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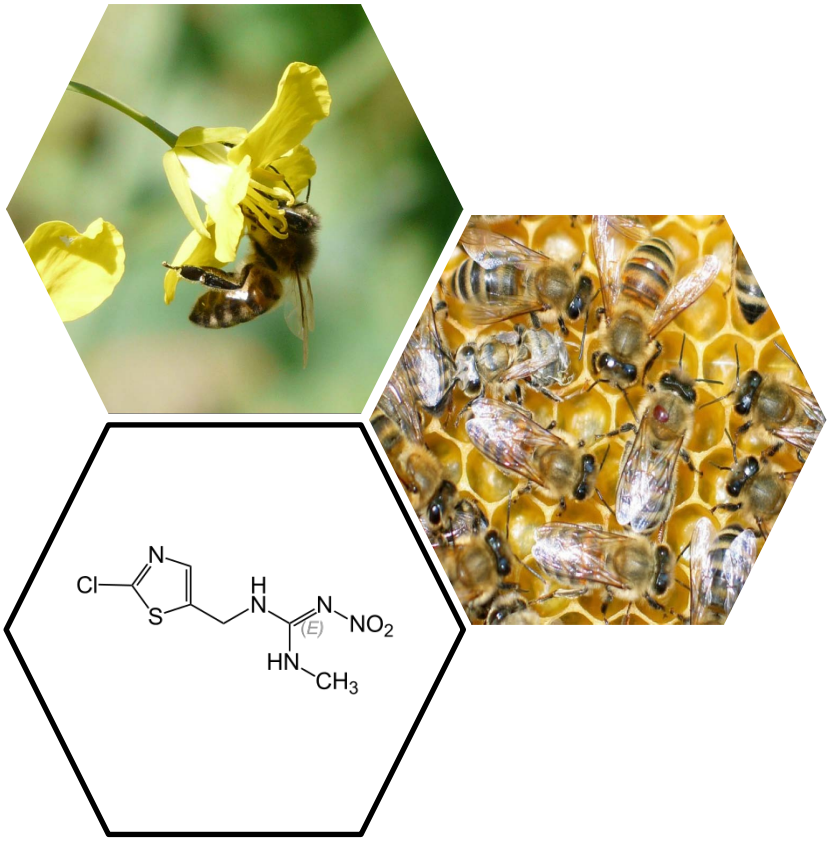
Neonicotinoids and honeybee health

The effect of the neonicotinoid clothianidin, applied as a seed dressing in *Brassica napus*, on pathogen and parasite prevalence and quantities in free-foraging adult honeybees (*Apis mellifera*)

Julia Goss



European Master of Environmental Science (EnvEuro)
Uppsala 2014

Independent project/Degree project / SLU, Department of Ecology 2014:4

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Credits:	30 hec
Level:	A2E
Course title:	Independent Project in Environmental Science – Master's thesis
Course code:	EX0431
Programme/education:	European Master in Environmental Science (EnvEuro)
Place of publication:	Uppsala
Year of publication:	2014
Cover picture:	Foraging honeybee, Julia Goss 2013
Title of series:	Independent project/Degree project / SLU, Department of Ecology
Part no:	2014:4
Online publication:	http://stud.epsilon.slu.se

Keywords: honeybees; pathogens; parasites; virus; *Nosema*; *Varroa*; neonicotinoids; clothianidin

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Department of Ecology

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Abbreviations

A	After
ABPV	Acute bee paralysis virus
ALPV	Aphid lethal paralysis virus
B	Before
BACI	Before-After-Control-Impact
BQCV	Black queen cell virus
C	Control
CBPV	Chronic bee paralysis virus
CCD	Colony Collapse Disorder
cDNA	Complementary deoxyribonucleic acid
Cq	Quantification cycle
D.f.	Dilution factor
DNA	Deoxyribonucleic acid
DWV	Deformed wing virus
E-DNA	Estimated copies of Nosema DNA
EFSA	European Food Safety Agency
GIS	Geographical information system
GLM	Generalized linear model
I	Impact
IAPV	Israeli acute paralysis virus
KBV	Kashmir bee virus
LD50	Median lethal dose
nAChR	Nicotinic Acetylcholine Receptor
N-DNA	Normalized copies of Nosema DNA
NOEC	No observed effect concentration
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT-qPCR	Reverse Transcription quantitative polymerase chain reaction
SBPV	Slow bee paralysis virus
SBV	Sacbrood virus
SQ	Starting quantity

Abstract

Sub-lethal doses of neonicotinoids have been shown to negatively impact the health of honeybees. However, most studies to date have exposed bees only artificially to these pesticides under laboratory conditions. There have been just a few well designed and replicated studies of the impacts of realistic neonicotinoid exposure on honeybees foraging under field conditions. In order to close this knowledge gap, and to test the influence of the neonicotinoid clothianidin on honeybees, we used a study system of 16 paired, spatially separated (>4 km) spring oilseed rape fields in the south of Sweden. The fields were paired according to land use, the surrounding landscape and geographical proximity, using GIS. Eight of the fields were randomly assigned to be sown with clothianidin dressed *Brassica napus* (oilseed rape) seeds and their corresponding pairs with undressed *B. napus* seeds, as controls. Six equally sized *Apis mellifera* colonies, with known queen origin, were placed at each field resulting in a total of 96 colonies. Samples of bees, pollen and nectar taken from the colonies showed that the honeybee colonies at the treated fields were exposed to several orders of magnitude higher clothianidin concentrations than the colonies at the control sites. To determine the effect of this neonicotinoid on pathogen and parasite prevalence and quantities in honeybee colonies samples of adult bees were taken from each colony both before and after the flowering period in the paired fields. The parasites studied included the ectoparasitic mite *Varroa destructor* and the microsporidian gut parasite *Nosema.* The pathogens studied included eight different honeybee viruses (BQCV, SBV, DWV, KBV, SBPV, CBPV, ABPV, and IAPV). Both the prevalence (proportion of positive colonies) and the amount of parasites/pathogens in each colony (infestation rate/titres) were analysed. The infestation with *V. destructor* was relatively low and the exposure to clothianidin had no significant impact on the *V. destructor* prevalence and infestation rate of the colonies. A seasonal effect was found where more mites were detected and more colonies were infested after the experiment than before. The exposure to clothianidin had no significant influence on the *Nosema spp.* prevalence or the amount of *Nosema* spores in infested colonies. However, the *Nosema spp.* infestation rate was significantly higher before than after the rape flowering season. Furthermore the proportion of the two *Nosema* species changed over time. Before the experiment, 33 % of the samples were not infested with *Nosema spp*., 6 % were infested with pure *N. apis*, 25 % with pure *N. ceranae* and 36 % with mixed infestation. In the mixed infestations, on average, one third of the DNA originated from *N. apis* and the rest from *N. ceranae*. After the experiment only *N. ceranae* was present in the colonies. Three out of the eight viruses studied were detected: DWV, SBV and BQCV. Both BQCV and SBV were detected in practically all colonies, both before and after the experiment, with consequently no difference in prevalence due to clothianidin exposure or season. There was also no difference in BQCV and SBV titres due to

clothianidin exposure, although for BQCV there was a significant reduction in titre as the season went along. SBV titres remained constant throughout the season. The DWV prevalence was relatively low; 4% and 36% of colonies infected, before and after the experiment respectively, resulting in a significant seasonal difference in DWV prevalence in contrast to the DWV titres in positive samples which showed no seasonal effect. The clothianidin exposure had no effect on the DWV prevalence or on the titres in DWV positive samples. The higher prevalence of DWV in the control group compared to the treated group can be explained by the different initial conditions. It can be concluded that in this experiment, clothianidin exposure had no effect on the prevalence or the amount of the studied pathogens and parasites in honeybee colonies.

Svensk sammanfattning

Subletala doser av neonikotinoider har visat sig ha en negativ inverkan på honungsbinas hälsa i laboratorieförsök. De flesta studier som hittills genomförts har emellertid exponerat bin på ett artificiellt sätt för dessa bekämpningsmedel, genom direkt fodring av preparaten. Det finns få, eller inga, väl utformade och replikerade studier med realistiska nivåer av neonikotinoid exponering av honungsbin under fältmässiga förhållanden. För att råda bot på denna kunskapsbrist, och för att testa effekten av neonikotinoiden clothianidin på honungsbin, använde vi i en studie 16, rumsligt separerade (> 4 km), vårrapsfält i södra Sverige. Fälten parades med avseende på markanvändning, läge och geografisk närhet till varandra, med hjälp av GIS. Hälften av fälten slumpades för sådd med clothianidin-betade *Brassica napus* (raps) frön och det andra fältet i paret fungerade som kontroll och såddes med obehandlade frön. Bredvid varje fält placerades sex jämnstarka *Apis mellifera* samhällen, varje par med syster-drottningar, med totalt 96 samhällen i experimentet. Prover av bin, pollen och nektar från dessa samhällen visade att bisamhällen vid de clothianidin-behandlade fälten exponerades med flera tiopotenser högre mängder av clothianidin än samhällen vid kontrollfälten. Prover av vuxna bin togs från varje samhälle både före och efter blomningsperioden, för att bestämma effekten av exponering till neonikotinoiden på förekomst och mängder av patogener och parasiter i bisamhällen. De parasiter som undersöktes inkluderade det ektoparasitiska kvalstret *Varroa destructor* och de intracellulära tarmparasiterna *Nosema apis* och *Nosema ceranae*. De patogener som undersöktes inkluderade också åtta olika honungsbivirus (BQCV, SBV, DWV, KBV, SBPV, CBPV, ABPV och IAPV). Både prevalensen (andelen positiva samhällen) och mängden av parasiter/patogener i varje samhälle analyserades. Angreppen av *V. destructor* var relativt låg under hela experimentet och det fanns ingen signifikant effekt av exponering av clothianidin på vare sig förekomsten eller angreppsnivån av *V. destructor* i samhällen En säsongseffekt påvisades, där fler kvalster påvisades när experimentet avslutades än när det påbörjades. Prevalensen av *Nosema* spp.

skilde sig inte signifikant mellan behandlingarna, men visade en säsongseffekt: angreppen var högre före än efter rapsblomningen. När experimentet påbörjades var 33% av proverna inte infekterade av *Nosema* spp., Sex % var infekterade med *N. apis*, 25% var infekterade med *N. ceranae* och 36 % var infekterade med blandinfektioner. I angrepp med båda parasiterna kom i genomsnitt en tredjedel DNA kom från *N. apis* och resten från *N. ceranae*. När experimentet avslutades kunde endast *N. ceranae* påvisas i angripna samhällen. Tre av de åtta virus som undersöktes för kunde påvisas: DWV, SBV och BQCV. Både BQCV och SBV påvisades i praktiskt taget alla samhällen, både före och efter försöket. Således kunde ingen skillnad i prevalens påvisas mot bakgrund av clothianidin exponering eller av säsong. Det fanns inte heller någon skillnad i BQCV- och SBV-titer på grund clothianidin exponering För BQCV fanns en signifikant säsongseffekt med en minskning av titer senare på säsongen. Titern av SBV förblev konstant under hela säsongen. Prevalensen av DWV var relativt låg; 4 % och 36 % av samhällen smittade, före respektive efter experimentet, vilket innebär i en betydande säsongsskillnad i DWV prevalens. Det fanns dock ingen signifikant skillnad i DWV-prevalens på grund clothianidin exponering, eller inte. Det kunde inte heller påvisas någon signifikant interaktion mellan säsong och clothianidin exponering på DWV-prevalens. Samhällen infekterade med DWV visade ingen signifikant skillnad i DWV-titer på grund av clothianidin exponering. Man kan därför dra slutsatsen att i det genomförda experimentet, hade clothianidin exponeringen ingen påvisbar effekt på förekomsten eller mängden av studerade patogener och parasiter i friflygande bisamhällen.

