Host-Parasite Adaptations and Interactions Between Honey Bees, *Varroa* Mites and Viruses

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Cover: *Varroa* mite on a honey bee
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Abstract

The ectoparasitic mite, *Varroa destructor*, has become the largest threat to apiculture and honey bee health world-wide. Since it was introduced 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. Currently, the apicultural industry depends heavily on chemical *Varroa* control treatments to keep managed colonies alive. Without such control the mite populations in the colony will grow exponentially and the honey bee colony will succumb to the development of overt virus infections that are vectored by the mite typically within three years.

Two unique sub-populations of European honey bees (on Gotland, Sweden and in Avignon, France) have adapted to survive for extended periods (over ten years) without the use of mite control treatments. This has been achieved through a natural selection process with unmanaged mite infestation levels enforcing a strong selection pressure. This thesis reveals that the adaptation acquired by these honey bee populations mainly involve reducing the reproductive success of the parasite, that the different populations may have evolved different strategies to do so, and that this mite-resistant trait is genetically inherited. In addition, results of this thesis demonstrate that chemical mite control treatments used by beekeepers to inhibit the mite population growth within a colony can actually worsen bee health by temporarily increasing the bee's susceptibility to virus infection.

The results of this thesis highlight the impact that apicultural practices otherwise have on host-parasite interactions and the development of disease in this system. Possible solutions to the threat of *Varroa* are discussed such as the potential to breed for mite-resistant honey bees, which may offer a sustainable long-term solution, and the need for better general beekeeping techniques that reduce the use of chemical treatments and inhibit the spread of disease.

Keywords: natural selection, coevolution, parasite resistance, host-parasite interaction, honey bee epidemiology, disease resistance breeding, acaricides, deformed wing virus, *Varroa destructor*, *Apis mellifera*

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In memory of my mother

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List of Publications

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

I **Locke, B.** & Fries, I. (2011) Characteristics of honey bee colonies (*Apis mellifera*) in Sweden surviving *Varroa destructor* mite infestation. *Apidologie,* 42(4), 533-542.

II **Locke, B.**, Le Conte, Y., Crauser, D. & Fries, I. (2012) Host adaptations reduce the reproductive success of *Varroa destructor* in two distinct European honey bee populations. *Ecology and Evolution,* 2(6), 1144-1150.

III **Locke, B.** Reducing *Varroa destructor* reproduction is a genetically inherited trait in a naturally adapted *Varroa*-resistant honey bee population. (Manuscript).

IV **Locke, B.**, Forsgren, E., Fries, I. & de Miranda, J.R. (2012) Acaricide treatment affects viral dynamics in *Varroa destructor*-infested honey bee colonies via both host physiology and mite control. *Applied and Environmental Microbiology,* 78(1), 227-235.

1 Introduction

The European honey bee (*Apis mellifera; Figure 1*) is the most valuable insect pollinator globally. An estimated 35 % of human food consumption depends on insect pollination and of those crops, over 90 % of them rely on honey bee pollination services (Klein *et al.*, 2007). Pollination services for agricultural crop production have been estimated at €22 billion in Europe and €153 billion world wide (Gallai *et al.*, 2009). Additionally, the value of honey production by honey bees in Europe alone is about €140 million. Even though the honey bee can be economically valuated for its services and products used by humans, the honey bee's role in sustaining natural plant biodiversity as an ecosystem service provider is immeasurable (Biesmeijer *et al.*, 2006; Potts *et al.*, 2010).

Recent catastrophic mass honey bee colony losses are causing overall population declines in the United States and Europe. This occurrence is drastically threatening the apicultural industry, while causing economic and ecological pressures on agricultural crop production and ecosystem services respectively. Although a variety of causal agents have been suggested to explain these colony losses, recent reports point to the spread of honey bee diseases and parasites as an explanation for these mass colony losses (Neumann & Carreck, 2010; Ratnieks & Carreck, 2010), which ironically is facilitated through intensified management practices of the beekeeping industry (Fries & Camazine, 2001).

The ectoparasitic mite, *Varroa destructor,* is at the core of colony losses worldwide and has been responsible for the nearly complete eradication of wild and feral honey bee populations in Europe and North America since it was introduced to this new honey bee host species (Guzman-Novoa *et al.*, 2010; Le Conte *et al.*, 2010). No other pathogen has had such a large impact on beekeeping or honey bee research through the history of apiculture. The mite weakens the honey bee's immunity and their susceptibility to other

environmental stressors and vectors lethal honey bee viruses (Boecking & Genersch, 2008). Currently, the apicultural industry depends heavily on chemical *Varroa* control treatments to keep managed colonies alive. These chemical controls can leave residues in hive products, have negative impacts on honey bee health, and remove selective pressures that would be required for host or parasite adaptations towards a stable host-parasite relationship (*see section 3.4*). Therefore, there is an urgent need for a sustainable solution to the threat of *Varroa* mites for the economic viability of apiculture and agriculture, as well as for honey bee health, conservation and for ecosystem services.

Understanding the interactions and adaptations between honey bees and *Varroa* mites is an essential first step towards achieving a long-term sustainable solution. This thesis presents aspects of host-parasite adaptations and interactions by investigating unique honey bee populations that, through natural selection, have adapted to be able to survive *Varroa* mite infestation without beekeeping management or *Varroa* control (**Papers I, II & III**). Further, interactions between honey bees, *Varroa* mites, the honey bee viruses vectored by *Varroa*, and the chemical *Varroa* control treatments used by beekeepers are explored in this work (**Paper IV**).

Figure 1. European honey bee (*Apis mellifera*) pollinating apple blossoms (Photo: B. Locke).

2 Honey bees

This section focuses on the specific features of honey bees that are important for understanding the host-parasite system presented in this thesis. For a more thorough description of honey bee biology, see for example *The Biology of the Honey Bee* (Winston, 1991).

2.1 Honey bee societies

Honey bees are eusocial insects living in perennial colonies with overlapping generations, cooperative brood care and a reproductive division of labor. The colony consists of three castes: female worker bees which can number between 15000-50000 depending on the time of year with a peak in the summer and a dearth in the winter; a few hundred male drones usually present in the spring; and one reproducing female queen bee (*Figure 2*). All three castes have four developmental life stages: egg, larva, pupa, and adult. All except for the adult stage occur in single hexagonal wax comb cells built by the bees within the nest. These immature stages (collectively referred to as brood) are immobile and completely dependent on the care of their sisters for their own survival. Due to the haplodiploid system, fertilized eggs become diploid females and unfertilized eggs become haploid male drones. Whether a fertilized egg will develop into a worker bee or a queen depends on the quality of the food they are fed by their sisters during larval development.

Worker bees rarely reproduce and instead devote their lives to helping raise their sisters. While worker bees still have the possibility to lay unfertilized eggs, the pheromones of the queen inhibit this behavior within the colony. The queen bee lays all the eggs for the colony at a rate of 2000 per day on average. The queen mates once in her life with several drones and is able to store all the sperm in her spermatheca for her entire life of about three years. Workers and drones have a life span of about 6 weeks with the exception of overwintering worker bees that can survive up to 8 months in a winter cluster. Drones have

only one purpose – to mate with virgin queens. Upon copulation the drone will die while any drones that did not manage to mate will be expelled from the colony in the autumn.

The female worker bees perform all the tasks of the colony (except for egg laying) based on a temporal division of labor that is loosely dependent on their age. Young adult workers begin with cleaning, building comb, and tending to brood, at which point they are called nurse bees. After two to three weeks the adult workers will begin foraging for pollen to feed their brood, and for nectar to concentrate into honey and store in the hive as the winter food supply.

2.1.1 The superorganism

Within a honey bee colony there is a reproductive division of labor, with a few reproductive individuals (queens and drones) and sterile workers that normally do not reproduce themselves but are essential for maintaining colony function. These two groups are analogous to the germ cell line and cells of somatic tissue of a multicellular organism respectively. Further, the division of labor within the worker caste specializing on different tasks is analogous to organ-like functions. As a single cell will not survive outside a human body and as the human body will not die without that cell, so the individual worker bee will not survive without the colony and it is not needed to maintain colony survival. The honey bee colony is analogous to a multicellular organism and for this reason has been referred to as a superorganism (Wilson & Sober, 1989).

The colony growth and reproductive fitness depend entirely on the reproductive efforts of a single queen bee. However, swarming is a process where the colony divides into two or more new colonies and produces new queens (*Figure 2*). This event is thus considered a form of colony-level reproduction where the colony reproduces as a single unit. Although some reproductive fitness may be achieved through the production of drones, swarming is fundamental for colony fitness (Moritz & Southwick, 1992). This colony-level reproduction makes studying honey bee host-parasite interactions and disease epidemiology complex, as they have two levels where selective pressures can have an effect – at the individual and the colony level. This is an important consideration both for the evolution of pathogen virulence but also for the evolution of host adaptations towards disease resistance or tolerance.

2.1.2 Honey bee epidemiology

Honey bee parasites and pathogens need to be successful at multiple levels within the honey bee superorganism in order to reproduce and disperse to new hosts. The first step requires successfully infecting an individual at which point the pathogen must then be able to infect additional individuals to assure a

sufficient parasite load within the colony. Finally, the pathogen must successfully gain access and infect new colonies. The ability of a pathogen to advance through these different levels depends on its virulence. Virulence is defined as the degree to which the infection decreases the host survival or reproduction and is adaptive only so far as to increase pathogen fitness. A virulence trade-off can occur when the virulence is either too high that the host dies before the pathogen is able to infect a new host, or when the virulence is too low that transmission opportunities for the pathogen are lost. The mode of disease transmission plays a significant role in the natural selection process of host-parasite interactions by influencing the evolution of pathogen virulence (Lipsitch *et al.*, 1996). Therefore, adaptations by either the bees or the parasite are affected by the availability of different transmission routes.

For honey bees, horizontal transmission can occur either between honey bee colonies or between individuals within the colony. Vertical transmission occurs through reproduction, either at the colony level from mother colonies to swarms or at the individual level from infected queens to eggs (Fries & Camazine, 2001). If vertical transmission is the main route for infections to spread, then it can be predicted that less virulent relationships between the host and parasite evolve because the pathogens depend on the success of host reproduction. However if transmission is mainly via horizontal routes then the pathogen can be expected to evolve with higher virulence since opportunities exist to spread to other hosts after the host dies and host death may even enhance transmission.

There is often a contrast between individual- and colony-level virulence that is seen with many honey bee pathogens and therefore requires that the interactions at both levels be studied. Pathogens that are virulent at the individual level are often not as virulent at the colony level and vice versa. An example of a honey bee pathogen that is not virulent at the individual level but can be very virulent at the colony level is deformed wing virus (DWV), described in detail in section 3.3.1 of this thesis as well as being the focus of **Paper IV**. In contrast, sacbrood virus (SBV) and the fungal disease chalkbrood, are examples of pathogens highly virulent at the individual level but not at the colony level.

2.2 Honey bee disease defense

The honey bee nest cavity maintains a relatively constant temperature and humidity providing an ideal environment for parasites and pathogens. Further, with thousands of individuals within a colony having close contact (from casual contact to trophallaxis), numerous and diverse opportunities for

pathogen transmission are possible. Despite this high potential for disease, the individual level honey bee immune response system is not well developed compared to other insects and lacks about 30% of the immune system genes that are known for various dipteran species (eg. *Drosophilia melanogaster*, *Anopheles gambiae*; Evans *et al.*, 2006). Instead, honey bees rely largely on colony-level adaptive pathogen resistance mechanisms for disease defense, which collectively have been coined social immunity (Cremer *et al.*, 2007).

2.2.1 Social immunity

Social immunity results from the cooperation of individuals within the colony to decrease the risk of disease transmission and has evolved due to selection at both the individual and colony level. This type of defense can consist of behavioral, physiological and organizational adaptations to prevent the entrance, establishment, and spread of disease within the colony. Examples of Social immunity are described in detail in the *Varroa* tolerance and resistance host traits section in relation to the work of this thesis (*see section 4.2*) and are investigated in **Paper I**.

