Swedish University of Agricultural Sciences
**Faculty of Natural Resources and Agricultural Sciences**

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# Påverkan av odlade honungsbin och kommersiellt framtagna humlor på förekomsten av patogener i vilda bin

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*Photo: Sofie Tinggren*

Independent project, 30 HEC
Animal Science - Master's programme
Department of Ecology
Uppsala 2020

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**Credits:** 30 HEC

**Level:** A2E

**Course title:** Independent Project in Biology - Master's thesis

**Course code:** EX0565

**Program/education:** Animal Science - Master's Programme

**Course coordinating department:** Department of Aquatic Sciences and Assessment

**Place of publication:** Uppsala

**Year of publication:** 2020

**Online publication:** http://stud.epsilon.slu.se

**Keywords:** honey bee, bumblebee, diseases, commercial bees, bee intensity
**Nyckelord:** honungsbin, humlor, sjukdomar, kommersiella bin, bi intensitet

Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences
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# *Special thanks to:*

***Supervisor - Joachim de Miranda***

***Co-supervisor - Barbara Locke***

***Biomedical technologist - Emilia Semberg***

***Lab technician - Piero Onorati***

*who made this thesis possible*
*through support and patience*

## Populärvetenskaplig sammanfattning

**Bin är viktiga insekter tack vare deras förmåga att pollinera våra grödor som t.ex. grönsaker och frukter. Men de senaste åren så har forskare upptäckt en minskning av antalet bin. I Nordamerika har man sett att mängden bin minskat med 59% (1947-2005), och i Europa med 25% (1985-2005). Däremot har odling av bin ökat så kraftigt i Kina att generellt så har mängden bin i världen ökat 45%. Men det är inte bara att antalet bin minskar utan även att antalet grödor som behöver pollinering samtidigt har ökat med 300% de senaste 50 åren. Så pollineringen av grödor blir inte tillräcklig, vilket leder till att skördarna minskar samt att vissa vilda blommor och bär riskerar att försvinna utan bin.**

Varför antalet bin minskar kraftigt på olika platser vet man inte exakt. Det kan finnas många olika anledningar. Landskapet har förändrats, intensivare jordbruk med bekämpningsmedel, minskning av dikeskanterna där bina har sina boplatser och där vilda blommor växte är borta. Odlingsarealerna blir större med ensidiga grödor som blommar och ger bina nektar och pollen under en kort period. Utan dikeskanternas blommor före och efter grödans blomning så finns det ingen mat att samla för bina. En annan viktig och troligtvis starkt bidragande faktor till minskningen av bin är sjukdomar.

I världen finns det ca 20,000 biarter, varav ca 6-7 stycken är honungsbin, 225 stycken är humlor och resten är vilda bin, också kallade solitära och lever inte i kolonier, utan som ensamma individer. Det nästan alla bin har gemensamt är att de samlar pollen och nektar till mat både till sig själva och sina larver.

Världens bin delar många gemensamma typer av sjukdomar och patogener, som både kan överföras mellan bin av samma, eller av olika arter. För att försäkra sig om att grödor såsom exempelvis jordgubbar och tomater ska bli tillräckligt pollinerade så kan humlebon köpas in och placeras ut i växthus och på fält, likaså kan honungsbin förflyttas och skickas mellan odlare. En ökad oro är att odlade eller konventionella bin kan föra över patogener till naturligt vilda bin med potentiellt förödande konsekvenser.

Syftet med detta projekt var att bedöma om patogener i vilda bin har någon relation till fördelningen av patogener i odlade bin. De sex undersökta patogenerna har valts ut utifrån att de alla är relativt vanliga och drabbar antigen både honungsbin och humlor eller de båda biarterna separat. De sex patogenerna är Nosema spp., Crithidia spp., Apicystis spp., Acute bee paralysis virus (ABPV), slow bee paralysis virus (SBPV) och deformed wing virus (DWV). Studien delades in i tre olika projekt. Det första var att se om det sker en förändring i patogenfloran hos vilda bin på samma plats under tre år. Det andra projektet var att se om patogenfloran förändras hos vilda bin i förhållandet till mängden bin och avståndet till honungsbin och deras kupor. Det sista projektet var att jämföra patogenfloran hos vilda bin i förhållande till förekomsten eller frånvaron av importerade humlekolonier.

Smittspridningen av patogener mellan bin kan ske på flera olika sätt. Antingen så kallat horisontell överföring mellan bina, då överföringen av patogener sker mellan bina i samma generation. Till exempel när bina inom samma koloni kommunicerar, kammar eller matar varandra. Men kontakten med andra bin kan ske genom kontakt med samma material så som utnyttjandet av samma blomma för nektar eller polleninsamling. Det kan också förekomma att främmande bin försöker stjäla mat från en annan koloni och kan på det viset ta med sig nya patogener in eller ut från kupan. Vertikal smittspridning sker mellan generationerna, från vuxet bi till larven. Men kan också ske genom att kolonin svärmar. Det vill säga att kolonin delar på sig och drottningen flyger iväg med en grupp arbetarbin och drönare, och letar upp ett annat boende. Medan de bin som är kvar föder upp en ny drottning.

Beroende på vilken patogen som drabbar bina så blir det olika symtom. Vissa symptom kan bli starkare om bina lever under stress, som till exempel när det finns dåligt med blommor och bina lever på svältgränsen. De vanligaste symptomen blir en ökad vinterdödlighet, långsam uppstart på våren, lägre honungsproduktion och kortare livstid. Inlärningssvårigheter, paralysering och outvecklade vingar kan också vara konsekvenser av patogenförekomst.

Resultaten i studien visar att det finns ett förhållande mellan fördelningen av patogener i odlade bin och i naturligt vilda bin. Den första studien visar att under de senaste tre åren på den undersökta platsen, har det skett en ökning av patogener både för honungsbin och vilda bin, men det har också skett en ökning av antalet bin. Vilket kan betyda att en ökning av bin ger en tätare kontakt mellan arterna genom delad miljö och blommor, som kan ha lett till en ökning av patogenförekomsten. Den andra studien visar att avståndet mellan honungsbin påverkar förekomsten och nivåerna av vissa patogener i vilda bin. På det avståndet där det inte förekom några honungsbin (2000m), så sjönk patogenförekomsten för två av de sex patogener. Varav en tredje patogen bara kunde hittas där det inte fanns några honungsbin. Resultaten för den tredje studien som visar förhållandet av patogener mellan importerade och icke-importerade är otydliga. Dock hade importerade humlor ett högre antal bin infekterade med en av patogenerna än de andra bina. Hur mycket dessa tre olika studiers förhållanden påverkar antalet bi och de olika biarternas hälsa skulle behöva undersökas ytterligare och i större skala.

Strategier över hur vi bäst ska kunna bevara bina och andra pollinatörer, speciellt inom jordbruket är en viktig uppgift. Ett odlingssystem där odlingen ger fler växter med olika blomningssäsong, minskning av bekämpningsmedel eller mer specifik tidpunkt när den ska användas, utan att riskera en ökning av skadegörare på grödorna. Försöka skapa system där flödet av mat är mer konstant och där det ska finnas utrymme för vilda bins boplatser. Avel på binas egen motståndskraft mot patogener skulle vara ett naturligt och hållbart sätt att bevara och skydda odlade bin. Mer kunskap och forskning kommer att behövas för att få veta mer om hur bina påverkas av patogener i kombination till olika miljöer. Bin är viktiga djur som genom sin pollinering av växter hjälper till av bevara den biologiska mångfalden och det naturliga ekosystemet, samt hjälper oss att odla fram nyttiga grönsaker, frukter och bär.

## Abstract

Bees are important beneficial insects due to their ability to pollinate our crops and vegetables, as well as being critically important for maintaining wild floral biodiversity. Bees are important from both an economic perspective and an environmental perspective, which highlights the importance of having healthy and sustainable bee populations. The world's bees share many of the same, or similar, pathogens, which can be transmitted between bees of the same or different species. One concern is that pathogens in imported or managed bees may spill over to native bees, with potentially devastating effects. The aim of this project was to assess if the pathogen distribution in wild bees has any relationship to the distribution of pathogens in managed bees. The pathogens investigated are: *Nosema* spp., *Crithidia* spp., *Apicystis* spp., acute bee paralysis virus (ABPV), slow bee paralysis virus (SBPV) and deformed wing virus (DWV). The study was divided into three separate projects.

**Super-B project:** The goal of this study was to determine if there is a change in the pathogen distribution in wild bees at the same location over three years.
**Lövsta project:** The goal of this study was to determine the pathogen distribution in wild bees in relation to the bee density and distance to honeybee hives.
**MSB project:** The goal of this study was to compare pathogen distribution and abundance in wild bumblebees in relation to the presence or absence of imported bumblebee colonies.

These three projects show that there is a relationship between the distribution of pathogens in managed bees and wild bees. During three consecutive years (2015 to 2017), there was an increase in overall pathogen pressure in both honeybees and wild bees, coinciding with an increase of the density of bees in the sampled area. These trends were however different for different pathogens. The distance to honeybee colonies affected the presence and levels of pathogens in wild bees, again in a pathogen-specific manner. Similarly, the effect of imported *Bombus terrestris* on the pathogen distribution in wild bumblebees, including wild *Bombus terrestris*, was different for different pathogens. Nosema, Crithidia and ABPV were present at higher levels in the strawberry farm without imported *Bombus terrestris*, SBPV was higher at the farm with imported *Bombus terrestris*, while Apicystis and DWV were roughly the same. More investigation is needed on the effect of these pathogens on different bee species and their function pathogen transmission.

## Sammanfattning

Bin är både viktiga och gynnsamma insekter tack vare deras förmåga att pollinera våra grödor. De är också essentiella för att upprätthålla den biologiska mångfalden. Bin är viktiga både ur ett ekonomiskt perspektiv, ett miljöperspektiv och från biets eget perspektiv att de är friska och har en hållbar framtid. Världens bin delar många patogener som kan överföras mellan bin av samma eller olika art. En ökad oro är att införda kommersiella humlor eller odlade honungsbin kan föra över patogener till vilda bin med potentiellt förödande konsekvenser. Syftet med detta projekt var att bedöma om förekomst och mängd patogener i vilda bin har någon relation till fördelningen av patogener i kommersiella bin. De undersökta patogenerna är: *Nosema* spp., *Crithidia* spp., *Apicystis* spp., Acute bee paralysis virus (ABPV), slow bee paralysis virus (SBPV) och deformed wing virus (DWV). Studien delades in i tre separata projekt.

**Super-B-projekt**: Syftet med denna studie var att se om det sker en förändring i patogenfloran hos vilda bin på samma plats under tre år.
**Lövsta-projektet**: Syftet med denna studie var att se om patogenfloran förändras hos vilda bin i förhållandet till binas förekomst och avståndet till honungsbin och dess kupor.
**MSB-projekt**: Syftet med denna studie var att jämföra patogenfloran hos vilda bin i förhållande till förekomsten eller frånvaron av importerade humlekolonier (*Bombus terrestris*).

Dessa tre projekt visar att det finns ett förhållande mellan fördelningen av patogener i odlade bin och i naturligt vilda bin. Under tre år i rad (2015 till 2017) har det skett en ökning av patogentryck i allmänhet, både för honungsbin och vilda bin, samtidigt som det har skett en ökning av antalet bin i området. Trenden är dock olika för olika patogener. Avståndet till honungsbisamhällen påverkar förekomsten och nivåerna av patogener i vilda bin, återigen med olika mönster för olika patogener. Likadant hade närvarande av odlade importerade *Bombus terrestris* olika effekt på olika patogenens förekomst i vilda humlor, inklusive vilda *Bombus terrestris*. Nosema, Crithidia och ABPV fanns i högre utsträckning på den jordgubbsodling som inte använda sig av importerade humlor, SBPV fanns i mycket högre grad i den odling som använde sig av importerade humlor, medan Apicystis och DWV fanns i lika grad hos både odlingar. Mer forskning behövs gällande effekten av dessa patogener på olika biarter och deras risk för smittspridnings.

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# Introduction

Bees are important beneficial insects due to their ability to pollinate our crops and vegetables (Goulson 2015), as well as being critical important for maintaining wild floral biodiversity. Although the production of honey from honeybees has significant direct economic value world-wide (Goulson 2015), by far their greatest economic value is through their pollination services to both arable and orchard crops (Gallaiab *et al.,* 2009). There are more than 20 000 bee species globally that has been acknowledged (Ascher *et al.,* 2015). Of them about 6-7 are honeybee species (Kotthoff *et al.*, 2011), and 225 species are bumblebees (Williams *et al.,* 2008), the rest are solitary bees. It is important both from an economic perspective, an environmental perspective and from the bee's perspective that they are healthy and have a sustainable population. It is a rising concern all over the globe that pollinators are decreasing (Goulson 2015), especially since pollination dependent crops have been increasing the last 50 years by 300% (Aizen *et al.,* 2009). In Europe, 84% of the crops grown are dependent of pollination (Williams 1994) and 80% of Europe's wild plants are pollinator dependent (Kwak *et al,.* 1998).

The reasons why bees are decreasing in many places such as Europe and the United States are unknown. The export and import of honeybees or bumblebees, both colonies and queens, have never been as large as it is today. The decline in pollinators is thought to originate from multiple factors, such as loss of habitat, pesticides, pollutants, climate change, diseases and pathogens (Goulson 2015).

## Aims of this Project

Even though the world's bees are classified in many different families and genera, they share many of the same, or similar, pathogens, which can be transmitted between bees of the same or different species. One concern is that pathogens in imported or managed bees may spill over to native bees with potentially devastating effects (Plischuk and Lange 2009; Tehel *et al.,* 2016).

The aim of this project was to assess if the pathogen distribution in wild bees has any relationship to the distribution of pathogens in managed bees sharing the same habitat area, considering both managed honeybees (*Apis mellifera*) and imported, commercially reared bumblebees (*Bombus terrestris*). The pathogens investigated are: *Nosema spp., Crithidia spp., Apicystis spp.,* acute bee paralysis virus (ABPV), slow bee paralysis virus (SBPV) and deformed wing virus (DWV). The study was divided into three separate subprojects:

**Super-B project**: The goal of this study was to determine if there is a change in the pathogen distribution in wild bees at the same location over three years, in an area with a long-term (>30 years) presence of honeybee colonies close to the sampling location

**Lövsta project**: The goal of this study was to determine the pathogen distribution in wild bees in relation to the bee density and distance to honeybee hives, in an area where honeybee colonies were newly introduced (< 1 year) after an absence of >30 years

**MSB project**: The goal of this study was to compare pathogen distribution and abundance in wild bees in relation to the presence or absence of imported bumblebee colonies

The pathogens investigated in this study were chosen because they are known to be naturally common to many bee species (Crithidia, Nosema, Apicystis), are known to be transmissible between bee species (all) and/or have a particular relationship with either honeybees (deformed wing virus) or bumblebees (slow bee paralysis virus) that makes them interesting for the research questions of this study.

# Literature review

## Background

Pollinating insects are essential to both the natural ecosystem and agricultural crop production (Goulson *et al.,* 2015). Insect pollination is directly beneficial to 75% of the global crop production (FAO 2018b). Over the last 50 years there has been a huge decline in wild bee and pollinator numbers, and even local extinctions of certain species. Crop yield has also decreased due to insufficient pollination (Goulson *et al.,* 2015), which has led to the recognition of a "pollination crisis" (Holden *et al.,* 2006). Interest for research in the area has increased during the last few decades. Common assumptions about the decline in bee species are being questioned, due to a lack of clear data about the decline in wild bee species in the world (Goulson *et al.,* 2015). Much of the data on wild bee declines comes from research on bumblebees, largely from studies in the United Kingdom, where some species have been confirmed extinct (Goulson 2008), and there are major knowledge gaps about other pollinator species and other areas in the world. Much research on bee declines is also heavily focused on seasonal losses of domesticated honeybee (*Apis mellifera*) colonies (Goulson *et al.,* 2015). In Europe these losses have averaged about 25% between 1985 and 2005 (Potts *et al.,* 2010), and in North America the annual colony loss has averaged 59% between 1947 and 2005 (NRC 2007). However, the global population of domesticated honeybee colonies has increased by 45% between the years 1961 and 2008, with much of the increase occurring in China (Aizen *et al.,* 2009).

While general bee abundance and diversity have experienced a decline, the demand for crop pollination services has risen by 300% during the last 50 years (Aizen *et al.,* 2009). World-wide, most of the pollination service of crops is performed by wild pollinators, rather than managed honeybees. Mallinger *et al,* (2014), showed that a high abundance of wild pollinators is more closely correlated to high crop yields than a high abundance of honeybees. Increased stocking with domesticated honeybee colonies to compensate for inadequate pollination of crops is unlikely to solve the problem (Goulson 2015; Mallinger *et al.,* 2014), while overdependence on a single pollinator species for full pollination service is always a risky solution (Kremen *et al.,* 2005).

There are several suggested explanations for the decline in bees. The progressive loss of habitat for the pollinators, particularly during the previous century, has contributed significantly to the long-term decline (Goulson 2008). Intensively managed farmlands have replaced much of the flower-rich natural and semi-natural habitats that most wild bee species need. During the 20$^{th}$ century, 97% of United Kingdom's semi-natural pastures where transformed into farmlands (Fuller 1984). Also urbanization and its associated infrastructure, such as the building of roads, has disturbed and reduced the habitats for pollinators (Baxter-Gilbert 2015).

Another important factor in bee declines are diseases. Bees suffer naturally from many parasites and pathogens, included bacteria, viruses, protozoans and fungi. Most of the research has been done on the diseases connected to honeybees, while the experimental research on bumblebee diseases has been conducted almost exclusively on *Bombus terrestris*, the buff-tailed bumblebee. Our knowledge about diseases of solitary bees is still very limited (Goulson 2015). The pathogens *Nosema ceranae* and deformed wing virus (DWV) are able to infect both honeybees and bumblebees (Genersch *et al.,* 2006b). Historically, the long-distance transportation and world trade in honeybee colonies, coupled with the absence of effective quarantine strategies, and has helped spread a number of bee diseases that were previously geographically restricted. A world-wide trading network for bumblebee pollination services developed during the 1980s and is now responsible for the production, sale and world-wide distribution of more than 1 million

bumblebee colonies annually (Goulson 2015). There is a real possibility that the pollination service companies that ship bumblebee colonies around the world for the pollination of greenhouse and tunnel crops may have contributed to the re-distribution of bee diseases.

Agrochemicals are also a possible factor in the decline in bees. Pesticides are an effective tool for reducing weed, insect and disease problems in agricultural farming systems, but also contribute to a lack of floral resources for bees in the agricultural landscape (Goulson 2008, Goulson 2015). Neonicotinoids are a group of insecticides that have a sublethal effect for wild- and solitary bees. They affect reproduction, colony growth for bumblebees, nesting behavior for solitary bees and the wild bee density (Rundlöf *et al.*, 2015). Rondeau *et al.,* (2014) showed that neonicotinoids had a lethal effect in high doses and sublethal effect in small doses for overwintering honeybees. However, newer studies of the effect of neonicotinoids on honeybees did not show any evidence of any harm for the colony as a whole (Zemeckis *et al*., 2019; Osterman *et al.,* 2019), even if individual bees may well be affected. The increasing use of monocultures on farmlands also has made the diet for the bees very monotonous compared to what the diet has been for bees in the past nearby farmlands (Goulson 2015). The pollen quality and the diversity of flowers producing the pollen affects the tolerance against diseases and influences bee longevity (Pasquale *et al.,* 2013).

Another pressure on the decline of native bees may occur with the introduction of non-native bees. Competition for floral resources and limited nesting sites may precipitate a decline of native bees in favor of non-native bee species (Goulson 2003). A major threat, not only to bees but for all biodiversity, is the on-going and rapidly accelerating changes in climate. There is little evidence that the change in climate have an impact on bees, but there is a potential effect that the changes will create a mismatch between plant and their specific pollinators. Geographic range shifts are one major type of response to changes in the climate, were species that have a certain level of mobility (*e.g.* animals and insects) can react quickly to the changes in their environment, while species that need a longer time to move and migrate (*e.g.* many plants) are likely to shift or reject the area first (Lundy *et al.*, 2010). Range shift has been shown in butterflies as a response to climate changes (Forister *et al.,* 2010), and something similar is also expected to occur in bees (Williams *et al.,* 2009).