Zusammenfassung

Unter Laborbedingungen wurde nachgewiesen, dass sub-letale Dosen von Neonikotinoiden sich negativ auf die Gesundheit von Honigbienen auswirken können. Feldstudien mit genügend Wiederholungen fehlen allerdings. Aus diesem Grund wurde im Süden von Schweden eine Studie durchgeführt, um den Einfluss des Pestizids Clothianidin auf die Prävalenz und die Quantität von Krankheitserregern und Parasiten zu untersuchen. Auf 16 räumlich voneinander getrennten (> 4km) Sommerraps Feldern wurden jeweils 6 gleich große Honigbienenvölker (*Apis mellifera*) platziert, wobei 8 Felder mit Clothianidin gebeizt wurden und 8 Felder als Kontrolle nicht mit Neonikotinoiden behandelt wurden. Die Bienenvölker neben den behandelten Feldern waren nachweislich höheren Konzentrationen von Clothianidin ausgesetzt als neben den Kontrollfeldern. Es wurden Proben von 100 adulten Bienen aus jeder Kolonie vor und nach der Rapsblüte genommen und anschließend der Befall von der Milbe *Varroa destructor*, dem Pilz *Nosema* und acht Viren (BQCV, SBV, DWV, KBV, SBPV, CBPV, ABPV, und IAPV) im Labor untersucht. Die Befallsrate und die Prävalenz von *V.*

destructor war durchweg sehr gering. Allerdings wurden nach der Rapsblüte signifikant mehr Milben gefunden als zuvor. Es konnte kein signifikanter Einfluss von Clothianidin auf die Befallsraten oder die Prävalenz von *V. destructor* nachgewiesen werden. Das Vorkommen und die Infektionsraten von *Nosema* spp. wurde durch Clothianidin nicht signifikant beeinflusst. Im Sommer wurde eine niedrigere Prävalenz nachgewiesen als im Frühjahr, wobei die Infektionsrate keinen saisonalen Unterschied aufwies. Die dominierende *Nosema* Art in beiden Proben war *N. ceranae* wobei im Sommer kein *N. apis* nachgewiesen wurde. Zu Beginn des Experiments waren 25% der Proben rein mit *N. ceranae* infiziert, 6% rein mit *N. apis*, 36% hatten eine gemischte Infektion. Der Rest, 33 % war nicht infiziert. Es wurden drei von 8 Viren in den Kolonien nachgewiesen: DWV, SBV und BQCV. BQCV und SBV wurden in nahezu allen Kolonien gefunden, sowohl vor als auch nach der Rapsblüte und folglich wurde auch kein Unterschied in der Prävalenz für diese beiden Viren im Hinblick auf Clothianidin Behandlung oder Zeit gefunden. Aber im Gegensatz zu SBV zeigt BQCV eine signifikante Reduktion der Befallsrate vom Frühling zum Sommer. Clothianidin hatte keinen Effekt auf die Infektionsrate von SBV und BQCV. Das Vorkommen von DWV war relativ niedrig; 4% und 36% der Kolonien waren infiziert, vor und nach dem Experiment jeweils, was zu einem signifikanten Unterschied in der saisonalen Verbreitung führt. Jedoch hatte Clothianidin keinen Einfluss auf die Prävalenz von DWV. Kolonien, die mit DWV infiziert waren, unterschieden sich nicht im zeitlichen Verlauf und auch Clothianidin hatte keinen Effekt auf die Infektionsrate. Daraus kann geschlossen werden, dass das Neonikotinoid, Clothianidin, keine Auswirkungen auf das Vorkommen und die Befallsraten der untersuchten Pathogene und Parasiten in dieser Studie hatte.

1. Introduction

The European or western honeybee *Apis mellifera,* commonly known as honeybee, belongs to the family *Apidae* and is one of seven species within the Genus *Apis*. Its original distribution extended from Asia throughout Europe and Africa, and the honeybee can now be found worldwide due to their use by humans for producing honey and for pollinating crops (Winston 1987; Seeley 1985). As for many other bee species, the European honeybee is a colony forming insect and it can be divided into three types of colony members: a single fertile queen, female workers and drones. These castes all develop through four stages: egg, larva, pupa, and adult. The size of a colony depends on the time of the year. With a peak in the summer and a decline in the winter, a colony can consist of between 15000 and 50000 female workers. During spring and summer, additionally, a few hundred male drones are normally present, in order to mate with the virgin queens.

As the honeybee is an eusocial insect, the labour within a colony is divided. The queen lays practically all the eggs, since this behaviour in female workers is restricted by pheromones produced by the queen. Adult drones and workers live up to 6 weeks during the summer, while workers in a winter cluster are able to survive up to 8 month. During their adult life, workers perform different colony tasks in relation to their age. The first tasks of an adult worker are building combs, cleaning and tending brood. After two or three weeks these bees start to forage outside the hive for pollen and nectar. The nutritional requirements of adults and brood are provided by these two plant produced substances and their converted form for young brood, which are collected within a 4 km range.

In order to ensure reproduction at colony level, during the swarm season, some young larvae within a colony are fed exclusively with royal jelly and this allows them to develop into new queens. During swarming, a majority of the workers leaves the hive together with the old queen and they settle at a suitable new place, while the remaining workers stay inside the old hive with the newly hatched queen. This queen starts laying eggs after mating with drones and forms a new colony (Winston 1987).

1.1 The importance of bees

The honeybee is an important, valuable and useful social insect for human beings. In addition to the production of honey and wax, honeybees are one of the main pollinators for the agricultural food production (Gullan & Cranston 2005). Around 35 % of all food is dependent on pollination by insects and honeybees account for 90 % of this pollination (Klein et al. 2007). This service has been estimated at € 22 billion per year in Europe and € 153 billion per year globally (Gallai et al. 2009). Furthermore honeybees, as key generalist pollinators, are important for biodiversity and for the ecosystem at large, as honeybees contribute greatly to the gene flow within and between plant communities (Jaffé et al. 2010). The general decline of pollinators, which are key elements for global biodiversity (Potts et al. 2010), could cause a reduced success in plant reproduction (Thomann et al. 2013). Among environmental scientists there is consensus that the protection of biodiversity should be a matter of great concern (Ehrlich 2002). Nature in all its levels such as species, population and communities, should be preserved for its own sake but also the wellbeing of humans (Ehrlich 2002). A loss of biodiversity influences the dynamics and functioning of ecosystems and their services for humans in many ways (Cardinale et al. 2012). Ecosystem services are defined as the benefits from for instance food, fresh water, regulation of the climate or cultural aspects provided by ecosystems (Millennium Ecosystem Assessment 2005). A target for the European Union is to stop the loss of biodiversity and the degradation of ecosystem services by 2020 and honeybees play a key role in that. The Member States of the European Union are also parties of the UN Convention on Biological Diversity which made the conservation and sustainable use of pollinators a priority (European Commission 2010). To sum up, a decline in the European honeybee population would be a threat to the conservation of biodiversity and human wellbeing as well as lead to an economic loss for beekeeping and agricultural production systems and should therefore be halted.

1.2 Colony losses

Even though the number of managed honeybee colonies has increased around 45 % during the last 50 years (Aizen et al. 2008), some regions experienced a decline (VanEngelsdorp & Meixner 2010; Aizen & Harder 2009a). The FAO (2009) estimated a decrease of honeybee colony of 14 % within Europe, for which economic reasons should also be considered as well as biological reasons (Aizen & Harder 2009a). Controversially to globally growing colony numbers the demand for pollination in a growing agricultural production industry has not been met (Aizen & Harder 2009b).

In the last few years, abnormally high colony losses have been reported, primarily in the United States, but also in Europe (VanEngelsdorp & Meixner 2010). During the winter of 2006/2007 beekeepers in the United States faced hives without adult bees, while brood and food was left behind (VanEngelsdorp & Meixner 2010; VanEngelsdorp et al. 2008). The term Colony Collapse Disorder (CCD) was introduced to name a phenomenon with an unclear and unknown cause (vanEngelsdorp et al. 2009). However, a literature study revealed that mass colony losses, often with symptoms similar to CCD, have occurred periodically since the late 19th century, in different parts of the world (Underwood & VanEngelsdorp 2007; Oldroyd 2007).

Winter mortality of honeybee colonies is well known to beekeepers. A rate of 5 to 10 % loss over the winter is seen as acceptable (Le Conte et al. 2010). However, during the winter 2007 – 2008 an estimation of 36 % of the colonies were lost in the United states (VanEngelsdorp et al. 2008). Normal winter mortality can be caused by many different reasons: lack of adequate food reserves, poor foraging conditions, queen losses, low fall population size or diseases and parasites (Le Conte et al. 2010). Factors such as the nutrition, the quality of the queen, pesticides and pathogens have been suspected to be the cause of CCD (Ratnieks & Carreck 2010). Pest and pathogens are one of the most important causes of honeybee losses (Ratnieks & Carreck 2010) but usually have clearly defined symptoms that are different from those of CCD (Oldroyd 2007). On their own pathogens and parasites cannot account for all the high unexpected mortality (VanEngelsdorp et al. 2008) and a causal connection between them and CCD has not yet been proven (Johnson et al. 2009). However, the reported association of parasites and pathogens with CCD could be due to a reduced bee immune system induced by pesticides and other environmental stresses (Di Prisco et al. 2013).

In general, reasons for honeybee colony losses are complex. Even though there is an ongoing replacement of infertile female workers within a colony and losses of a few individuals can be compensated, the number of individuals living together is vital for the survival and the reproduction. For instance swarming, comb construction or rearing a new queen is dependent on the number of bees within a hive (Michener 1974). The health of honeybees is influenced by many different stress

factors which can also interact with each other, which is illustrated in Figure 1. Pesticides, pathogens and parasites are two of them.

