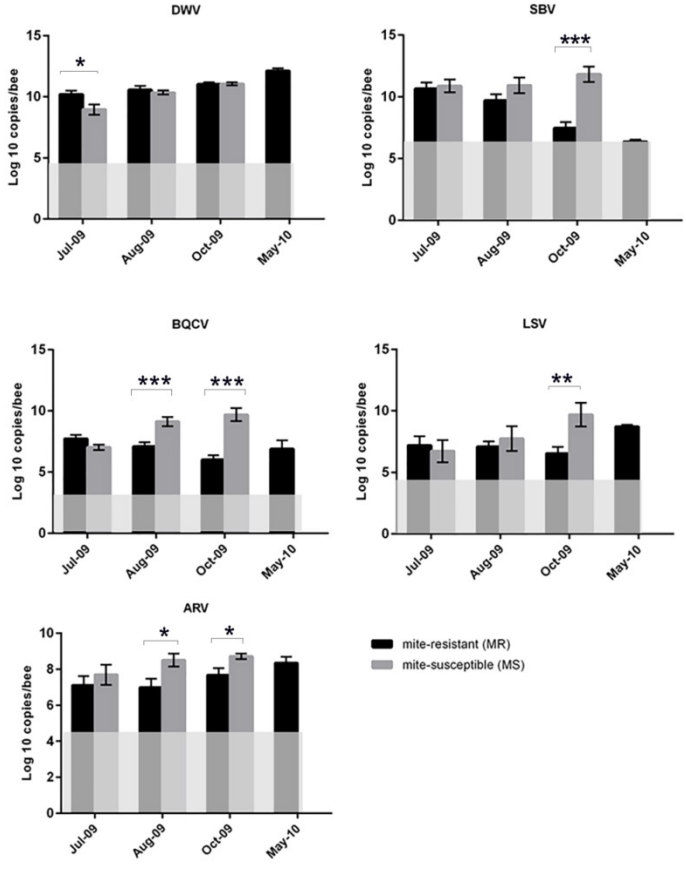

Figure 6. RT-qPCR data of major bee viruses. Virus titres in the 2009–2010 season for colonies in the MR and MS honeybee populations. Data are presented on a logarithmic scale for DWV, SBV, BQCV, LSV and ARV-1. The opaque area in each graph (May-2010 for DWV, BQCV,

SBV, and all seasons for LSV and ARV) represents the RT- qPCR detection threshold. Error bars indicate the standard deviation at each time-point. The asterisks indicate statistically significant differences, as determined by Welch's t-test (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$) (Thaduri *et al.*, 2018).

In order to determine the viral titres in the MR and MS honeybees, RT-qPCR based virus assays were performed. The virus assay results of ARV-1 and LSV shown a strong decrease in the MR titres compared to MS titres during the 2009 season, followed by a large increase in titres between October 2009 and May 2010 for the surviving MR colonies (Figure 6). No significant difference observed in LSV and ARV-1 virus titres in MR and MS population in July, but as the season progressed from summer to autumn the viral titres were significantly higher in the MS population. Further, the DWV and BQCV titres were much higher in May 2010 than at any time during 2009. In contrast to this SBV was almost disappeared from the MR colonies by May 2010, from a high during autumn 2009. This suggests that MR bees may have also developed resistance to LSV and ARV-1, as observed in the previous study in the case of BQCV and SBV (Locke *et al.*, 2014).

4.2 Microbiome differences between mite-resistant and mite-susceptible bees (Chapter II)

The honeybee gut microbiota has a primary role in food metabolism, neutralizing dietary toxins, biosynthesis of essential nutrients, and protection against pathogenic agents (Engel *et al.*, 2016; Kwong & Moran, 2016). By considering the protective function of bee microbiome against pathogenic agents, we hypothesized that the honeybee microbiome could have a role in the enhanced survival of Gotland mite-resistant honeybees. To investigate this, bacterial community differences between Swedish MR and MS honeybee colonies were studied by using 16S rDNA sequencing technology. We found that the bacterial microbiome in both MR and MS bees was dominated by three major phyla, *Proteobacteria*, *Firmicutes,* and *Actinobacteria*. In particular, bacteria belonging to *Lactobacillaceae*, *Bifidobacteriaceae*, *Snodgrassella alvi* and *Gilliamella apicola* were highly abundant which is consistent with previous observations (Kwong & Moran, 2016; Martinson *et al.*, 2012).