It is likely that it is the interaction and combination of different stressors that is more harmful to honeybee colonies than any one single stressor (Shi *et al.,* 2004). Wu *et al.,* (2012) for instance show that honeybees are more susceptible to the pathogen *Nosema ceranae* if they have been exposed to neonicotinoid insecticides during their development. Exposure to pesticides has also been shown to weaken the immune system of insects (Doublet *et al.,* 2014; James *et al.,* 2012).

## Pollinators & Pollination

The bees are estimated to account for €153 billion in pollination services world-wide. This represents 9.5% of the value of foods for human consumption produced by the agriculture industry in the world every year (Gallai *et al.,* 2009). The country with the most developed pollination service is probably the USA (Aizen *et al.,* 2009). It estimated that about 20% of the honeybee hives in the USA are used primarily for pollination service, rather than for honey production (Morse *et al.,* 2000). Foraging is a challenging task for the bee to learn. The bee needs to learn how to locate the flowers and how to forage as effectively as possible. Some flowers are more complex to manipulate to get access to the nectar or the pollen. Flowers have a huge variation in scent, color and morphology. Plants also vary in the extent to which their nectar and pollen nourishes the bees (Lavert 1994). It is easier for bees to learn to forage for nectar than to forage for pollen (Raine *et al.,* 2006).

## Honeybees

The honeybee is a social bee living in a large colony with many thousands of sterile female worker bees, a few hundred males (drones), and a single reproductive female; the queen. The queen can live for several years while the other bees live for a few weeks during the active season, and several months during winter. After a queen has mated outside the hive in the air, she goes back to the hive and stays there. Her main purpose will be to lay eggs, up to 2000 eggs a day during spring and summer. The worker bees will feed her and the colony's larvae, they will clean the hive and produce a store of food for when no forage is available, which in the temperate regions mostly is the winter. When the winter comes, the queen will be at the center of the cluster of bees, where she is kept warm by her workers, who will shiver to keep the temperature in the center of the cluster close to 32°C. The following spring the queen will start building up a new young population of bees and the worker bees that kept her warm during winter will be replaced (Gornert 1969).

The global honeybee industry has increased during the last few decades. This is due to the increased demand for pollination services for agriculture crop production. The honeybee is regarded as the most important pollinator of agricultural crops (McGregor 1976). World-wide, commercial honeybee colonies have increased by 45% during the last 50 years (Diagram 1a). In Europe and United states there has been a decline in honeybee population, but this drop is off-set by increases in beekeeping the rest of the world (Aizen *et al.,* 2009). During the last 50 years, the average honey yield per hive has increased between 30% and 50%. Globally, the average honeybee hive produces about 35 kg honey per year (Diagram 1b). Pollination-dependent crops have increased by 100% during 30 years from 1961 to 1990, and again by a further 200% during the subsequent 20 years (Diagram 1c). This rapidly increasing need of pollinators has led to a shortage of honeybee colonies during much of this time. This lack of honeybee colonies for pollination started to recover during the beginning of the 21st century (Diagram 1d; Aizen *et al.,* 2009).

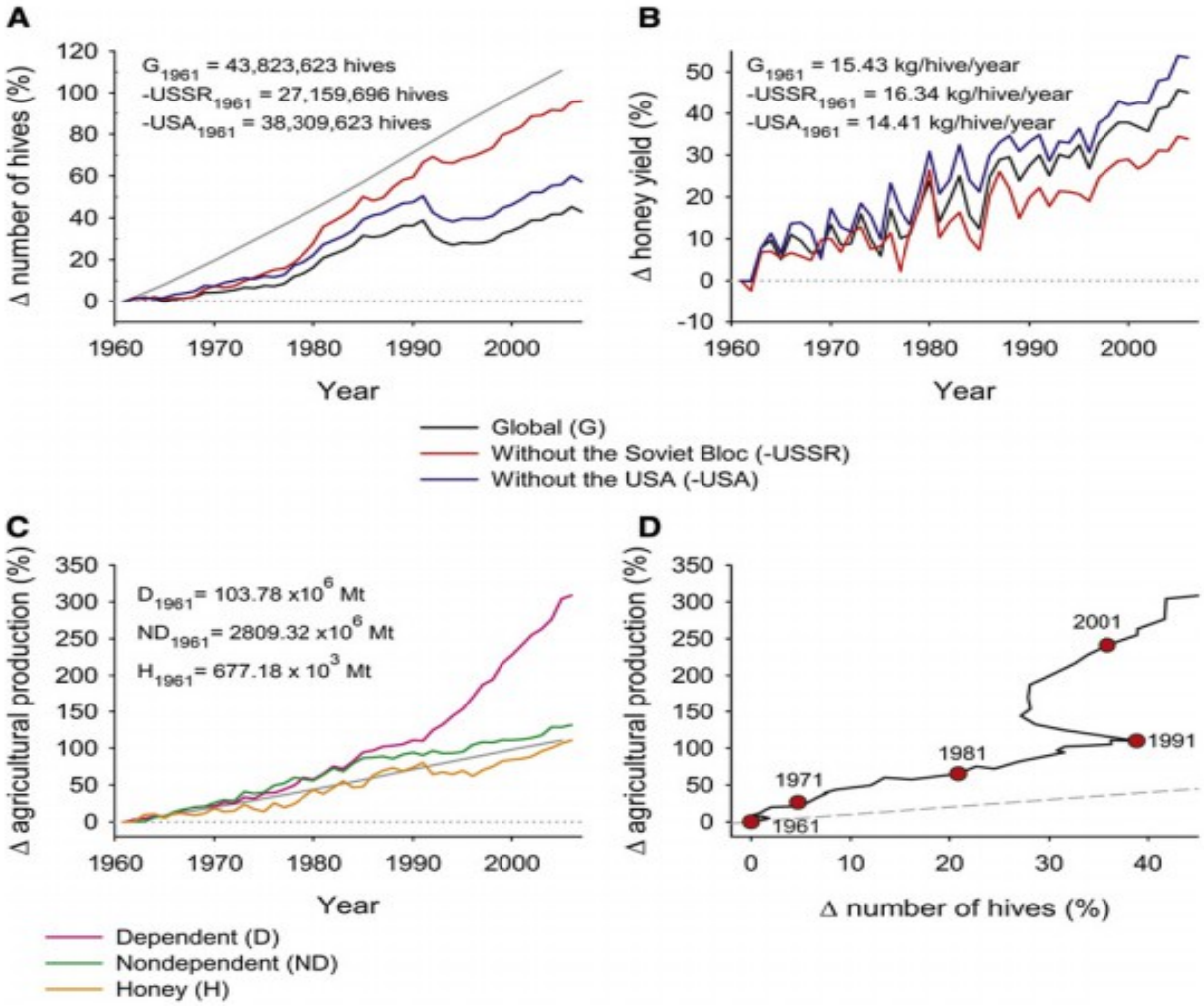



Figure 1: Change in honeybee productivity from the year 1961 to 2007. A) The global change in number of honeybee hives, relative to data from 1961; B) The global change in honey production, relative to data from 1961; C) Change in agricultural production over time, relative to data from 1961; D) The change relative to 1961 in agricultural production that is dependent on pollinators in relation to the change in number of bee hives during the same period (Aizen *et al.,* 2009).

One of the advantages of honeybees as pollinators are that they form large colonies. One honeybee colony can have up to 60,000 individuals (Gornert 1969), compared to 50 to 400 individuals per colony for bumblebees, depending on the species. However, honeybees are more sensitive to bad weather and low temperatures than bumblebees. Nielsen *et al,* (2017) showed that honeybees have a reduced foraging activity if the temperature is above their optimum of 24.1°C. An optimal foraging temperature was not found for bumblebees. The study used flower visits as an indicator of activity. The study showed that the change in flower visits in relation to temperature was higher (46%) for honeybees than for bumblebees (2%) (Nielsen *et al.,* 2017). Winston (1987) claims that honeybee foraging activity starts at 12-14 °C, but also decreases with more wind.

## Bumblebees

The bumblebee is a social bee living in small colonies of a few hundred individuals. The bumblebee life cycle starts when the bumblebee queen emerges from hibernation, sometime between early spring and the beginning of the summer, depending on species, to look for a place to build up her colony. After building a nest she starts laying eggs and forages for both herself and her first batch of larvae. When the first workers bees emerge, they start to help to collect

food and building out the nest. The colony will consist of a few dozen to a few hundred worker bees, depending on species and foraging resources. From this moment forward, the queen will stay in the nest to focus on laying eggs. The population will grow bigger during the summer and the colony will start producing some drones and new queens. At the end of the season, the new queens emerge from the nest to mate, after which they immediately start looking for a place to hibernate until next spring. Only the mated queens will go in hibernation: the old queen, workers and drones will gradually die. The old queen lives for about one year, and the workers just a few weeks (Mossberg and Cederberg 2012).

The bumblebee is the most popular non-Apis bee to be commercialized for pollination. The commercialization of bumblebees for pollination started in 1987, and ever since the yearly sale of colonies has increased (Figure 2). The main bumblebee species produced are *Bombus terrestris* and *Bombus impatiens*; the latter specific for the USA market. Bumblebee species are divided into two groups, "pollen storer" and "pocket maker" based on how they provision the pollen for their offspring (Sladen 1912). The foragers of "pollen storer" type bumblebees leave the collected pollen in storage pots for the house bees to collect and distribute to the brood cells, while the foragers of "pocket maker" type bumblebees press down the pollen by themselves in pockets in the base of the brood cells. This habit makes the pocket maker bumblebees more difficult to rear artificially. The house bees of pollen storer bumblebees readily accept honeybee pollen that is provided externally by the staff of the rearing facilities while pocket maker bumblebees are much less inclined to do so (Velthuis *et al.,* 2006).

Bumblebees from the pocket maker group often have a longer tongue than those of the pollen store group. This has the advantage that they can reach down in flowers with deep corollas, such as red clover. Unfortunately, long tongue bees cannot be reared artificially because of the difficulty in supplying them with pollen (Hobbs *et al*., 1961).

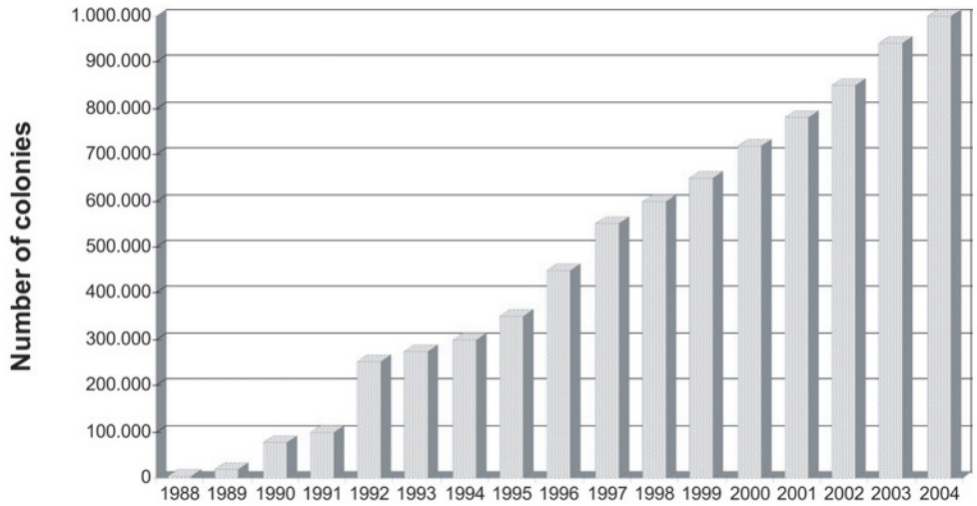



Figure 2: The increase of worldwide sales of bumblebee colonies between the years 1988-2004 (Velthuis *et al.,* 2006).

The optimum climate for rearing bumblebees is 27°C and 65% relative humidity (Yoon *et al.,* 2002). The most suitable hibernation temperature for queens is between 1-4 °C for nine months. Horber (1961) compared the temperature -1°C and +1°C where the queen survival rate differed from 53%-87%. An 8 hour period of daylight was optimal for stimulating colony initiation by the nesting queens (Tasei *et al.,* 1994). Commercial bumblebee colonies are given pollen collected by honeybees. Pollen that has been frozen fresh is preserved better than dried pollen (Ptacek

2001). When the colony has reached a size of 50 workers, the nest is ready for sale. Three to five weeks later the colony size peaks to around 200 individuals (Velthuis *et al.*, 2006).

To keep commercial bumblebee colonies pathogen-free is an important task not only for internal health routines and quality control, but also to minimize the risk of spreading diseases to the local native bees. The national veterinary service controls the standards of the rearing facilities. Certificates are issued that include checks for honeybee pathogens, including some honeybee pathogens that are not easily transmitted to bumblebees (Velthuis *et al.,* 2006). Many pathogens infecting honeybees are also detected in bumblebees, especially many viruses. DWV is known to infect both species (Genersch *et al.,* 2006b; Martin *et al.,* 2019). Another pathogen is SBPV which has been found in both honeybees (de Miranda *et al.,* 2010) and bumblebees (McMahon *et al.,* 2015). De Sousa Pereira *et al.,* (2019) did a pathogen screen of honeybee-collected pollen which had not been irradiated. The study identified seven parasites and four viruses that could be infectious to bumblebees, including *Apicystis bombi*, *Crithidia mellificae, Nosema ceranae* and DWV. Several of these pathogens are specific to bumblebees, with different incidences in different bumblebee species, while others have a wider host-range including also other bee genera and families. With the increased contact and coexistence between wild bees and ever increasing numbers of commercial and domesticated bees these last few decades, the risk of horizontal spread of pathogens between bee species has increased (Graystock *et al.,* 2014).

### Solitary bees

Most of the world's 20,000 bee species are solitary bees (FAO 2018a). They do not live a social life with other bees, but they can live as neighbors in the same area, in aggregations, where the environment is beneficial for certain bee species. In general, a solitary female bee builds her nest by herself, lays her eggs and collects the pollen that she deposits in a cell together with an egg. The female does not feed her larvae continuously, in the way that social bees do. When the larva hatches it stays in the cell and feeds on the pollen. At some point development is paused and the larva or pupa enters diapause, from which it emerges after winter in response to an environmental cue, usually temperature. From this moment forward development continues until the bee emerges as an adult, usually during spring or early summer. The male bees emerge first from their nests, and will then wait until the females are ready to come out and mate. After mating, the male dies and the female will start building a new nest and lay her eggs (Naturskyddsföreningen).

Due to the increase in pollination-dependent crops and the high cost and irregular availability of pollination services from managed honeybees, the attention has turned to some extent back to the on-site management of wild bees as an alternative, or additional, source of pollination service (Mallinger *et al.,* 2014). Wild bees could serve as an insurance for pollination of farmed crops in case of a problem with the delivery or supply of the managed bees, reducing the dependency on managed pollinators which then can be used just to supplement pollination needs during peak blooming. However, to rely on wild bees entirely for pollination services is also a risk, since it requires perfect timing between the emergence of the bees and the main blooms, at a time when the diversity and the abundance of the bees is changing between the seasons and regions (Mallinger *et al.*, 2014).

There are three species of solitary bees that are produced commercially for pollination services: *Nomia melanderi, Megachile rotundata, Osmia cornifrons.* These are mainly used in the US and New Zealand for alfalfa, fruit and nut farming. However, the biggest groups of wild bees providing natural pollination service to farmed crops are the bumblebees.

Wild bees are in some cases better pollinators for certain crops than honeybees (Woodcock *et al.,*

2013; Mallinger *et al.,* 2014). In a study by Woodcock *et al,* (2013), honeybees, bumblebees and wild bees were compared for their pollen collection capacity in a field with oilseed rape. Visitations likely to result in a pollination was 34.0% for honeybees and 35.1% for bumblebees, while for wild bees the pollination rate was 71.3%. Honeybees showed a higher visitation rate when the landscape had a higher quality with a large foraging habitat in the surrounding area. The visitation rates for bumblebees and solitary bees were not affected by the structure of the landscape (Woodcock *et al.,* 2013). Also for pollination service in fruit orchards did wild bees have a higher visitation rate than honeybees. Mallinger *et al.,* (2014) measured the fruit set between orchards with and without the presence of honeybees. As a negative control, the study used a very fine mesh screen wrapped around apple tree branches to prevent pollinators from reaching the control flowers. They then compared fruit development and number of bulbs with the experimental, non-screened flowers. They also recorded the identity and number of visits per flower by different pollinator species included in the study. The results showed that the presence of honeybees in the orchards had no effect on fruit set (Figure 3) but that there was a large and significant effect of wild bee species richness on fruit set. It was also seen that the number of flowers on the apple branches affected the visitation rate for the honeybees. They had a higher visiting rate on branches with a higher density of flowers, while the wild bees did not show any preference for the density of the flowers of the apple threes. The number of wild bees and the species richness did not show any significant relationship to the number of honeybees (Mallinger *et a*l., 2014).

This agrees with the meta-analysis by Garibaldi *et al.*, (2013), were 29 studies from around the world were investigated. This analysis showed that fruit sets increased significantly with both species richness and visitation rate of solitary and wild bees.

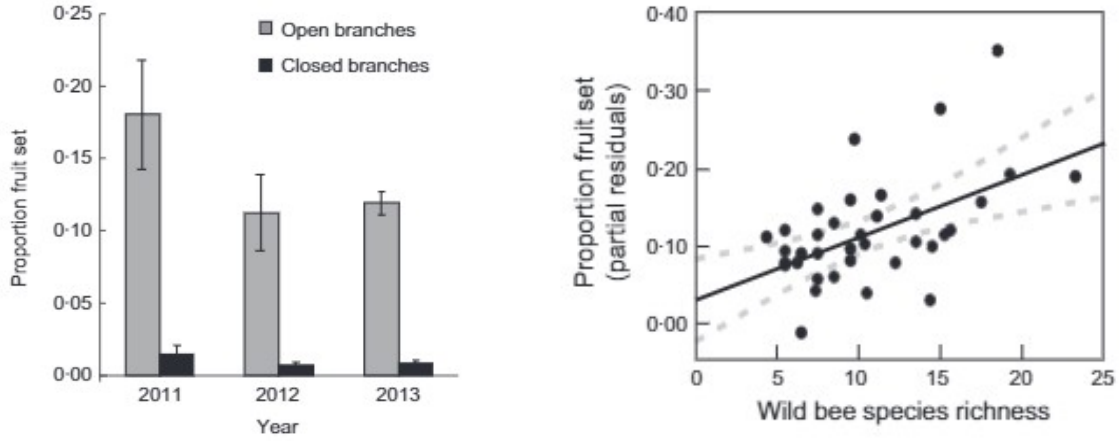



Figure 3: a). The proportion of apple flowers that developed into fruit on "open branches", which were available for all pollinators or "closed branches" were a fine mesh was wrapped around the branches to keep flying pollinators unable to pollinate. b) The proportion of amount of developed fruits in relation to the wild bee species richness (Mallinger *et al.,* 2014).

The wild bee species frequently recorded in apple orchards were *Andrena spp.*, which are ground nesting mining bees (Park *et al.,* 2015). One of the biggest and most diverse group of wild pollinators are the *Halictidae* (Pesenko *et al.,* 2000), which makes them an important group of pollinators (Dikmen 2007). Their social condition ranges from eusocial to solitary and they forage on a wide range of flowers.

## Floral resources

The color of the flowers can play an important role for the interaction between bees and plants. The color of the flower attracts pollinators and help them identify which flower to visit (Lee 2007). Often colors are more vibrant if they are dependent on animal pollination than if the flower is a wind pollinated. Also, the color of the flower can be seen differently by different pollinators, depending on the ability of their visual organs to process infrared, ultraviolet and visible light (Miller *et al.*, 2011). The flowers which are the most attractive for bees are often yellow or blue, and very rarely are they red. Other pollinator species, such as butterflies or beetles, have their own color preferences for flowers. For pollinating moths, the flowers need to stand out during the evening and dusk light, so they are often white or yellow. Pollinating flies prefer red or brown colors, which imitates the color of meat. Beetle pollinators are believed to have a poor visual sense and therefore prefer flowers that are white and dull in colors (Miller *et al.,* 2011).