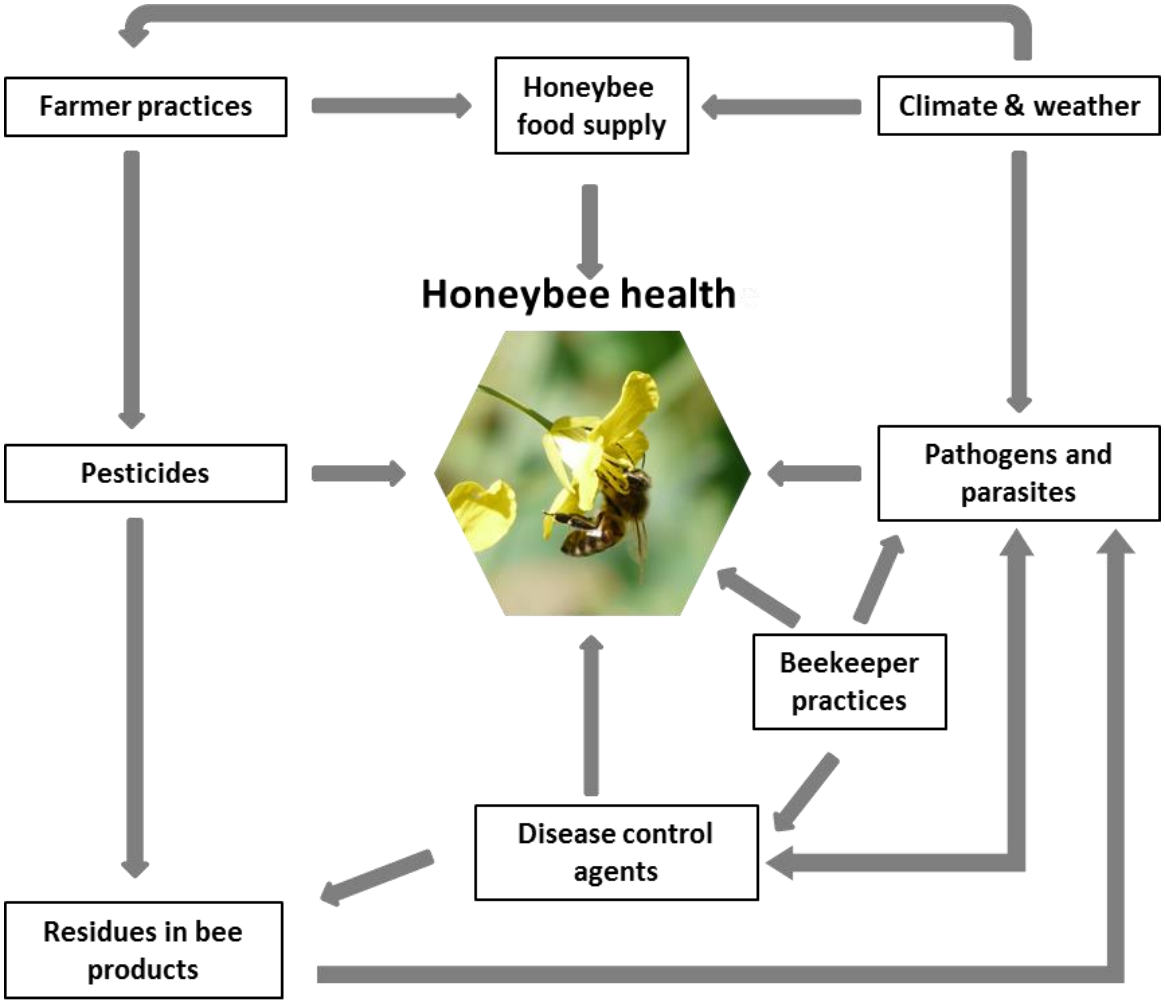

Figure 1. Honeybee health and its influences adapted from (OPERA 2013).

1.3 Honeybee immunity

Within a colony, thousands of individual honeybees live in a confined space and have close contact (Winston 1987). This, and the fact that the honeybee hive maintains a constant temperature and humidity, makes a honeybee colony an ideal environment for disease transmission (Evans et al. 2006). In order to cope with their parasites and pathogens, honeybees have several behavioural, physiological, morphological, and immune-based defences (Evans 2006).

Certain behaviours of honeybees within the colony, such as nest hygiene, grooming and minimizing the entry of infectious agents, can be labelled as a "social immunity"(Evans & Spivak 2010). In addition to these group level defence mechanisms against pathogens, honeybees also have an immune system at the individual level (Evans et al. 2006). For instance, the cuticle and the epithelial layers, and mechanical barriers avert microbial organisms from entering or adhering to the body. Furthermore, the insect gut is protected by physiological and chemical barriers from microbial invasion (Crailsheim & Riessberger-Gallé 2001).

Insects lack an adaptive immune system of the type found in vertebrates (Hoffmann 1995). Insects have instead only an innate immune response (Azzami et al. 2012), involving a wide range of physiological, molecular and biochemical actions such as melanization, enzymatic degradation of pathogens, local blood clotting, phagocytosis of bacteria and the secretion of antimicrobial peptides (Hoffmann 1995; Hultmark 2003). However, only one third of genes involved in the immunity of other insects, such as *Drosophila* and *Anopheles,* have been found in the honeybee genome (Evans et al. 2006). This suggests that honeybees rely to a large degree on social immunity mechanisms, for instance the cooperation between individual group members to defend themselves from pathogens and parasites (Cremer et al. 2007).

1.4 Parasites and Pathogens

Honeybee health is affected by parasites and pathogens such as the Varroa mite (*Varroa destructor,* Figure 2) as well as fungal, bacterial and viral diseases (VanEngelsdorp et al. 2008; Bailey 1967). A pathogen can be defined as a microorganism that can cause a disease, for instance morphological, behavioural, physiological or molecular damage, in a host (Pirofski & Casadevall 2012). In contrast, a parasite does not always damage its host but it is never beneficial for it. Parasites obtain their nutrition from another living organism. Some parasites are obligate, which means that they are unable to survive entirely apart from their host, while others are facultative, meaning they can also life independently (Drisdelle 2010). Examples of some pathogens and parasites, mentioned in the context of colony losses, are described below.

1.4.1 *Varroa destructor*

The ectoparasitic mite *Varroa destructor* is currently considered to be the most damaging threat to honeybees (Dietemann et al. 2013; Rosenkranz et al. 2010; Boecking & Genersch 2008). *V. destructor* evolved from *V. jacobsoni,* whose occurrence was restricted to Asia and to its original host *Apis cerana* (Anderson & Trueman 2000). After a host shift to *Apis mellifera*, probably in mixed *A. mellifera/A. cerana* apiaries in Asia during the first part of the 20th century (Oldroyd 1999; Jong et al. 1982) the mite was first found on *A. mellifera* in Eastern USSR in the 1950´s, Western USSR by the 1960`s, in Europe and South America by the 1970`s (Ruttner & Ritter 1980; Jong et al. 1982) and in North America by the late 1980`s (Rosenkranz et al. 2010).

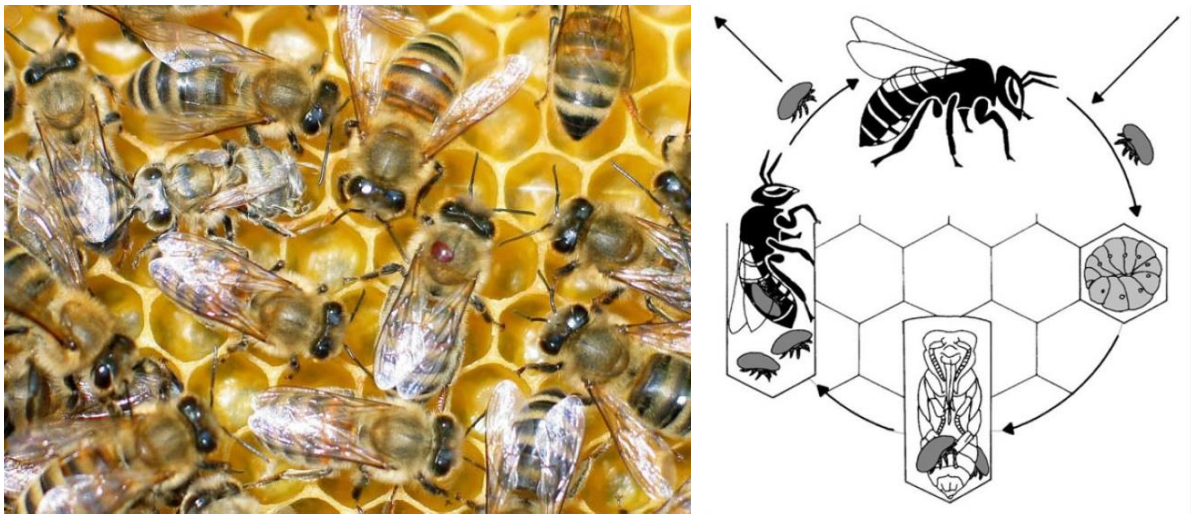

Figure 2. Varroa mite on the thorax of a honeybee (left, from Rosenkranz et al. 2010) and its lifecycle in its host *Apis mellifera* (right, from Boecking & Genersch 2008).

The *Varroa* mite reproduces in the brood cells and feeds on the bee haemolymph of both adults and larvae (Rosenkranz et al. 2010; see Figure 2). An uncontrolled infestation can, within a few years, lead to colony death (Buechler 1994), reducing the production and reproductive capacity of the colony throughout this decline (Rosenkranz et al. 2010). This gradual decline results mainly from the fact that *V. destructor* is a biological vector for several of honeybee viruses (described under 1.4.3). This has been shown for Deformed wing virus (DWV), Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV) and Acute bee paralysis virus (ABPV) (Boecking & Genersch 2008).

1.4.2 *Nosema*

Nosema apis and *Nosema ceranae* are related, but distinct obligate intracellular microsporidian parasites, which disperse between hosts as spores (Fries et al. 2006), causing infections in the ventriculus (Bailey 1955). Only *N. apis* was known to infect the European honeybee, until in 1996 *N. ceranae* was identified in *Apis cerana*, the Asian hive bee, and both Nosema species were found to be capable of cross-infecting each other's original hosts (Fries et al. 1996). Infection with *N. apis* leads to a reduced lifespan of the workers and heavily infected colonies are weakened (Fries et al. 1984). Although *N. ceranae* was thought to be restricted to *A. ceranae* and Eastern Asia (Fries 1997), it has been detected as natural infections in *A. mellifera* colonies around the world (Huang et al. 2007; Fries et al. 2006). *N. ceranae* is thought to play a major role in colony losses (Higes et al. 2009; Cox-Foster et al. 2007; Oldroyd 2007; Martín-Hernández et al. 2007) and has currently a nearly world-wide distribution (Fries et al. 2006; Genersch et al. 2010).

1.4.3 Viruses

Viruses are intracellular, molecular life forms that are entirely dependent on the host for reproduction. Although many viruses are pathogenic, many others can be entirely asymptomatic (de Miranda et al. 2012). Currently over 18 different viruses infecting honeybees are known, such as deformed wing virus (DWV), black queen cell virus (BQCV) and Sacbrood virus (SBV) (Chen & Siede 2007; see Figure 3). Most honeybee viruses have a single-stranded RNA genome and several can be grouped into complexes of closely related viruses (de Miranda & Genersch 2010; de Miranda et al. 2012). Viruses are common in bee populations (de Miranda et al. 2013). At low levels of infection, most honeybee viruses cause no symptoms (de Miranda et al. 2013), the colony seems healthy even if a several viruses are present. Some viruses can be identified by characteristic symptoms, such as BQCV, while in other cases the symptoms are less clear (de Miranda et al. 2012). Many of the

symptoms are behavioural changes, such as inability to fly, trembling, crawling, learning difficulties or disorientation (de Miranda et al. 2012). Furthermore, most virus infections reduce the lifespan of adult honeybees to different extents (de Miranda et al. 2013). A honeybee colony is able to compensate the loss of a few infected bees. However, when too many individuals are infected, the colony can no longer compensate it and collapses gradually (de Miranda et al. 2012). A list of some viruses, their symptoms and effects can be found in Table 1.

Higher virus titres have consistently been associated with colony losses during the winter, particularly those of the DWV and ABPV species-complexes. In a study during the winter 2007/2008 lower proportions of DWV were found in colonies, which survived the winter (Dainat & Neumann 2013). Furthermore, a metagenomic survey, the Israeli acute paralysis virus (IAPV) was correlated with CCD colonies in the United States (Cox-Foster et al. 2007).