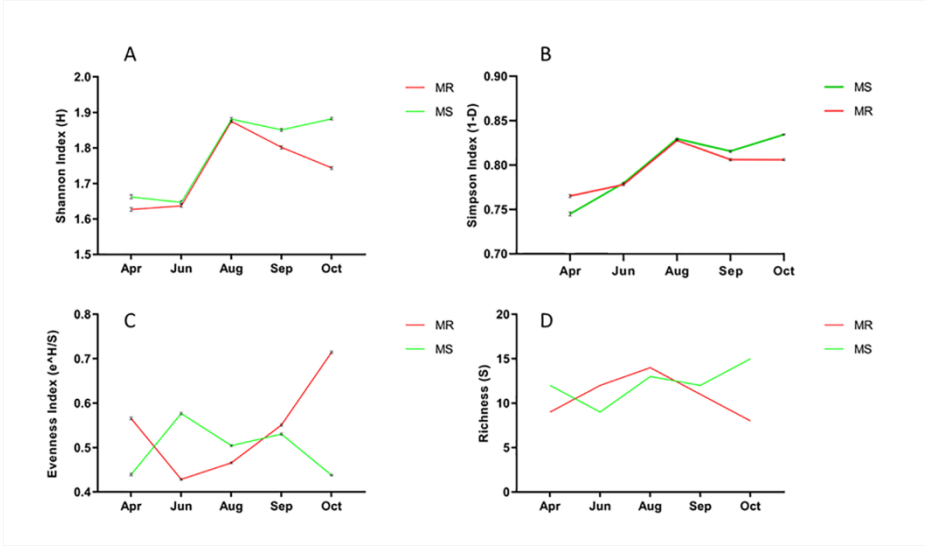

Figure 7. A comparison of four different alpha-diversity estimates. A) Shannon H-index; B) Simpson 1-D index; C) Evenness (e^H/S); D) Richness for the MR (red) and MS (green) colonies between April and October 2015. Illustration adapted from Chapter II.

The bacterial richness and diversity were measured by Shannon H-index, the Simpson 1-D index, the Evenness e^H/S index and the Richness S index. The results demonstrated a general increase in microbial biodiversity as the season progresses and a separation between the MR and MS samples in microbial diversity towards the end of the season, with a higher diversity in the MS colonies than in the MR colonies at the end of the season (Figure 7). This observation was further supported by species richness with more OTU's recovered from the MS samples towards the end of the season than from the MR samples. Although the species richness and diversity increased in MS towards the end of the season, the evenness (e^H/S) index was increased in the MR population (Figure 7). This suggests that the bacterial communities in the MR colonies were more evenly distributed than the MS colonies especially at the beginning and the end of the season, *i.e.* the critical parts of the year for colony survival, whereas the MS colonies have greater balance during the middle part of the season. An overabundance of non-core bacteria could be a sign of dysbioses associated with a disease, pathogen or stress (Napflin & Schmid-Hempel, 2018; Cariveau *et al.*, 2014; Hamdi *et al.*, 2011). In this regard, the presence of the genus *Serratia* only in MS colonies and its complete absence in the MR colonies during the critical early spring and late autumn parts of the season was particularly interesting. *Serratia* is an opportunistic pathogen of mammals and insects, reproducing primarily in the hemolymph

that has associated with varroa transmission and individual bee mortality in overwintering colonies (Raymann *et al.*, 2018; Burritt *et al.*, 2016). Furthermore, in a recent study *Serratia* isolated from bees caused increased mortality following treatment with agrochemicals and antibiotics (Raymann *et al.*, 2017).