The reduced innate individual immunity mentioned above suggests that honey bees either rely more heavily on colony level adapted behaviors for defense since the death by disease of an individual does not greatly effect the reproductive success of the colony, or that during their natural evolution they have not been exposed to heavy pathogen pressures.

Although there are a variety of colony level mechanisms such as social immunity that limit disease transmission within the colony, there are very few described mechanisms where honey bees limit disease transmission between colonies (Fries & Camazine, 2001). The lack of adaptive strategies for combating horizontal transmission between colonies may demonstrate that throughout honey bee evolution this type of disease transmission has not induced a strong selection pressure. However, intensified apicultural practices are changing the adaptive pressures on honey bees and their diseases.

2.3 Apiculture

Apiculture, or the craft of keeping bees, is dated back as far as 2500 BC and probably started when bee swarms settled in a basket, clay pot, or tree log that could be taken and placed together with other colonies in what we call an apiary or a bee garden. Honey bee colonies were historically kept with little disturbance until it was time to harvest honey, which in most cases entailed the complete destruction of hives. For this reason, swarms were encouraged and then collected to provide a next generation of bee colonies. Although hive

structures have adapted through the centuries to suit local conditions, relatively little change occurred in beekeeping techniques until the invention of the movable frame hive in 1852 by Rev L.L. Langstroth where wax combs, still built by the bees but in wooden frames, could be individually removed from the hive structure (*Figure 2*). This new hive caused a revolution in beekeeping practices as it enabled beekeepers to base their management methods on swarm prevention instead of swarm encouragement and eliminated the necessity to destroy the hive when harvesting honey. A movable frame hive meant that wax comb could be removed to harvest honey and put back into the hive without damage. There was no longer a need to keep a close watch over swarming colonies since controlling the hives at predetermined dates could now prevent swarming. Apiaries could be kept away from the home or transported to forage on distant agriculture crops and if a colony was to die, the framed wax combs could be kept and stored for re-use in new colonies.

For a full description of the development of apiculture, see Crane (1983).

2.3.1 Apiculture and honey bee epidemiology

Today, apiculture is a threatened industry largely due to the spread of honey bee diseases. Paradoxically, apicultural management practices actually encourage the spread of disease and increase pathogen virulence by facilitating pathogen transmission routes (Fries & Camazine, 2001). For example, beekeeping methods often involve preventing natural swarms, which reduces colony level vertical transmission opportunities for pathogens that would encourage low virulence. When colonies are kept in large numbers in close proximity and colony equipment and contaminated hive material is exchanged between colonies, horizontal transmission opportunities for pathogens increase dramatically encouraging increased virulence. To make matters worse, the pesticides and antibiotics that are administered to colonies by beekeepers to treat infections have been shown to actually cause additional damage to bee health (Haarmann *et al.*, 2002; Johnson *et al.*, 2009; **Paper IV**).

Figure 2. Queen bee (top left); worker bees with *Varroa* mites on their thoraxes (top middle); drone bee between worker bees (top right); swarm of bees hanging in a tree branch (middle left); hive frame with wax-sealed honey cells on the periphery surrounding central papery-capped worker pupae (middle right); four Langstroth movable frame bee hives (bottom; Photos: B. Locke).

3 *Varroa* mites

The *Varroa* mite (*V. destructor*) is an exotic and relatively recent invasive species to parasitize the European honey bee (*A. mellifera*). The mite was first described as *Varroa jacobsoni* Oudemans (Acari: Varroidae) from its natural host the Asian honey bee (*Apis cerana*) in Java, Indonesia (Oudemans, 1904). The *Varroa* mite did not receive much attention by scientists until a host shift occurred and it became a pest on *A. mellifera* in Europe. The mite was first found in Europe in 1977 and in North and South America in 1977 and 1971 respectively (Ruttner & Ritter, 1980). Since then it has spread throughout the world with the help of honey bee importations (Oldroyd, 1999; Boecking & Genersch, 2008). Today only Australia (Anderson & Trueman, 2000; Rosenkranz *et al.*, 2010), Northern Scandinavia (Anon., 2010), and some extremely isolated island populations (Tentcheva *et al.*, 2004; Shaibi & Moritz, 2010) remain free of *Varroa*. Based on DNA analysis of *V. jacobsoni* mites collected in Asia, genetic variation revealed a "species complex" and a new distinct species was described as *V. destructor* (Anderson, 2000; Anderson & Trueman, 2000). This species is the only identified *Varroa* species parasitizing European honey bees and is therefore the only mite species discussed from here onward throughout this thesis. Several mitochondrial haplotypes of *V. destructor* have been identified, but only two are able to reproduce in *A. mellifera* colonies: the Korean haplotype which has a world-wide distribution; and the Japanese/Thailand haplotype which has only been reported in Japan, Thailand, and North and South America, and is considered less virulent than the Korean type (de Guzman *et al.*, 1998; de Guzman & Rinderer, 1999; Anderson & Trueman, 2000; Garrido *et al.*, 2003; Munoz *et al.*, 2008). By use of microsatellite markers, Solignac *et al* (2003, 2005) found a lack of genetic variation within these two haplotypes and considered them to have a quasi-clonal population structure.

3.1 Mite biology

The *Varroa* mite is a highly specific brood parasite that relies completely on its host's biology for its own survival and propagation by feeding on bee hemolymph and by reproducing in brood cells. A bee independent life stage does not exist. This section focuses on the aspects of mite biology that are important to this thesis. A more detailed review of mite biology can be found in Rosenkranz *et al* (2010).

Adult female *Varroa* mites are reddish brown in color and are flat and oval in shape (1.1mm x 1.6mm; *Figure 3*). They have two distinct life stages: a phoretic phase spent on the adult bees traveling within or between colonies; and a reproductive phase that occurs in the capped brood cells during honey bee pupal development. Generally, mites are significantly more often found in brood cells than on adult bees, with up to 90 % of the colony's mites found within the brood (Boot *et al.*, 1993; Rosenkranz & Renz, 2003). *Varroa* mites have a distinct sexual dimorphism and males, being significantly smaller and less sclerotized than females, are unable to survive outside the protection of the brood cell (Ifantidis, 1983; *Figure 3*).

3.1.1 Phoretic phase

During the phoretic phase the mite can be found between the abdominal segments of the adult bee where they can reach the intersegmental membrane for feeding. To optimize the ability of finding an appropriate brood cell for reproduction, female mites preferentially travel on nurse bees (Kraus, 1993). The female mite enters brood cells of 5th instar larvae just before cell capping (Boot *et al.*, 1992) and is attracted by chemical volatiles from the larval cuticle (Le Conte *et al.*, 1989; Aumeier *et al.*, 2002). Mite infestation occurs at a higher frequency in drone brood than in worker brood (Fuchs, 1990). Possible explanations for this occurrence are:

- Drone pupae require three days more for development, which could allow the maturation of more mite offspring,
- Nurse bees more frequently visit drone larvae, thereby increasing the opportunity for mite infestation (Calderone & Kuenen, 2003),
- Drone larvae produce slightly higher quantities of certain esters involved in larvae attractiveness to mites, and over a longer time (Le Conte *et al.*, 1989; Calderone & Lin, 2001).

3.1.2 Reproductive phase

During the reproductive phase, the female mite synchronizes her egg laying with the development of the bee pupa. Oogenesis is triggered by volatiles of

the host larva (Garrido & Rosenkranz, 2004) and the first egg is laid approximately 60-70 hours after cell capping (Ifantidis, 1983). The first egg is normally unfertilized and develops into a male since *Varroa* are haplodiploid. The mother mite continues to lay fertilized eggs at 30-hour intervals that develop into female offspring (Rehm & Ritter, 1989; Martin, 1994). A normally reproducing mother mite is able to lay up to five female eggs in worker brood and up to six female eggs in drone brood (Martin, 1994, 1995). The mite offspring develop through proto- and deutonymph mobile stages and proto- and deutochrysalis immobile stages (*Figure 3*). The developmental time takes about 5.8 days for females and 6.6 days for males from hatching until the adult molt (Ifantidis, 1983; Rehm & Ritter, 1989). Both female nymphal stages and males are soft-bodied mites lacking sclerotized chelicera strong enough to pierce the pupa cuticle to feed and so rely completely on their mother to provide a feeding site on the developing bee during their development (Donzé & Guerin, 1994). Mating takes place within the cell between adult brothers and sisters (Donzé *et al.*, 1996). Only mature adult female mites will survive outside the brood cell and immature female mites, along with the adult male, will die when the bee emerges.

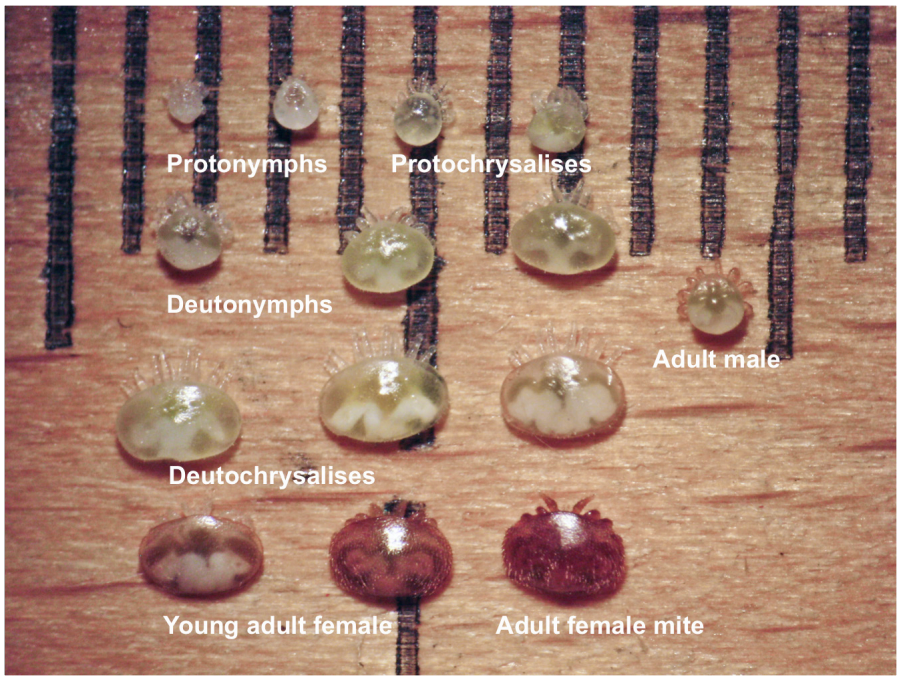

Figure 3. Female *Varroa* mite developmental stages with an adult male mite to the right (Photo: B. Locke).

Mite reproductive success is defined as the ability of a mother mite to produce at least one viable, mature, mated female offspring before the developing bee pupa hatches as an adult. Successful mite reproduction therefore requires the maturation of at least two eggs laid by the mother mite inside the brood cell: a male mite and a sister female mite, who must mate before bee eclosion. Mating takes place immediately after the last molt of the female offspring. All mature mated daughter mites will enter the colony's mite population along with their mother to find a new brood cell for reproduction. A mother mite that lays no eggs, lays only one egg, produces no male offspring, or begins laying eggs too late in relation to larval development, will not contribute any progeny to the mite population.

The reproductive rate of the mite in European honey bee colonies is approximately 1.45 in worker brood (Martin, 1994) and 2.2 in drone brood (Martin, 1995). Because of these rates and the ability of mother mites to reproduce multiple times during their life span (Fries *et al.*, 1994), the mite population growth can be exponential.