Figure 3. Honeybee (in the centre) with symptoms of DWV: shortened abdomen and deformed wings (left, from Locke 2012) and larvae infected by SBV (right, Photo: S. Camazine)

Table 1. List of some honeybee viruses and their symptoms, effects, association with V. destructor and seasonal occurrence from de Miranda et al. (2013); ~ uncertain; + = present;

Virus	Abbr.	Virus Complex	Symptoms	Effect	Association with V. destructor	Season		
						Spring	Summer	Autumn
Acute **b**ee **p**aralysis **v**irus	**ABPV**	ABPVC	Sharply reduces adult longevity. Trembling and inability to fly after artificial virus infection	Can be lethal at individual and colony level	+	+	+++	+++
Black **q**ueen **c**ell **v**irus	**BQCV**	ABPVC	Infection of the queen, their larvae and pupae; Walls of the queen cells can be black		+	+	+++	+++
Chronical **b**ee **p**aralysis **v**irus	**CBPV**	CBPV	Trembling of the body and wings; adults with inability to fly		~	++	++	+
Deformed **w**ing **v**irus	**DWV**	DWVC	Pupal death; discolorations, deformed wings and shortened abdomen of adult bees		+	+	++	+++
Israeli **a**cute **p**aralysis **v**irus	**IAPV**	ABPVC	Sharply reduced adult longevity. Trembling and inability to fly after artificial virus infection	Can be lethal at individual and colony level	+	+	++	++
Kashmir **b**ee **v**irus	**KBV**	ABPVC	No visible symptoms. Strongly reduced adult longevity.	Can be lethal at individual and colony level	+	+	++	+++
Slow **b**ee **p**aralysis **v**irus	**SBPV**	DWVC	No natural symptoms. Paralysis of the two front leg pairs of adults after artificial infection.		+	+	+	+
Sac**b**rood **v**irus	**SBV**	DWVC	Dead larvae are sac-like (see figure 2); uneven brood		~	+++	++	+

1.5 Neonicotinoids

Besides pathogens, the potential role that pesticides might play in the unexpected high loss of colonies, is controversially discussed (Blacquière et al. 2012). Plant protection products such as chemical insecticides contribute significantly to ensure that the food demand for a fast growing human population continues to be met. One fifth of the worldwide crop yield are defended by the application of chemical insecticides (Oerke & Dehne 2004). Neonicotinoids, which were introduced in 1991 by Bayer CropScience with the launch of imidacloprid, are the most important chemical class of insecticides in the world today. In 2008 seven different neonicotinoids were produced commercially which can be divided into two groups (Iwasa et al. 2004; see Table 2). Combined, these chemicals accounted for around 24 % of the total global market for modern crop protection (Jeschke et al. 2011). Neonicotinoids are systemic insecticides, when they are added to the soil or used as seeds dressing. The chemicals are absorbed by the plant (Blacquière et al. 2012) and with their outstanding plant systemic activity they are dispersed within the plants (Tomizawa & Casida 2005). For several months, plants are protected from insects (Goulson 2013). Although neonicotinoids are also used as foliar sprays, the most common application is as a seed dressing (Goulson 2013).

Even though neonicotinoids have a low toxicity to fish, birds, and mammals (Tomizawa & Casida 2005) they are extremely toxic to insects. This group of pesticides binds permanently to neonicotic receptors of acetylcholine (nAChr) and block the nerve system in insects (Tomizawa & Casida 2005). Beside pest insects, non-target organisms such as insect pollinators can also be affected through a translocation within the plant (Blacquière et al. 2012). Neonicotinoids are detectable in the nectar and pollen, the main food sources for insect pollinators, throughout the flowering period. Therefore bees are particularly exposed to these pesticides during this period (Reed et al. 2010).

Table 2. List and features of neonicotinoids

Feature	Neonicotinoids
Nitro group	Imidacloprid, clothianidin, thiamethoxam, nitenpyram and dinotefuran
Cyano group	Acetamiprid and thiacloprid

However, the toxicity of neonicotinoids is very diverse and complex. In general pesticides can affect honeybees in four different ways: Either lethal or sub-lethal effects from either acute or chronic exposure (van der Sluijs et al. 2013). Lethal effects are mostly described by the LD50 value, which is

defined as the dose at which 50% of the honeybees are dead within 48 hours. After a persistent exposure to neonicotinoids lethal effects are called chronicle. The time honeybees are exposed to this pesticide magnifies the poisonousness (van der Sluijs et al. 2013). In addition to lethal effects, low concentrations of neonicotinoids can cause sub-lethal effects, which means that the exposure will not directly cause collapse of the colony or the death of individuals but might make the colony more sensitive or even become lethal in time. As well as lethal, sub-lethal effects can be caused from acute or chronic exposure. While effects are called acute when honeybees are only exposed once to neonicotinoids, chronic effects are a synonym for multiple exposures (van der Sluijs et al. 2013). The route of exposure is another important factor in the toxicity of pesticides. For instance, an oral intake of the pesticides is usually more toxic for honeybees than only the contact (Blacquière et al. 2012). Not all neonicotinoids are equally poison to honeybees, the group of neonicotinoids with a nitro-group, including clothianidin, is more toxic than acetamiprid and thiacloprid (Iwasa et al. 2004; see Table 2).

The European Food Safety Authority (EFSA) summarized several studies of the effects of three neonicotinoids in 2013 for a request from the European Commission to provide a better basis for decision making. All in all, the usage of clothianidin is a high risk for honeybees in some applications. Nevertheless, there are several data gaps for instance the risk from the consumption of contaminated pollen and nectar or from the exposure via guttation's fluids (EFSA 2013). Hence, three neonicotinoids (clothianidin, imidacloprid and thiamethoxam) were restricted for seed treatment, soil application and foliar treatment on bee attractive plants and cereals with some exceptions in the European Union from the first of December 2013. However, there will be a revision of this status within the next two years, as soon as new information is available. Relevant scientific and technical developments will be taken into account for further regulations (European Commission 2013). The already existing studies about lethal and sub-lethal effects on honeybees will be described below.

1.5.1 Lethal effects of neonicotinoids on honeybees

It has been proven that clothianidin is toxic for honeybees by contact or oral intake. The LD50 value for oral intake per bee was specified to be 3.79 ng clothianidin per bee and 44.26 ng clothianidin in contact per bee (European Commission 2006). The No Observed Effect Concentration (NOEC) was calculated in a ten-day experiments where honeybees where fed with a sugar solution containing clothianidin in order to test which threshold leads to chronic lethal effects. A concentration of 10 µg/l is considered to have no observed influences (EFSA 2013). The NOEC concentration in the diet varies between 20 and 40 µg/l (EFSA 2013).

In Germany 2008, colonies within several apiaries collapsed in the Rhine valley. The dead honeybees and bee bread contained high contents of clothianidin. This neonicotinoid was released during maize sowing due to a poor seed dressing bonding, the use of pneumatic seed drills and a delayed sowing conducted during the rape flower season (Rosenkranz & Wallner 2008).

1.5.2 Sub-lethal effects of neonicotinoids on honeybees

Studies have found that sub-lethal doses of the neonicotinoid thiamethoxam have a negative impact on honey foraging success (Henry et al. 2012) and imidacloprid on the development of brood and adults (Decourtye et al. 2005).

In addition to that synergistic interactions between neonicotinoids and pathogens have been found to influence honeybee health. For instance, a sub-lethal oral doses of the neonicotinoid thiacloprid can enhance the harmful effects of the microbial pathogens *Nosema ceranae* and the black queen cell virus, on honeybee larvae and adults (Doublet et al. 2014), although only at very high infection levels. It has also been shown that the interaction between imidacloprid and a mixture of *N. apis* and *N. ceranae* infestations weakened honeybees (Alaux et al. 2010). Furthermore neonicotinoids were found to affect the loads of pathogens and parasites. Honeybees exposed to clothianidin had a decreased immune defence under laboratory conditions, which stimulated the replication of deformed wing virus (Di Prisco et al. 2013). Imidacloprid was found to increase level of the gut pathogen *Nosema spp.* (Pettis et al. 2012).

Although there have been numerous laboratory studies on the effects of neonicotinoids on honeybees, the influences and exposures under field conditions are poorly known. Laboratory experiments are important to analyse the possible toxicity of pesticides on the individual honeybee, which might be hard to determine under field conditions. Nevertheless, honeybees, as social insects, might buffer the loss of individuals as a “super organism”. Therefore it is important to conduct field studies. One field study has been conducted already in Canada where no influence of the exposure

to clothianidin seed-treatment of canola on worker longevity, brood development, honeybee mortality, honey yield or colony weight was found (Cutler & Scott-Dupree 2007). However, the methodology of this study can be questioned in many ways. Honeybee colonies were placed at 1 ha clothianidin treated and control canola (oilseed rape) fields (Cutler & Scott-Dupree 2007) and the size of the fields is seven times smaller than the average of spring oilseed rape fields in Skåne (Rundlöf et al. 2013). Furthermore the distance between control and treated fields was kept as a minimum of 295m (Cutler & Scott-Dupree 2007). The foraging range of honeybees can be much larger (Winston 1987) and consequently honeybees forage at the other field as well. Most importantly, the influence of clothianidin on the prevalence and infestation rate of pathogens and parasites was not included in this field experiment. Therefore a field study should be designed consisting of sufficient large fields', representative for the south of Sweden, where control and treated sites are separated far enough from each other and with a sufficient number of replicates.

1.6 Aim

The aim of this study is to investigate the impact of clothianidin in flowering oilseed rape (*Brassica napus*), as required systemically through seed dressing, on pathogen and parasite prevalence and infestation or infection rate in free-foraging honeybees under field conditions in Sweden. It will contribute to the ongoing search of reasons for the unexplained high rate of honeybee colony losses noticed in some parts of the world. Field experiments give a better view on real conditions in the nature and this study can contribute to a decision for further legislations in the European Union.

1.7 Hypothesis

Within this thesis, the hypothesis to be tested is whether the occurrence and levels of infection of different pathogens and infestation of parasites in free-foraging honeybees (*Apis mellifera*) are affected by the exposure to the neonicotinoid clothianidin during the oilseed rape (*B. napus*) flowering period, as delivered systemically through seed coat dressing. The null-hypothesis is that there is no effect of clothianidin exposure on the prevalence and loads of parasites and pathogens in free-foraging honeybees.

2. Methods

2.1 General structure of the study

The study consisted of 16 spring oilseed rape (*Brassica napus*) fields located in Skåne (Figure 4), in the south of Sweden, which has a temperate humid climate. The fields were paired according to land use in the surrounding landscape and geographical proximity, using GIS (Rundlöf et al. 2013). Eight of the fields were randomly assigned to be sown with *Brassica napus* (oilseed rape) seeds dressed with Elado, in which clothianidin is the active neonicotinoid, and their corresponding pairs with undressed *B. napus* seeds (controls). Within each pair, the fields were as close to each other as possible, but at least 4 km apart in order to minimize the chance of honeybees foraging in both fields. The average size of the fields was 8.2 ha, ranging from 4 to 12 ha, which is representative for spring oilseed rape fields in Skåne (7.0 ha). Only one field consisted of 27 ha. Sowings were carried out between the 6th of April 2013 and the 18th of May 2013 (Rundlöf et al. 2013).