A non-metric multidimensional scaling (NMDS) analysis with Bray-Curtis dissimilarity measure was used to illustrate the differences in bacterial community composition between the MR and MS colonies for each of the five sampling occasions during the season. An analysis of similarity (ANOSIM) demonstrated that these patterns were significantly different for the five sampling occasions (ANOSIM: $P = 0.0001$), but that the patterns for the MR and MS colonies overlapped at each sampling occasion. Then, a *Post hoc* non-parametric multivariate analysis of variance (NPMANOVA) was conducted to make pairwise comparisons of bacterial community structure between MR and MS colonies. The results showed significant differences in bacterial community composition across the season, for both the MR and the MS colonies, but that there was no great difference in bacterial community composition between the MR and MS colonies at each sampling occasion. We have also tested the abundance previously identified honeybee viruses, DWV, SBV, BQCV, LSV, and ARV-1. The results showed no significant difference between the MR and MS colonies for any of the viruses at any point during the season, although SBV titres were significantly lower in the MR colonies relative to the MS colonies in April 2015 and also trending lower in autumn 2015. These results suggest that the bacterial and viral microbiome may play only a minor role in the enhanced survival of the Gotland mite-resistant honeybees.

4.3 Virus infectivity studies (Chapter III & IV)

Although *Varroa destructor*, in combination with the viruses it transmits, is inarguably the leading cause of honeybee mortality world-wide (Rosenkranz *et al.*, 2010), several studies also showed viruses by themselves were also implicated in honeybee colony mortality in temperate regions (Dainat *et al.*, 2012; Berthoud *et al.*, 2010; Highfield *et al.*, 2009). Therefore, it is imperative to study virus host interactions and adaptations under natural selection especially at the individual level, independent of the confounding effects of varroa mite parasitism. In this regard, **Chapter III & IV** investigated how oral inoculation with DWV and ABPV affects the laboratory reared larvae and caged adult bees from MR and MS colonies, through studies of the virus infection time course and adult bee mortality rates.

In the larval infection experiments, both the DWV and ABPV inoculations were successful at establishing an infection, in that the virus titres increased after the zero time point and was significantly elevated throughout the time-course compared to the un-inoculated controls (Figure 8). Most importantly, no differences were detected between the MR and MS larvae in either DWV or ABPV susceptibility at any time during the experimental time course. Furthermore, no significant differences were detected between the MR and MS larvae in DWV or ABPV background infection levels, at 0 hours post-infection (hpi), before the experiment started.