3.2 Effects of *Varroa* on bee health

By feeding on bee hemolymph, the mite is a significant stressor on honey bee health causing a variety of physical and physiological effects for both individual bees and the colony. The bee pupa is injured physically by the repeated piercing of its soft body tissue by the mite's chelicerae during *Varroa* feeding. At the same time, the loss of hemolymph interferes with organ development (Schneider & Drescher, 1987). As an adult, the bee has a reduced body weight and lifespan (De Jong *et al.*, 1982) while foragers suffer from a reduced learning capability (Kralj *et al.*, 2007) with a prolonged absence from the hive and a lower return rate, possibly due to reduced navigational abilities associated with *Varroa* parasitization (Ruano *et al.*, 1991; Kralj & Fuchs, 2006).

The honey bee colony-level fitness is reduced in two ways by mite infestation: drones have a decreased flight performance and therefore a lower chance to mate (Duay *et al.*, 2002) and the colony suffers from a reduced ability to produce swarms (Fries *et al.*, 2003; Villa *et al.*, 2008).

The most devastating colony-level effects of *Varroa* mite infestation are actually caused indirectly by honey bee viruses that are vectored by the mite. It is the development of overt viral infections associated with *Varroa* infestation that ultimately cause the honey bee colony to collapse (Ball & Allen, 1988; Bailey & Ball, 1991; Boecking & Genersch, 2008). This relationship between

Varroa and viruses is discussed in detail in the following section and is an important relationship studied in **Paper IV**.

Varroa infestation can affect honey bee colony performance by a reduction in colony growth and honey production before clinical symptoms are recognized by beekeepers. Therefore damage thresholds have been established to help identify a mite infestation level where irreversible colony damage and economic loss occurs. Fries *et al* (2003) and Rosenkranz *et al* (2006) found independently, that if mite infestation rates exceeded 30% of the adult bee population during the summer, the colony would not survive the following winter. When the mite was first introduced to Europe over 30 years ago, studies found 7000 to 11 000 mites in a colony 4 years after the initial infestation (Ritter & Perschil, 1982; Fries *et al.*, 1994). At that time the economic threshold was determined to be 200 fallen mites per day in July (Ritter *et al.*, 1984). Now it is unusual to find such high mite infestation and control treatments are required to avoid colony losses when the natural mite drop exceeds 10 mites per day in July. Colony mite loads that exceed 3000 mites indicate the colony is close to collapse (Boecking & Genersch, 2008). Such thresholds however vary in seasonality, colony brood production and the presence of associated viruses. Poor beekeeping skills can also affect colony losses and the build up of the *Varroa* populations due to a lack of mite treatment or poor timing of treatments (Delaplane & Hood, 1997; Currie & Gatien, 2006, *see section 3.4*).

The presence of viruses and their interactions with *Varroa* is key to this changing mite infestation threshold over the years and is essential in understanding colony collapse. It is the viral infections vectored by *Varroa* that ultimately kill a colony within 1-3 years if the mite population in the colony is not reduced by beekeepers (Boecking & Genersch, 2008). Because of this, wild and feral honey bee populations in Europe and North America have been nearly completely eradicated since the introduction of the mite and control methods are essential for keeping managed honey bee colonies alive.

3.3 *Varroa* and honey bee viruses

Before the arrival of *Varroa*, most if not all viruses were mainly present as covert infections in *A. mellifera* colonies (Bailey & Ball, 1991). Covert infections are defined as conditions in which the virus is present in the host without clear disease symptoms and have the ability to remain fully competent and reemerge later to cause overt infections (Burden *et al.*, 2003). The ‘normal situation’ with honey bee viruses, of an absence of disease symptoms, changed when the *Varroa* mite was introduced.

There are over 18 identified and characterized honey bee viruses, many of which are suggested to be associated with mite infestation to various degrees (Bailey & Ball, 1991; Martin, 2001; Tentcheva *et al.*, 2004; Shen *et al.*, 2005; Chen & Siede, 2007; Ribière *et al.*, 2008; Carreck *et al.*, 2010; Martin *et al.*, 2010). While feeding on bee hemolymph the mite vectors some of these honey bee viruses, which can cause severe disease or mortality at the individual and/or colony level (Bailey & Ball, 1991; Martin, 2001; Shen *et al.*, 2005; Chen *et al.*, 2006; de Miranda & Genersch, 2010). The mite is not just a mechanical vector for viruses but also functions as an alternative replicative host or biological vector for certain viruses (Ongus *et al.*, 2004; Yue & Genersch, 2005; Gisder *et al.*, 2009). This significantly enhances the epidemiology potential and lethality of the virus infection (Moeckel *et al.*, 2011). In fact, the viruses play an important role in colony collapse due to mite infestation as shown by field observations and supported by modeling approaches (Bowen-Walker *et al.*, 1999; Nordström *et al.*, 1999; Martin, 2001; Sumpter & Martin, 2004; Tentcheva *et al.*, 2004; Todd *et al.*, 2007; Berthoud *et al.*, 2010; Carreck *et al.*, 2010; Martin *et al.*, 2010).

In order to prevent colony mortality, management strategies are implemented by beekeepers to reduce the mite population within the colony and thereby limit the transmission opportunities for potentially lethal virus infections. Without such treatment the exponential mite population growth would lead to increased virus transmission causing overt viral infections that ultimately result in colony mortality (Martin, 2001; Boecking & Genersch, 2008).

3.3.1 Deformed wing virus

The best studied relationship of a virus vectored by *Varroa* is that between the mite and deformed wing virus (DWV), which today has become the most prevalent honey bee virus and is highly associated with *Varroa* mite infestation and colony collapse (de Miranda & Genersch, 2010; Genersch & Aubert, 2010). DWV is a positive single-stranded RNA virus (Lanzi *et al.*, 2006) pathogenic to both honey bees and bumble bees (Genersch *et al.*, 2006) and can be detected in all life stages of honey bees whether visible disease symptoms are present or not (Chen *et al.*, 2005; Yue & Genersch, 2005; Tentcheva *et al.*, 2006). DWV can be transmitted horizontally within the colony through trophallaxis, feces, and salivary gland secretions and between individuals through venereal transmission from drones to queens during mating, as well as vertically from infected queens to their progeny (Chen *et al.*, 2006; Chen & Siede, 2007; Yue *et al.*, 2007; De Miranda & Fries, 2008). These transmission routes however do not cause overt symptoms but instead

only maintain the virus in the colony. According to theories in evolutionary epidemiology, vector-borne transmission often results in more virulent infections (Ewald, 1994). In fact, DWV usually exists within a colony with no apparent symptoms when *Varroa* mite infestation is low or absent (Nordström *et al.*, 1999) and overt viral infections depend on a severe infestation with a large mite population (Martin *et al.*, 1998; Martin, 2001). As the mite population grows within a colony, increased opportunity for viral transmission will lead to the development of an overt infection that ultimately kill the colony. Therefore, the virulence of the mite is related to its ability to vector these viruses. Consequently, the viruses with a new vector transmission route will become more virulent, as the virus' virulence is in general a measure of mite abundance.

The clinical symptoms of DWV include adult bee deformities such as deformed wings, shortened body size and abdomen, and reduced vigor and longevity (Bowen-Walker *et al.*, 1999; Martin, 2001; Tentcheva *et al.*, 2006; *Figure 4*). Although deformed bees usually have higher DWV titers than non-deformed bees, it is not the titers alone that result in deformities but rather how and when DWV is transmitted. Although DWV is the sole cause of the symptoms (Moeckel *et al.*, 2011), they only appear naturally during circumstances of *Varroa*-mediated transmission during the pupal stage (Bowen-Walker *et al.*, 1999; Yue & Genersch, 2005; Gisder *et al.*, 2009). The mite also weakens the bee's immune system, suppressing the expression of immune related genes and increasing viral titers in the bee, both of which reduce worker survivorship and colony fitness (Yang & Cox-Foster, 2005, 2007). Not only is DWV transmitted by the mite, but it also replicates within the mite and individual bee symptoms seem to be related to whether the virus was replicating or not in the infesting mite during the pupal stage of bee development (Yue & Genersch, 2005; Gisder *et al.*, 2009).

Figure 4. Honey bee in the center of photo with symptoms of DWV: deformed wings and shortened abdomen (Photo: B. Locke).

The paradox of the virus-mite-bee interaction is that it is the otherwise 'normally' low virulence of DWV that enables *Varroa* infested pupae to complete development despite the virus infection resulting in the bee emerging, releasing the mite to infect another pupa to reproduce again and sustaining the virus epidemic that ultimately becomes lethal at a colony level when the majority of the individuals are damaged. This contrast between individual- and colony-level virulence is seen for other bee pathogens as well, emphasizing the need to study interactions at both levels.

3.4 Control of *Varroa*

A major obstacle to the development of mite tolerance in the European honey bee is intensive beekeeping practices including mite control. Since the mite has been introduced to the western world, beekeepers use methods to remove the mite from colonies, therefore eliminating the selective pressure of mite infestation that would be required for adaptations towards parasite tolerance or resistance in the bees, or towards lower virulence in the mites (Fries & Camazine, 2001). Further, these mite control methods are often based on chemicals and can be problematic for several reasons:

- Chemical residues that are fat soluble can build up in hive products and especially in wax comb (Bogdanov *et al.*, 1998; Wallner, 1999; Bogdanov, 2006; Martel *et al.*, 2007).
- Mites can develop resistance to effective acaricides rendering them ineffective as a class of miticide (Milani, 1995, 1999; Hillesheim *et al.*, 1996; Sammataro *et al.*, 2005).
- Some methods can cause damage to bees (Imdorf *et al.*, 1990, 1999; Charriere & Imdorf, 2002).

The honey bee is unusually sensitive to a range of chemical insecticides (Stefanidou *et al.*, 2003; Thompson, 2003; Barnett *et al.*, 2007), likely due to a relative deficit of detoxification enzymes (Yu *et al.*, 1984; Claudianos *et al.*, 2006). Little is known about the interactions between pesticides, the bees, and bee pests or pathogens. Pesticides and/or pathogens when studied in isolation or at the individual bee level may not appear to cause harm but sub-lethal effects may accumulate or interact to become significant at the colony level. A single infection may cause no harm to a colony but when exposed to a pesticide at the same time it may cause colony death (Pettis *et al.*, 2012). The quantification of the interactions between parasites, virus infections, and the pesticides used to control *Varroa* at the various developmental stages of the honey bee is central to **Paper IV**.

Chemical treatments of honey bee diseases, even if successful at the colony level in the short term, have not eradicated the problem of pathogens at the population level. This is particularly important if the pathogen has a high infectivity and transmission rate (Moritz *et al.*, 2010). Treatments targeting a single pathogen will not cure the problem if the real difficulty exists through interactions between multiple pathogens or pathogens and other stressors.

4 Host-parasite interactions

Honey bee societies, the *Varroa* mites that infest them, and the honey bee viruses that are vectored by the mites, together form a complex system of host-parasite interactions. Coevolutionary theories in the study of host-parasite interactions indicate that antagonistic reciprocal selection pressures will lead to an "arms race" with a series of adaptations and counter-adaptations by the host and the parasite (Thompson, 1994). Such antagonistic interactions actually accelerate molecular evolution compared to selection pressures of environmental changes (Paterson *et al.*, 2010). The evolutionary dynamics of host-parasite coevolution can lead to a relatively stable relationship between the host and parasite with fitness optimality for both by means of a natural selection process (Schmid-Hempel, 2011). However, this coevolutionary process has been hindered for the European honey bee host since apicultural practices remove the mite and consequently the selective pressures required for such a process.

4.1 Honey bee tolerance and resistance to *Varroa*

In host-parasite interactions, host tolerance is defined as the ability to reduce the effect of the parasite, while host resistance is the ability to reduce the fitness of the parasite and in most cases resistance and tolerance are correlated (Lipsitch *et al.*, 1996; Schmid-Hempel, 2011). Coevolution theory predicts that parasites will have an evolutionary advantage over their host due to their faster evolution through a shorter generation time (Hafner *et al.*, 1994; Schmid-Hempel, 2011). However, in this particular study system, the *Varroa* mite is of clonal origin with low genetic variation (Solignac *et al.*, 2005) and the honey bee has a 10-fold higher recombination rate than any other higher order eukaryote (Beye *et al.*, 2006). These aspects provide the honey bee host with an evolutionary advantage in the arms race with *Varroa,* as the mite's options

for genetic adaptation are limited compared to those of the bee. For this reason, adaptations of resistance or tolerance due to coevolution are most often discussed in general literature from the host's perspective (the honey bee), in contrast to adaptations of virulence by the parasitic mite.