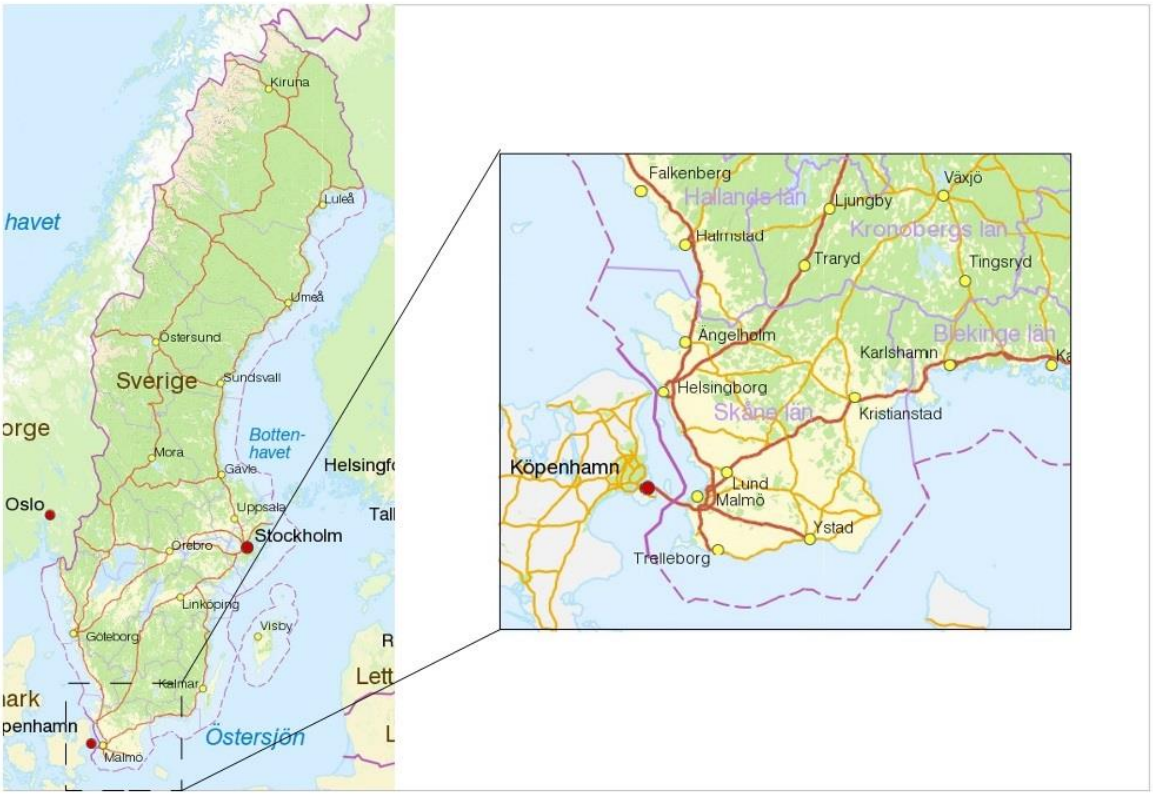

Figure 4. The study area Skåne in the south of Sweden.

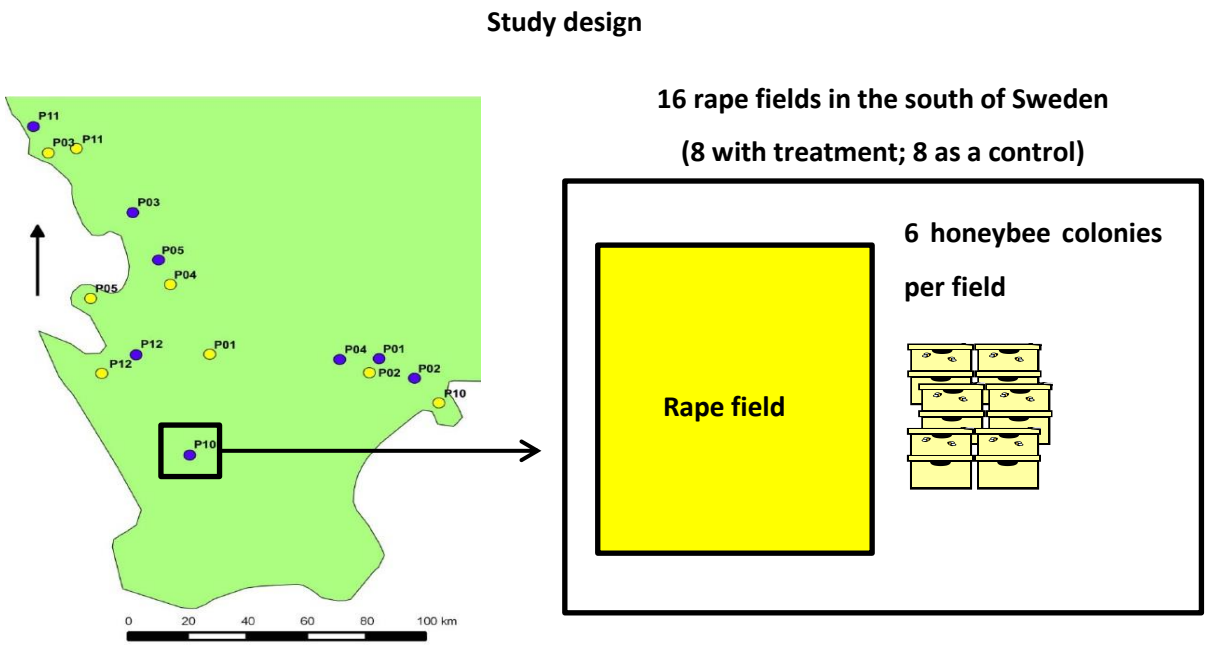

Figure 5. Study design: distribution of the 16 spring oilseed rape fields in the south of Sweden where blue dots mark treatment A and yellow dots treatment B (left) (adapted from Rundlöf et al. 2013) and study design on field level with six honeybee colonies per field (right).

The seed dressing dose was 25 ml Elado per kg seeds (Rundlöf et al. 2013). Elado contains 400g clothianidin and 80 g beta-cyflutrin per litre (Bayer CropScience 2013). In order to have approximately 150 spring rape plants per square meter, 7.5 kg/ha untreated seeds and 7.7 kg/ha Elado-dressed seeds were sown on the fields. The seeds were delivered to the farmers from Lantmännen (Rundlöf et al. 2013).

At each study site six equally sized honeybee colonies were placed, equating to a total of 96 colonies in the experiment (

Figure 5). In order to minimize the influence of genetic differences between colonies, the queen origin of all colonies was known and sister queens of the same age were randomly distributed to the twelve colonies of each pair of fields (Rundlöf et al. 2013).

To confirm that the bees were exposed to clothianidin, pollen pellets and honey sacs, where the gathered nectar is stored, were collected from foraging bees and analysed for neonicotinoid residues, as was the remainder of the honeybee bodies. For each of these three matrices samples were taken on apiary level, resulting in a total of 48 pooled samples (3*16). Clothianidin, acetamiprid, imidacloprid, thiacloprid and thiamethoxam were detected in these samples, although

only clothianidin was detected in all three matrices (honeybee, pollen, and nectar) and in considerably higher concentrations than the other neonicotinoids. Most importantly, very large and highly significant differences were found in the levels of clothianidin in bee samples collected from treated fields and those from untreated fields (Figure 6). The mean concentrations of clothianidin from treated fields were 2.44 ng/g in the honeybees, 10.31 ng/ml in the nectar and 13.94 ng/g in the pollen while the concentrations in the corresponding samples from the untreated fields are negligible (honeybee: 0.13 ng/g; pollen: 0.11 ng/ml; nectar: 0.0 ng/g) (Rundlöf et al. 2013; Figure 6). According to the classification established by the European Food Safety Agency (EFSA) these levels are not lethal doses but theoretically harmful levels of clothianidin (EFSA 2013).

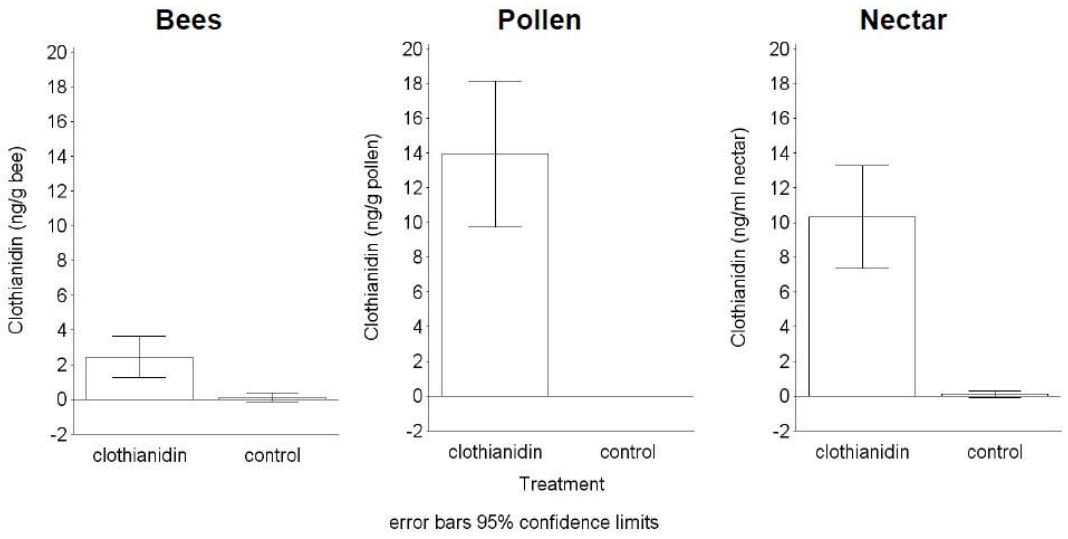

Figure 6: Clothianidin detection in three matrices (honeybees, pollen, and nectar) comparing treated and control sites (Rundlöf et al. 2013).

The proof that the honeybees were exposed to clothianidin under these field conditions allowed an investigation of the effect of clothianidin on honeybee health. Samples of around 100 adult bees were taken from each colony before and after the flower period of the rape fields. The first sample was collected before the colonies were placed at the fields (06-07.06.2013) and the second sample was collected before honey harvest and autumn miticide treatment (25.07.2013 and 05.08.2013). The samples were taken from the outer comb covered by bees. Hence, the samples consist of a mixture of older house bees and flight bees. Samples were stored at -20°C until the laboratory work was performed. Both field and laboratory work were conducted blinded regarding the treatment.

2.2 Laboratory work

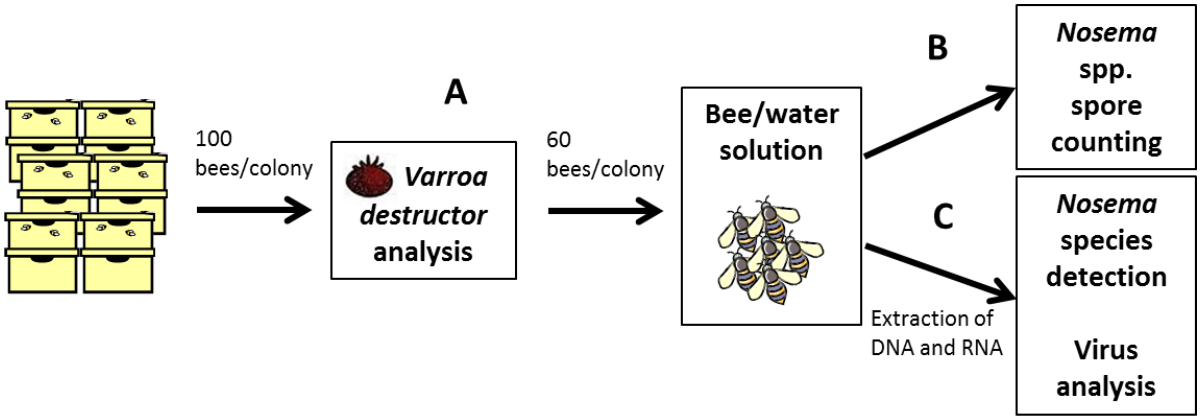

Figure 7. Workflow for the laboratory investigations on individual colony level.