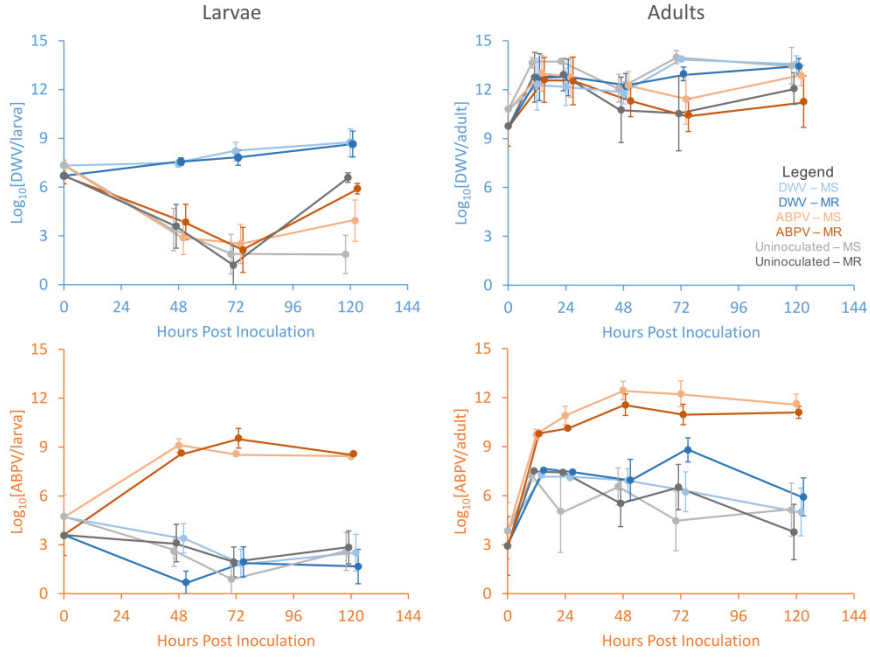

Figure 8. Graphical representation of the raw data from the deformed wing virus (DWV; blue panels - top) and acute bee paralysis virus (ABPV; orange panels - bottom) inoculation experiments in honeybee larvae (left panels) and adult worker bees (right panels). The line diagrams represent bees inoculated with either ABPV (orange lines), DWV (blue lines), or un-inoculated bees (grey lines) from either mite-susceptible (MS; light-shaded colours) or mite resistant (MR; dark-shaded colours) colonies. Shown are the average DWV (blue) and ABPV (orange) virus titres plus standard errors across all replicate trials and colonies of each population, in relation to the time post-inoculation (hours) (Thaduri *et al.*, 2019).

In the adult bee infection experiments also no significant differences were observed between MR and MS bees in any of the infection time-courses, for either the DWV infection experiment or the ABPV infection experiment, or for

both the inoculated bees and the un-inoculated/alternate virus-inoculated control bees (Figure 8). For DWV infection in adults, the background titres in newly hatched adult bees prior to inoculation were extremely high in both MR and MS bees, making it difficult establish whether DWV inoculation had any additional effect on the DWV titres. However, after 72 hpi, the DWV titres were increased in the DWV-inoculated bees compared to the un-inoculated/ABPV-inoculated controls and show marginal evidence that the DWV-inoculation has established an infection. In contrast to this, the ABPV inoculation experiment showed much earlier and much clearer separation in ABPV titres between the ABPV-inoculated bees and the un-inoculated/DWV-inoculated control bees demonstrating an infection developed due to inoculation. In both larval and adult bee infection studies, oral inoculation with DWV has no effect on the background infection dynamics of ABPV and *vice versa*, that indicates that viruses did not appear to compete or interfere with each other. This supports earlier research showing that viruses develop equally well in isolation or when co-inoculated with other viruses, and also induce entirely different transcriptional responses (Ryabov *et al.*, 2016).

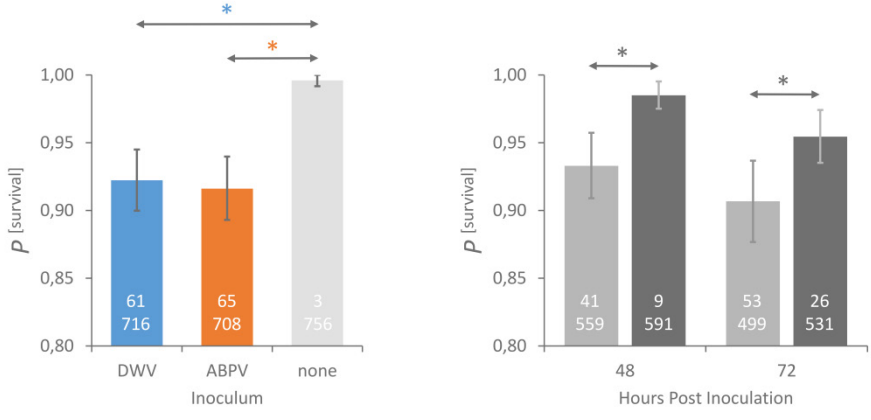

Figure 9. Left panel: The predicted marginal mean and 95% confidence intervals across all sampling time points, colonies and populations of the probability of survival for the adult bees inoculated with either DWV (blue), ABPV (orange) or uninoculated (grey). Right panel: The predicted marginal mean and 95% confidence intervals across all virus inoculation trials of the probability of survival for the adult bees from either the MR (dark grey) or MS (light grey) colonies, at two different time-points post-inoculation. Statistically significant differences are marked with an asterisk. The absolute numbers of dead (top number) and live bees (bottom number) involved in the estimations are shown in each column (Thaduri *et al.*, 2019).