4.2 *Varroa* tolerance and resistance host traits

A variety of honey bee characteristics have been suggested or shown to influence and regulate mite population dynamics. The following sections describe some important traits that have been linked with *Varroa* tolerance or resistance in bees and are investigated in **Papers I & II**. Environmental conditions have also been suggested to play a role in the development of the *Varroa* mite population within a colony (Dejong *et al.*, 1984; Moretto *et al.*, 1991b), however it is more likely that this is only observed through the indirect effect of environmental factors that regulate honey bee brood amounts or the activeness of certain host defense behaviors.

4.2.1 Behavioral defense

A well-known behavioral defense is grooming behavior, where honey bees groom themselves and other nestmates resulting in the capturing and damaging of adult mites (Peng *et al.*, 1987; Moosbeckhofer, 1992; Boecking & Ritter, 1993; Moretto *et al.*, 1995). Hygienic behavior, another well-known behavioral defense of honey bees, is the ability to detect and remove dead or diseased brood (Rothenbuhler, 1964; Spivak, 1996; Boecking & Spivak, 1999). A variation of hygienic behavior involves the ability to detect and remove mite-infested brood and has been termed *Varroa*-sensitive hygienic behavior (Spivak, 1996; Ibrahim & Spivak, 2006; Harris, 2007). The removal of mite-infested brood however does not necessarily include the death of the mite and most mites escape during the removal process (Boecking & Spivak, 1999). Nevertheless, this behavior results in an interruption of the mite's reproductive cycle that ultimately could slow down the mite population growth in the colony.

It is not well known how the bees are able to recognize the mite on nestmates during grooming behavior or within brood cells during hygienic behavior since the *Varroa* mite has a similar cuticular hydrocarbon profile to their host bee (Nation *et al.*, 1992) used for chemical mimicry especially within the brood cells (Martin *et al.*, 2001; Salvy *et al.*, 2001). It may be that mite-infested brood are recognized by an unspecific stress reaction of the pupae (Aumeier & Rosenkranz, 2001).

Bee defense behaviors are highly variable between bee species and races (Fries *et al.*, 1996; Moretto, 2002) and quantifying the trait accurately depends strongly on the methods used. Recently it has been discovered that damage to the dorsal surface of the mite, a characteristic that has been used to quantify grooming behavior, is actually a normal birth defect of mites (Davis, 2009). Further, mutilated mites may have been damaged after they have died naturally or by other insects such as ants that scavenge on bottom boards in colony debris (Rosenkranz *et al.*, 1997).

4.2.2 Population dynamics of the host

Colony size and temporal dynamics are honey bee characteristics that are known to greatly influence the mite population given the importance of brood amounts for mite reproduction (Fries *et al.*, 1994; Calis *et al.*, 1999) and include the incidence of swarming, brood production, the ratio of drone to worker production, and the ratio of adult bees to brood (Boot *et al.*, 1993, 1994; Wilkinson & Smith, 2002).

Drone brood provides better reproductive conditions for the mite and the amount of available drone brood in a colony therefore greatly influences the population dynamics of the mite (Calis *et al.*, 1999). The duration of the capped pupal phase of bee development is a limiting factor for the development of mite offspring and could reduce the mite population dynamics in the colony (Buchler & Drescher, 1990). However, a shorter capped pupal phase may result in negative effects on the vitality of the hatching worker bee (Bienefeld & Zautke, 2007), and a shorter developmental time may also result in more brood cycles per season (Martin, 1998), which would provide the mite with more reproductive cycles.

4.2.3 Control of mite reproduction

One effective host strategy to prevent the growth of the *Varroa* population from reaching devastating levels within the honey bee colony, would be to control and limit the parasite's reproductive ability (Fries *et al.*, 1994; Rosenkranz & Engels, 1994). This can be achieved through host adaptation that either adjust the host's population demographics important for mite reproduction (as mentioned above, such as reducing drone brood availability), or alter the chemical ecology of volatiles that are important for initiating (or inhibiting) mite oogenesis (Nazzi & Milani, 1996; Trouiller & Milani, 1999; Garrido & Rosenkranz, 2004; Nazzi *et al.*, 2004) and influencing the attractiveness of the brood for infesting mother mites (Aumeier *et al.*, 2002). Mite reproduction is important for *Varroa* population dynamics and variable reproductive rates have been observed since the first infestation on European

honey bees (Anderson, 2000). The ability of the host to reduce the reproductive success of their infesting mites is the main trait investigated in **Papers I, II & III.**

4.3 Breeding for mite resistance

Breeding *Varroa*-resistant bees is considered to be the only real long-term solution to the *Varroa* mite problem in contrast to the short-term *Varroa* chemical treatments. For this reason, many different tried by bee researchers and bee breeders in the beekeeping industry have attempted to produce mite-resistant lines of European honey bees for commercial use (Buchler *et al.*, 2010; Rinderer *et al.*, 2010). A well-known attempt for selecting mite resistance was the introduction to the United States and subsequent selective breeding of 'Russian bees' that apparently, by natural selection, developed mite tolerance or resistance in Eastern Russia where the natural boundaries between *A. cerana* and *A. mellifera* meet (Rinderer *et al.*, 2001). Hygienic behavior and *Varroa*-sensitive hygienic behavior are other well-known traits involved in selective breeding programs in the United States (Spivak, 1996; Boecking & Spivak, 1999; Harbo & Harris, 2001, 2005; Spivak & Reuter, 2001; Ibrahim & Spivak, 2006). Various reports have confirmed at least partial tolerance and a slower increase of the *Varroa* mite population in these different breeding programs with Russian bees (De Guzman *et al.*, 2007; Tarpy *et al.*, 2007; de Guzman *et al.*, 2008), hygienic bees (Ibrahim *et al.*, 2007) and *Varroa*-sensitive hygienic bees (Harris *et al.*, 2003). However, none of these breeding programs offer sustainable long-term solutions and they still require regular mite population monitoring and periodic mite control treatment (Tarpy *et al.*, 2007; Rosenkranz *et al.*, 2010).

In Europe, breeding strategies take a different direction with more emphasis placed on maintaining the local genetic diversity in bee races that have adapted to the different environments and selection for mite resistance is based on low natural mite infestation rates produced through natural selection rather than selecting for specific traits (Buchler *et al.*, 2010). Investigating the breeding potential of a naturally evolved mite-resistant trait was the objective of **Paper III**.

Selective breeding programs often involve simultaneous selection for a variety of traits such as increased honey production and gentleness, which may reduce the efficacy of specific selection for disease resistance. Natural selection acting on both the bees and the mites is a process towards co-adaptation in the host-parasite system and is therefore likely to produce more sustainable results.

Although some breeding efforts have been successful at enhancing *Varroa* tolerance, none have shown convincing evidence of producing a honey bee stock with long-term sustainability of mite tolerance or resistance without the need of mite control treatment.

4.4 True mite-resistant honey bees

4.4.1 Asian honey bees

The *Varroa* mite is not considered a threat to its natural host the Asian honey bee (*A. cerana*) for several reasons. *A. cerana* is known to detect and remove worker pupae that are infested with mites, thereby limiting mite reproduction to the small proportion of drone pupae present in the colony (Koeniger *et al.*, 1981; Boot *et al.*, 1999; Rath, 1999). Single drone pupae infested with multiple mites become too weak from the intense parasitization to open their hard cocoon cap themselves and die within the brood cell causing entombing of the mites that consequently become trapped in the cells and die with the pupa (Boecking *et al.*, 1999; Rath, 1999). Furthermore, the adult grooming behavior towards phoretic mites is very active in *A. cerana* (Peng *et al.*, 1987; Boecking, 1992). These adaptations prevent the mite population from reaching large numbers. The mite population growth rate produces a negative feedback loop in *A. cerana* colonies that rarely results in damage to infested colonies.

Unfortunately, the co-existent relationship between *Varroa* and its original host species is of limited use when the European honey bee is considered. In *A. mellifera* colonies, the mites are able to reproduce in worker brood and "entombing" of mites in drone pupae is not seen. The grooming behavior found in *A. cerana* also exists in *A. mellifera* but to a much lower extent and is much less pronounced (Fries *et al.*, 1996), even though it is a trait widely used for selective breeding with the European bee (*see section 4.3*). Interestingly, the mite appears to be better adapted to reproduction within *A. cerana* drone brood than in *A. mellifera* brood, yet mite population growth is faster and exponential in *A. mellifera* due to the exploitation of the worker brood (Boot *et al.*, 1995, 1997). Mite resistance in the Asian honey bee has evolved through natural selection over many years.

4.4.2 African and Africanized honey bees

Varroa mite resistance has also been demonstrated for specific honey bee races of the new host, *A. mellifera*; for example *A. m. scutellata* in Africa (Allsopp, 2006) and honey bees of African origin (Africanized bees) in South and Central American (Correa-Marques *et al.*, 2003; Carneiro *et al.*, 2007; reviewed in Rosenkranz, 1999).

Africanized bees in tropical America are well documented to have a stable host-parasite relationship with *V. destructor* and apiculture in this area does not need mite control treatment (Moretto *et al.*, 1993; Rosenkranz & Engels, 1994; Guzman-Novoa *et al.*, 1999; Mondragon *et al.*, 2005). However, no single clear explanation for their resistance exists. *Varroa* tolerance and resistance traits have been well studied in these bees and a variety of defense mechanisms, such as behavioral traits (Moretto *et al.*, 1991a; Correa-Marques & De Jong, 1998; Boecking & Spivak, 1999; reviewed in Rosenkranz, 1999) and reduced mite reproductive ability (Medina *et al.*, 2002; Martin & Medina, 2004; Mondragon *et al.*, 2006), have been suggested to explain their resistance. The mite reproductive success in South America has shifted from about 50 % of the mites reproducing (Dejong *et al.*, 1984) to over 80 % (Carneiro *et al.*, 2007) and the initial lower mite fertility on Africanized bees compared to European honey bees (Rosenkranz & Engels, 1994) has not been observed subsequently (Garrido *et al.*, 2003) although they maintain their overall resistance to mites. This change in *Varroa* reproductive success may be due to a replacement by the more virulent mite haplotype in the region, but it nevertheless demonstrates the difficulty in isolating a single major factor explaining the resistance of Africanized bees to mites, especially since an 80 % successful mite reproduction rate is an equivalent to that seen for mite susceptible European honey bee races (Medina & Martin, 1999; Correa-Marques *et al.*, 2003; **Papers I & II**).

The *Varroa* mite situation is far less documented in Africa compared to South America. Nevertheless, since the mite was first detected in South Africa in 1997, a stable host-parasite relationship has developed and these bees do not need mite control treatment (Allsopp *et al.*, 1997).

In tropical South America and in Africa, the wild and feral populations of honey bees comprise a much larger proportion of the overall honey bee population than in temperate North America and Europe where the majority of bees are managed (Moritz *et al.*, 2007). This means that most of the honey bee population in South America and Africa is subject to natural selection pressures towards adaptive resistant mechanisms to *Varroa* infestation. These naturally adapted traits can then be passed to managed colonies through natural mating events between the wild and managed bees and could be an explanation for the overall mite-tolerance seen in both South America and in Africa.

Africanized bees are more or less genetically identical to the original *A. m. scutellata* race from Africa that was first introduced to the continent (Schneider *et al.*, 2004). This bee race has a variety of characteristics that have contributed to their successful invasion and replacement of the European honey bee race genetics in the population (Schneider *et al.*, 2004). The African and

Africanized bee races may also have different genetic or environmental advantages compared to the European races for survival in tropical climates that can indirectly affect *Varroa* infestation and reproduction, making direct comparison of individual tolerance or resistance traits between them difficult.