The workflow in the laboratory consisted of three steps, illustrated in Figure 7. First the *Varroa destructor* infestation rate was determined using 100 bees per colony (A). Of these, 60 bees were then used to prepare an extract for counting the spores of *Nosema spp*. under the microscope (B) while simultaneously an aliquot of this extract was retained for DNA and RNA extraction (C), for molecular *Nosema* and virus analyses.

2.2.1 *Varroa destructor*

Samples consisting of 100 worker bees (drones removed) per colony were examined for the number of *Varroa destructor*. Due to small sample sizes, fewer than 100 bees were used in some cases. 10 bees were kept apart for another analyses and examined by eye for the presence of mites (Figure 2). The remaining 90 bees were washed with soapy water to remove the mites from adult bees, as follows: The bees were placed in a bowl of an electric household blender mixed with 1 litre of soapy water and blended at medium speed for approximately one minute. The content of the bowl was poured through a sieve with two parts and rinsed with a large amount of water. The first sieve, with a mesh of 3 to 4 mm, retained all the bees while the second sieve underneath (mesh < 0.5 mm) retained the mites (Figure 8). With the size of 1.5 mm in width and reddish-brown colour adult female mites are easy to distinguish as well as males and female nymph stages which are smaller and cream or white in colour (Dietemann et al. 2013). The number of the mites washed off from the 90 bees was added to the number of mites counted on the sample of 10 bees for the final infestation rate.

Figure 8. Double sieve with different aperture widths including a sample of bees (left, Photo: Julia Goss) and mites collected in the sieve after washing (right, Photo: Julia Goss).

2.2.2 Preparation of a bee/water solution

Out of the 90 bees washed with soupy water, 60 honeybees were used to prepare a bee/water solution to determine *Nosema* spp. by counting the spores and extract DNA. The abdomen of the honeybees were removed and put in a polyethylene bag with an inner mesh bag. After grinding the abdomens with a pestle, 30 ml nuclease-free (Milli-Q) water (0.5 ml per bee) was mixed thoroughly with the samples to create a homogenous suspension. 1 ml of this suspension was removed immediately and frozen for further DNA and RNA extraction.

2.2.3 Determining the *Nosema* spp. prevalence and infestation rate

For counting the *Nosema* spp. spores using a haemocytometer it is recommended that the solution should contain 5 to 50 spores per haemocytometer square (Human et al. 2013). A higher number of spores would increase the errors while counting. Therefore, before the spores were counted accurately, an approximate assessment of the spore concentration was made in order to determine if a further dilution of the extract was necessary. This was done by examining a drop of the ground bee abdomen under a microscope (16 x 40). The infection rate was divided in three groups (see Table 3) and high infected samples were diluted appropriately.

Table 3. Categorical Nosema infection rate (spore count) as determined by Microscopy (adapted from Cantwell 1970)

Infection rate	Spores in one field of view	Spores per bee
Light infection	Between 1 to 4	< 1 200 000
Medium infection	Between 5 to 40	1 200 000 – 12 000 000
High infection	More than 40	> 12 000 000

The *Nosema* spores can be identified by their shape, which is similar to a grain of rice, and by their specific reflection of light (Figure 9). The size of a spore of *N. apis* is 3 x 6 µm (Zander & Böttcher 1984) and of *N. ceranae* is 2.7 x 4.7 µm (Fries et al. 2006). Although there are differences between *N. apis* and *N. ceranae* in the size and the shape of their spores, it can be difficult to distinguish them using light microscopy, especially when analysing mixed infections (Fries 2010). Therefore DNA analyses were carried out to determine the species.

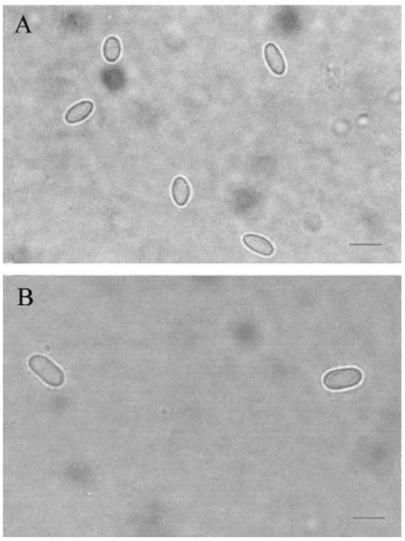

Figure 9. Spores of *N. ceranae* (A) and *N. apis* (B) under a light microscope (from Fries et al. 2006).

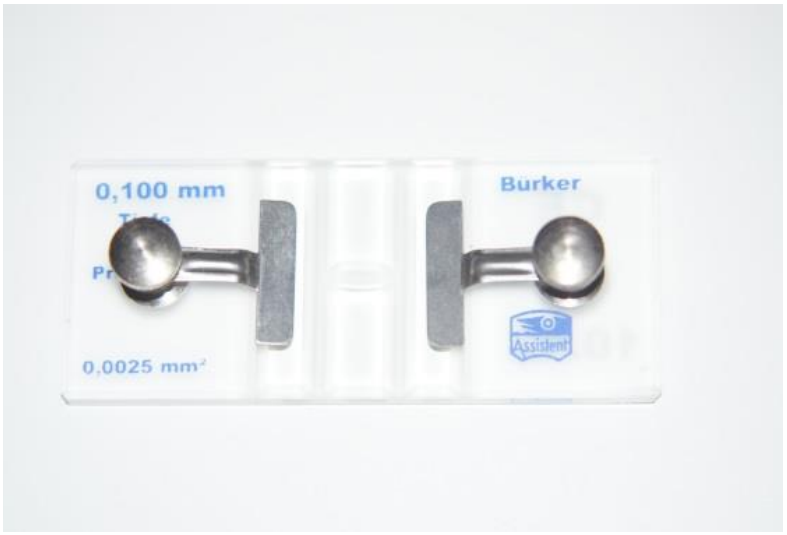

Figure 10. Haemocytometer model Bürker with 0.1 mm depth (Photo: Julia Goss).

As recommended by Human et al. (2013), a haemocytometer (model Bürker with 0.1 mm depth) was used to determine the infection rate of *Nosema apis* and *Nosema ceranae* spores. A haemocytometer consists of a counting chamber with a known volume. Therefore it is possible to count particles under the light microscope and extrapolate this number to the total sample volume and calculate the spores per bee. The recommendations for *Nosema* spore counts are to use 1 ml of water per bee. Since the primary bee abdomen suspensions were made with 0.5 ml water per bee, these were first diluted with an equal volume of water prior to analysis.

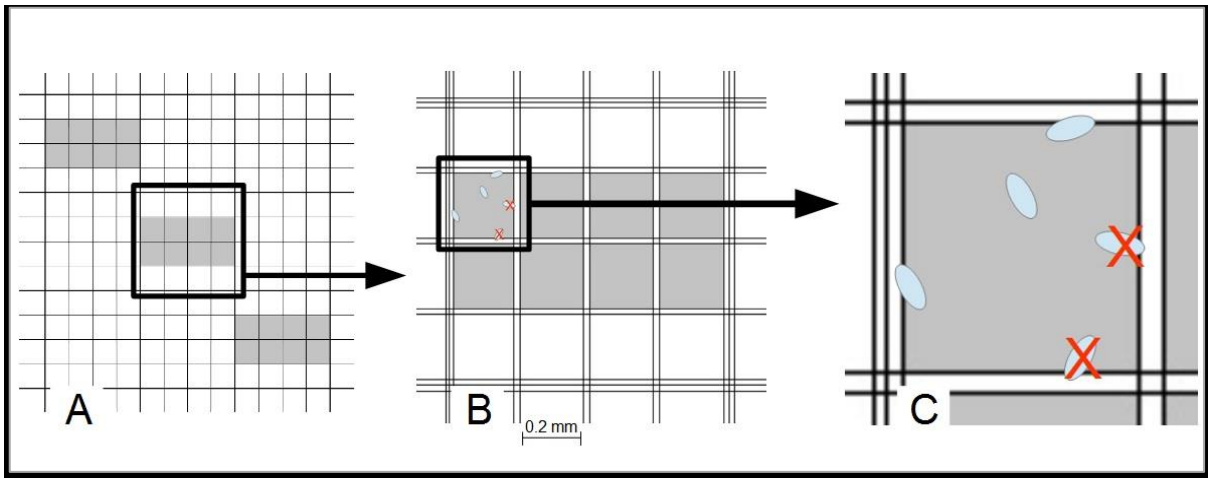

Figure 11. Counting grid of a haemocytometer illustrated in three levels (model Bürker). (A) Out of nine big squares, three (two out of 4 rows are marked in grey) where taken into consideration for the counting. (B) Each of the squares in part A are divided in 16 smaller squares, where eight where used for counting Nosema spores (marked in grey). (C) Spores within the square and spores touching the left and upper boundary lines were counted. The X marks the spores which are excluded.

10 µl of this diluted suspension was applied to the counting grid of the haemocytometer, such that with capillary action, the area under the cover glass was filled with the suspension. Counting of the spores was done two minutes after applying the liquid to the haemocytometer, to allow the spores to settle in the counting chamber before counting (Human et al. 2013). The grid area of a haemocytometer is divided into 9 big squares (Figure 11A), which are delimited by triple lines. Each of these squares is further divided into 16 squares (Figure 11B). Eight small squares where used to count the *Nosema* spores per big square within three out of the nine big squares (Figure 11A). If one of the small squares was blocked and impossible to use, the one above or the one below was used. This means that for each sample a total of 24 small squares were investigated for *Nosema* spores. In order to correct for double counting, spores touching the bottom and right boundary line were excluded (Figure 11C). The spore count was extrapolated to spores per bee using Equation 1. And the resulting infestation rate can be divided into a three grades (Cantwell 1970; Table 3).

Equation 1. Calculation of *Nosema* spores per bee (modified from Human et al. (2013) and Cantwell (1970))

$$spores\ /\ bee = \frac{number\ of\ spores\ counted\ \times\ dilution\ factor}{number\ of\ squares\ counted\ \times volume\ of\ the\ square}$$

$$spores\ /bee = \frac{number\ of\ spores\ counted}{24\ \times 0.004\ mm^3 \times \frac{ml}{1000\ mm^3}} = \frac{number\ of\ spores\ counted\ \times 250\ 000\ \frac{1}{ml}}{24}$$

2.2.4 Nucleic acid detection

Nucleonic sequences such as DNA or RNA signatures can be used to determine the presence and abundance of an organism (Phillippy et al. 2007). Therefore DNA and RNA were extracted and then analysed for target sequences using (RT-)qPCR in order to determine the *Nosema* species and detect viruses, respectively.