Goulson *et al*., (2007) gave the bumblebees and honeybees a choice of yellow or orange flowers (*Tropaeolum majus*). Both bee species chose to visit the yellow flowers even though both flowers contain the same amount of nectar. This also shows that the survival of orange colored flowers (*Tropaeolum majus)* depends on humankind. Yellow flowers with a defect were also dismissed. The reason could be that defective flowers could be older and produce a lower amount of nectar (Goulson *et al.,* 2007). The most important color signal for attracting pollinators seems to be the color of the petals. Plants not only attract pollinators with color, they can use several multi-component signals that include shapes, patterns and scents (Kulahci *et al.,* 2008). Kulahci *et al.,* (2008) showed that the foraging behavior of pollinators became enhanced with the increased number and intensity of attraction signals that the plant used. Bumblebees had a higher visitation rate when provided with flowers that attracted pollinators through both shape and scent, as compared to either shape or scent (Kulahci *et al.,* 2008). By getting more information from a flower through signals, the bee can be more sure about its identity and what kind of reward that specific type of flower could give, rewards such as nectar and pollen (Hebets *et al.,* 2005).

Pollen foraging is a very complex motor skill to learn for bees. Raine *et al.,* (2006) showed that bumblebees needed to visit flowers three times more often for learning how to collect pollen effectively, compared to learning to collect nectar, even for flowers with simple morphological features. To collect nectar, the bee needs to learn how to get into the flower and suck up the nectar. However, to collect pollen the bumblebee needs to learn how to groom itself to remove pollen grains and pack these together in special structures on their legs (Heinrich 1976).

It has been shown that the foraging preferences and choices of pollinators affect the evolution of flowers that are dependent on insect pollination (Chittka *et al.,* 2006). Small differences in flower species and changes in the ecosystem can affect the relationship between pollinator and flower (Miller *et al.,* 2011).

## Threats to bees

### Introduced exotic bees on native ecosystems

Every year, over a million bumblebee colonies are produced commercially and transported around the world. It is difficult to prove that introduced exotic bees compete with native bees in the native ecosystem but there has not been any clear evidence that non-native bees have had a significant negative effect on the native bees (Goulson, 2003). Flowers containing nectar and pollen are the exclusive diet for bees (Michener 1974), but also a large number of other

organisms are dependent on nectar and/or pollen for their nutritional needs. Most of these are also insects, primarily flies (*Diptera*), butterflies (*Lepidoptera*), beetles (*Coleoptera*), wasps and bees (*Hymenoptera*). In addition some species of mammals, bats and birds also collect nectar and/or pollen for their diets. Within a natural ecosystem is there a large distribution of native species competing with bees for nectar and pollen resources. This means that exotic bees that are introduced add to this intense competition (Goulson 2003).

Large-scale introduction of commercial pollinators also raises concerns about possible genetic pollution of local bee populations through the dispersal of drones and new queens that would then mate with bees from the local populations (Velthuis *et al.,* 2006). However, this only applies to cases where the bee species in question is already endemic, and thus not exotic. It is unlikely that an already mated queen would escape and succeed to start a colony in the environment outside the greenhouse. If that were to happen, the new colony would start to develop outside the natural bumblebee season and probably not be able to withstand challenges such as unfavorable weather and lack of seasonable flowers (Velthuis *et al.,* 2006).

### Agricultural homogenization

Biodiversity in agricultural landscape has decreased due to the intensification and expansion of harvest crops (Krebs *et al.,* 1999). Wide open grasslands, hedges, trenches and field margins have disappeared due to the consolidation for efficient mass production of annual crops (Benton *et al.,* 2003). However, non-crop habitat oases are needed for the protection, nourishment, and living space for many species (Öckinger *et al.,* 2007). The alternative to intensive farming systems is extensive farming systems, including organic farm management. One of the aims of organic farming is to preserve the biodiversity and to affect the environment as little as possible (Krebs *et al.,* 1999). Organic farming normally uses a crop rotation system with a larger variety of crops and does not use any herbicides, pesticides or non-organic fertilizer. Organic farming can enhance biodiversity in a farmland (Bengtsson *et al.,* 2005).

An intensive conventional farming strategy will lead to a homogeneous agricultural landscape, while an extensive farming system naturally leads towards a more heterogeneous agricultural landscape (Holzschuh *et al.,* 2006). The main reason for the differences amongst conventional and organic farming is the use and non-use of pesticides and herbicides. Agro-chemicals in intensive conventional agricultural systems prevent the establishment and maintenance of flowering weeds and their cover (Bengtsson *et al.,* 2005). The conventional and intensive field often do not leave enough room for perennial weeds, whereas in organic fields, weeds can to be self-sustaining to a limited degree (Roschewitz *et al.,* 2005).

Flower cover increases the abundance for a few bee species, while a flower high diversity is related to a high diversity of bee species (Steffan-Dewenter *et al.,* 2001). In a landscape with a large area of mass flowering-crop, the overall density of bumblebees increased (Westphal *et al.*, 2003). Holzschuh *et al.,* (2007) showed that in organic fields, bee diversity was correlated with proportion of land under flower cover, whereas in conventional fields the bee diversity was correlated to the number of flowering plant species (Figure 4). Even if organic farming is promoted to increase the diversity of bee species, there also needs to be a sufficient area of semi-natural environment for nesting sites (Kremen *et al.,* 2005). Holzschuh *et al.,* (2007) observed that fields in heterogeneous landscapes had a higher diversity in bee species. The explanation could be that fields in homogeneous landscapes lack suitable semi-natural nesting sites. Steffan-Dewenter *et al.,* (2001) found that the diversity of local arthropods are positively correlated to landscape heterogeneity. For wild bees in particular, a diversified habitat is important for the wide range of sites and materialts that they use for nesting, such as holes in woods, burrows in soil, hollow plants etc. By leaving edges of fallow fields unmanaged or adding hedge rows and

promote the floral diversity, the nesting sites for bees are also likely to increase (Garibaldi *et al.,* 2013).

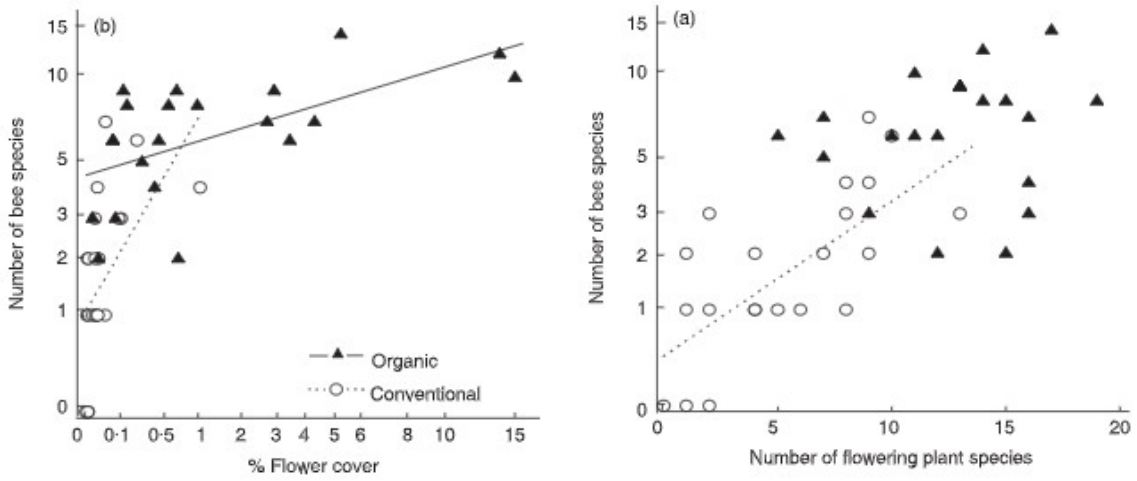



Figure 4: The association between the number of bee species and % in flower cover, and the association between the number of bee species and number of flower plant species, between organic and conventional farming systems. (Holzschuh *et al.,* 2007)

In contrast to Holzschuh *et al.,* (2007), Bengtsson *et al.* (2005) found that the effect from organic farming on species richness both increased and decreased, depending on the study. Bengtsson *et al.,* (2005) divided the organisms in agricultural landscapes in various organism groups and analysed the effect of organic and conventional agriculture on their abundance. The result showed that organic systems where positive for birds, plants, soil organisms and predatory insects, while pests and insects that were non-predatory did not respond positively. In general 50% of the organisms increased with organic farming. The data from the collected studies varied a lot between the different agriculture landscapes. Bengtsson *et al.* (2005) suggested that the farming system (conventional or organic) together with the type of landscape affected the species richness more than only the agriculture system itself.

The landscape homogeneity can also affect the agricultural farming system. There are bigger differences between conventional and organic farming in a homogeneous landscape than in a heterogeneous landscape (Holzschuh *et al.,* 2007). Happe *et al*.,(2018) analysed the change in species richness and abundance of bumblebees and solitary bees at conventional and organic farms, in small-scale and large-scale agricultural landscapes (Figure 5). These revealed a larger variation between small scale and large scale agricultural landscapes than between conventional and organic. Also, bumblebees were shown to be more sensitive to the scale of the agricultural landscape, rather than whether it is a conventional or an organic farm.

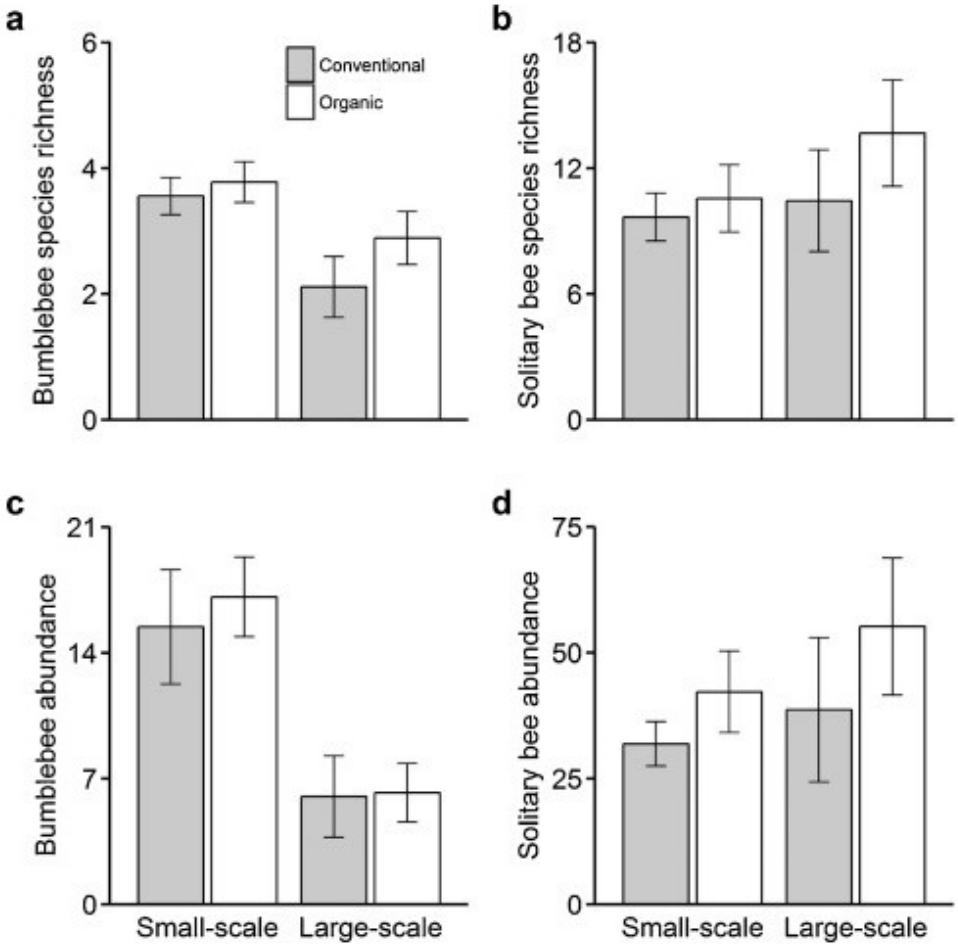



Figure 5: Comparison of species richness and abundance of bumblebees and solitary bees at conventional (grey) and organic (white) production, in small-scale and large-scale agricultural landscapes (Happe *et al.,* 2018).

## Agrochemicals

Agrochemicals are used to protect crop against pests and pathogens that can infect the plant, prevent the crop from reproducing, inhibit the growth, or kill it. for example, pathogens that infect the blossom of the plant often causes abortion of the fruit or nut and inhibit the plant from reproducing (Mussen *et al.,* 2004). Pesticides, herbicides or fungicides are used either one time or several times a year during different developmental stages of the crop plants.

Agrochemicals not only protect the plant from disease and pathogens, they can also have a negative effect on essential plant pollinators. Pesticides can have a sublethal effect on bee health and functionality. For instance, the bee reproductive system, learning ability, the ability to manipulate flowers and flower visiting rate can be affected. There is limited knowledge about the realistic field exposure levels of pesticides for a bee. It has been confirmed that pesticides have an effect on learning ability for both bumblebees (Stanley *et al.,* 2015) and honeybees (Williamson *et al.*, 2013)

For instance, fungicides are used a lot in the almond orchards of California. They are applied when the almond trees are in blossom. Atkins *et al.,* (1981) showed that fungicides do not affect adult bees, but Vandame *et al.,* (1998) determined that the application of the fungicides could trigger hypothermia in adult honeybees. A study by Mussen *et al.,* (2004) investigated the effect of eight common used fungicides and their effect on bee brood. The result showed that fungicide-contaminated pollen the adult honeybees are bringing back to the hive to feed the

larvae with can interfere with the development of the larvae and pupal stages. Osterman *et al.,* (2019) showed that at colony-level, honeybees are relatively resistant against clothianidin, a commonly used neonicotinoid insecticide, when applied as directed at field level. No negative impacts were found on honeybee colonies in the vicinity to fields sown with clothianidin-treated oilseed rape seeds.

Neonicotinoids have been shown to harm bumblebees directly (Rundlöf *et al.,* 2015). Both the colonies growth and the queen production have been decreased. The levels of neonicotinoids that are damaging to bumblebees were between 0.7 and 6.0 ppb (Whitehorn *et al.,* 2012), which is the same levels found in crops that are claimed to be safe for bees (Blacquière *et al.,* 2012). In honeybees level of 2.5 ppb can lead to neuronal inactivation, affection of the brains learning center (Palmer, 2013). Wintermantel *et al*., (2018) showd that at field level, neonicotinoid clothianidin coating impacted *Bombus terrestris* directly, at individual level, both through bumblebee size and reproduction. The bumblebee microbiome was also analyzed but neonicotinoid exposure did not affect the levels of beneficial microbiota, or the intracellular parasites and viruses.

There is little updated knowledge about how wild bee communities react to the pesticides in the agricultural landscape. In New Brunswick, Canada it was shown that the failure in blueberry crops, that are dependent on outcrossing through pollinators, was caused by a reduction in wild bees, who in turn were affected by the spread of the insecticide Fenitrothion. The insecticide had been used against spruce budworm in the surrounding forest to the blueberry fields (Kevan 1975). Rundlöf *et al.,* (2015) also showed a strong effect of neonicotinoid exposure on wild bee communities surrounding the affected fields.

More than 120 different pesticide residues have been found in honeybees and their hives (Mullin *et al.,* 2010). The amount of pesticide residues that could be found in wild bees communities, is largely unknown. Also, the exposure and synergistic effects of different pesticides on wild bees are difficult to predict (Park *et al.,* 2015). Brittain *et al.,* (2010) showed that the richness in wild bees decreased when a second and third application of insecticides were applied on fields, but that they were largely unaffected by a single field application of insecticide. The wild bee richness could be seen to decline in fields where insecticides where applied, while either cultivated or uncultivated fields with no application of insecticides did not show any decline in wild bee species. Brittain *et al.,* (2010) also examined if pesticide use on intensive farms affected pollinators. They showed that the species richness for butterflies and bumblebee was lower than for solitary bees.

Natural habitat provides both foraging and nesting areas. Park *et al.,* (2015) showed that natural areas up to 2 km from the crop / orchards could function as a buffer, and dampen the effect of pesticides on wild pollinators. Both the abundance and the species richness of wild bees increased with increasing proportions of natural habitats (Figure 6; Park *et al.,* 2015). The natural habitat can also provide nourishment both before and after the crop have blossomed (Watson *et al.,* 2011). The higher the variation and range of flowers in an area; the greater the pollinator diversity and richness (Tscharntke *et al.,* 2012).

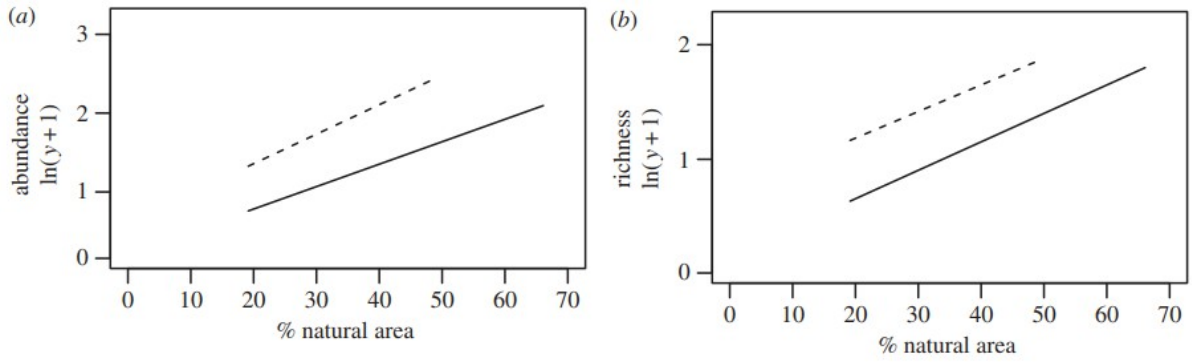



Figure 6: The relationship between the abundance (a) and the richness (b) of bees with an increased natural habitat within 2km from the apple orchards that using pesticides. The increase in natural habitat around the orchard were significantly positive for the wild bees. Dotted line is results from 2011, and the solid line is results from 2012 (Park *et al.,* 2015).

Tests for chronic effects from pesticide exposure in semi natural habitats on the individual honeybees have not been standardized (Pisa *et al.,* 2015). However, the honeybee colony as a whole may be more resistant to exposure of pesticides than individual bees (Henry *et al.,* 2015; Osterman *et al.,* 2019). There is a lack of studies on pesticide effects on bees in the wild. The studies on individual honeybees are currently the only approximation for possible effects on solitary wild bees, as well as honeybees. Bees make decisions about the available food source, transport the food and communicate with the other bees when foraging (Tison *et al.,* 2016). The bee will only consume and metabolize a small part of the sucrose solution on the transport back to the hive and therefore only consume a small amount of pesticide. In a study by Tison *et al.,* (2016), two groups of honeybees where studied. One control and one fed with nectar containing thiacloprid. It was seen that low (4.5 ppm) concentrations of thiacloprid consumed by foraging bees gave sublethal effects. Communication, foraging and navigation were all negatively affected (Tison *et al.,* 2016).

## Pathogens

Honeybee pathogens include viruses, bacteria, fungi and parasites. Bee diseases have been known for a many years but have increased in prevalence in the latest decades causing increased colony losses, which has had devastating consequences for both apiculture and crop growers. A possible explanation for the increase in bee disease could be related to the change in beekeeping practices that have become more intensified including transporting bees within and outside continents (Goulson 2015).

Social bees, such as honeybees and bumblebees, may be more sensitive to the spread of diseases because they live in colonies of many individuals sharing a small space and interact with each other through social behaviors (FAO 2006). Trophallaxis is a social behavior where individuals share food orally, along with pheromones that provide information on the colony status. Because of the increased risk of disease for social bees an increasing worry is that bee colonies may function as pathogen reservoirs causing disease spill over to wild solitary bees. To have bees that themselves could remove disease from the colony, through a hygienic behavior, is an important contribution for their own survival and success in many different environments (FAO 2006).