4.5 European honey bee races surviving with *Varroa* mites

Around the world and in different climates, there have been periodic reports of unique feral and unmanaged European honey bee races surviving mite infestation for long periods without mite control treatment (DeJong & Soares, 1997; Kefuss *et al.*, 2004; Fries *et al.*, 2006; Le Conte *et al.*, 2007; Seeley, 2007). These reports suggest that some level of tolerance to the pest has been established and even possibly a sustainable host-parasite co-adaptation between *A. mellifera* and *V. destructor*. Although these populations have been described, the nature of their co-adaptation process between *V. destructor* and its new host species still remains to be fully explained. These surviving populations may hold an answer to achieving a stable relationship between European races of *A. mellifera* and *V. destructor*. What all these populations, in different climates and in different geographic regions, have in common is that they are not managed by beekeepers or are wild or feral, and that they are therefore exposed to natural selective forces. Two populations, of which are in focus of this thesis (**Papers I, II & III**) are described in detail below.

4.5.1 Gotland, Sweden

In 1999, an isolated honey bee population of 150 colonies was established on the southern tip of Gotland. The colonies came from a variety of locations around Sweden with different genetic backgrounds and were equally infested with an average of 50 *Varroa* mites in each colony. These colonies were to be part of a selection experiment to evaluate if the mites would eradicate an isolated population of bee colonies under natural Nordic conditions. For this purpose, the colonies were unmanaged, allowed to swarm freely and did not receive any mite control treatments. The experiment was called the "*Bond Project, Live and Let Die*" as some colonies would live and some would be let to die. The bees in this project have thus become known as the 'Bond Bees'. A central hypothesis to the Bond Project was that beekeeping management strategies inhibited the natural development of mite resistance in two main ways:

1. Swarm prevention inhibits colony level vertical transmission pathways, increasing the emphasis on horizontal transmission pathways, which may result in the evolution of more virulent mites.
2. Mite population control treatments remove the selective pressure of heavy mite infestation that would be required for natural selection to shape host adaptations towards tolerance and resistance.

The Bond Bees have been continuously monitored for swarming, winter losses, mite infestation rates in the fall, and bee population size in the spring since the beginning of the project (Fries *et al.*, 2003, 2006). Many of the bee colonies swarmed in the first two years of this project, but by the third year the increased mite infestation had weakened the colonies and the swarming rate decreased significantly (Fries *et al.*, 2003).

Within the first three years more than 80 % of the colonies in this project died (from 150 to 21 by 2002) due to the rapid build up of mite infestations rates well over the winter mortality threshold (Fries *et al.*, 2003, 2006). Nevertheless, more than ten years post mite introduction, a small number of colonies still remain that have survived without mite control and have established themselves as a hybrid sub-population (**Paper I**).

After the initial losses, the mite infestations rates in the fall decreased, winter mortality decreased and the incidence of swarming increased again as colonies were again strong enough to do so (Fries *et al.*, 2006). Although swarming reduced the mite infestation in the mother colonies of this population, it was not enough to prevent the development of high mite levels in the fall. Therefore, it was concluded that the ability to swarm probably does not limit the mite population growth enough to fully explain the survival of the Bond Bees (Fries *et al.*, 2003).

The recovery of the population suggested that an adaptive process has occurred in the bees, the mites, or both through natural selection and co-evolution. In order to determine whether it was the bees or the mites that have adapted, a cross transfer experiment was performed in 2007 to test if the source of mites affected the mite population growth in the Bond Bees, using a group of unrelated colonies previously treated with mite control and with regular beekeeping management as a control group (Fries & Bommarco, 2007). Results demonstrated an 82 % lower mite population growth rate in Bond Bees compared to control colonies, irrespective of the mite source. It was concluded that the low mite growth rate was linked to characteristics of the host and not of the parasite (Fries & Bommarco, 2007).

The next step in understanding how the Bond Bees are able to survive with mite infestation was to identify colony characteristics that may be involved in

resistance or tolerance towards the mite (**Paper I**) and examine the heritability of any such traits to determine the practical use of the population for breeding mite resistance in managed colonies (**Paper III**).

4.5.2 Avignon, France

Another well-known population of European honey bees that have survived *Varroa* mite infestation for an extended period is found in Avignon, France. Here, Le Conte *et al* (2007) established a collection of feral colonies and abandoned managed colonies from different locations around France where no mite control treatments have been used for 2 to 3 years previously on any of the colonies. The *Varroa* mite infestation and mortality was monitored and compared to a group of unrelated, managed control colonies. Swarming was not prevented and management was limited to harvesting honey. For over 7 years (1999-2005) there was no significant difference in the mortality of the collected mite-tolerant colonies compared to the control colonies. Other observations of this study include an initial difference in swarming which was higher in the collected mite-tolerant colonies but seemed to level out over the study. A significant difference in honey production was observed, with the control colonies producing almost twice the amount of honey of the mite-tolerant colonies (Le Conte *et al.*, 2007).

Like the Bond Bees, exposure to the selective pressure of natural mite infestation has resulted in adaptations either by the host, the parasite, or both in this Avignon honey bee population, enabling their long-term survival despite mite infestation. The mite tolerance or resistance traits of the Avignon population and how they compare to traits of the Bond Bees on Gotland is the topic of **Paper II**.

Figure 5. Locations near the two mite-surviving honey bee populations investigated in this thesis. The Bond Bee research 'laboratory' at Skåls Gård on Gotland, Sweden (left) and the Sénanque Abbey lavender fields near Avignon, France (right).

5 Thesis aims

The overall aim of this thesis was to gain a deeper understanding of the host-parasite interactions between honey bees and *Varroa* mites in order to establish a sustainable solution to the threat of *V. destructor* infestation in apiculture. Reports of honey bee colonies surviving *Varroa* mite infestation without treatment has presented a possible way to study *Varroa* and honey bee coevolution through natural host-parasite adaptations in European honey bees. An additional aim of this thesis was to identify potential effects that current *Varroa* control acaricide treatments have on the host-parasite interactions within this system, including the viruses that are so closely associated with *Varroa* infestation.

The more specific aims of this thesis can be broken down according to the four Papers:

I. Although a few honey bee populations surviving extended periods with *Varroa* mites have been documented, none of these populations have been characterized for *Varroa* resistance or tolerance mechanisms that may have evolved to explain their long-term survival. **Paper I** was an exploratory investigation of the population on Gotland, Sweden known as the Bond Bees that has been surviving *Varroa* infestation for over ten years without beekeeping management. The aim was to identify any honey bee colony-level defense mechanisms that may be linked to, or suggested to be important for mite tolerance or resistance.

II. The results of the study presented in **Paper I** indicated that the bees on Gotland Sweden were able to survive *Varroa* infestation for such a long time without *Varroa* control by beekeepers because the mite's reproductive success was reduced in some way by unknown host factors. The aim of **Paper II** was to investigate mite reproductive success in another documented population of mite surviving honey bees in Avignon, France and compare data with the Gotland populations to identify

similarities or differences between these two geographically and genetically separate populations that have both been experiencing similar selective pressure of uncontrolled, natural mite infestation levels.

III. Since a potential sustainable solution to the threat of *Varroa* is to breed mite-resistant honey bees, the aim of **Paper III** was to determine the breeding potential of the mite-resistant trait identified in the population on Gotland. The inheritance of the reduced mite reproductive success demonstrated in this population was investigated by examining the phenotypic variation of this trait that can be attributed to genetic variation in the F1 generation of artificially inseminated crosses of the Bond Bees and control colonies along with reciprocal hybrid crosses.

IV. Finally, **Paper IV** aims to gain deeper understanding of the interactions between the bee, the mite, the viruses associated with the mite, and the chemical acaricides that the apicultural industry regularly uses to control mite infestation, both at the colony level and individual level. In order to investigate the impact of a common mite control substance (tau-fluvalinate) on honey bee virus infections, the virus infection dynamics before and during a mite removal treatment were examined and compared to control colonies that were not treated with this substance.

6 Methods

This section summarizes some of the research methods used in the four papers of this thesis. For a more complete description of the experimental designs used for each study, refer to the individual papers in the following chapters of this thesis.

6.1 Experimental colonies

The control colonies used in the first three studies of this thesis (**Papers I, II & III**) were unrelated to the mite-surviving colonies on Gotland or Avignon but were in the same general location, had previously been treated for mite control and were exposed to regular beekeeping management. The control colonies in **Paper IV** were in the same location as the test colonies and had similar mite infestation rates but where not treated with Apistan™ (strips of tau-fluvalinate) during the investigation, while test colonies were treated with Apistan™.

6.2 Estimating honey bee and *Varroa* mite population dynamics (Papers I & IV)

Mite infestation rates in pupae and on adult can be combined with estimates of colony size to calculate the total number of mites in the colony. From this information, the proportion and distribution ratio of mites in brood or on adult bees can be determined.

6.2.1 Colony size estimations

Population estimates of adult bees, worker brood and drone brood were made using the Liebefeld Estimation Method in order to determine colony size

(Imdorf *et al.*, 1987). These estimates were performed over time intervals to determine temporal dynamics of colony size.

6.2.2 Mite infestation rates

Samples of approximately 200-300 worker bees were taken from the brood chamber of the hive to determine the phoretic *Varroa* mite infestation rate on adult bees. The bees from each sample were counted and then washed in soapy water to dislodge the mites. Using a strainer the mites were separated from the bees and both were counted to calculate the proportion of mites per bee (De Jong *et al.*, 1982; Fries *et al.*, 1991).

The proportion of mite-infested pupae was determined by opening 100-200 randomly selected pupal cells and counting the number of pupae infested with at least one mite, either in the field or from cut pieces of comb brought to the laboratory.

6.2.3 Calculating mite distribution

Samples for determining mite infestation rates were taken on the same day as the colony size measurements. The total number of mites on the colony's adult population was estimated by multiplying the mite infestation rates of adults by the number of adult bees in the colony. The total number of mites in brood was calculated similarly, using the colony brood estimations. The total numbers of mites on adults and in brood were added to estimate the total number of mites in the colony. The mite distribution was calculated as the proportion of the total mite population within the colony on either adult bees or in brood. The mite distribution was used as a measure for brood attractivity.

6.3 Investigating behavioral defenses (Paper I)

6.3.1 Hygienic behavior

The method used for determining hygienic behavior was chosen for its practicality in the field. One hundred pupae in each colony were marked and killed by piercing the pupal capping with a small insect pin (Palacio *et al.*, 2000). One hundred cells in the same brood area were marked without being killed to serve as a within-colony control. The proportion of pupae that were removed 12 and 24 hours after pin killing was recorded. These proportions expressed the brood removal rate, or hygienic behavior of the colony.

6.3.2 Grooming behavior

Colony debris, including damaged mites, was collected using bottom board metal slide-in trays. The proportion of total mites found in colony debris that

were damaged was recorded and used as a quantitative measure of adult bee grooming behavior (Bienefeld *et al.*, 1999).

6.4 Assessing *Varroa* mite reproductive success (Papers I, II & III)

Brood frames containing worker bee pupae that have been sealed for approximately 190 hours but before pupal eclosion, which occurs at approximately 280 hours of bee development, were brought into a laboratory for dissection. There are four main stages of pupal development during this time frame that can be categorized based on their appearance. These are: i) the yellow thorax stage (between 190-235 hrs); ii) the grey wing pad stage (235-260 hrs); iii) the grey thorax stage (260-270 hrs); iv) the resting adult (270-280 hrs). These stages are depicted in *Figure 6*. The yellow thorax stage of the pupae is the longest stage and is the earliest stage of pupa exocuticle sclerotization. The male mite typically does not become an adult until the pupal age is around 210 hours (Martin, 1994). Immature male mites are extremely difficult to distinguish from early stage immature female mite offspring so any yellow thorax stage infested pupa where the male mite was not positively identified was recorded as 'uncertain' to eliminate biased recordings.