2.2.4.1 RNA and DNA extraction

For each colony bee sample, 500 µl of the retained honeybee-water suspension was extracted for *Nosema* spore DNA detection, following the methodology described by Fries et al. (2013), starting with centrifugation for 5 min at 16 060 x g. The supernatant was discarded and the remaining pellet was frozen with liquid nitrogen. The pellet was crushed with a sterile sealed pipette tip in order to destroy the walls of the Nosema spores. This step was repeated until the pellet was pulverized. Subsequently, 400 µl AP-1 buffer and 4 µl RNase A was added to each sample and the mixture was incubated for 10 minutes at 65°C. During that time the samples were mixed by inverting the tube one to two times. After the incubation, 130 µl of AP-2 buffer was added and mixed and then incubated for 5 min on ice. Finally, the lysate was centrifuged for 5 min at 20 000 x g. The DNA was then extracted from 500 µl of the supernatant by a Qiacube automated extraction robot (Qiagen) using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions for plant tissues.

For the RNA extraction, 100 µl of the honeybee-water suspension was mixed with 350 µl RLT buffer (containing 10 µL/mL β-mercaptoethanol) and the RNA was extracted by a Qiacube automated extraction robot (Qiagen) according to the protocol for plant tissues using RNeasy plant mini kit (Qiagen). The RNA concentration was determined by NanoDrop. All RNA samples were diluted for further analyses to a final concentration of 20 ng/µl.

2.2.4.2 Real time quantitative polymerase chain reaction (qPCR)

Polymerase chain reaction is a molecular technique to amplify DNA. The procedure consists of three different iterative elements. In the first step, the DNA strands are denatured by heat so that primers can bind to the single-stranded DNA target sequences. In this step two complementary oligonucleotides are used to prime DNA synthesis under certain temperature conditions. In the next step an extension of the primers is carried out, completing one cycle. This process is then repeated for 30~40 cycles, thus artificially increasing the amount of target DNA. In order to measure the abundance of particular DNA or RNA sequences in an original sample, real-time quantitative PCR (qPCR) was used. This process includes a PCR product detection step after each amplification cycle using fluorescence-detecting thermocyclers (Sambrook & Russell 2001). The initial amount of the product can be calculated very accurately by the quantification cycle (Cq) value, which is the number of amplification cycles required for the amplification products to reach a defined fluorescence detection threshold. Quantification is achieved by running qPCR on a series of 10-fold dilution of an external standard of known concentration, normally a plasmid clone of the target sequence, to establish a calibration curve which is used to estimate the amount of target DNA in the experimental samples. Each qPCR run included one negative H_2O control and three dilution standards (10^{-3}; 10^{-5}; 10^{-7}) for each DNA or RNA target. Using the calibration curve derived from the dilution standard series, the starting quantity (SQ value) was calculated for each experimental essay reaction. All DNA assays were run in duplicate, with the mean value of these two duplicates used in further calculations.

In this study, the real-time PCR detection system from BIO-RAD (CFX Connect™) and the CFX Manager™ Software (Version 2.1) were used.

2.2.4.3 DNA analysis for *N. apis* and *N. ceranae*

The reaction mixtures for *N. apis* and *N. ceranae* detection consisted each of 18 µl PCR master mix and 2 µl DNA each. The PCR master mix contains 6.4 µl of RNAse free water, 0.8 µl of forward primer, 0.8 µl universe reverse primer and 10 µl of EvaGreen™ super mix. The primers and their sequences used for the *Nosema* species detection can be found in the appendix 1. The amplification of the DNA was undertaken with the following thermal conditions: one initial step at 98°C for 2 min to activate enzymes, followed by 40 cycles of (98°C for 5 seconds denaturation; 60°C for 10 seconds to annealing extension) followed by a Melting Curve analysis for product specificity, consisting of a gradual increase in temperature from 65°C to 95°C in steps of 0.5°C for 5 seconds per step with fluorescence absorbance reading after each step; and one hold temperature of 10°C until removal of the samples.

The amount of DNA, represented by the SQ mean, determined in the extraction has to be converted in order to get the amount of target DNA per bee, using equation 2. The extraction for DNA analysis should consist of approximately the same amount of DNA and therefore the estimated copies of Nosema DNA was normalized using the DNA concentration of the sample itself and the average DNA concentration within the analyses. The species composition of *Nosema* was calculated using the DNA from positive samples during the manual counting procedure. Pure infections were set at the threshold level of >99%.

Equation 2. Transformation from SQ mean values to number of DNA copies per bee

$$\frac{\boldsymbol{E-DNA}}{\boldsymbol{bee}} = \frac{SQmean * D.f.}{2\ \mu l\ \frac{\text{DNA}}{reaction}} * \frac{100\ \mu l\ DNA\ eluted}{500\ \mu l \frac{\text{extract}}{\text{colum}}} * \frac{36\ 000\ \mu l\ (\text{bee extract})}{60\ bees}$$

$$\frac{\boldsymbol{N-DNA}}{\boldsymbol{bee}} = \frac{E-DNA}{DNA\ conc./sample} * average\ DNA\ conc./sample$$

Dilution factor = D.f.

Estimated copies of *Nosema* DNA = E-DNA

Normalised copies of *Nosema* DNA = N-DNA

2.2.4.4 RNA analysis for different viruses

To test the impact of clothianidin exposure on the prevalence and titres of honeybee viruses, eight different viruses were analysed (BQCV, SBV, DWV, KBV, SBPV, CBPV, ABPV, and IAPV; for further description see Table 1). These viruses consist of single stranded RNA genomes (de Miranda et al. 2012) and therefore the RNA fraction of the nucleic acids were used to detect and quantify the viruses. Since RNA is easily degradable, an assay for the mRNA of the internal reference gene (RP49) was also included, in order to correct the quantitative data for differences between the samples in the quantity and quality of the extracted RNA (de Miranda et al. 2013).

The reaction mixtures consisted of 8.5 µl PCR master mix and 1.5 µl RNA each. The PCR master mix contains 2.975 µl RNAse free water, 0.2 µl of a 10 µM solution of each primer (one pair of primer per reaction), 5 µl of iTaq universal SYBR Green RT-mix and 0.125 µl of Script reverse transcriptase. A list of the primers and controls used in the analyses can be found in appendix 1. The Amplification of the RNA was undertaken with the following thermal conditions: one initial incubation at 50°C for 10 min to synthesis cDNA; one incubation at 95°C for 5 min to inactivate the reverse transcriptase; 40 cycles for PCR cycling and detection at 95°C for 10 seconds followed by 58°C for 30 seconds followed by a data collection; The PCR reaction was followed immediately by a Melting Curve analysis, consisting of 60 fluorescence measurements at gradually increasing temperatures, from 65°C to 95°C in increasing steps of 0.5°C, with 5 seconds per step for fluorescence reading. As with the qPCR analysis of the DNA samples, a series of 10-fold dilutions of external standards of known concentration was included for each RNA target, to establish calibration curves for the quantification of the amount of target in each reaction. These resulted in the SQ values for the different RNA targets in each sample.

The amount of virus per bee was calculated starting with the SQ value and the various dilution factors of the experimental steps. The efficiency of producing cDNA under these conditions is approximately 0.1 (10%).

Equation 3. Transformation from SQ mean value to number of RNA copies per bee

$$\frac{\boldsymbol{E-RNA}}{\boldsymbol{bee}} = \frac{SQ * Dilution\ factor}{1.5\ \mu l\ \frac{\text{RNA}}{reaction} * 0.1\ cDNA\ efficiancy} * \frac{50\ \mu l\ RNA\ eluted}{100\ \mu l \frac{\text{extract}}{\text{colum}}} * \frac{36\ 000\ \mu l}{60\ bees}$$

$$\frac{\boldsymbol{N-RNA}}{\boldsymbol{bee}} = \frac{E-RNA/bee}{RP\ 49\ RNA/bee} * average\ RP\ 49\ RNA/bee$$

Estimated copies of target RNA = E-RNA

Normalised copies of virus RNA = N-RNA

2.3 Statistical Analysis

Before statistical analyses were performed, the distribution of the data was explored, in order to detect outliers, the presence of zeroes and to avoid common statistical problems (Zuur et al. 2010).

The raw data was transformed before analyses were performed according to different research questions and biological patterns of the pathogens and parasites. To test the impact of clothianidin exposure on the prevalence of pathogens and parasites in honeybee colonies, the binominal data (presence-absence) was evaluated. Those viruses which were nearly present in all colonies were not analysed for their occurrence patterns. In a second step the raw data was transformed to test the impact of clothianidin on the infection or infestation rate of honeybee pathogens and parasites. All virus titres and the level of *Nosema* infestation were logarithmic transformed (Log_{10}) using the formula Log_{10} (value + 1) in Microsoft Excel 2010, as the data was not normally distributed.

The study was designed as a Before-After-Control-Impact (BACI) experiment, which is used to evaluate whether or not a stress factor disturbed or changed the environment (Smith 2002). In our case, the impact of clothianidin on the health of honeybees was tested. To determine possible effects over time, samples of honeybees were taken before and after the clothianidin exposure of honeybees (before-after). However, trends seen over time do not have to be causally linked to human activity and therefore control sites were included (impact-control) (Smith 2002). Furthermore the difference between sites might be due to several factors and not necessarily from the environmental impact. In this design (Equation 5), particular interest lies in the differential change of period*treatment, which, if significant, implies an impact of clothianidin over time. The differential change is expressed by the interaction of spatial and temporal values (Equation 4).

Equation 4. Interaction effects in a BACI model

$$BACI = (\mu(CA) - \mu(CB)) - (\mu(IA) - \mu(IB))$$

To compare both the prevalence and the quantities of pathogens and parasites between the treatments, a generalized linear model (GLM) was used. This model allows the combination of fixed factors and random effects, which control for correlations in the data arising from grouped observations. In the GLM three independent variables were set as fixed factors: (1) period; (2) treatment; (3) period*treatment (Equation 5). As explained before particular interest lies in the last factor. A first-order temporal autocorrelation within sites was included in the model to exclude any

possible effect of season or groups (treatment). The dependent variables (Y) in this analysis were the infestation rate and the prevalence of pathogens and parasites. The repeated measurement of the colonies, nested within a field and a pair, were fitted as random effects to control for the non-independence of observations within sites and time.

Equation 5. Generalized linear model used in SAS

$$y = period\ treatment\ period * treatment$$

Statistical significance of the three fixed factors, period, treatment and the period*treatment interaction, were tested using F-statistics. We used p = 0.05 as a threshold value for significance.

For the virus data subset, the values for 2 samples (both before and after the treatment period) were excluded, as the assay for the internal reference gene RP49 had failed for before-treatment samples, such that the data could not be normalized as described in the previous section. The excluded samples came from the same field-pair, with one belonging to the clothianidin exposed field and the other to the corresponding control fields.

All statistical data analyses were performed in SAS 9.3 for Windows with previous conversion steps in Microsoft Excel 2010. Graphs were created using Microsoft Excel 2010. The code used in SAS can be found in the appendix 2 and the output of these tests under the appendices 3.

3. Results

3.1 Varroa destructor

The prevalence in percent and the mean infestation rate of *Varroa destructor* increased significantly over time (see Figure 12). Overall, the treatment and the control groups did not differ from each other, regarding the prevalence and the infestation rate of *V. destructor* and therefore site effects can be excluded. Changes in the prevalence and the infestation rate did not seem to be related to the exposure to clothianidin, as the interaction of treatment and period did not significantly differ (Figure 12; Table 4).