The most interesting observation in this study was a clear difference between the MR and the MS bees in the survival rates of the virus-inoculated adult bees (Figure 9). In both DWV and ABPV inoculated bees there was a significantly higher survival rate for MR bees than for MS bees, both at 48 hpi and 72 hpi. Despite having near identical DWV and ABPV viral levels across the infection time courses, MS adults had significantly higher mortality than MR adults. This suggests that host tolerance, instead of resistance, is an important component of the naturally adapted survival mechanisms of this population. Further, the observed individual level tolerance response to virus infections in Gotland MR bees complements earlier work demonstrating a colony-level tolerance to DWV (Locke *et al.*, 2014).

In Chapter III we observed that Gotland MR bees have adapted a natural tolerance to DWV and ABPV at the individual level. Considering this, in **Chapter IV** we investigated whether this tolerance was specific for the Gotland MR population, or could also be found in three other mite-resistant populations, from Norway, The Netherlands, and France. As **Chapter III**, individual larvae and newly emerged adult bees from the different MR populations, and a Control MS population were tested for susceptibility to oral ABPV or DWV infections, in laboratory virus infection time-course studies. Virus susceptibility was determined by comparing the virus titres of virus-inoculated bees relative to both the pre-experiment background virus titres and the natural infection development in un-inoculated control bees, across the time-course. Further, adult bee mortality overtime was also documented.

The virus infection time course for the two viruses followed the same trends as in **Chapter III**. For the DWV infection experiments in larvae, a clear increase in DWV viral titres was observed after the virus inoculation in all five populations, and no increase in DWV titre was observed in either the non-inoculated control or the ABPV-inoculated samples, except for the French population and, to a lesser degree, the Dutch population. However, DWV infection experiments in adults it was difficult to establish a successful infection due to the very high background DWV titres in the populations, as was also found in **Chapter III**. For the ABPV infection experiments, there was clearer evidence of a slow progressive increase in ABPV titres; in both larvae and adults that suggests an active infection by ABPV.

Although both inoculated and background virus titres tended to increase slightly over time in both the larval and adult experiments and for all populations, these increases were not large enough with respect to the replicate error variance to be significant. Therefore, in the remaining analysis, values from the time-course were pooled, effectively treating time post-inoculation as a random factor in the GLMM analyses. This meant that the data from the

entire time-course were compressed into a single value, which can be taken as a measure of the overall susceptibility of the population to DWV or ABPV infection over the entire time-course, as well as the susceptibility to background DWV or ABPV infections due to inoculation with the alternate virus. The results showed a clear difference between the different populations in susceptibility to DWV and ABPV infection, either through inoculation and/or as background infections, where the MS-Control population exhibited the highest susceptibility.

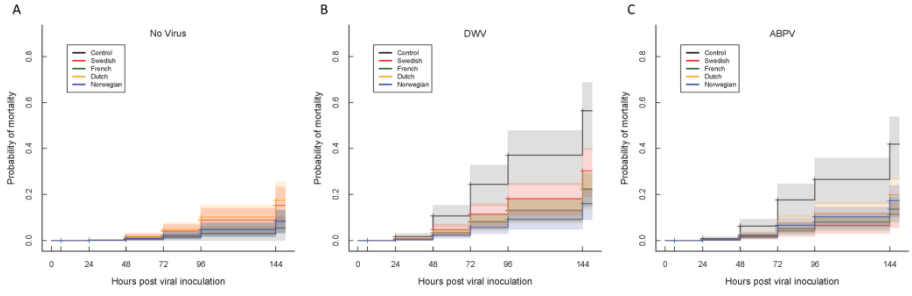

Figure 10. Cox Proportional Hazard curves for the Dutch, French, Norwegian and Swedish varroa-resistant honeybee populations and the varroa-susceptible Control population for the non-inoculated (Figure 2a), DWV-inoculated (Figure 2b) and ABPV-inoculated (Figure 2c) adult bee virus infection experiments. The shaded areas represent the 95% confidence intervals for the proportional hazard lines, based on the data from four replicate honeybee colonies for each population. The effects of varroa infestation and apiary origin of the colonies have been accounted for in the models. Illustration adapted from Chapter IV.