Each pupal cell was opened carefully using forceps. The pupa was removed from the cell and examined under a stereomicroscope to ensure that no mite progeny were accidentally discarded with the pupa. The developmental stage of the pupa was determined based on the appearance description given by Martin (1994).

Complete mite families from cells infested with a single mother mite were removed using a fine artist's paintbrush and examined under a stereomicroscope. For each pupal cell, the following information was collected: i) whether the mother mite had reproduced; ii) the total number of offspring; iii) the developmental stage of each individual mite offspring; iv) whether an alive male mite was present or absent; v) the number of dead mite offspring.

The collected information was used to determine the reproductive success of the mother mite, measured as the ability to produce at least one viable mated female offspring when the developing bee hatches from the cell. A typical mite family that may be seen when the pupal age is roughly 260 hours and the mite has reproduced successfully is depicted in *Figure 7*.

Figure 6. Pupal developmental stages examined for mite reproduction from left to right: yellow thorax; grey wing pads; grey thorax; resting adult (Photo: B. Locke).

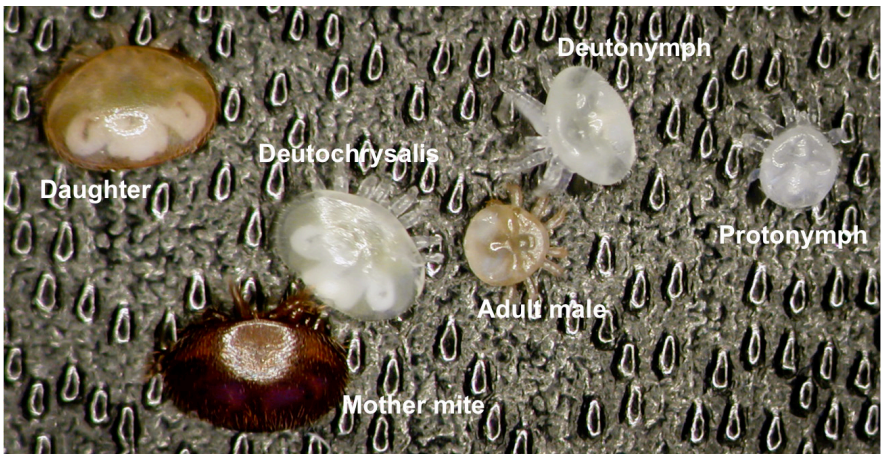

Figure 7. Typical *Varroa* mite family seen when the pupal age is approximately 260 hours and the mother mite has reproduced successfully (Photo: B. Locke).

The information collected was also used to identify which parameter was most often responsible for any reproductive failure. Such failure could depend on infertility, absence of male offspring, high proportion of mite offspring mortality, or delayed egg-laying by the mother mite. Delayed egg-laying was determined by comparing the developmental stage of the pupae with the developmental stage of the mite offspring using the mite ontogenic development chart found in Martin (1994). This chart made it possible to determine the number of present mite offspring, if any, that will potentially develop to maturity before the bee hatches (*Figure 8*).

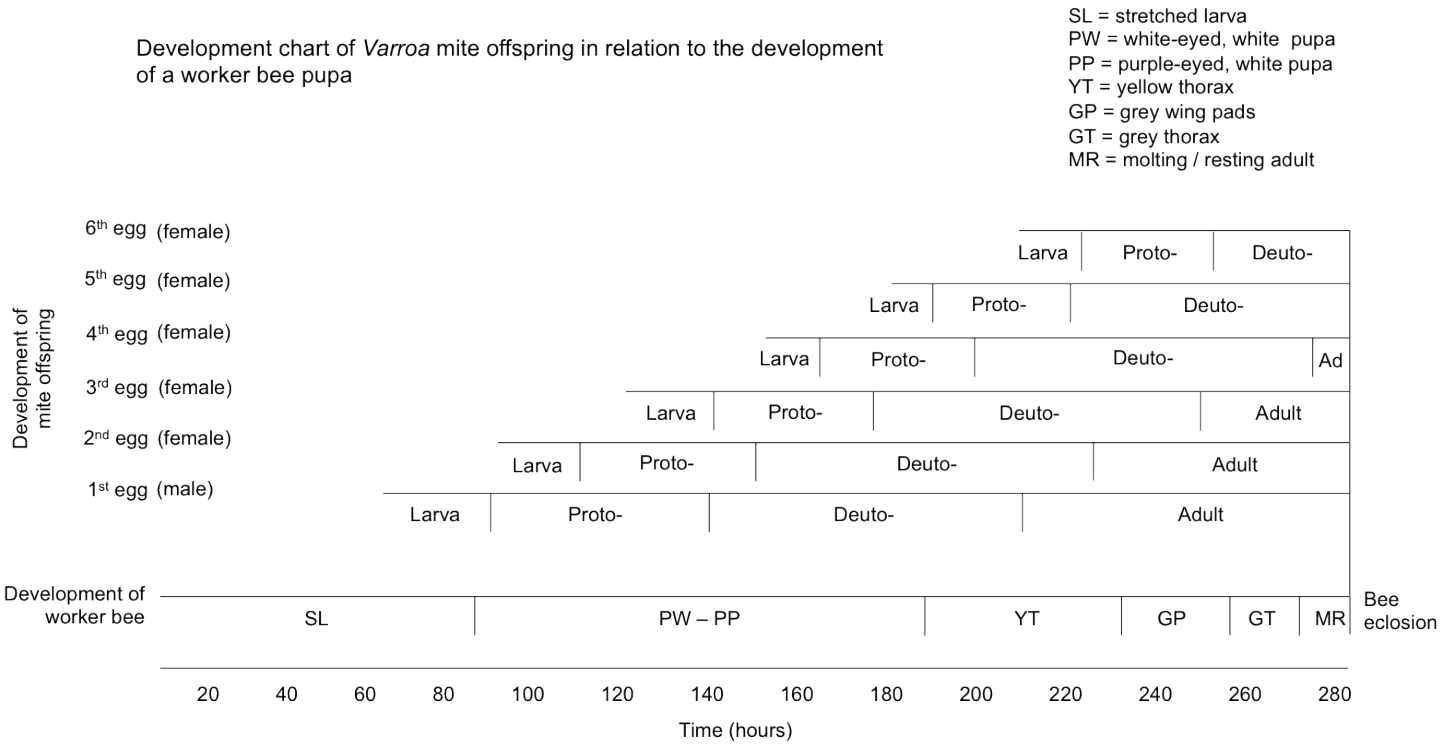

Figure 8. Simplified *Varroa* ontogenic developmental chart from Martin (1994). Proto- and deuto- stages encompass both nymphal and chrysalis forms (chart interpretation by B. Locke).

6.5 Instrumental insemination of queen bees (Paper III)

Instrumental insemination is a reliable method to control honey bee mating and allows the creation of specific crosses that would otherwise be difficult to obtain naturally. It is used widely in apiculture for breeding and honey bee stock improvement and has become an essential tool for research. For our purposes, instrumental insemination was used to create crosses of mite resistant and mite susceptible honey bee populations and vice versa. Queens were inseminated with semen collected from single drones from the desired population. Replicates of different crosses were difficult to obtain in our study due to low survival of inseminated queens after introductions to new colonies (**Paper III**). Similar difficulties have been experienced by other studies using similar techniques (Perez-Sato *et al.*, 2009; Unger & Guzman-Novoa, 2010).

6.6 Molecular detection of honey bee viruses (Paper IV)

The honey bee viruses that we were interested in detecting are positive single-stranded RNA viruses and, thus, the molecular detection techniques we used were based on RNA extraction (Grabensteiner *et al.*, 2001; Chen *et al.*, 2005; Genersch *et al.*, 2006; Blanchard *et al.*, 2007, 2008).

6.6.1 RNA extraction

RNA was extracted from bulk samples of four different sample types: adult bees; pupae without *Varroa* mites; pupae that were infested with *Varroa*; and the associated infesting *Varroa* mites. A QIAcube® automated extraction robot (QIAGEN®) was used to extract the RNA from 100 µl of each bulk sample homogenized in RLT buffer (QIAGEN®). The RNeasy® protocol for plant tissues (QIAGEN®) was used. RNA is easily degraded so the storage methods prior to virus detection are crucial for maintaining the quality of the extracted nucleic acid. For this reason, the bulk samples were stored at -20°C until RNA could be extracted after which the extracted RNA was stored as two 50 µl aliquots at -80°C until subsequent virus analysis (Chen *et al.*, 2007).

6.6.2 RT-qPCR

The polymerase chain reaction (PCR) is a molecular technique used to exponentially amplify copies of a fragment of a DNA molecule. Two complimentary oligonucleotide fragments, known as primers, are used to bind to the target region on the DNA molecule. An enzyme is used to copy the DNA molecules together with the primers in a repetitive reaction during cycles of

repeated heating and cooling to amplifying the target fragment. Honey bee virus detection was performed using a variant of the PCR technique known as RT-PCR, referring to the detection of RNA by converting it into complementary DNA (cDNA) with reverse transcriptase (RT) followed by standard PCR. Real-time quantitative PCR (qPCR) methods detect the product as it accumulates (in 'real' time) and determines the number of new DNA molecules formed in each reaction. The number of cycles required for a sample product to reach a particular threshold is called the quantification cycle (Cq) value and differences in the Cq value reflect the differences in initial amount of the product. Real time RT-qPCR is a standard technique for quantitative diagnosis of honey bee pathogens.

An additional assay for β-actin, a commonly used internal reference gene (Lourenco *et al.*, 2008) using intron-spanning primers (De Miranda & Fries, 2008) was used to normalize the RT-qPCR data for differences between samples in the quality and quantity of the RNA. The amounts of virus and β-actin in each sample were determined using the Bio-Rad iScript™ One-Step RT-qPCR Kit with SYBR® Green as the detection chemistry, 96-well optical qPCR plates, and the Bio-Rad Chromo4™ thermocycler. Each assay was performed with one negative H_2O control and five positive controls, obtained from 10-fold serial dilutions of purified PCR product of known concentration and covered 6 orders of magnitude.

6.6.3 Data conversion, transformation and normalization

Cq-values were used to obtain the absolute amounts of virus and β-actin RNA in each reaction, which was then converted to estimate amounts per bee through the different reaction and extraction dilution factors. Log transformation was used to render the data suitable for parametric analysis. The virus titer and mite infestation rate data for each colony were normalized to the average pre-treatment value for all colonies to avoid statistically significant effects purely due to natural, preexisting differences between colonies.

7 Results and discussion

Through a natural selection process with unmanaged mite infestation levels enforcing a strong selection pressure, honey bee colonies have adapted mechanisms enabling them to survive for extended periods without the use of mite control treatments. By investigating two distinct populations of such mite surviving colonies (on Gotland and in Avignon), this thesis reveals that the adaptation acquired by the colonies to survive *Varroa* infestation mainly involves reducing the reproductive success of the parasite in some way (**Papers I & II**), that the different populations may have evolved different strategies to do so (**Paper II**), and that this ability is genetically inherited (**Paper III**). In addition, the results of this thesis demonstrate that chemical mite control treatments used by beekeepers to inhibit the mite population growth within a colony can actually worsen the health situation for the bee colony by temporarily increasing the bee's susceptibility to virus infection (**Paper IV**).

7.1 Honey bee adaptations reduce mite reproductive success (Papers I & II)

A clear and significant reduction in the reproductive success of the *Varroa* mites was observed in the Bond Bees on Gotland and in the mite surviving population in Avignon when compared to the control colonies in the same locations (*Figure 9*).