### Colony collapse disorder

Colony collapse disorder (CCD) is a condition of honeybee colonies that first appeared in the United State in 2006. The characteristics of CCD are an abundance of brood, pollen and honey but the absence of adult bees, with no dead bees found in or outside the hive (VanEngelsdorp *et al.,* 2007). When CCD first emerged it was described as “catastrophic losses of unknown origin”

by Johnson *et al.,* (2009). CCD seems to be related to a high prevalence of several different pathogens affecting honeybees, which could have affected the efficiency of the bees' immune system making them extra vulnerable for adult depopulation: the main symptom of CCD (Johnson *et al.,* 2009). Another theory related to CCD is the impact of agricultural pesticides affecting bee colonies while individuals forage on crops. Even the pesticides used as disease control of honeybee parasites may have an involvement in the unexplained disappearance of bees. Higes *et al.,* (2009), showed that bees that have been exposed to neonicotinoid pesticides fail to return to the colony due to disruptions in their orientation and flight behavior. Initially CCD had only been reported and identified in United States but in October 2009, a Swiss beekeeper reported sudden colony losses with similar characteristics to CCD (Dainat *et al.,* 2012).

### Varroa mite

The Varroa mite (*Varroa destructor*) is an ectoparasite of honeybees and the main cause of colony death worldwide (Crane 1978). The mite feeds on the hemolymph of adult bees, and developing pupae (Sammataro *et al.,* 2000). Affected individuals are weakened, leading to early death (De Jong 1997). The mites reproduce in the brood cells of developing worker or drone pupae (Boot *et al.,* 1992). The harmful effect of the mite comes through its role as a vector for several lethal honeybee viruses through feeding on the bees during the sensitive developmental stage of pupation while the mite is reproducing (Bowen-Walker *et al.*, 1999). These viruses normally have a relatively harmless effect on honeybees but when vectored by the mite gave lethal results (Hung *et al.,* 1995; Nordström *et al*., 1999).

### American foulbrood

American foulbrood (AFB), caused by the bacterium *Paenibacillus larvae*, is one of the most serious diseases affecting honeybee colonies (Ellis and Munn 2005). AFB infections are spread through contaminated food and are extremely contagious and lethal (Genersch *et al.,* 2006a).

The antibiotic treatment Terramycin (oxytetracycline) has been used against AFB for more than 50 years around the world (Gochnauer 1951). Argentina, Canada and the U.S. use antibiotics routinely as a preventive treatment against AFB, while in the EU, antibiotic use is forbidden as a treatment for honeybees due to the potential for residue contamination in honey (Johnson *et al.,* 2010). In recent years, resistance against the antibiotic has increased dramatically (Miyagi *et al.,* 2000).

Breeding efforts to improve the honeybee's natural defense against AFB would be a more sustainable solution to the disease by reducing the need for prophylactic antibiotic use. Spivak *et al.,* (2001) study the behavior of honeybees that has been classified for hygienic and non-hygienic behavior. The bees had been selected and breed on from their ability to clean out freeze killed brood. The colonies that cleaned out 95-100% of the dead brood within 48h were judge as hygienic. In Spivak study the hygienic colonies were seen to have a significant lower level of mummies on the brood due to AFB (Figure 7).

Honeybees have a hygienic behavior where bees are quick to detect and remove infected individuals from the hive before the bacteria spores become infectious. This behavior has a genetic component and can be enhanced through apicultural breeding efforts (Rothenbuhler 1964).

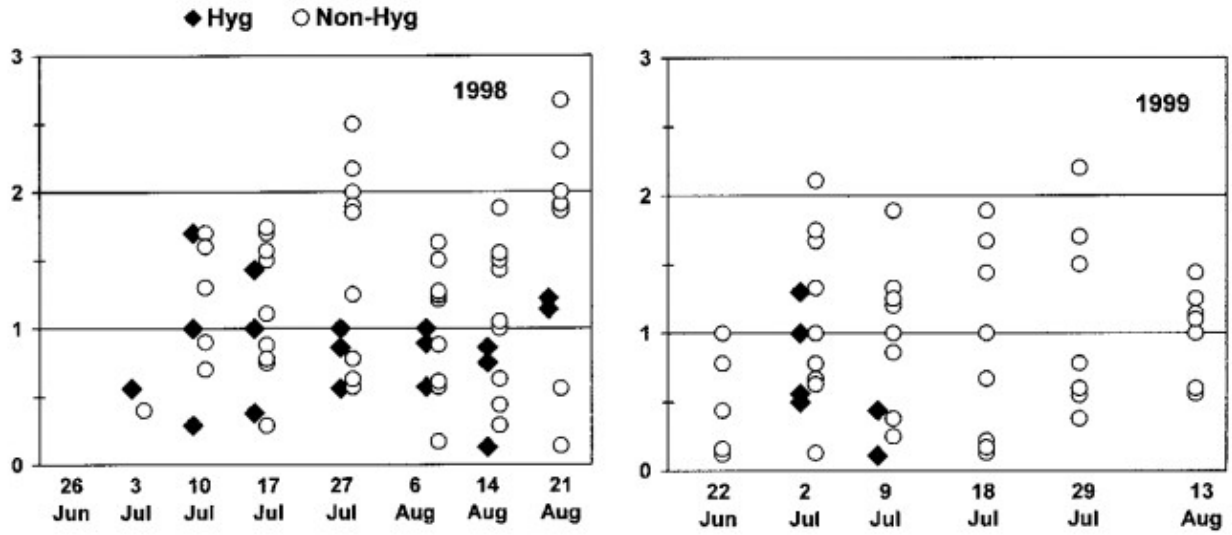


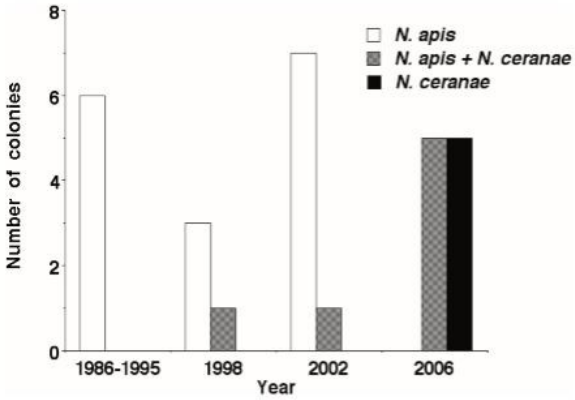

Figure 7: The scores of the level of American foulbrood (AFB) in hygienic colonies (♦) and non-hygienic colonies (♀) showing symptoms for both 1998 and 1999. Score 0 = no mummies on the brood, 1 = 1–5 mummies on the brood (light symptom), 2 = 6–25 infected cells (moderately symptom), 3 ≥ 25 cells (strong symptoms) (Spivak *et al.,* 2001).

## Nosema

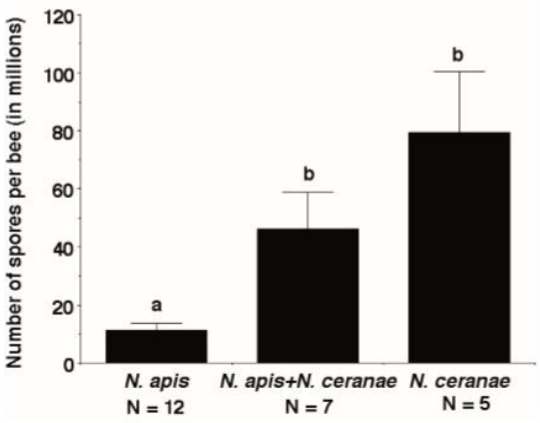
Two species of microsporidians, an intracellular parasite, have been recognized in honeybees, *Nosema apis* (Zander 1909) and *Nosema ceranae* (Fries *et al.*, 1996). Nosema infects adult honeybees through the ventriculus by ingestions of spores. This may happen when the workers are cleaning out combs containing infected faeces (Fries *et al*., 1996). The symptoms from the infections of Nosema on a colony is an increased winter mortality, poor startup in the spring, reduced honey production and a shorter lifespan for infected honeybees (Fries *et al.*, 1984).

Of the two Nosema parasites that affects honeybees, *N. ceranae* species are relatively new to the western world, originally from East Asia (Frise *et al.*, 1996) and in 2003, was recognized with having a globally distribution (Paxton *et al.,* 2007). Nosema is generally not lethal for honeybees but can have serious consequences in more temperate climates (Fries 1993).

Paxton *et al.,* (2007) compared the effects of *N. apis* and *N. ceranae* on caged honeybees that were given the same dose of infection spores from the both species. Their study found that the honeybees infected with *N. apis* had a higher amount of infected spores during a shorter time than the honeybees infected with *N. ceranae* (Figure 8a) However, when the number of spores from the two Nosema species were investigated, *N. ceranae* had a higher number of spores per individual (Figure 8b). Honeybees that were infected with *N. ceranae* had a quicker mortality rate than *N. apis*. In day 15, 14 of 25 *N. ceranae* infected cage bees were alive, compared with bees infected with *N*. a*pis*, where 23 of 25 bees stayed alive (Paxton *et al.,* 2007).




(a.)




(b.)

Figure 8: (a) The data of infected colonies from Finland between the years 1986 to 1995. Honeybee (*Apis mellifera* infected with either *N. apis* (white bars), *N. ceranae* (black bars) or a mixed infection of both *N. apis* and *N. ceranae* (grey bars). (b) The means of the number of spores from *N. apis* and *N. ceranae* found in inviduall bees between the years 1986 and 2006 in Finland. Where *N. ceranae* had a higher number of spores per individual bee and a higher mortality (Paxton *et al.,* 2007).

## Crithidia

The gut trypanosomes are a group of bee parasites often found in managed bees, such as honeybees and bumblebee. Trypanosomatidae flagellates are commonly found as parasites in insects (Podlipaev 2001), and were first found and described by Lipa *et al.,* (1988). The protozoa *Crithidia spp.* is found both in honeybees and bumblebees. Crithidia is a well known and studied worldwide parasite that infects *Bombus spp.* with *Crithidia bombi* being the most studied species (Lipa *et al.,* 1988). A new species, *Crithidia expoeki,* has been discovered in bumblebees through the use of *molecular* markers (Schmid-Hempel *et al.,* 2010).

*Crithidia bombi* can only live a short period outside the host, where it normally exist in the hindgut (Tognazzo *et al.,* unpubl. observ; Schmid-Hempel *et al.,* 2010; Schmid-Hempel *et al.,* 1999). Transmission occurs either with contact of infected materials in the nest, contact with infected bees in the nest or through visitations to the same flowers as infected bees (Durrer *et al.,* 1994). Infection with *C. bombi* limits the fitness of the queens (Brown *et al.,* 2003). There is a concern that *C. Bombi* will spill over from commercially used bumblebee colonies to infect other species of wild bees (Colla *et al.,* 2006).

*Crithidia Bombi* on *Bombus terrestris* are more critical in conditions were starvations occurs (Brown *et al.,* 2000). While infections with *Crithidia mellificae* on honeybees are affecting the winter mortality of the hives (Ravoet *et al.,* 2013)

## Apicystis

The neogregarine parasite *Apicystis bombi* is found in most places around the world (Colla *et al.,* 2006). *A. bombi* is a single-cell intracellular parasite existing in the fat bodies of the host. Outside the host the parasite is found in feces or on flowers, which means an oral-faecal transmission route (Schmid-Hempel 1998).

*A. bombi* infection causes the bumblebee to obtain a sensitivity to sucrose (Graystock *et al.,* 2015). A heightened sensitivity to low sucrose levels has been confirmed to affect learning ability and hunger in bees. A bee with a higher hunger level is less willing to share its food with others, which will affect the feeding of the brood (Naug and Gibbs., 2009). *A. bombi* infection occur in the fat bodies reducing the levels of stored fat in bumblebees. Graystock *et al,* (2015)

showed a reduction of 17% in average of fat/lipid content in bumblebees infected with *A. bombi.* A reduction in fatty tissue will lower the chances of surviving hibernation for bumblebee queens (Graystock *et al.,* 2015). It has also been shown that the survival of post-capture bumblebee queens infected with *A. bombi* had a shorter life span (12-31 days) than non-infected queens (52-91 days). Infected queens were not able to establish a colony or produce any brood (Jones and Brown, 2014). Also the workers in the colony will be affected by the reduced fatty tissue. The fat bodies are important for biochemical reactions that control the bee's metabolism and immunity (Arrese *et al.,* 2010).

The increase in prevalence of *A. bombi* has been suggested to come from spillovers by introduction of non-native strains of honeybees and bumblebees (Plischuk *et al.,* 2009). The presence of *A.bombi* in manage honeybee colonies in Belgium detected through molecular screening has shown that more than 40% of sampled bees are infected (Ravoet *et al.,* 2013).

### ABPV

Acute bee paralysis virus (ABPV), is commonly found in honeybee colonies. ABPV seems to be enhanced and activated by a stressful environment, such as mite- or bacterial infections, insecticides and other agro-chemicals or environmental pollution (Bakonyi *et al.,* 2002). ABPV infection in adult bees leads to paralysis, inability to fly, loss of hair from abdomen and thorax and rapid death (Tantillo *et al.,* 2015). ABPV can affect honeybees in all stages in life but multiplies during the host pupation phase (Sanpa *et al.,* 2009). Bee larvae can be infected by adult bees that transmit ABPV particles through salivary gland secretions when feeding (Benjeddou *et al.*, 2001; Ball 1984). Yue *et al.,* (2006) have shown that venereal transmission of ABPV is possible through semen of healthy drones. If the larva is heavy infected it will die and be removed from the colony halting the further spread of the infection. However, if the infection is mild the larva can survive and become an infectious adult bee who can then transmit the virus to other bees in the colony (Bailey & Ball 1991).

ABPV has been detected in Varroa mites (Allen *et al.,* 1986; Bakonyi *et al.,* 2002). Due to the increase in Varroa infected apiaries in Europe the ABPV has increased in prevalence during the last decades. Because of the spread and the serious consequences of virus infections which can lead to collapse of entire colonies (Berènyi *et al.,* 2006).

### SBPV

Slow bee paralysis virus (SBPV), is primarily a virus of bumblebees (McMahon *et al.,* 2015) and are very rare in honeybee colonies (de Miranda *et al.,* 2010). SBPV infections in honeybees are most critical when the colony is also infected with Varroa mites, which also act as a host for the virus (Manley *et al.,* 2017). SBPV was first discovered in England in 1974 during experiments with other bee viruses. The virus paralyses the front legs of adult bees and expands later into the abdomen (Bailey and Woods 1974). As with many honeybee viruses, SBPV is an infection that exists naturally but latent in honeybees. The transmission of the virus occurs orally, when nectar is passed from one individual to another (Bailey and Ball 1991). However, the Varroa mite presents a new, highly efficient transmission route that can result in a lethal infection for the colony (Santillán-Galicia *et al.,* 2010). Unlike many other honeybee viruses, SBPV is not common and colony mortality due to SBPV has only been registered in the United Kingdom (Carreck *et al.,* 2010).

During starvation conditions, SBPV can result in reduced longevity for infected bees (Manley *et al.,* 2017). Honeybees can usually compensate for this by having a storage of food. However starvation periods affect bumblebees greater than honeybees due to a lack of stored energy (Moret and Schmid-Hempel 2000). It is seen that 5% of UK bumblebees that are considered healthy are carrier of SBPV (McMahon *et al.,* 2015).

DWV

Deformed wing virus (DWV) can occur both in honeybees and bumblebees. In honeybees DWV needs a vector to be able to reproduce and spread. Without the vector, the honeybees do not normally show any symptoms. This vector is the ectoparasitic mite *Varroa destructor* and the symptoms of DWV are deformed wings, reduction in size and death. This will develop if the bee is infected during the period between larva to pupa (Ryabov *et al.,* 2014). Bumblebees can also acquire DWV and have displayed symptoms without the Varroa mite as a vector. In laboratory tests, single infections with DWV induce bumblebee death much faster than un-infected bees (Figure 9); Fürst *et al.,* 2014).

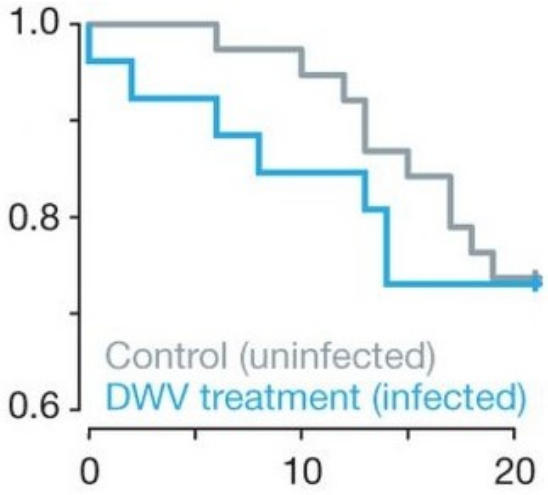



Figure 9: The survival rate of uninfected (grey) and DWV infected (blue) bumblebee queens during 21 days. The y-axis describe the survival probability (Fürst *et al.,* 2014).

For bumblebees, the oral transmission route is most likely to be the source of transmission of DWV (Genersch *et al.,* 2006b). Genersch *et al,* (2006b), showed that DWV affected the body tissue in different ways for clinically diseased honeybees and bumblebees. In crippled honeybees, DWV could be found in RNA from the head, while there was no detection of DWV in the head of bumblebees.

## Transmission

The expansion and distribution of pathogens can occur between individual bees with contact to each other, through communication, grooming, feed exchange or contact with the same material. These are considered horizontal transmission routes. Transmission of pathogens from one generation to the next is known as vertical transmission. Depending on a pathogen's ability to transmit horizontal or vertical, will dictate how virulent it will become (Fries *et al.,* 2001). The virulence is the severity or harmfulness of the disease. For honeybees, horizontal and vertical transmission can occur both inside the colony but also outside the colony. Horizontal pathogen spread to other colonies, happens when bees are drifting between or robbing other colonies. Vertical spread at the colony level occurs through swarming. For solitary bees, vertical transmission is likely to be the most efficient way for pathogens to spread (Fries *et al.,* 2001).

Several factors could impact a pathogen's virulence. Usually pathogens that are mainly vertically transmitted have a lower virulence than pathogens that are horizontally transmitted or transmitted by a vector (Ewald 1994). This is because vertically transmitted pathogens require a host to survive to adulthood and reproduce. A factor that can influence transmission and thus the virulence of a pathogen is the density of individuals or colonies. If there is a high density of

individuals the horizontal transmission opportunities increase, which could select for pathogens with higher virulence. If there is a low density of colonies, transmission opportunities will be reduced and this would select for pathogens to have a lower virulence since their transmission opportunities is reduced to vertical transmission (Bull 1994).

Pathogen transmission can also occur by visiting the same flowers. A parasite or pathogen can be deposited onto the pollen or nectar carrying structure of a flower by an infected foraging bee and acquired by another bee visiting the same flower (Durrer *et al.,* 1994). Importation and exportation of honeybee colonies around the world have been documented as a way to transfer diseases. For example it is a concern that commercially produced *Bombus terrestris* could amplify *Crithidia spp.* as a host and then transmit this parasite to other species of bees (Colla *et al.,* 2006). Also the beekeeper could be spreading pathogens by using or borrowing contaminated tools or equipment (FAO 2018b).

The aim of this project was to assess if the pathogen distribution in wild bees has a relationship to the distribution of pathogens in managed bees, considering both honeybees (*Apis mellifera*) and imported bumblebees (*Bombus terrestris*), sharing the same habitat area. The pathogens that have been investigated are: Nosema, Crithidia, Apicystis, ABPV, SBPV and DWV. The study was divided into three more specific projects with independent specific objectives to support the overall aim of this study.

**Super-B project**: The goal of this study was to determine if there is a change in the pathogen distribution in wild bees at the same location over three years, in an area with a long-term (>30 years) presence of honeybee colonies close to the sampling location

**Lövsta project**: The goal of this study was to determine the pathogen distribution in wild bees in relation to the bee density and distance to honeybee hives, in an area where honeybee colonies were newly introduced (< 1 year) after an absence of >30 years

**MSB project**: The goal of this study was to compare pathogen distribution and abundance in wild bees in relation to the presence or absence of imported bumblebee colonies

# Materials and Methods

## Study area

The field-work for this study was conducted between the end of June and the end of July 2017 in the county of Uppsala. The study included seven collection sites, relating to the three separate sub-projects (Figure 10).

The **Super-B project**, concerning the seasonal variation in pathogen distribution in different bee species, focused on the SLU campus university gardens in Ultuna (N59.817744; E17.657820). The site consisted of three local patches within 1 hectare (100 x 100 m²).