The effect of virus inoculation on adult bee mortality was also tested. The mortality data was generated by counting the number of dead bees every day until the completion of the experiment, *i.e.* 144 hours post-infection. The mortality data was analysed with reference to the population of origin and the inoculation treatment, using Cox's Proportional Hazard analyses. The results showed that after the oral inoculation with either DWV or ABPV, adults from the varroa-susceptible Control population were much more likely to die than bees from any of the varroa-resistant target populations (Figure 10). However, without virus inoculation there was no difference between any of the populations in background mortality. This clearly shows that oral infection with DWV and ABPV is tolerated better in Varroa-resistant bees than the Varroa-sensitive bees, and these results are in line with the previous studies, tolerance of DWV at the colony level (Locke *et al.*, 2014), and tolerance of ABPV and DWV at the individual level (Thaduri *et al.*, 2019). Tolerance is a host defense strategy that reduces the negative impact of infection on host

fitness, and the concept of tolerance as a defense strategy was observed in plant immunity, animal immunity and in invertebrates (Raberg *et al.*, 2009; Ayres *et al.*, 2008).

The confounding effect of colony level varroa infestation on the virus susceptibility was also studied. The results showed no significant relationship between colony-level varroa infestation and DWV or ABPV susceptibility in larvae, and which is no unexpected because varroa reproduces on developing pupae and has no direct interaction with young brood (Rosenkranz *et al.*, 2010). In contrast, higher colony-level varroa infestation was associated with greater susceptibility of adult bees to both DWV and ABPV infection **(Chapter IV)**. Furthermore, varroa infestation significantly increased the mortality of both non-inoculated and ABPV-inoculated bees, but not of DWV-inoculated bees. This absence of an independent effect of varroa infestation on adult bee mortality in only DWV-inoculated bees may explain the co-evolution of DWV with the mite, and how its association with mite helped the spread of DWV around the world (Wilfert *et al.*, 2016; Locke *et al.*, 2014; Mondet *et al.*, 2014; Martin, 2001).

5 Conclusions

5.1 Viral metagenomics of Swedish mite-resistant honeybees

- Viral metagenomics has been successfully applied to comprehensively analyze the virome of Swedish mite-resistant honeybee population.
- *Apis rhabdovirus-1* (ARV-1) and *Lake Sinai virus* (LSV) were identified for the first time on Swedish honeybees and near complete genome sequences of these two viruses were obtained.
- Gotland mite-resistant bees appear to have developed a colony-level resistance to ARV-1, LSV, SBV, and BQCV and tolerance to DWV.
- Phylogenetic analyses showed a consistent separation of all the identified bee viruses between the MR and MS seasonal isolates by their population of origin.

5.2 Microbiome differences between mite-resistant and mite-susceptible bees

- The bacterial microbiome in both MR and MS bees was dominated by three major phyla, *Proteobacteria*, *Firmicutes,* and *Actinobacteria*.
- The mite-resistant honeybee bacterial community was more evenly distributed than MS honeybees during the early and late parts of the season.
- A non-metric multidimensional scaling (NMDS) analysis showed no great difference in bacterial community composition between the MR and MS colonies at each sampling occasion.

- The previously studied honeybee viruses DWV, SBV, BQCV, LSV, and ARV-1 also showed no significant differences in abundance between the MR and MS colonies at any of the sampling occasions, except for SBV.

5.3 Virus infectivity studies

- Oral inoculation with viruses was successful at establishing an infection, the only exception was with DWV infection in adults where high background DWV titres precluded any conclusive evidence of infection.
- In both larval and adult bee infection studies, oral inoculation with DWV had no effect on the background infection dynamics of ABPV and *vice versa*, that indicates that viruses did not appear to compete or interfere with each other.
- Adult bees from the mite-susceptible Control population had much higher mortality after oral DWV or ABPV inoculation than Gotland mite-resistance bees and bees from the three other varroa-resistant populations
- Varroa infestation had a significant effect on virus susceptibility; higher colony-level varroa infestation was associated with greater susceptibility of adult bees to both DWV and ABPV infection.