The reduced fecundity and reduced ability to produce viable female offspring clearly had an impact on the mite population growth rate and could explain the lower mite infestation rates in the mite-surviving population. Further, this ability to suppress the mite's reproductive success and thus the mite population growth will consequently delay the virus infection build up in the colony, ultimately avoiding colony mortality caused by the virus. Fewer

mites in the colony to vector the viruses means that virus transmission is reduced to less effective transmission pathways within the colony such as vertical or horizontal oral transmission, which rarely leads to colony mortality (de Miranda & Genersch, 2010; *see section 3.3*). This secondary effect of the adaptation to reduce the mite's reproduction may help explain the long-term survival of both these populations without *Varroa* control treatment. However, the observed 30 % reduction in the mite reproductive success is not enough to prevent the mite population from reaching mite infestation mortality threshold levels.

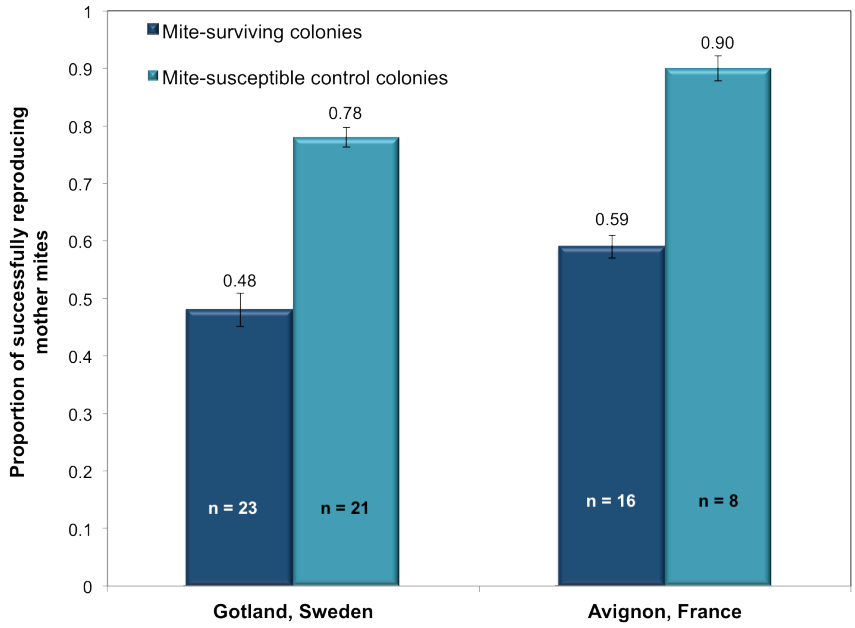

Figure 9. Mean proportions with standard error bars of successfully reproducing mother mites in the *Varroa* mite-surviving colonies and the mite-susceptible control colonies on Gotland, Sweden and in Avignon, France.

7.2 Differences between two distinct *Varroa*-resistant populations (Paper II)

Both the Avignon and the Gotland populations of mite surviving colonies have experienced similar selection pressures through natural mite infestation. This is a unique feature of these colonies compared to most other European honey bee populations whose mite population is controlled by apicultural management and treatment. Through these similar selection pressures, both populations have evolved a similar colony-level mite-resistant trait, namely the

ability to reduce the overall reproductive success of their infesting mites (*Figure 9*). However, when the mite reproductive parameters were investigated more closely, differences between these two populations became apparent. In the Bond colonies on Gotland, delayed egg-laying by the mother mite was the most frequent cause of failure to produce a mature mated female offspring before eclosion of the bee pupa. The second major cause of reproductive failure was the high proportion of dead mite offspring (Table 1). In contrast, the mite surviving population in Avignon had a significantly larger proportion of infertile mites compared to the local control colonies and delayed egg-laying was a secondary contributing factor to the majority of mite reproductive failures (Table 1). The proportion of infertile mites was also significantly different between the two mite surviving populations, demonstrating a distinct difference between them regarding the major parameters involved in reduced mite reproduction. Furthermore, there were no drastically significant differences between the control colonies on Gotland and in Avignon, highlighting that these surviving population are in fact unique among the larger managed population of honey bees in Europe.

It has been demonstrated that bee colonies expressing *Varroa*-sensitive hygiene (VSH) may selectively remove pupae with reproducing mites resulting in a bias in the estimated proportion of infertile mites recorded from the remaining infested cells (Harbo & Harris, 2005; Ibrahim & Spivak, 2006). This may be the mechanism or an explanation for the observed high proportion of infertile mites in the Avignon population. On Gotland however, the delayed egg laying may be caused by differences in pupal volatiles that can inhibit the initiation of egg-laying of mother mites (Nazzi & Milani, 1996; Garrido & Rosenkranz, 2004; Nazzi *et al.*, 2004).

Although both populations on Gotland and in Avignon experienced similar natural selection pressure, these populations have different life history traits and different environmental factors that would also be involved in their adaptive response to mite pressure. In general, it can be expected that different traits would be favored in different populations living in distinct environments especially in traits involved in a coevolutionary relationship (Thompson, 1999).

Table 1. *Mean values and standard errors (SE) of mite reproductive parameters investigated in Avignon, France and Gotland, Sweden including probability values of significant differences between the surviving colonies (SC) and the control colonies (CC) within locations and the probability values of significant differences between all surviving and control colonies (between locations). Levels of significance are denoted with increasing number of asterisk.*

	Within locations, mean (SE), *P*							
	Avignon, France			Gotland, Sweden			Between locations, *P*	
Reproductive parameters	SC	CC	*P*	SC	CC	*P*	SC	CC
Infertility	0.15 (0.02)	0.04 (0.01)	0.0002***	0.08 (0.01)	0.04 (0.01)	0.0259*	0.0002***	0.8679
Dead progeny	0.08 (0.01)	0.02 (0.01)	0.014*	0.13 (0.01)	0.06 (0.03)	0.0050*	0.0203*	0.0554
Absence of male	0.04 (0.01)	0.004(0.01)	0.0186*	0.07 (0.01)	0.03 (0.01)	0.0104*	0.0653	0.1590
Delayed egg-laying	0.13 (0.02)	0.03 (0.02)	0.0015**	0.20 (0.02)	0.08 (0.01)	<0.0001***	0.0131*	0.0410*
Fecundity	3.1 (0.09)	4.1 (0.01)	<0.0001***	3.7 (0.09)	4.3 (0.08)	<0.0001***	0.0006***	0.2810

7.3 Heritability of colony-level reduced mite reproductive success (Paper III)

The mean proportion of successfully reproducing mother mites was drastically lower in all F1 generation colonies that contained genetic material (either maternal or paternal) from the Bond Bees compared to the crosses between control queens and control drones (*Figure 10*). This result suggests that the ability of the Bond Bees to suppress the mite's reproductive success is genetically heritable. Recently three quantitative trait loci (QTL) have been identified in a genomic screen of drones produced by F1 hybrids of the Bond Bees and found to have a highly significant impact on the reproductive success of the mite, primarily through epistatic effects (Behrens *et al.*, 2011).

Due to a low survival of inseminated queens in this heritability experiment, it was not possible to establish replicate colonies of different F1 cross combinations. Therefore, it was not possible to differentiate the mode of inheritance, maternal vs. paternal, which requires further study. However, the expression of this trait in the F1 colonies with only paternal origin from the Bond Bees is indicative of a genetic basis for its expression, as opposed to a maternal or an environmental effect.

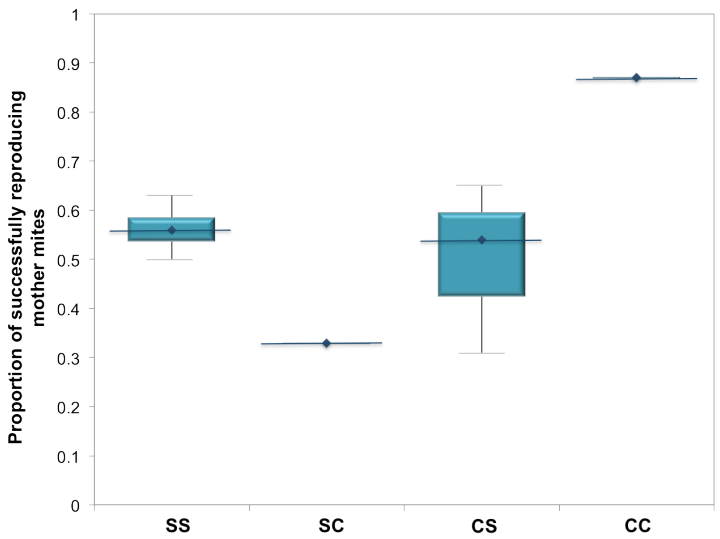

Figure 10. The distribution of mite reproductive success in the colonies of each of the four genotypic groups: SS, Surviving x Surviving; SC, Surviving x Control; CS, Control x Surviving; and CC, Control x Control. The median values (dark blue diamonds with lines), the maximum and minimum values (grey lines); and the 25th to 75th percentile of the data (boxed area), are presented.

Individuals within a honey bee colony have variation in relatedness and heritable traits due to the polyandrous mating behavior of the queen. This results in different patrilines, which can reduce the probability of individuals in a colony sharing alleles. Although some individuals may possess mite resistant genes, if not enough individuals share this genotype it will not influence the resistance at the colony level (Perez-Sato *et al.*, 2009). Therefore, colony level phenotypes are less consistent in the expression of traits in the next generation and may require strong selection on drones in the population to ensure that many patrilines share a particular trait. Controlling the paternal source is the most difficult part of selective breeding programs with honey bees. This may explain why artificial selection has not yet been sustainably successful at producing mite-resistant honey bees and why natural selection, that includes selection on the drones in the population, has provided long-term mite surviving populations on Gotland and in Avignon.

7.4 Additional adaptations of the mite-surviving honey bee population on Gotland (Paper I)

In 2008, the control colonies on Gotland were also not treated against *Varroa* in order to allow a natural mite population development for comparison with the Bond Bees. During the summer of 2009, the control colonies had a significantly faster mite population growth rate than the Bond Bees (*Figure 11*) and all the control colonies died the following winter.

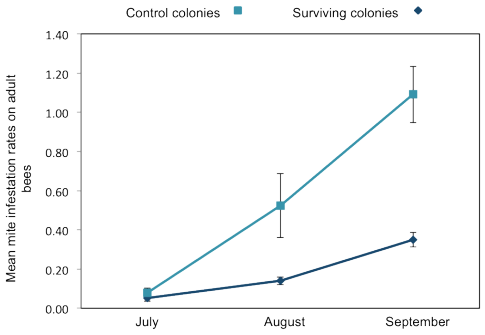

Figure 11. Mean *Varroa* mite infestation rates in surviving Bond colonies and control colonies during the late summer of 2009 with standard error bars.

Neither hygienic nor grooming behavior was found to be significantly higher in the Bond colonies than in control colonies and therefore they are not considered likely explanations for the survival of this population. This result suggests that the attention given by mite-resistance breeding programs to these behavioral defense traits (Buchler *et al.*, 2010; Rinderer *et al.*, 2010; *see section 4.3*) may be misplaced. These behavioral traits were not favored in the

adaptation process through natural selection pressures, as opposed to these artificial breeding programs. Artificial breeding programs aimed at increasing the expression of these behavioral traits for mite resistance are challenged with the difficulty of accurately measuring hygienic or grooming behavior, which usually results in an overestimation, as was likely the case for this study as well for grooming behavior. For example, dimples on the dorsal shield of the mite, which are usually considered to be a sign of damage caused by bees during grooming behavior, have recently been shown to actually be mite birth defects (Davis, 2009). Bees may also damage already dead mites in colony debris (Rosenkranz *et al.*, 1997).