The **Lövsta project**, concerning the effect of distance from honeybee colonies on the pathogen distribution in wild bees, consisted of four sites near SLU's Lövsta animal research station; Lövsta-1 (N59.835314; E17.828165), Lövsta-2 (N59.837588; E17.802941), Lövsta-3 (N59.847385; E17.810563) and Lövsta-4 (N59.890625; E17.810555). Sites Lövsta-1 and Lövsta-2 were located in mixed agricultural landscapes, while sites Lövsta-3 and Lövsta-4 were located in more natural, wooded landscapes. Both areas contained a high density of honeybee colonies, but with the significant difference that in Ultuna beekeeping had been present in the area for >30 years, while in Lövsta beekeeping was re-introduced to the area in 2016 (<1 year prior to sampling) after an absence of >30 years.

The **MSB project**, concerning the effect of imported bumblebees on pathogen distribution in wild bumblebees, took place in Fredrikslund (59.763657; 17.644592) and Ulva kvarn (N59.913253; E17.575508).

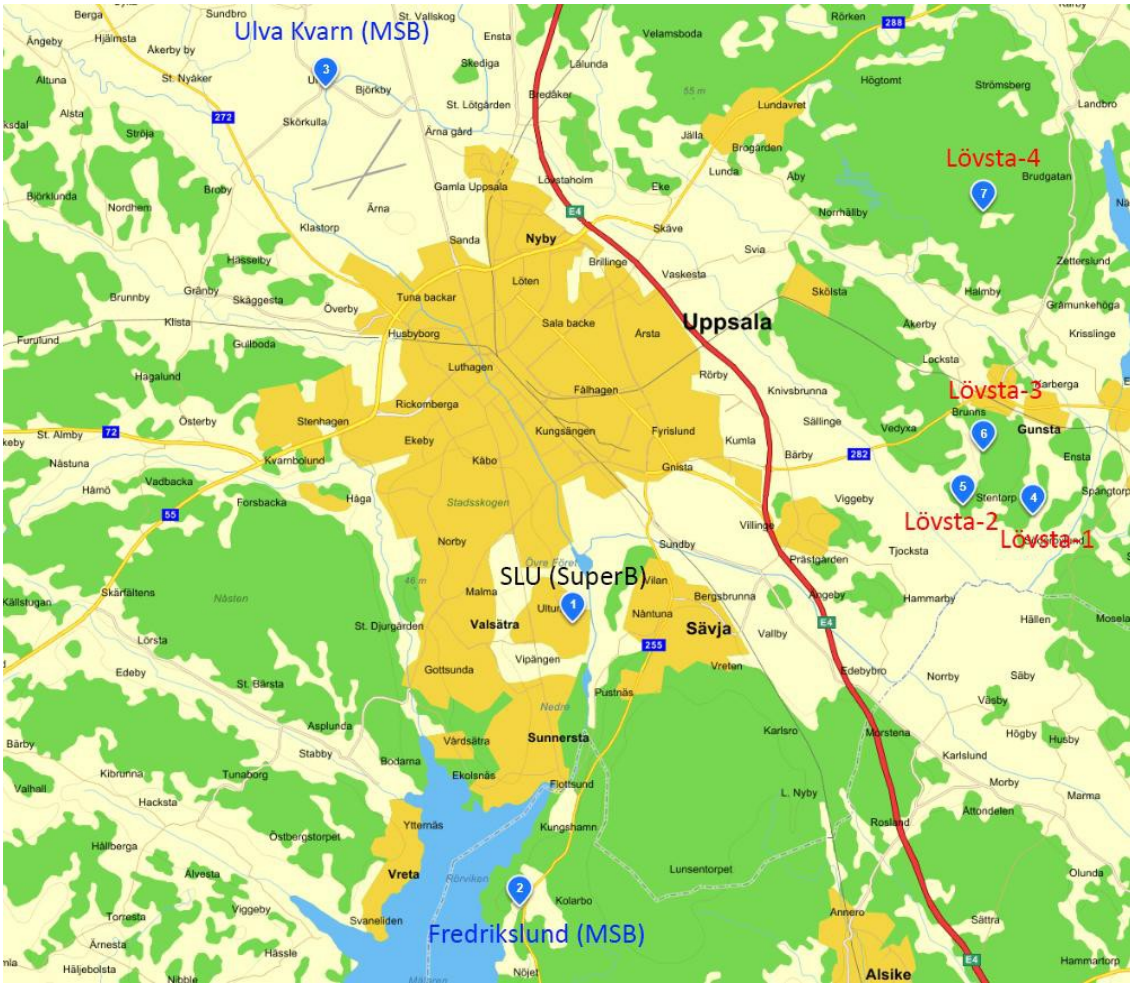



Figure 10: Maps of the collection sites, near Uppsala, Sweden, identified by number, site name and project (black = Super-B project; blue = MSB project; red = Lövsta project).

## Experimental designs

### *1. Super-B project*

The experimental design for the Super-B project was a three-year survey where bees were collected on the same day and same place for three consecutive years: 2015, 2016 and 2017. Collection started at midday and continued until 30 honeybees, 30 wild bees and 15 additional samples of the most common wild bee were collected. No more than 5 bees of any specie were collected from a single plant, flowerbed or patch. The samples were numbered sequentially, in the order in which they were collected, and the precise time of collection recorded. The sample order and time can therefore be regarded as proxies for the relative and absolute bee density respectively.

### *2. Lövsta project*

The experimental design for the Lövsta project was an intensity gradient based on the proximity of honeybee colonies. The intensity gradient involved were four sub-sites at different local distances from the nearest honeybee colonies (<20 m; 200 m and >2000 m), corresponding to the different foraging ranges of honeybees, bumblebees and solitary bees. Lövsta-1 was located 200 m from the nearest honeybee colonies; Lövsta-2 and Lövsta-3 were located <20 m from honeybee colonies, while Lövsta-4 was >2000 m from any honeybee hives (Figure 10). The sampling strategy at each sub-site was the same as for the Super-B project, and the same metadata were collected. The honeybee colonies were introduced to Lövsta in September 2016, after more than 30 years absence of honeybees from the area, and after the end of the 2016 wild bee and bumblebee reproductive season. Summer 2017 was therefore the first time in 30 years that the local wild bees were again exposed to honeybees and their pathogens.

### *3. MSB project*

The MSB project was a pilot study for a larger project financed by the Swedish Civil Contingencies Agency (Myndigheten för Samhällsskydd och Beredskap, or MSB) on exotic biological threats to Swedish pollinators. The pilot study was for a sub-project assessing the possible biological threats posed by imported commercial bumblebee colonies. The experimental design for the MSB project was a paired-landscape design involving two strawberry farms near Uppsala, 'Fredrikslund' and 'Ulva Kvarn', with one farm (Ulva Kvarn) supplementing natural pollination with imported commercially reared bumblebees (*Bombus terrestris*) while Fredrikslund relied exclusively on natural pollinators for pollination. Fifty bumblebees were collected from each site, again ordered sequentially and timed. At Ulva Kvarn the collection of *Bombus terrestris* continued until 20 were collected, after which only non-*terrestris* bumblebees were collected. The same metadata was collected as for the Super-B project.

## Sample collection

The samples were collected according to a precise protocol developed by the Super-B consortium, which either controls or records the most important factors that influence foraging in bees. These factors are described in the metafile associated with the data (see below) and include: time of collection, weather conditions, type of environment, floral abundance and diversity, presence of honeybee colonies in the area, and the species of flower the bee was collected on. The samples in all three projects were collected on warm and mostly sunny days with little wind. All the bees collected at any one sampling site (Figure 10) were sampled on the same day within an area of 100 m x100 m. The bees were collected with nets, transferred to individual sterile tubes and stored on ice. The tubes were labeled with the sample number, the field identification of the bee and the date-time of collection. After the sampling the bees were transported immediately to SLU and stored at -20°C until processing.


Photos 1, 2 and 3: Images of the sampling procedures and locations. Clockwise from top left: Catching net next to a flower bed at SLU; an imported “Tripol” bumblebee box at Ulva kvarn; flowers in bloom at SLU.

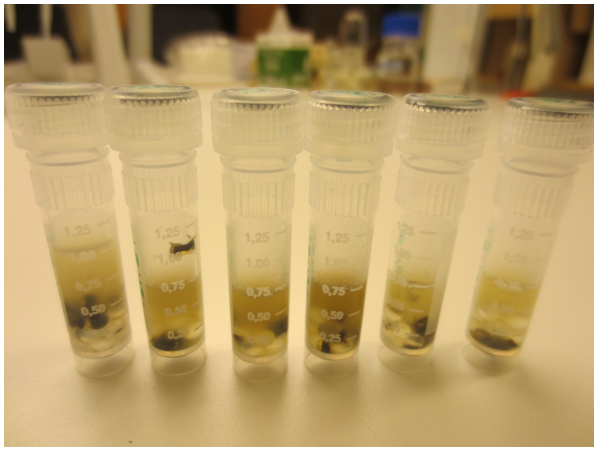
Photos 4 and 5. Images of the Super-B collection site: the kunskapsträdgården (left) and nearby honeybee colonies (right) at SLU campus.

Photos 6, 7, 8 and 9. Images of the Lövsta collection sites. Clockwise from top left: Lövsta-1, Lövsta-2, Lövsta-3 and Lövsta-4. Lövsta-1 and Lövsta-3 were located in mixed agricultural landscapes while Lövsta-2 and Lövsta-4 were located in more wooded landscapes.

Photos 10 and 11. Images of the MSB collection sites: Fredrikslund (left) and Ulva Kvarn (right).

## Sample processing

The samples were processed the same way for all three projects in the molecular laboratory at the Ekologicentrum at SLU, Ultuna in Uppsala. First, a second identification was made of each bee, using a LEICA binocular microscope, at 10x magnification and an identification key. Subsequently, the abdomen of each bee was separated from the rest of the body using a pincets and a scalpel, and placed separately in 2 ml screw cap micro centrifuge tubes with 10-12 glass beads of 2mm in diameter. The remaining parts of the bee (head, thorax, wings and legs) were stored separately at -20oC for possible future studies. A primary homogenate was prepared from each individual abdomen in TBS-RNA250 buffer, which consisted of 50 mM TRIS.CL pH 7.5/150 mM NaCl, with RNA250 (ThermoFisher; Waltham, MA, USA: cat. no: AM7155) added immediately prior to use to a final concentration of 10 ng/mL (based on the concentration given in the product sheet). The RNA250 was added as a exogenous reference RNA to assess the success of the RNA extractions and cDNA synthesis. Different amounts of TBS-RNA250 buffer were added depending on the size of the bee: 800 µL for bumblebee abdomens, 500 µL for honeybee abdomens (and similar-sized wild bees) and 200µL for sweat bees and similar sized smaller bees. The abdomen was homogenized in the TBS-RNA250 buffer in a screw-cap 2 mL micro centrifuge tube using a bead mill (photo 13), shaking for 2 minutes at 30 Hz.

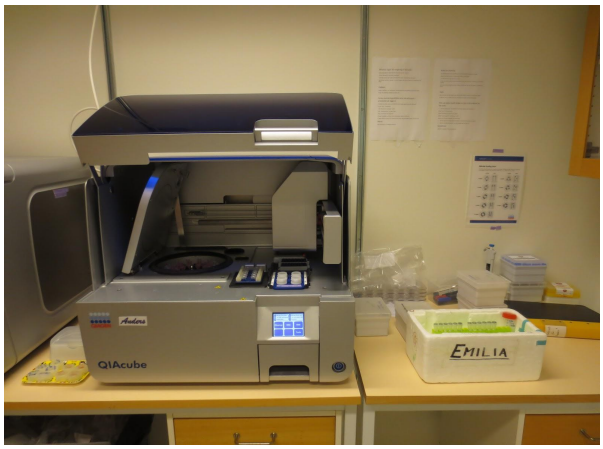


Photos 12 and 13: The 2 ml micro centrifuge tubes containing the homogenized individual bee abdomens (photo 12) and he “Bead mill” used to homogenize the bees (photo 13).

## DNA and RNA extraction

Both the RNA and DNA extractions were performed using a QIA-cube extraction robot (photo 4). For the RNA extraction, 100µL of the abdomen homogenate was mixed with 350µL of a RLT buffer containing 1% β-mercaptoethanol (added the day the buffer was used) in a 2ml micro centrifuge tube. After this, the Qiagen protocol for “Plant RNA extraction” was followed, as adapted for the QIA-cube. The RNA was eluted in a final volume of 50 ul sterile water.

For the DNA extraction, 100µL of the abdomen homogenate was mixed with 180 µL 20 mg/ml lysozyme (Sigma-Aldrich, St Louis, MI, USA) and incubated for 30 min at 37°C, followed by the addition of 20 µL 20 mg/mL proteinase-K (Qiagen, Heidelberg, Germany) and incubated for 30 min at 56°C. These digestion steps are necessary to break down the hard Nosema spores and release their DNA. After this, the Qiagen protocol for “Blood & Tissue DNA for Gram-positive bacteria” was followed, as adapted to the QIA-cube. The DNA was eluted in a final volume of 50 ul sterile water.



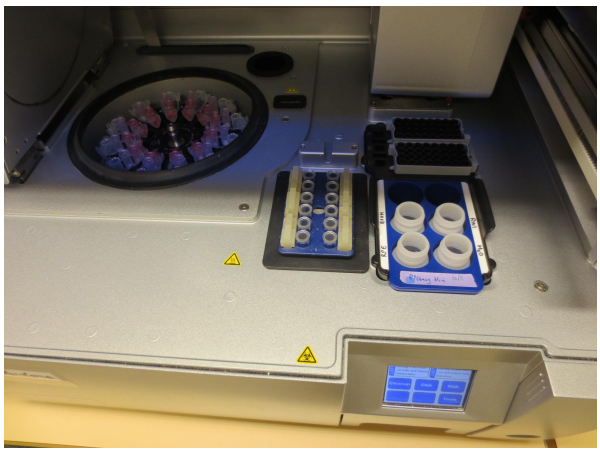


Photo 14 and 15: The Qiagen QIAcube robot used to extract the DNA and RNA.

After the extraction, the DNA and RNA concentration (ng/µL of nucleic acid) and purity (260/280 nm and 260/230 nm absorbance ratios) were determined using the NanoDrop (Thermo Fisher) spectrophotometer. Based on the NanoDrop data, the RNA and the DNA samples were then diluted with sterile water to a constant concentration of 100 ng/µL.

## cDNA synthesis

For the cDNA synthesis, 10 µL of each diluted RNA sample (1 ug) were added to 10 µL of a master mix solution from a M-MLV First strand cDNA synthesis kit (ThermoFisher, Waltham, MA, USA: cat. no: K1612) consisting of: 4 µL 5x Reaction Buffer, 1 µL Ribolock, 2 µL 10mM dNTPs mix, 1 µL 200 ng/µL random hexamer primers and 2 µL M-MuLV reverse transcriptase. Enough master mix was prepared for 100 cDNA reactions, i.e. one 96-well PCR plate. The master mix was aliquoted first to the 96 well plate, followed by the individual RNA samples. The cDNA reactions were then incubated according to the following protocol: 5 min at 25ºC followed by 60 min at 37ºC. After the incubation, the cDNA was diluted 10x fold with sterile water and stored at -20ºC.

## Polymerase chain reaction (PCR)

The bees were screened for six pathogens: three pathogens with a DNA genome (Nosema, Crithidia and Apicystis) and three pathogens with an RNA genome (ABPV, SBPV, DWV). The DNA samples were used to screen for the DNA pathogens, while the cDNA samples were used to screen for the RNA viruses, as well as for the passive exogenous reference, RNA250.

The PCR reactions were run in 10 ul volumes containing 2 ul template (DNA or cDNA) and 8 ul PCR mastermix. The reaction composition and PCR thermocycling profiles differed slightly for the DNA and the cDNA templates. However in both the mastermixes the dye EvaGreen is used, which become highly fluorescent upon binding to dsDNA. The details for all assays used, including primers and thermocycling profiles, are shown in table 1. For the DNA templates, the mastermix contained 5 µL 2x EvaGreen reaction buffer (BioRad, Hercules, CA, USA), 0.4 µL each of 10 uM forward primer and 10 uM reverse primer, and 2.2 uL water. The PCR thermocycling profile was: Initial enzyme activation step for 2 min at 98ºC, followed by 40 PCR cycles of: denaturation for 10s at 98ºC, extension and data collection for 30s at 58ºC. This was followed by a Melting Curve analysis, to confirm PCR product integrity, where the reaction mixture was heated in 0.5ºC increments, with 5s holds and data collection, until 95ºC was reached (see Table 1). For the cDNA templates, the mastermix contained 5 uL 2x EvaGreen reaction buffer, 0.2µL each of 10 uM forward and 10 uM reverse primer and 2.6 uL water. The PCR thermocycling profile was: Initial enzyme activation step for 30s at 95ºC, followed by 40 PCR cycles of denaturation for 5s at 95ºC, extension and data collection for 10s at 58ºC, and followed by a Melting Curve analysis as described above.

Each plate of PCR assays contained both negative (no-template) and positive controls for the PCR reactions, as well as negative (no-template) controls for the cDNA reaction. The positive controls consisted of a 10-fold dilution series of a (cloned) PCR product of the assay with known concentration. This dilution series was used to construct a standard curve for the assay with which to estimate the absolute amount of target template in the PCR reaction.

The PCR reactions were first examined by the Melting Curve profile, to confirm that the quantitative signal was derived from true assay product, rather than amplification artifacts. Once confirmed, the amount of each target template in the reaction was calculated automatically by the Bio-Rad software, using the calibration curve established by the assay standard dilution series. The data were converted to a per-bee basis by multiplying with the various dilution factors incurred during the sample processing, nucleic acid extraction and cDNA synthesis. For the RNA pathogens, this calculation was simplified by using the corresponding, sample-specific data for the RNA250 exogenous reference as a conversion factor.

| Target | Primers | Sequence '5-'3 | Size (bp) | Reference |
|---|---|---|---|---|
| ABPV complex | KIABPV-F6648 (F)<br>KIABPV-B6707 (R) | CCTTTCATGATGTGGAAAC<br>CTGAATAATACTGTGCGTATC | 98 | *Mondet et al., 2014* |
| DWV complex | DWV-F8688 (F)<br>DWV-B8794 (R) | GGTAAGCGATGGTTGTTTG<br>CCGTGAATATAGTGTGAGG | 143 | *Mondet et al., 2014* |
| SBPV complex | SBPV-F3177 (F)<br>SBPV-B3363 (R) | GYGCTTTAGTTCAATTRCC<br>ATTATRGGACGTGARAATATAC | 226 | *de Miranda et al., 2010* |
| RNA250 | RNA250 (F)<br>RNA250 (R) | TGGTGCCTGGGCGGTAAAG<br>TGCGGGGACTCACTGGCTG | 227 | *Mondet et al., 2014* |
| | | | | |
| *Nosema spp.* | Nosema (F)<br>Nosema (R) | TATGCCGACGATGTGATATG<br>CACAGCATCCATTGAAAACG | ~250 | *Ravoet et al., 2014* |
| *Crithidia spp.* | Crithidia (F)<br>Crithidia (R) | CTTTTGGTCGGTGGAGTGAT<br>GGACGTAATCGGCACAGTTT | 417 | *Meeus et al., 2010* |
| *Gregarine spp.* | Gregarine (F)<br>Gregarine (R) | CCAGCATGGAATAACATGTAAGG<br>GACAGCTTCCAATCTCTAGTCG | 260 | *Meeus et al., 2010* |
| *COI gene* | LCO-1790 (F) | GCTTTCCCACGAATAAAATAATA | 408 | *Folmer et al., 1994* |
| | LCO-1490 (F)<br>HCO-2198 (R) | GGTCAACAAATCATAAAGATATTGG<br>TAAACTTCAGGGTGACCAAAAAATCA | 708 | |

Table 1. Details of the different PCR assays used in the study, including the assay target, the name and sequences of the primers used, the expected size of the PCR product the literature reference for the assay. The green assays use RNA (cDNA) as the target template. The orange and blue assays use DNA as the target template. The blue assay is for the barcode analysis used to identify bee species.