Overall, this thesis reveals that Gotland mite-resistant honeybees at the colony level have adapted virus tolerance and survive better with high levels of DWV. Further, mite-resistant honeybees at the individual level are also tolerant to orally inoculated DWV and ABPV. By considering the threat of viruses on economically important species such as honeybees, it is important to study virus-host interactions. Especially, honeybee molecular antiviral defense mechanisms and immune functions would give a better idea about host-pathogen interplay.

6 Popular science summary

The Western honeybee, *Apis mellifera*, is the most versatile, ubiquitous, and economically important managed pollinator worldwide. In addition to commercial pollination services and products, honeybees play a significant role in sustaining natural plant biodiversity as an ecosystem service provider. The ectoparasitic mite, *Varroa destructor*, together with its associated viruses is the most significant threat to honeybee (*Apis mellifera*) health world-wide. Since the introduction of varroa to the new host species, The European honey bee (*Apis mellifera)*, it has been responsible for the near complete eradication of wild and feral honey bee populations in Europe and North America. Without mite control strategies the mite populations in the colony will grow exponentially and the honey bee colony will succumb to the development of overt virus infections ultimately leading to the death of the colony in 2-3 years. However, a unique honeybee population on the island of Gotland, Sweden, has acquired resistance to the mite through a natural selection process and has survived without active mite control for more than 20 years. Besides having adapted resistant traits that reduced the mite infestations in these colonies, this population also seems to have adapted tolerance and resistance to virus infections, surviving winter with lower virus infection levels compared to unselected mite-susceptible colonies. This suggests that virus-host interactions may play a key role in the long-term survival of this population.

The aim of this thesis was to investigate the role of the viral and bacterial microbiome in the enhanced survival of the mite-resistant honeybees on Gotland, to compare the role of virus tolerance and resistance in other naturally selected mite-resistant honeybee populations, similar to the Gotland population, and to unravel individual level virus-host interactions in honeybees.

First, we have used viral metagenomics, a genomic sequencing method useful tool to identify all viruses present in a sample, to identify all the viruses

in Swedish mite-resistant honeybees. This method uses a combination of high-throughput sequencing and different bioinformatics tools. By using this methodology, we found that along with previously known honey bee viruses two new viruses, Lake Sinai virus and Apis rhabdovirus-1, in Swedish honey bees. Further molecular studies showed that Gotland mite-resistant bees have developed a colony-level resistance to these viruses, and tolerance to *Deformed Wing Virus* (DWV), the virus most commonly associated with a mite infestation. Earlier studies found that microbiome plays a significant role in protecting the host against pathogens. In this regard, we hypothesized that honeybee microbiome may play a role in the enhanced survival of mite-resistant honeybees. To investigate this we have used 16S rDNA amplicon sequencing technology to study the bacterial community difference between mite-resistant mite-susceptible bees. The results indicated little differences between MR and MS bees throughout the season. Finally, individual level susceptibility of MR and MS honey bees to oral virus infection was tested for DWV virus and Acute bee paralysis virus (ABPV). The results demonstrate that DWV and ABPV infection dynamics were nearly identical in MR and MS bees, but that bees from the MR honeybee populations had significantly lower mortality rates than bees from the MS population.

In conclusion, the results of this thesis present strong evidence that naturally adapted mite-resistant honeybees have also adapted through a natural selection process, tolerance and resistance to virus infections at both the colony and individual level. The bacterial microbiome did not appear to play a role in the enhanced survival of Swedish mite-resistant honeybees but more studies are required to investigate potential bacteria-virus interactions on honeybee health. Future work should aim to identify key genomic regions associated with virus resistance and tolerance that can be incorporated into honeybee breeding programs to improve honeybee health.