The amount of adult bees, worker brood, and drone brood were significantly lower in the Bond colonies than in control colonies (*Figure 12*). These parameters did not correlate with the mite infestation rates and were therefore not considered to be a consequence of mite infestation. Since mites reproduce in brood cells with a preference for drone brood (Fuchs, 1990), the reduced brood availability of the Bond colonies, particularly the reduced drone brood amounts, drastically limits mite reproductive opportunities. A model developed by Calis *et al* (1999) predicts that more brood, longer brood rearing, and a larger number of drone brood, dramatically increases the mite population growth. Therefore, the reduced population size of the Bond Bee colonies may be an adaptive characteristic to limit the mite population growth and could perhaps be an even greater adaptive strategy than their ability to reduce mite reproduction.

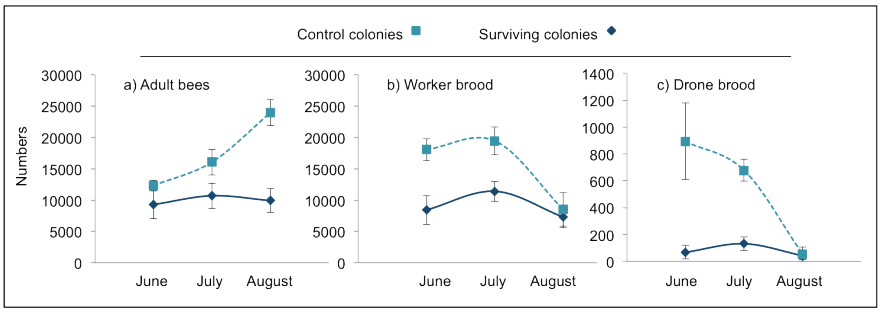

Figure 12. Mean amounts of a) adult bees, b) worker brood and c) drone brood with standard error bars for surviving Bond colonies and control colonies on Gotland in 2008.

7.5 Effects of a chemical *Varroa* control treatment on honey bee virus infections (Paper IV)

Given the strong influence that *Varroa*-mediated transmission has on colony level deformed wing virus (DWV) titers, the expectation of this study was that

removal of the mites from the colony using an acaricide treatment would in turn reduce the DWV titers in the colony (Bowen-Walker *et al.*, 1999; Martin *et al.*, 2010). On the contrary, the results of this study showed an initial increase in DWV titers in adult bees, *Varroa* mites, and both mite infested and uninfested pupae of the colonies that received the tau-fluvalinate treatment (*Figure 13*). This increase in DWV coincided with the most potent chemical effect of the treatment and could potentially be a consequence of debilitating direct effects of tau-fluvalinate on honey bee physiology and/or immune system responses that cause an increased susceptibility to DWV infection. A subsequent progressive decrease in DWV was observed in the treated colonies after the initial increase and is at least partly due to the removal of *Varroa*-mediated transmission (as a result of the treatments effectiveness of mite removal) but may also be a recovery of the host's immune system to the presence of the tau-fluvalinate after an initial shock. Black queen cell virus (BQCV) and sacbrood virus (SBV) also slightly increased in titers following the initial treatment application in some host stages, however much less pronounced than for DWV. Furthermore, their subsequent dynamics appeared random rather than directional. This study only demonstrates the colony level changes in viral infections during an actual acaricide treatment but can not make inferences on the long-term dynamics of virus infections in colonies that are either treated or not.

An interesting additional observation from this study was the large difference in DWV titers between *Varroa* mites in treated and untreated colonies at the end of the treatment period (*Figure 13*). At the cellular and biochemical level, fluvalinate blocks the voltage-gated sodium ion transport channels (Narahashi, 1996; Rosenkranz *et al.*, 2010) that regulate osmotic pressure. The channels are frequently a target for viruses that use osmotic pressure to burst cells to release newly formed virus particles (Stauffer & Ziegler, 1989; Kunzelmann *et al.*, 2000; Hoffmann *et al.*, 2008). A variable proportion of *Varroa* mites are themselves a host for DWV (Yue & Genersch, 2005) and have higher elevated DWV titers compared to mites that do not replicate DWV. Synergistic interactions between fluvalinate and DWV at the sodium transport channels may cause mortality for mites with replicating DWV. Mites that do not replicate DWV and thus have lower titers could survive fluvalinate treatment. This argument, however plausible, is based on the observations of only one colony that still had mites remaining at the end of the treatment and needs further investigations.

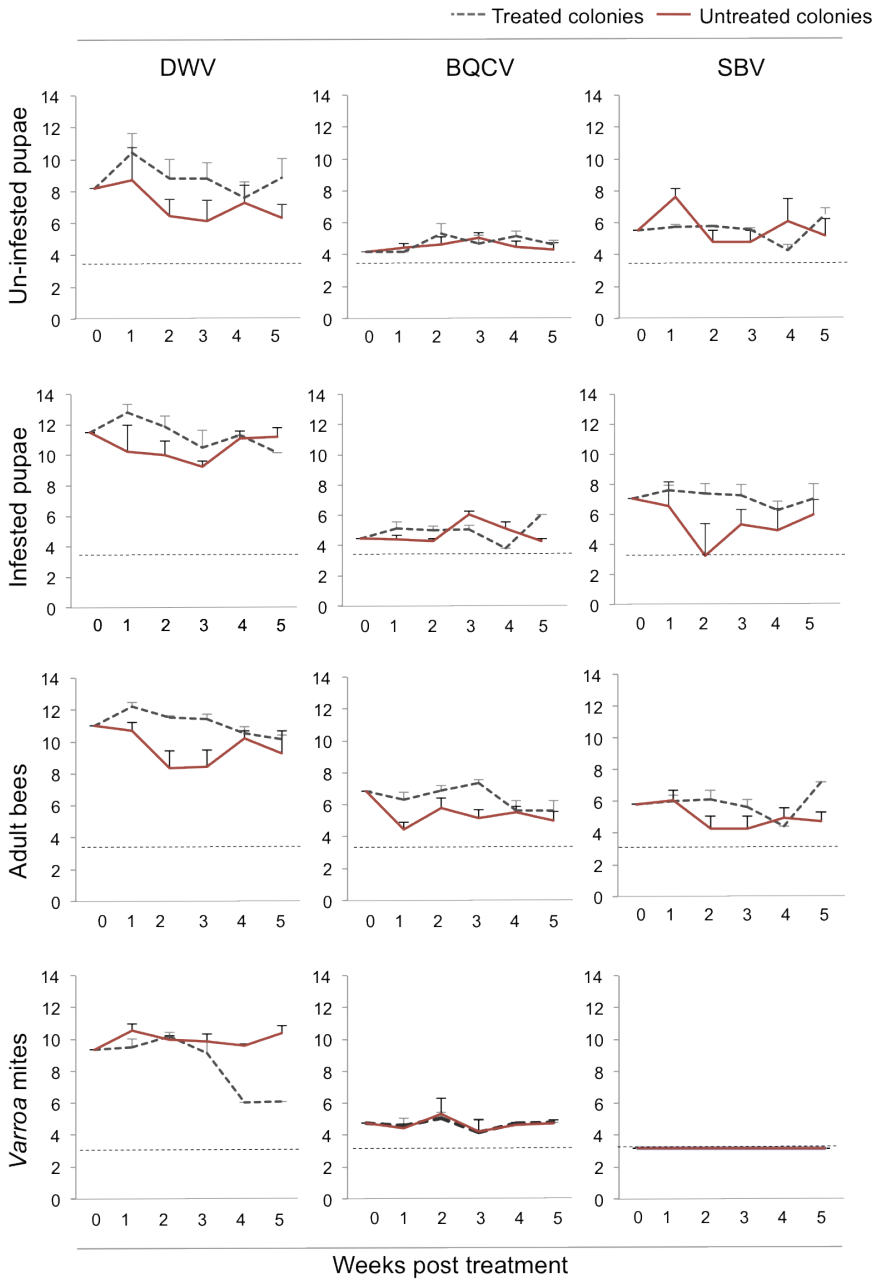

Figure 13. Pre-treatment normalized DWV, BQCV and SBV titers of uninfested pupae, infested pupae, adult bees and *Varroa* mites in acaricide treated colonies and untreated colonies for the duration of the acaricide treatment. Week 0 represents the pre-treatment sample. Titers are given on a $\log_{10}$ scale and the dotted straight lines indicate the limit of detection of the RT-qPCR assays. The error bars denote standard errors.

8 Conclusions and future challenges

The only documented sustainable tolerance to *Varroa* mites in European honey bees are of colonies that have not been selected by humans, but have been exposed to natural selection pressures. Both the Gotland and Avignon populations presented in this thesis share the fact that they have been unmanaged, enabling natural selection (as opposed to artificial) to shape the evolution of their mite resistance and ability to reduce the mite's fitness. This highlights the impact that apicultural practices otherwise have on these host-parasite interactions (Fries & Camazine, 2001), and suggests a human interference in coevolution between species. Further, this adapted resistance has evolved incredible fast by natural selection.

A deeper understanding of how honey bee colonies naturally coevolve with parasites and understanding the mechanisms behind such coevolution, is necessary for establishing long-term sustainable honey bee health management strategies in apiculture. Further, deeper knowledge of virus-vector epidemiology and interactions will be important in order to implement effective techniques for managing different virus infections.

Further work is required on the interactions between the many honey bee colony stressors in this complex system, including effects of *Varroa* mites, the viruses associated with *Varroa*, the variety of harsh treatments used to control *Varroa*, and intensified apicultural management practices. Some open questions developed from the studies included in this thesis are:

- What are the mechanistic explanations behind the bees' ability to suppress mite reproductive success and how does it differ between the Gotland and Avignon population?
- What is the relative importance of the reduced colony size compared to the reduced mite reproduction for the long-term survival of mite-infested colonies?

- What is the mode of inheritance for the reduced mite reproduction trait and can this trait be used in breeding mite-resistance?
- Since mite infestations remain quite high in the mite-surviving populations and visible virus symptoms are present, have these populations also evolved a level of resistance or tolerance to the actual virus infections?
- How does the adaptation of reduced mite reproductive success influence the coevolution of the viruses vectored by the mite in these populations?
- How does the chemical control treatment interact with the virus at a biochemical level in both the bee and the mite?
- Does the virus infection have any adverse effects on the mite while it is replicating?

8.1 Practical aspects

The disadvantage of natural selection is that it neglects features that are important for apiculture. The mite-surviving bee populations, although possessing a mite-resistant trait that can be genetically inherited (**Papers I, II & III**), may have evolved other characteristics that are undesirable for beekeepers such as small colony size resulting in small honey yields (as with the Gotland population) or may be overly aggressive (as with the Avignon population). A practical next step to the work achieved in this thesis is to introduce genetics of the mite-surviving bees into established breeding programs to determine if they can maintain their resistant traits under artificial selection for commercially viable traits in apiculture.

Selecting for reduced mite reproduction requires tedious examinations of mites in brood and may not be a practical selection-breeding avenue for beekeepers without laboratory facilities. The results from this thesis would perhaps indicate that a more realistic approach for breeding mite resistance would be to select for colonies with low mite population growth. This would eliminate the bias for certain traits that could be difficult to maintain in the next generations or are of relative little significance compared to other traits, and avoid the need for tedious inspections in all colonies.

Studying the synergistic interactions between pathogens and pesticides is a research field in its infancy. However, the few studies that have been conducted regarding this aspect of bee health (Alaux *et al.*, 2010; Pettis *et al.*, 2012; **Paper IV**), have all shown that these interactions have a negative impact on bee health. Therefore, we need not only to develop strategies that increase bee tolerance or resistance to specific disease but we also need to develop

strategies that reduce potentially harmful interactions between multiple pathogens or pathogens and pesticides.

As apicultural management techniques greatly influence the health of the honey bee colonies, strategies for better beekeeping practice in general are urgently needed that can reduce pathogen virulence by inhibiting the critical infection pathways that management otherwise induces. This could be accomplished through integrative management, disease awareness education and monitoring, and improved (disease conscious) beekeeping methods. This will not only improve colony health but also ensure the quality and safety of honey and other honey bee products by reducing the need for chemicals or antibiotics as disease control treatments.

Figure 14. A Beekeeper's smoker plugged up after a hard days work with the bees (Photo: B. Locke).

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