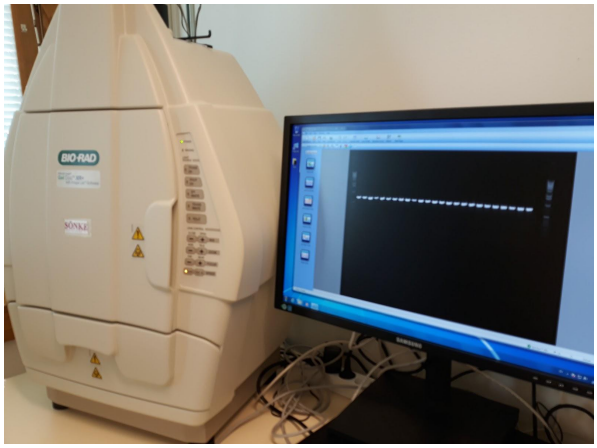
## Identification of the bee species

The wild pollinators were identified in three stages. The first stage was a field identification, based on the pollinator's general appearance and a handbook for identification. The second stage was a more detailed laboratory examination of specific morphological features using a microscope. The final identification step was a genetic barcode analysis based on the mitochondrial Cytochrome Oxidase I gene. Since honeybees (*Apis mellifera*) can be easily identified in the field with 100% accuracy, the barcode analysis was primarily conducted on the wild pollinators, with a few individual *Apis mellifera* samples included as positive controls for the functioning of the barcode analysis methodology. Similarly, when multiple specimens of the same wild pollinator were collected at the same time and place, and whose morphological features were unique and unambiguous, only a few samples were processed for barcode analysis. All bumblebees in the three projects were processed for barcode analysis. This was to limit the cost and effort of the barcode analyses.

A Mix with Chelex was prepared for the incubation with the DNA from selected bees. A Chelex mix was made of 5% Chelex and sterile water. The mixture was stirred for 30 minutes. The legs from the bees were removed with a scalpel and tweezers. The legs were cut into as small pieces as possible with a pair of scissors. The legs were transferred into 8-striped tubes and centrifuged down to the bottom of the wells. Then 100µL Chelex were pipette into each well and then 5µL proteinase K was added. A careful centrifugation was done to get all the reagents down to the bottom of the wells. The tubes were then incubated in the PCR machine following the program:

55ºC for 60min, 99ºC for 15min, 37ºC for 1min, 99ºC for 15min, hold at 14ºC until removal.

The barcode analysis involved amplifying the mitochondrial COI gene in 10 ul volumes containing 1 ul template DNA and 0.4 uM each of the LCO-1490 and HCO-2198 primers (Table 1) denaturing for 180 seconds at 93oC and then amplifying for 35 cycles denaturing for 30s at 95oC, annealing for 45s at 51oC and extending for 60 s at 72oC, followed by 8 min extension at 72oC (Folmer et al. 1994). The PCR products were then analyzed by agarose gel electrophoresis, using a 1% agarose gel prepared in TBE buffer, containing 4 ul GelRed solution to visualize the DNA bands. The wells were loaded with 2,5µL of the PCR reaction alongside a 1 kb reference ladder. The PCR products were visualized under UV light using a, Bio Rad molecular Imager and a digital record was obtained using the, Gel Doc XR+ software. This was done to confirm that only one product of the correct size had been amplified by the PCR reaction. In those instances where no product was produced, the PCR reaction was repeated, but using the LCO-1790 forward primer instead of the LCO-1490 primer. This gives a slightly smaller product. All successful COI gene amplifications with either the LCO-1490 or LCO-1790 forward primers were submitted to Macrogen-Europe BV (Amsterdam, The Netherlands) for Sanger sequencing with the HCO-2198 reverse primer. The resulting sequences were matched against the GenBank nucleotide database using BLAST for bar-code species identification. The final species identity was established using the results of all three identification methods. Conflicts were resolved by adopting the likely identification based on the three identification methods that is consistent with the known geographic distribution and features of the pollinator.




Photos 16 and 17: The electrophoresis with the gel in a TBE solution (photo 16) and a gel showing the results from the electrophoresis (photo 17).

# Results

## The bees in the collections

All together 492 bees were collected in the various subprojects, with 39 different species identified through a combination of field, laboratory and genetic barcoding techniques. The most common bees collected were *Apis mellifera*, *Bombus lapidarius*, *Bombus pascuorum*, *Bombus terrestris* and *Bombus soroeensis* (Figure 11), with bumblebees in general making up the bulk of the wild bee collection. Although these figures will inevitably be skewed due to the sample collection strategies employed in the various subprojects, especially with respect to *Apis mellifera* (see materials and methods) overall these data are an accurate reflection of the relative abundance of different wild bee species around Uppsala during June and July in any given year.

Photo 18: Composite photo of some collected bee species. Clockwise from top-left: *Apis mellifera*, *Bombus lapidarius,* Bombus *soroeensis, Bombus terrestris, and Bombus pascuorum*.

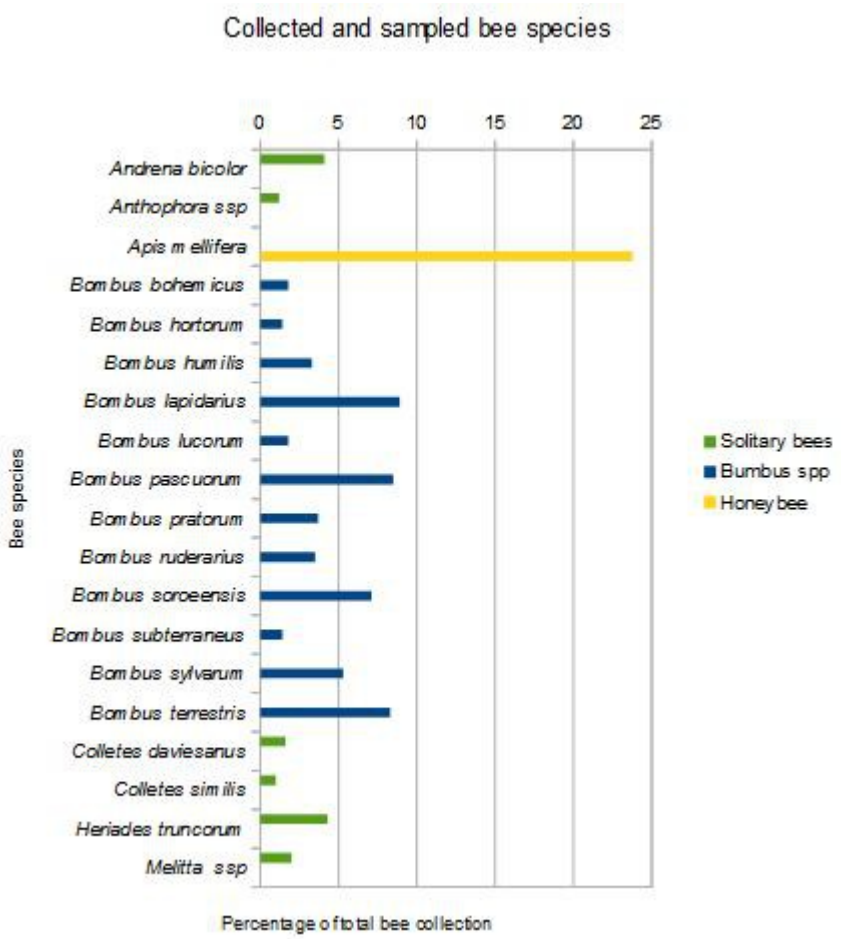



Figure 11: The percentage of different bee species collected and analyzed in the present study (only species occurring at >1%). Solitary bees are indicated by green bars, bumblebees by blue bars and honeybees by a yellow bar.

## Bee distribution

In the **Super-B project**, the distribution of the different bee species collected changed over the years (Figure 12). Year 2017 had a greater pollinator diversity with 17 different species among the 25 bee samples collected, compared to 11 species from 25 bee samples for year 2016 and 14 species for 2015. There are many environmental factors that affect the presence of wild bees in any particular location and at any one time (Öckinger et al., 20017; Steffan-Dewenter et al., 2001), including several related to pathogens and disease.

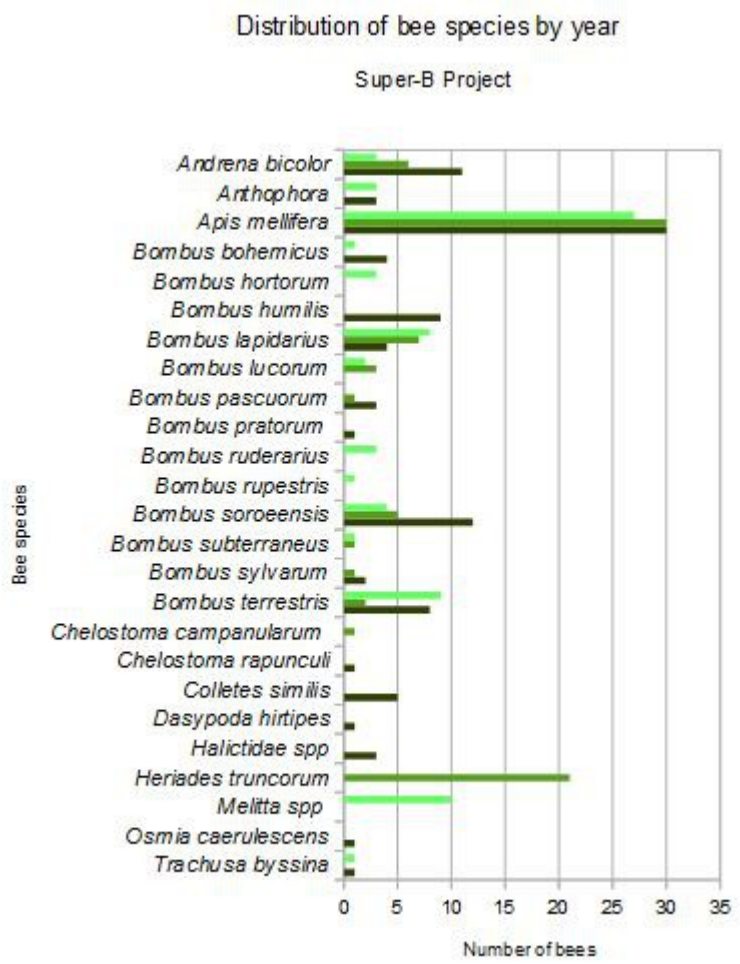



Figure 12: The bee species collected from SLU, Ultuna for Super-B project showing the difference in bee distribution between the years 2015 (light green), 2016 (green) and 2017 (dark green).

In the **Lövsta project** the distribution of the different bee species collected changed with the distance to honeybees. The diagram shows that there are species that are found on a distance of 2000m away from honeybee hives. Seven bee species were collected beyond honeybees, in an area close 20m to the honeybee hives, twelve species were collected at a distance of 200m from honeybee hives and six bee species with no presents of honeybees at Lövsta-4 2000m from honeybee hives. Also the absence of some bee species close to honeybee hives (<20m) could indicate that the honeybees are pushing some wild bee species away.

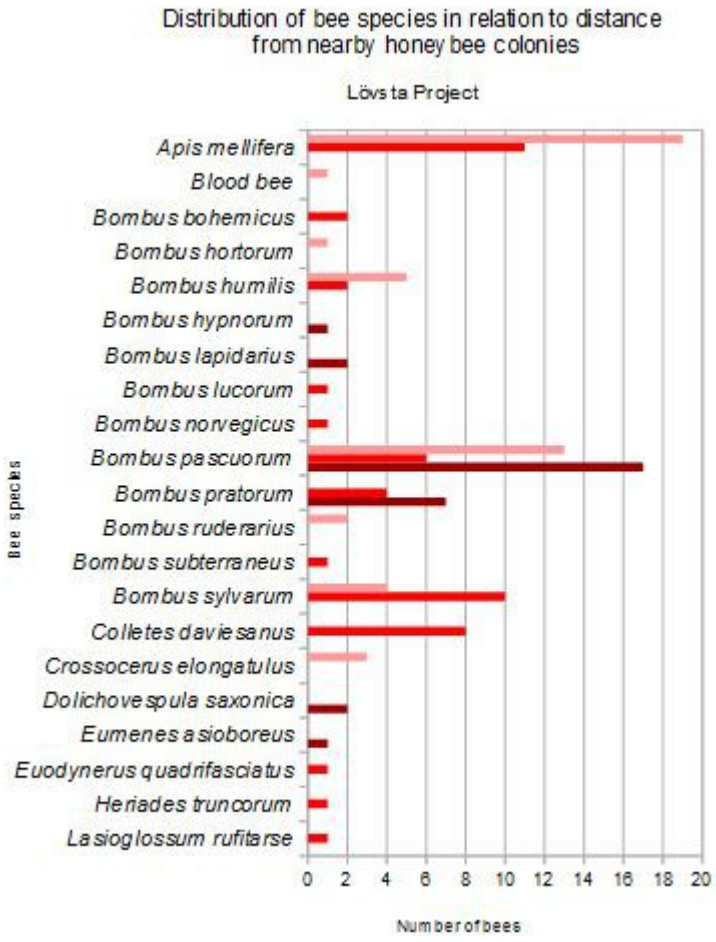



Figure 13: Bees collected from Lövsta, showing the difference in the number of bee species collected at the sites with the distance of 20m (dark red), 200m (red), and 2000m (pink) from honeybee hives.

In the **MSB project**, the figure 14 shows Fredrikslund that used natural wild pollinators and Ulva kvarn that used imported bumblebees. Ulva kvarn had a higher amount of *Bombus terrestris* than Fredrikslund, which is not surprising due to the importation of the *Bombus terrestris* colonies. It was seen that there were a slightly higher diversity of bee species at Fredrikslund (10 species) than Ulva kvarn (7 species). This could indicate that, with a lack of *Bombus terrestris*, other bombus species with smaller colonies sizes could benefit and appear. The two most common bombus ssp at Fredrikslund were *Bombus terrestris* and *Bombus lapidarius* and at Ulva kvarn it was *Bombus ruderarius* and *Bombus soroeensis*.

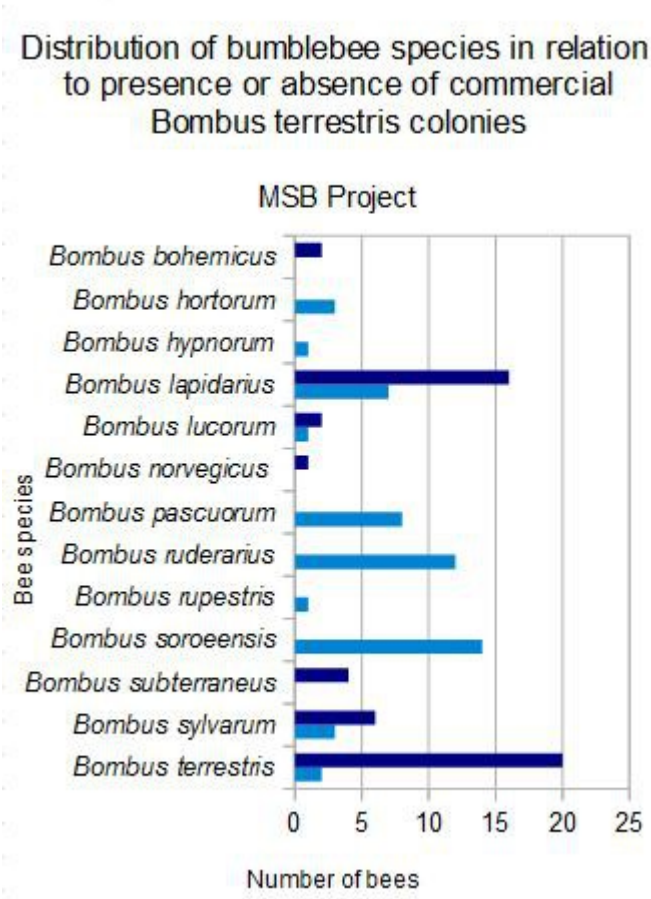



Figure 14: Bees collected from Fredrikslund (light blue) and Ulva Kvarn (dark blue) as part of the MSB project, showing the differences in bumblebee composition in two Uppsala strawberry farms.

## Collection rates

The time it took to collect the different samples is a rough indication of the absolute abundance of bees during the collection period (Figure 15). The most straightforward comparison is between the three collection years in the **Super-B project**, since these collections were done in the exact same location and date of the year: July 28th. The collecting effort took longer in 2015 than in 2016 and 2017. This was partly due to lower overall bee density in 2015 and partly because this was the first time the basic collecting protocol had been used, and the sample handling per bee took more time than in later years, or in the other projects, when we were more proficient at collecting. At this area honeybees have been present for a long time >30 years. The collecting area is the same. The difference can depend on a more beneficial environment. The

second type of comparison is between sites. In general, the collection rate for both sites in the **MSB project**, Fredrikslund and Ulva Kvarn, was lower than in the Super-B and Lövsta projects. Part of the reason for this is that a lot of honeybees were sampled in both Super-B and Lövsta, from nearby colonies. Both the collecting and the field identification of these honeybees were much simpler and faster than for the wild bees. For the MSB project, the collection took less time at Fredrikslund strawberry farm, which did not have any imported bee as pollination reinforcement, than at Ulva Kvarn farm, which did have imported *Bombus terrestris*. This could well be because the bumblebee density a Fredrikslund was considerably higher than at Ulva kvarn. For the **Lövsta project**, there seem to be an inverse relationship between the proximity of honeybee colonies nearby and the speed of collection, with the site furthest away from honeybee colonies (Lövsta-4) having the fastest collection rate and the two sites within 20 m of many bee colonies (Lövsta-2 and Lövsta-3) having the slowest collection rates.