7 Populärvetenskaplig sammanfattning

Det europeiska honungsbiet, *Apis mellifera*, är den mest mångsidiga, utbredda och ekonomiskt viktiga hanterade pollinatören världen över. Förutom kommersiella pollineringstjänster och -produkter, spelar honungsbin en viktig roll för att upprätthålla den naturliga biologiska mångfalden genom att tillhandahålla ekosystemtjänster. Det parasitiska kvalstret, *Varroa destructor*, tillsammans med dess tillhörande virus är det viktigaste hotet mot honungsbiets (*Apis mellifera*) hälsa världen över. Sedan varroa introducerades till den nya värdarten, det europeiska honungsbiet (*Apis mellifera*), har den orsakat en nästan fullständig utrotning av vilda och förvildade honungsbipopulationer i både Europa och Nordamerika. Utan strategier för att kontrollera och hålla ner kvalstermängden i bisamhället ökar kvalsterpopulationerna exponentiellt och virusinfektioner utvecklas och sprids bland bina vilket leder till att bisamhället dukar under och slutligen, inom 2-3 år, dör. Emellertid har en unik honungsbipopulation på ön Gotland, Sverige, utvecklat en motståndskraft mot kvalstret genom en naturlig urvalsprocess. Denna population har överlevt utan aktiv kvalsterkontroll i mer än 20 år. Förutom att ha anpassade resistenta drag som minskat kvalsterangreppen i dessa bisamhällen, verkar denna population även ha en anpassad tolerans och resistens mot virusinfektioner och överlever vintern med lägre virusinfektionsnivåer jämfört med oselekterade kvalsterkänsliga bisamhällen. Detta antyder att virus-värdinteraktioner kan spela en nyckelroll för den långvariga överlevnaden hos denna population.

Syftet med den här avhandlingen var att undersöka vilken roll det virala och bakteriella mikrobiomet har för den förbättrade överlevnaden hos de kvalsterresistenta honungsbin som finns på Gotland, att jämföra vilken roll virustolerans och resistens har i andra naturligt utvalda kvalsterresistenta honungsbipopulationer liknande Gotlandspopulationen och för att upptäcka virus-värdinteraktioner på individnivå hos honungsbin.

För att identifiera alla virus hos svenska kvalsterresistenta honungsbin har vi använt viral metagenomik, en genomisk sekvenseringsmetod vilken är ett användbart verktyg för att identifiera alla virus som finns i ett prov. Denna metod använder en kombination av sekvensering med hög kapacitet och olika bioinformatiska verktyg. Genom att använda denna metodik fann vi tillsammans med tidigare kända honungsbivirus två nya virus, Lake Sinai-virus och Apis rhabdovirus-1, i svenska honungsbin. Ytterligare molekylära studier visade att gotländska kvalsterresistenta bin på samhällsnivå har utvecklat en resistens mot dessa virus och tolerans mot Deformed Wing Virus (DWV), det virus som oftast är associerat med kvalsterangrepp. Tidigare studier fann att mikrobiomet spelar en viktig roll för att skydda värden mot patogener. I detta avseende antog vi att honungsbi-mikrobiomet kan spela en roll i den förbättrade överlevnaden hos kvalsterresistenta honungsbin. För att undersöka detta har vi använt sekvensering av 16S rDNA för att studera skillnaden i bakteriefloran mellan kvalsterresistenta (KR) och icke-resistenta (IR) bin. Resultaten indikerade små skillnader mellan KR- och IR-bin under hela säsongen. Slutligen testades känsligheten på individnivå hos KR- och IR-honungsbin för oral virusinfektion med avseende på DWV-virus och Acute bee paralysis virus (ABPV). Resultaten visar att DWV- och ABPV-infektionsdynamiken nästan var identisk hos KR- och IR-bin, men att bin från KR-honungsbipopulationerna hade signifikant lägre dödlighet än bin från IR-populationen.

Sammanfattningsvis visar resultaten från denna avhandling att naturligt anpassade kvalsterresistenta honungsbin också har anpassats genom en naturlig selektionsprocess, tolerans och resistens mot virusinfektioner både på samhällsnivå och individnivå. Bakteriemikrobiomet tycktes inte spela någon roll i den förbättrade överlevnaden hos svenska kvalsterresistenta honungsbin men fler studier krävs för att undersöka potentiella bakterie-virusinteraktioner inom honungsbihälsa. Framtida studier bör studera och identifiera viktiga genomiska regioner hos honungsbin förknippade med virusresistens och tolerans och som också kan integreras i avelsprogram för att förbättra honungsbihälsan.

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