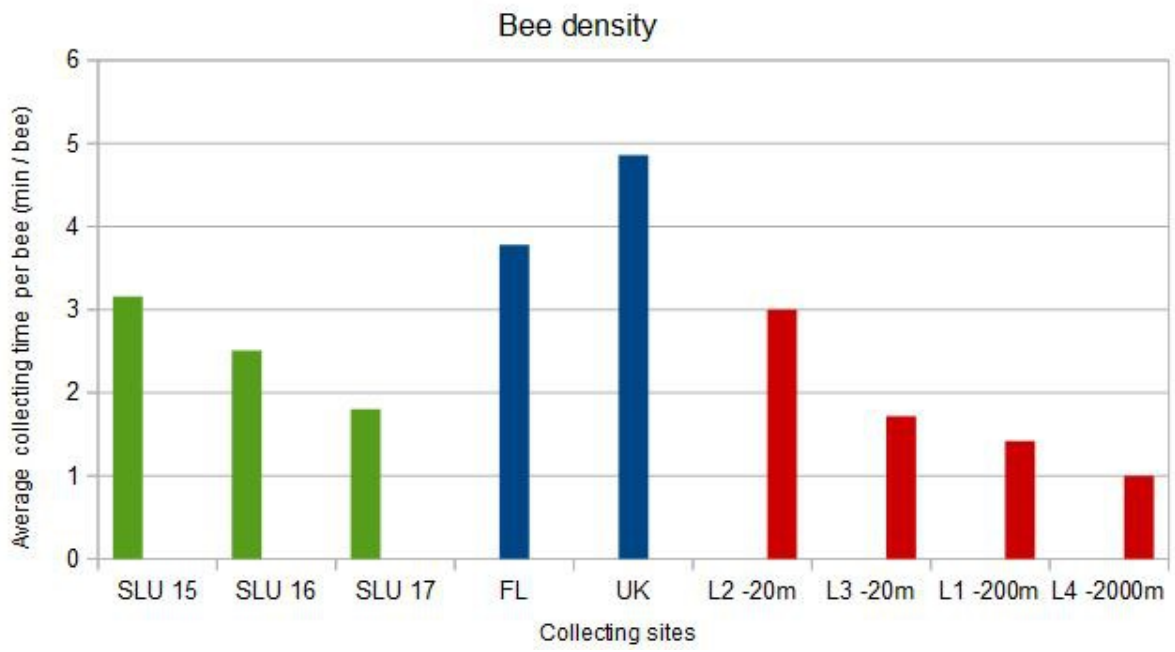
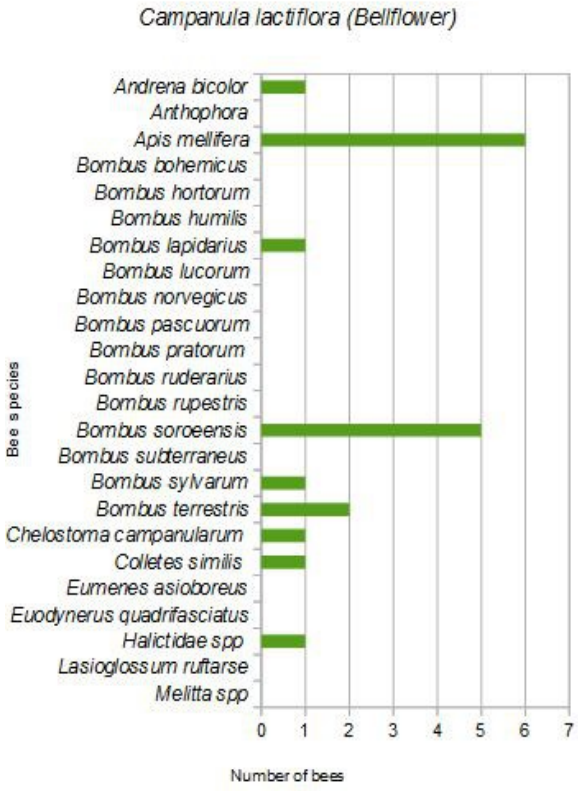


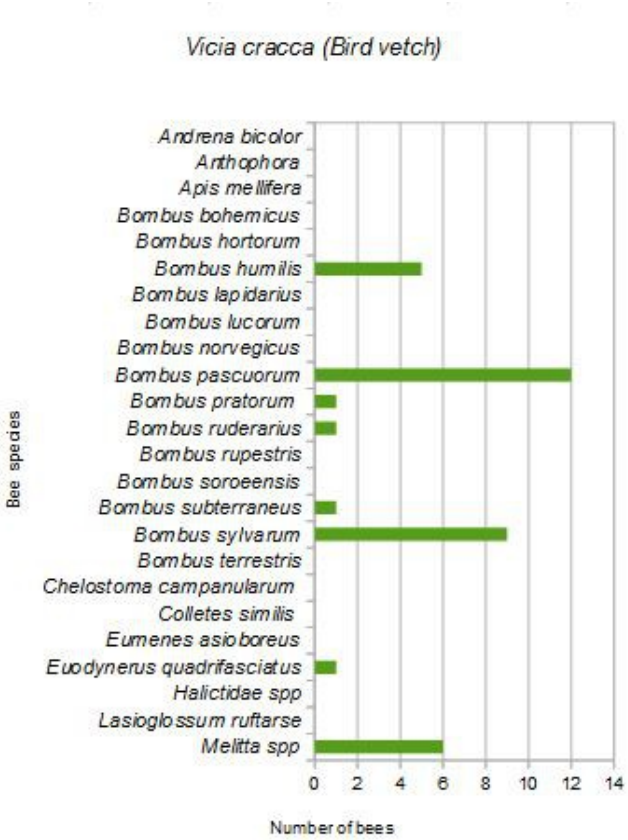

Figure 15: The density of bees at each site, as represented by the average time to collect individual bees.

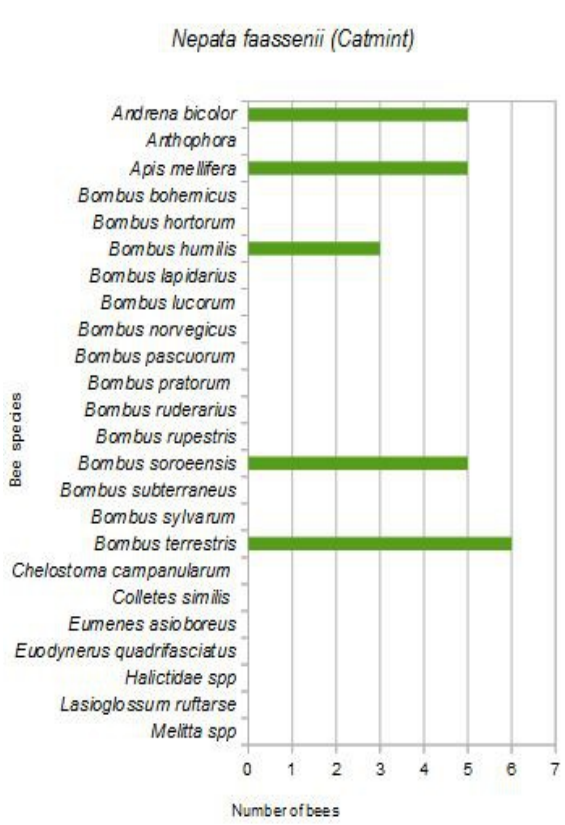
The **Super-B project** shows the different collecting seasons, SLU-2015, SLU-2016 and SLU-2017. Figure 15 indicates an increase in the density of bees from the year 2015-2017.
The **MSB project** had a longer collecting time per bee at the site Ulva kvarn (UK), which had imported bumblebees, than at the site Fredrikslund (FL), without imported bumblebees. This could indicate that it was less wild bumblebees in the presence of a larger amount of imported *Bombus terrestris* colonies. The **Lövsta project** refer to the four collection sub-sites, L2-20m, L3-20m, L1-200m and L4-2000m from honeybees hives. The density of bumblebees seems to increase further away from hives the sampling is done. This suggests that there may be more bumblebees in the absence of honeybees.

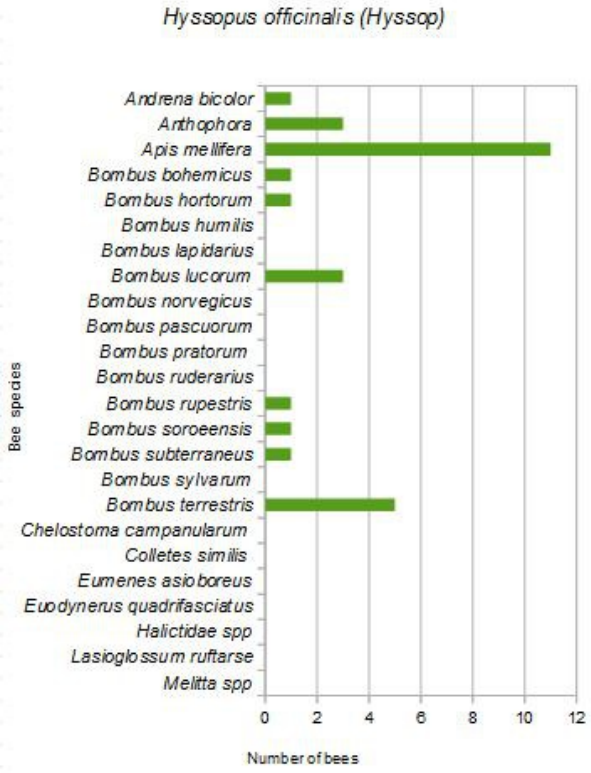
## Flower distribution

Distribution of bee species on six commonly visited flowers



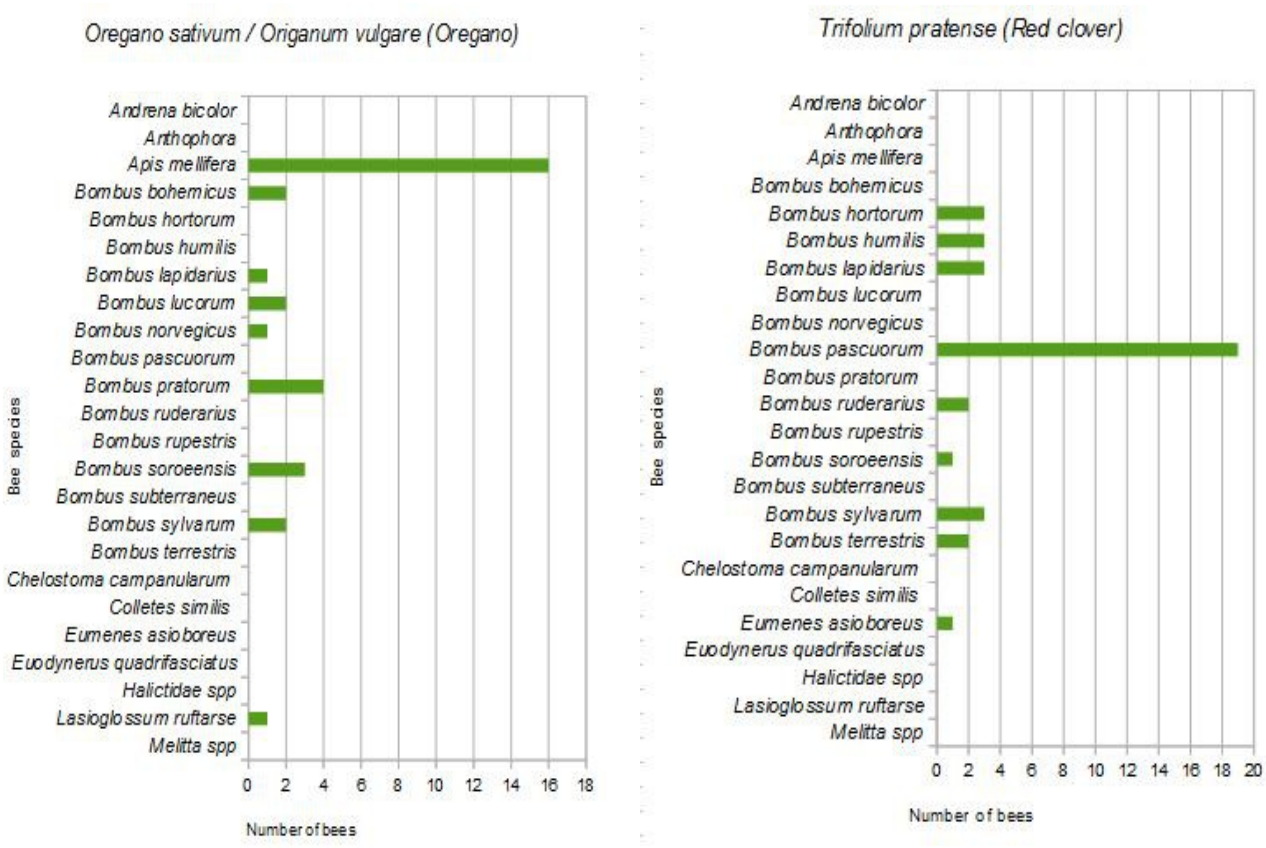



Figure 16: The six most visited plants in the three projects, and the distribution of the bee species on the plants.

Figure 16 separates the distribution of the different bee species collected in **all projects** (Super-B, MSB, Lövsta) by the flower they were collected on, focusing on the six most frequently visited flowers: bellflower, hyssop, catmint, oregano, red clover and bird vetch. In principle, wild bees, bumblebees and honeybees can and will visit the same flowers if necessary, which can facilitate transmission of pathogens between bee species. In practice however, many bee species have clear preferences for certain flowers, which can significantly reduce the contact rate between bee species, through the diversity of the floral contact network. The diagram shows that certain bee species have a clear preference for certain types of flowers (*e.g. B. pascuorum*, *B. sylvarum*, *A. mellifera*) while other bees are more cosmopolitan. Such preferences are driven by a number of factors, such as the compatibility between the type of resource offered by the plant and the nutritional requirements of the bee (Kulach *et al.,* 2008), and the ease of access to the resource. For instance, red clover has a long corolla thus favoring long-tongued bees (e.g. *B. pascuorum*) over short-tongued ones (most others) (Hobbs *et al.,* 1961). Oregano is an excellent nectar plant with only moderate pollen production, thus favoring nectar foragers (e.g. *Apis mellifera*) over pollen foragers (most solitary bees). This information about the shared preference of flowers is extremely relevant for all the three projects in the present study, where pathogen transmission is investigated.

## Pathogen distribution

As with bee species-specific differences in local distribution, and floral preferences, so are there also species-specific differences in the prevalence and amounts of different pathogens. The overall prevalence of the six pathogens studied here (Nosema, Crithidia, Apicystis, ABPV, SBPV and DWV) between all bee samples collected, is shown in Figure 17.

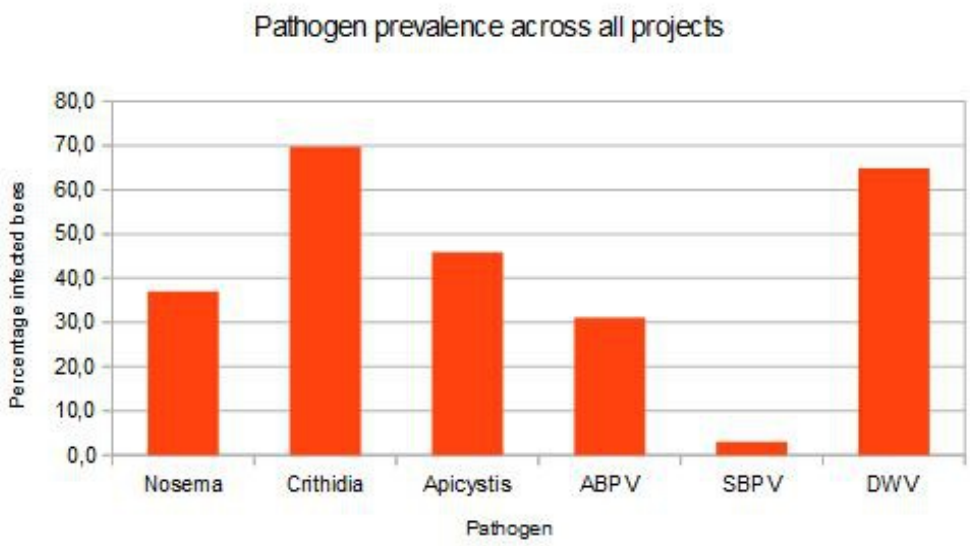



Figure 17: The prevalence of different diseases in percentage in all the collected and sampled bees.

Figure 17 shows the pathogen distribution in **all projects** (Super-B, MSB, and Lövsta). Nearly all sampled bees (97,8 %) had at least one of the six studied pathogens, while most bees (82,3%) had multiple infections of two or more pathogens. The two most common pathogens were Crithidia and DWV, while SBPV was the least common. This figure showing the global prevalence's of these pathogens in all pollinators and projects will help the interpretation of the further diagrams on the pathogen distribution for individual projects.

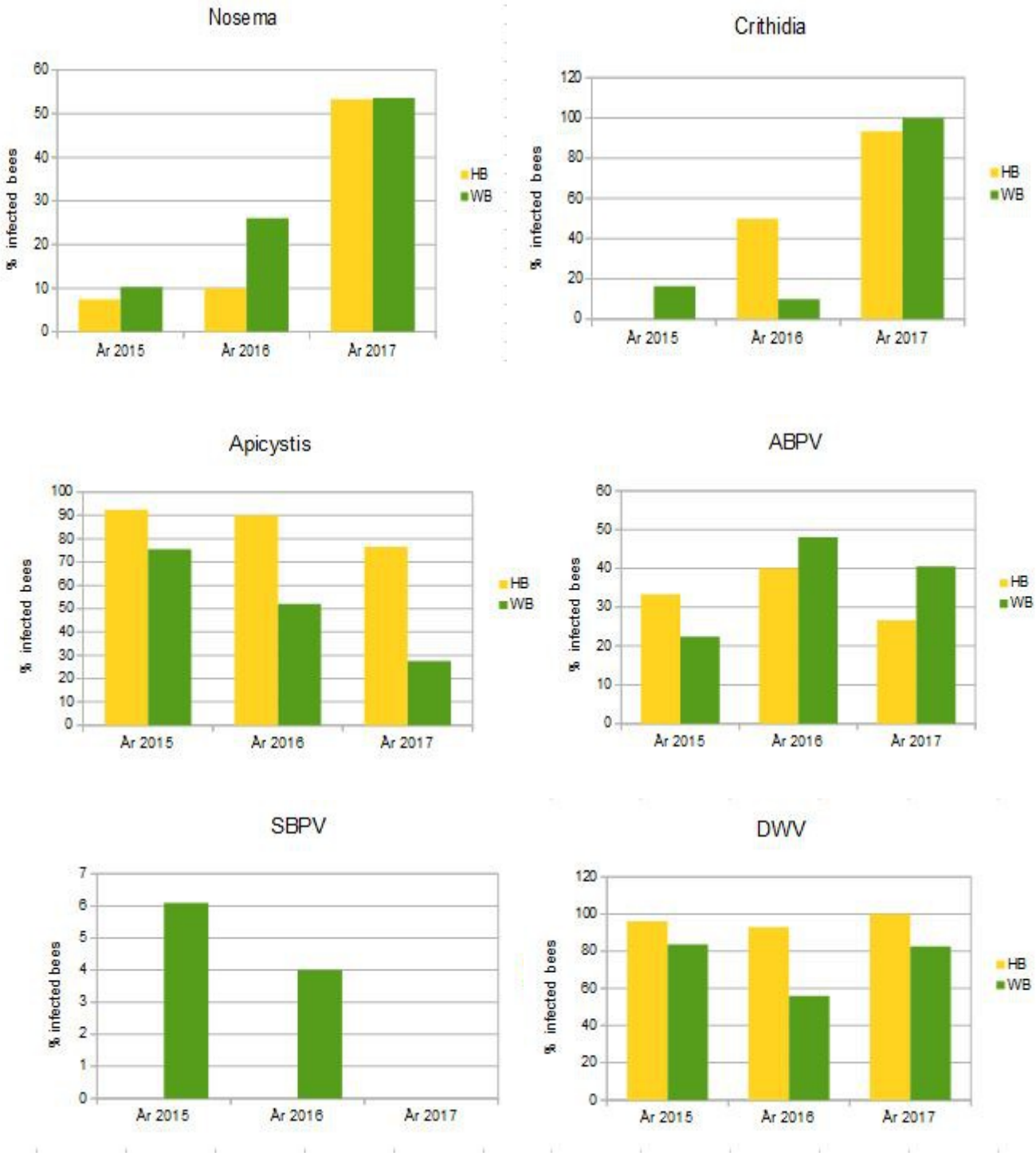



Figure 18: The comparison of change over time in the prevalence of six pathogens in honeybees (yellow) and wild bees (green) in the Super-B project at SLU, Ultuna from year 2015-2017.

The **Super -B project** shows that the prevalence of Nosema increased seven-fold in both honeybees and wild bees between 2015 and 2017 (Figure 18). Also Crithidia have increased year 2017, for both honeybees and bumblebees. The levels of Apicystis are decreasing during the three years, mostly for wild bees. However the levels of ABPV are increasing during the three years for wild bees, while for honeybees the levels of ABPV are lower year 2015 and 2017 and higher 2016. The pathogen SBPV was only found in wild bees, both in year 2015 and 2016 but not in year 2017. The levels of DWV are seen to be even, through the years for honeybees and wild bees, except for a lower level in 2016 for wild bees.

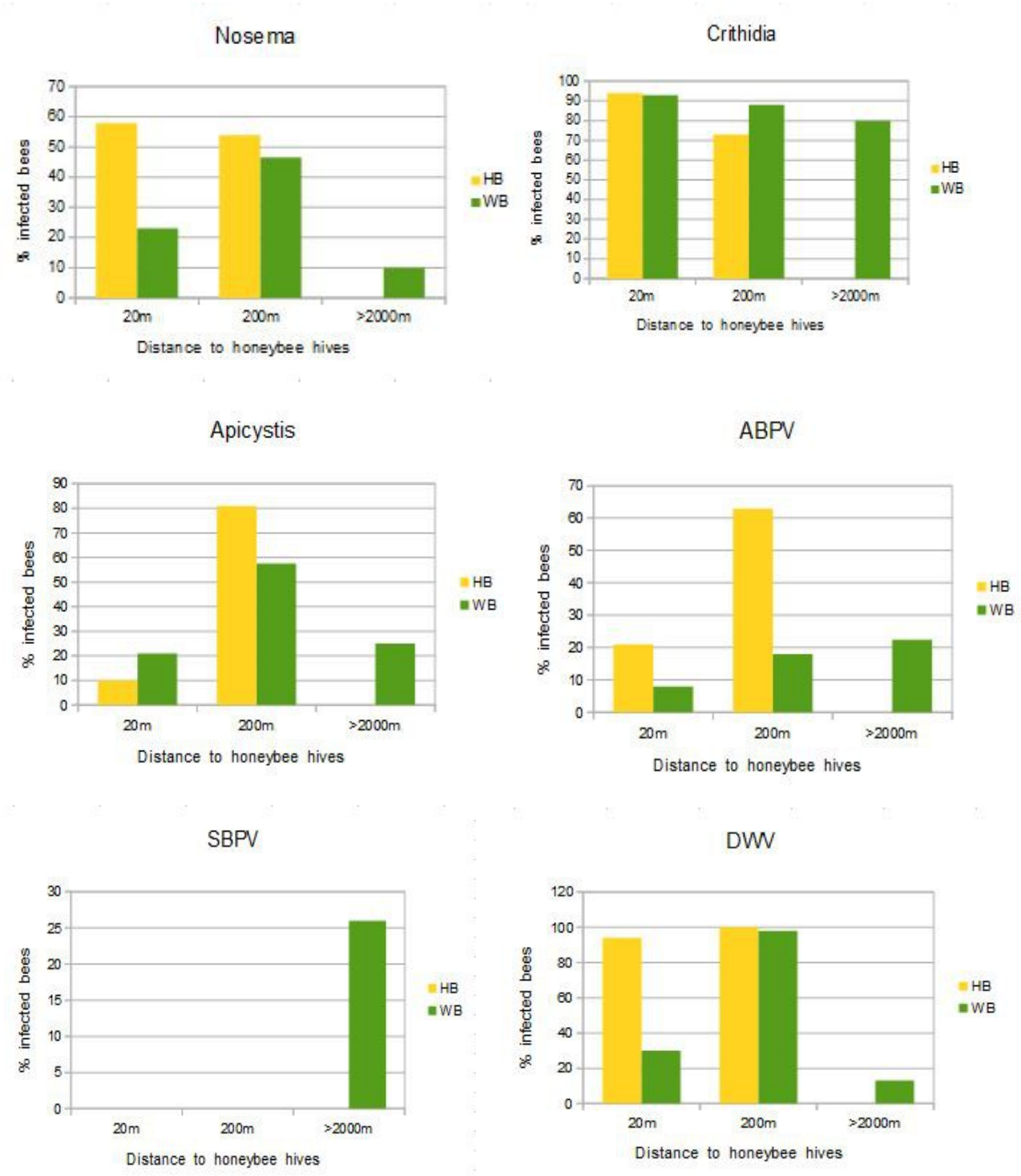



Figure 19: The comparison of the prevalence of diseases between honeybees (yellow) and wild bees (green), in relation to the distance from honeybee colonies at Lövsta.

The **Lövsta projects** show a comparison of the prevalence of diseases between honeybees and wild bees depending on distance from honeybee hives (Figure 19). For wild bees there is a decrease in the pathogens Nosema and DWV when there is no contact with honeybees, which could indicate that honeybees drive the transmission of these pathogens. By contrast, for the pathogens Crithidia, Apicystis and ABPV no relationships are seen between the presence of honeybees or distance from honeybee hives and the level of infection in wild bees. The pathogen SBPV does not seem to infect honeybees, only wild bees.

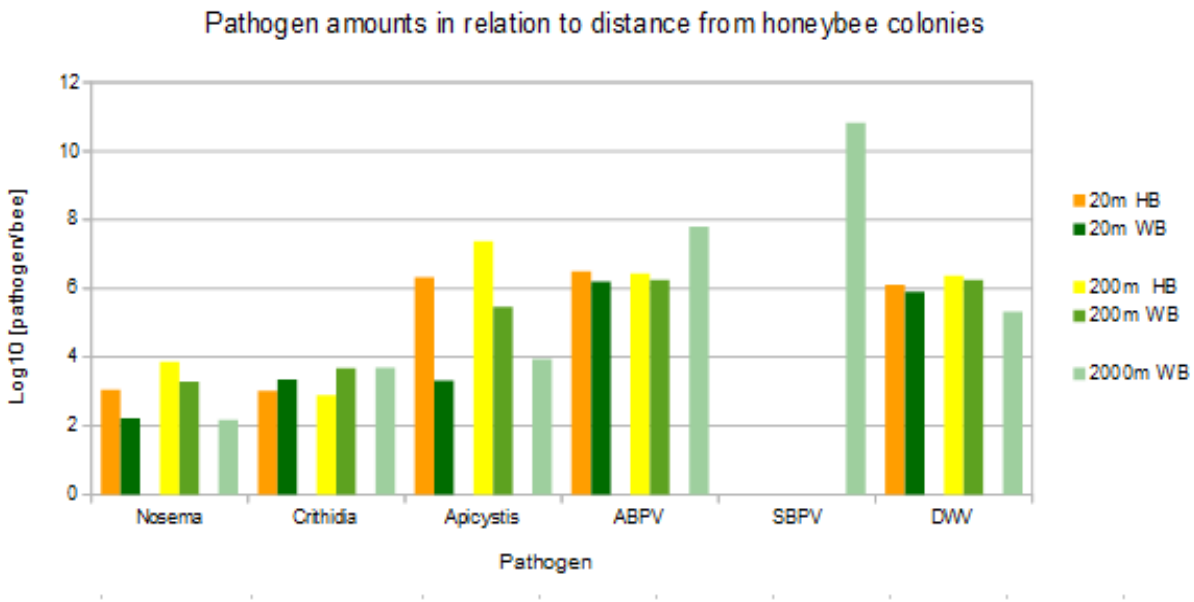



Figure 20: The average amount of different pathogens in wild bees (dark green, green, light green) and honeybees (orange, yellow) in relation to the distance from honeybee colonies (darker colors = closer to honeybee colonies).

The **Lövsta project** doesn't show any strong indication of a connection between the distance or the presence or non-presence of honeybees (Figure 20). The levels of Nosema and Crithidia in either wild bees or honeybees do not show any relationship to the distance from honeybee hives. For the pathogen Apicystis the levels were a bit higher for honeybees than wild bees. The absence of honeybees did not affect the level of Apicystis in wild bees as it was the same as for the wild bees that had honeybee hives 200m away. The levels of ABPV were higher at the control site without honeybees than at any of the sites with honeybees. SBPV was effectively only found at Lövsta-4 where no honeybees are present. The absence of honeybees had no real effect on the level of DWV in wild bees. Wild bees that are found 200m and 20m from honeybees have the same infection level.

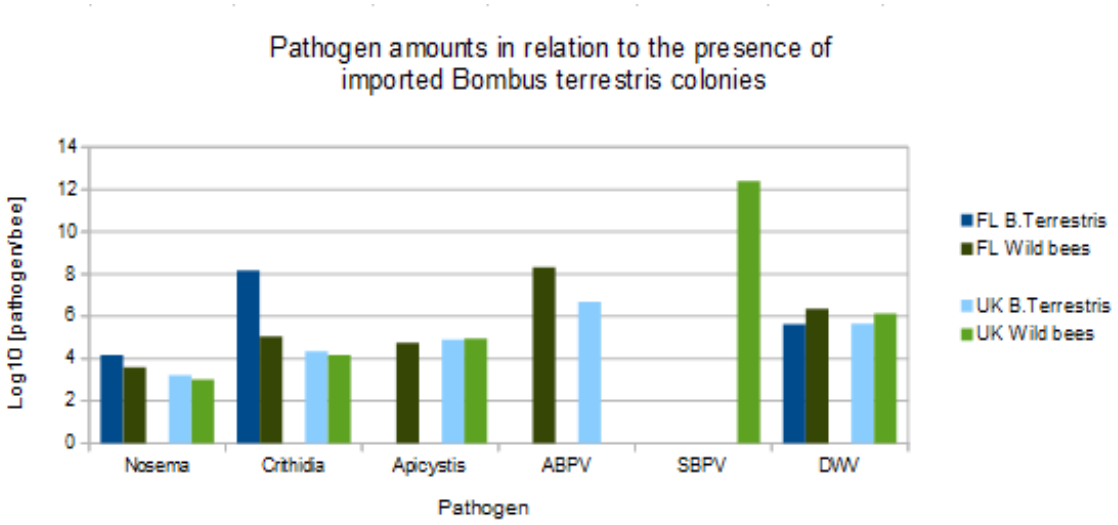



Figure 21: The average content of pathogens in bombus terrestris imported (UK, Ulva Kvarn), and non-imported (FL, Frederikslund), and the average content of pathogens in the surrounding *Bombus spp* at Frederikslund and Ulva Kvarn.

The **MSB project** did not find any big differences between Nosema infected bees at Fredrikslund and Ulva kvarn (Figure 21). The pathogen Crithidia did however show a high level of infections in wild *Bombus terrestris* at Fredrikslund, compared to the imported reared *Bombus terrestris* at Ulva kvarn, as well as to wild non-terrestris bumblebees at both locations. The pathogen Apicystis is absent in wild *Bombus terrestris* at Fredrikslund but present in other wild bumblebees, as well as in imported *Bombus terrestris* at Ulva kvarn. Both imported *Bombus terrestris* and the two groups of wild bees have the same levels of infection. The pathogen ABPV shows infection in imported *Bombus terrestris* bees but not in the surrounding wild bees. While the opposite is shown for non-imported bees, that had no infection but their surrounding wild bees where infected. SBPV is only present in wild bumblebees from Ulva kvarn. Infections of DWV did not show any significant differences between where imported and non-imported bumblebees existed neither for *bombus terrestris* nor wild bees.

## Discussion

Pathogens in apiculture constitute a threat both on colony level and for bees in shared habitat and for diversity of vegetation. The aim of this project was to assess if the pathogen distribution in wild bees have any relationship to the distribution of pathogens in managed bees. In this study the subject was investigated from three different angles in three different projects.

**Super-B project:** The goal of this study was to determine if there is a change in the pathogen distribution in wild bees in the same location over three years, with the presence of well-established honeybee colonies. Figures 12 and 15 shows the changes over time, in the diversity and density of the wild bee population, in a single location. Figure 15 suggest that the density of bees (or the collection proficiency) increased between 2015 and 2017, using the average collection time as a rough proxy for absolute density. Figure 12 shows that also the diversity of bees increased during this time, with six more bee species collected 2017 than year 2016. Figure 18 shows that both Nosema and Crithidia have increased in prevalence in both honeybees and wild bees from year 2015 to 2017. This could be due to the increase of the density of bees.

**Lövsta project:** The goal of this study was to determine the pathogen distribution in wild bees in relation to honeybee density and distance to honeybee hives. In Figure 13 and 19 it can be seen that some wild bees sharing the same habitat with honeybees. Some in the distance of 20 meter, some within the flying distance of honeybees 200 meters and some species of bees do not share the same habitat with the honeybees on a distance of more than 2000 meters. Those bees are not likely to cross-infect pathogens with honeybees. The pathogens Nosema and DWV have the lowest levels of infections in wild bees when no honeybees are around. In Figure 20 the pathogen Apicystis also show a higher level of infection in honeybees close to the hive, than in the surrounding wild bees.

**MSB project:** The goal of this study was to compare pathogen distribution and abundance in wild bees in relation to the presence or absence of imported *Bombus terrestris*. Bumblebee colonies, show that non-imported *Bombus terrestris* are not infected with Apicystis. However both the wild bees that share the same area are infected with Apicystis, and the imported *Bombus terrestris* and the surrounding wild bee species (Figure 21). The pathogen Crithidia have a higher infection level in non-imported *Bombus terrestris* than in the wild bees that share the same area are infected with Crithidia, and the imported *Bombus terrestris* and the surrounding wild bee species. The pathogen ABPV only exists in imported *Bombus terrestris* but not in the surrounding wild bee species. On the contrary in the area with non-imported bumblebees, only the surrounding wild bee species were infected with ABPV. However in Figure 14 it is seen that the collected sample size of *Bombus terrestris* from Fredrikslund are too small to give any clear indications.

The results are not clear whether the pathogen distribution and abundance in imported and non-imported *Bombus terrestris* are related. In some cases the pathogen seems to stay within one species and not transmit to the surrounding bee species. In some other cases all the bees in the same area are carrier of the same pathogen. The commercialization of bees is a high risk for spreading diseases. And in this study we can only show that some of the pathogens are found both in imported bees and in surrounding wild bees. But what's not known is, if the imported and the surrounding wild bee pathogens are genetically equal or if there is a difference. Further research is needed to know if the diseases have the same origin. To be sure if it is the imported bees and nonnative that transfers the diseases to the surrounding wild bees.

One aspect of imported bees could be that by human choice of imported bees that could indirect affect the biodiversity of the landscape. For example bumblebees from pocket makers often have

a longer tongue than those of pollen storers. This has the advantage that they can reach down in flowers with deep corollas, such as red clover. Unfortunately long tongue bees cannot be reared artificially because of the difficulty in supplying them with pollen (Hobbs *et al*., 1961). By importing bees that only can pollinate shallow types of corolla flowers. That could affect the natural balance of shallow- and deep corolla flowers.

If the density of bees gets higher, ether it is from one season to the next as for **Super-B** or it is between sites as for **Lövsta** and **MSB**, the bees will likely share the same feed sources and their by transmit diseases to other species. As seen in Figure 16, different bee species sometimes prefer the same plant. A study by Durrer *et al.,* (1994) confirmed that a pathogen transmission can occur and an infection can develop when pollinators use the same flower resources. However both ten- and twenty-five years later the researchers are not sure. Goulson, (2003) claim that it has not been any clear significant evidence that nonnative bees have a negative effect on native bees. In agreement with Goulson, (2003) there is updated research by Chandler *et al.,*(2019) that says "we conclude that there is currently not enough reliable, consistent evidence to support claims that the current use of managed *Bombus terrestris* in Europe is harmful to wild populations of *Bombus terrestris* and other bumblebees, and therefore the issue remains unresolved".

The risks with a decrease in bee density and species richness are the effect on the natural ecosystem and biodiversity. Also the economical aspect were 75% of the global crop production are beneficial by pollinating insects are at risk if there is a decline in bee density (FAO, 2018b) But to increased stocking with domesticated honeybee colonies as a prevention for inadequate pollination of crops is not likely to solve the problem (Goulson, 2015; Mallinger *et al.,* 2014). As it is seen in the present study bee colonies and species are changing from year to year. Also pathogens affect the species different. Overdependence on a single pollinator species for full pollination service is a risky solution (Kremen *et al.,* 2005).

Strategies to preserve bees and pollinators of all kind, especially in agricultural landscapes are an important task but a difficult one. Intensive conventional farming systems will lead to a homogenized agricultural landscape, where there is no room for natural and semi natural environments which is a possible habitat for pollinating bees. An organic farming system is giving a more heterogenized agricultural landscape, which is more beneficial for pollinating insects (Holzschuh *et al.,* 2007). It will be a more constant flow of feed with flowering plants replacing each other, instead of only one big peak of resources. However there are bigger differences between conventional and organic farming in a homogeneous landscape than in a heterogeneous landscape (Holzschuh *et al.,* 2007), where a heterogeneous landscape can provide much more habitat and nesting sites.

A big crucial difference between conventional and organic farming are the application of insecticides. The wild bee richness could be seen in a study by Brittain *et al.,* (2010) to decline in fields where insecticides where applied, while fields either with crop or uncultivated fields with no application of insecticides did not show any declines in wild bee species. But it was also seen that butterflies and bumblebees richness were lower than for solitary bees. However it was not until the second and third application of insecticides that affected the richness of wild bees. This information may give room for studies where pesticides could be applied adapted to the generation gap of wild solitary bees.

The decline in bee density and bee species is not sure to be due to a single factor. Different factors and different stressors could together hurt the bees more than just one factor. In Figure 17 it can be seen that several of the studied pathogens in this study had a high percentage level in

the sampled bees. More than 82% of the bees had the prevalence of more than one disease. Honeybees are more sensitive to the pathogen *Nosema ceranae* when they have been exposed to pesticides. Other factor that could affect the fitness of bees is the climate, cold conditions slows down the process of foreign food, lack of nesting sites and introduction of exotic bees, not only for the potential of new pathogens but also competing about resources. However Goulson, (2003), express that the native bees already have several thousands of other native pollinating insects to compete with, so one or a few new species will not make any difference.

One interesting work against bee diseases are the development around breeding on the bees' natural resistance against diseases. This would be a more sustainable way of treatment, and the need for antibiotics could maybe be reduced or removed. It has been suggested that the resistance against diseases depends on the bees' hygienic behavior (Spivak *et al.,* 2001).

## Conclusion

This study indicates that it is a relationship between the distributions of pathogens in managed bees and the pathogen distribution in wild bees. The distance to honeybees do affect the presence and level of pathogens in wild bees. Also the last years (2015 to 2017), there has been an increase in diseases both for honeybees and wild bees but it has also been an increase of the density of bees, in the sampled area. The relationship between imported and non-imported *Bombus terrestris* do not show any clear results except for that the imported bees have a higher number of bees infected with Crithidia than the other bees in the MSB project. However how much this relationships affect the number of bee and different bee species fitness need more investigation.

What we know today is that a more heterogeneous agriculture landscape would be beneficial for the wild bees. Also a decrease in pesticides or an accurate planning schedule for when to apply the pesticides to avoid sensible times in the generation cycles of bees would help wild bees. Imported bumblebees do help secure sufficient pollination, but the importation should be done with care and consideration since it is a risk of spreading diseases to surrounding environment. Breeding on natural resistance against diseases would be a sustainable way of treatment. Further research is needed to secure correct information about how much the increase of pathogens affect the different species of bees and their ability to pollinate, and thereby preserve the biodiversity of the pollinating dependent plants and the natural ecosystem.

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# Annex I

**Metafile**

| | |
|---|---|
| SAMPLE-ID | Sample ID number. Format XX:000, where 'XX' is the location code and '000' the individual sample number |
| DATE | Date of sampling |
| TIME-START | Time of sampling |
| LOCATION | Name of the location and sub-location. |
| LATITUDE | Latitude of the location (decimal) |
| LONGITUDE | Longitude of the location (decimal) |
| IMPORT_BB | Presence (yes) or absence (no) of imported bumblebee colonies |
| BB_PRODUCER | Producer of imported bumblebee colonies |
| HB_HISTORY | History of beekeeping in the immediate area (< 1km radius) |
| 20M_HB_COL | Number of honey bee colonies within 20 meters of the sample when taken |
| 200M_HB_COL | Number of honey bee colonies within 200 meters of the sample when taken |
| 2000M_HB_COL | Number of honey bee colonies within 2000 meters of the sample when taken |
| FLORAL_DIVERSITY | Subjective estimation of the floral diversity in the area (low, medium, high) |
| TEMP_SHADE | Temperature in the shade during sampling |
| TEMP_OPEN | Temperature in the open during sampling |
| CLOUD | Estimated cloud coverage during sampling |
| LANDSCAPE | Type of landscape |
| FLOWER | Flower species the sample was collected from |
| FINAL-ID | Final insect ID, distilled from field-ID, lab-ID and barcode analysis |
| ANTECKNINGAR | Notes taken during the sampling |
| Nosema.prev | Prevalence of Nosema - Broad assay for *N. apis*, *N. ceranae* and *N. bombi* |
| Crithidia.prev | Prevalence of Crithidia - Broad assay for *C. mellifica*, *C. bombi* and *Lotmaria passim* |
| Apicystis.prev | Prevalence of Apicystis - Broad assay for *Apicystis bombi* and close relatives |
| Nosema.amount | Estimated amount of Nosema per bee (log-normal distributed) |
| Crithidia.amount | Estimated amount of Crithidia per bee (log-normal distributed) |
| Apicystis.amount | Estimated amount of Apicystis per bee (log-normal distributed) |
| ABPV.prev | Prevalence of Acute bee paralysis virus - Broad assay for all strains within the ABPV-complex |

| | |
|---|---|
| SBPV.prev | Prevalence of Slow bee paralysis virus - Broad assay for all strains within the SBPV-complex |
| DWV.prev | Prevalence of Deformed wing virus - Broad assay for all strains within the DWV-complex |
| ABPV.amount | Estimated amount of ABPV per bee (log-normal distributed) |
| SBPV.amount | Estimated amount of SBPV per bee (log-normal distributed) |
| DWV.amount | Estimated amount of DWV per bee (log-normal distributed) |

Table 2:. Metafile of descriptions and definitions for the data and metadata collected for each individual bee.