Table 4. F values and levels of significance testing Varroa destructor prevalence and infestation rate

	***Varroa destructor* prevalence**			***Varroa destructor* infestation rate**		
	df	**F**	**P**	**df**	**F**	**P**
Period	94	11.22	0.0012	94	9.10	0.0033
Treatment	94	0.14	0.71	94	0.34	0.56
Period*treatment	94	1.38	0.24	94	1.57	0.22

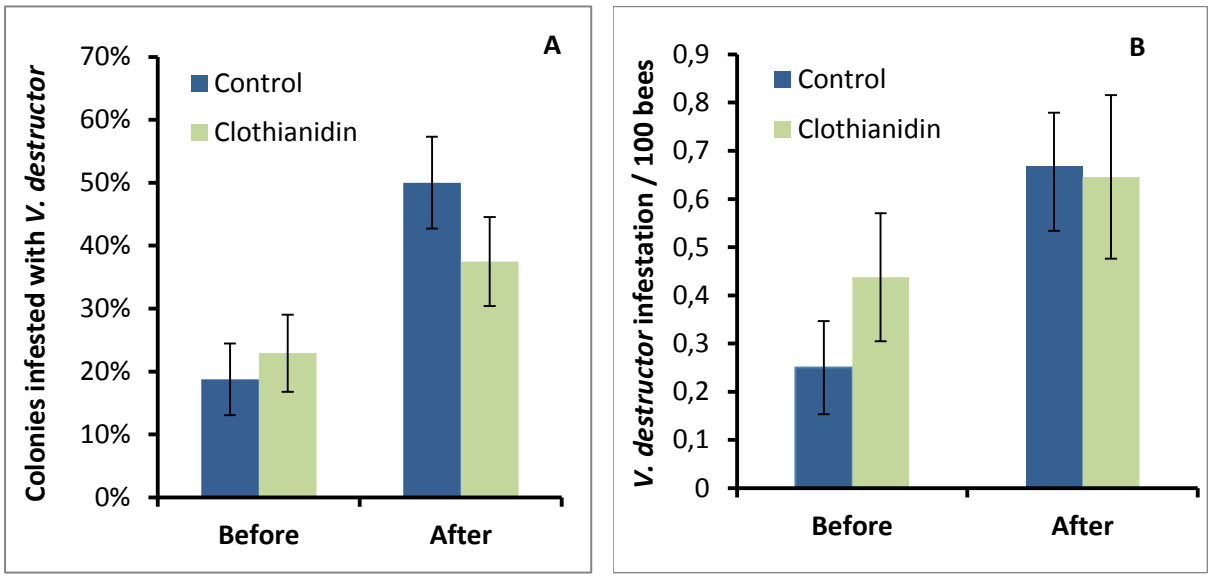

Figure 12. Colonies infested with V. destructor in percentage ± standard error (A) and mean *Varroa destructor* infestation per 100 bees ± standard error (B).

3.2 *Nosema* spp.

The percentage of colonies infested with *Nosema spp*. decreased significantly over time (Figure 13 A; Table 5). However, differences in the prevalence of *Nosema spp*. did not seem to be influenced by the treatment groups nor the interaction of treatment and time, which clarifies that the exposure to clothianidin had no effect on the prevalence of *Nosema spp.*in honeybee colonies (Figure 13A; Table 5).

The infestation rate of *Nosema* spp. showed no significance change over time or between the treatment and control group (Figure 13B; Table 5). The exposure of the honeybee colonies to clothianidin had no effect on the infestation rate in *Nosema* positive samples, as the interaction of period and treatment was not significant (Figure 13B; Table 5).

Table 5. F values and levels of significance testing *Nosema spp*. prevalence

	***Nosema spp.* prevalence**			***Nosema spp.* infestation rate**		
	df	**F**	**P**	**df**	**F**	**P**
Period	94	46.27	< 0.0001	6	3.01	0.13
Treatment	94	0.14	0.71	6	4.57	0.07
Period*treatment	94	0.81	0.37	6	3.21	0.12

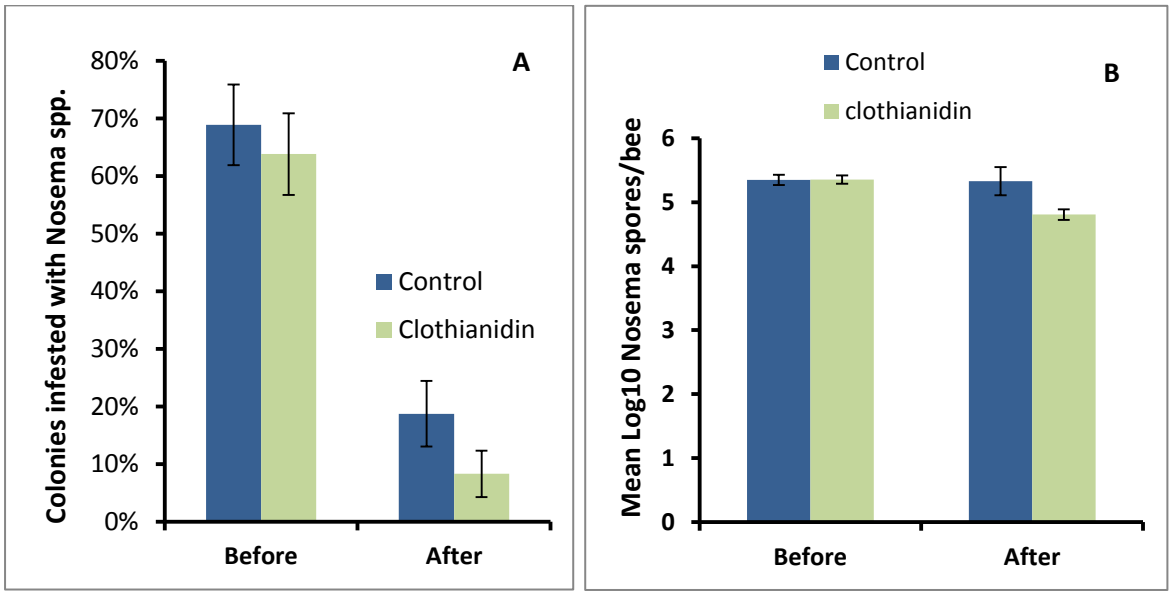

Figure 13. Honeybee colonies infested with *Nosema* spp. in percentage ± standard error (A) and mean (LOG10) Nosema spp. Infestation in positive samples (B) before and after the experiment comparing control hives with treated hives ± standard error.

Samples positive for *Nosema spp*. infection were examined for the proportion of *N. apis* and *N. ceranae*. Prior to the experiment, *Nosema* spores could be detected in 67 % of the samples (Figure 14) of which 6 % were only infested with *N. apis*, 25 % only with *N. ceranae* and 36 % of the samples had a mixed infestation with both *Nosema* species. On average, about 1/3 of the DNA in mixed infestation belonged to *N. apis* and 2/3 to *N. ceranae*.

After the experiment spores could be detected only in 13 % of the colonies (Figure 15) of which 5 % were a pure *N. ceranae* infection and for the remaining 8 % no *Nosema* DNA was detected at all. No *N. apis* was found in any of the colonies at the end of the experiment.

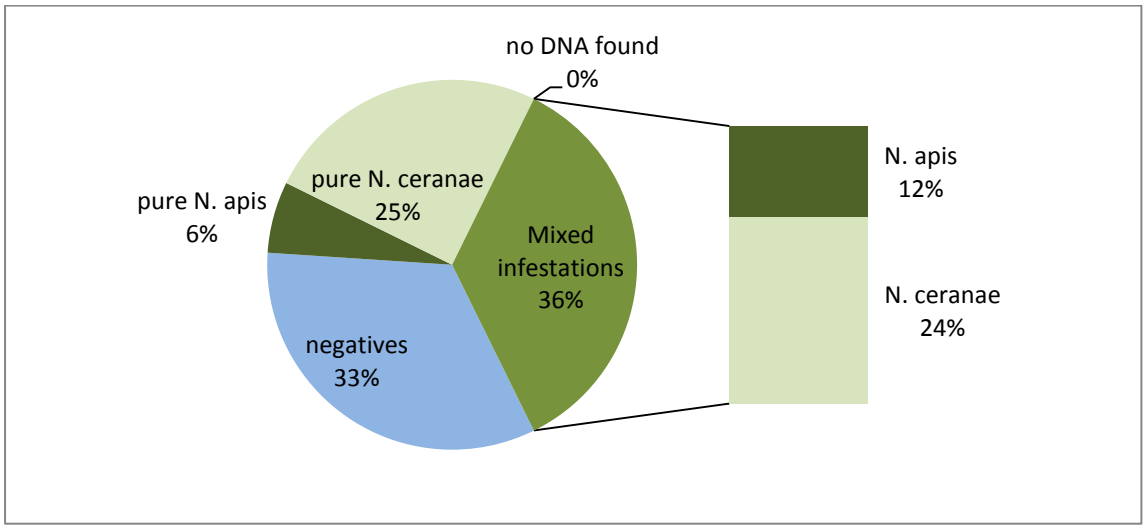

Figure 14. Nosema species proportion (percentage) in positive samples before the experiment. Control and treated sites are combined.

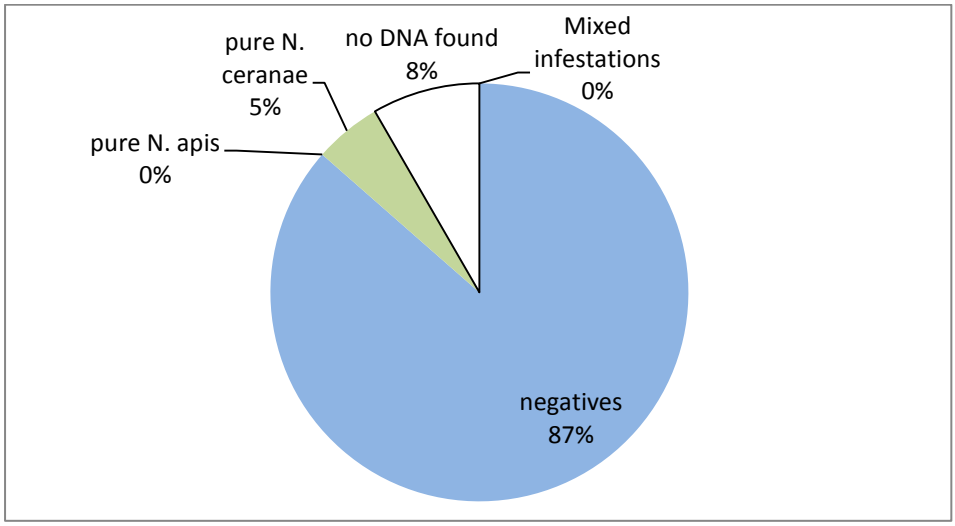

Figure 15. Nosema species proportion (percentage) in positive samples after the experiment. Control and treated sites are combined.

3.3 Comparison of two methods to quantify *Nosema spp.*

The prevalence and the infestation rate of *Nosema* spp. was detected by counting spores under the microscope. However, the amount of Nosema in a sample can also be calculated by qPCR from the DNA samples through the qPCR protocols for distinguishing the two *Nosema* species. The two methods are compared in Figure 16 using the data from all 192 samples. 64 samples were negative by both methods (16A) and 69 samples were positive by both methods. This means that in 69 % of all analyses, the results were consistent between the two methods. However, for 31 % of the samples only one of the methods produced a positive result. For 8 samples (4.1 %) *Nosema* spores were counted even though no *Nosema* DNA was found (16B) and for 51 samples (26.5 %) *Nosema* DNA could be amplified even though no *Nosema* spores were seen under the microscope (16C).

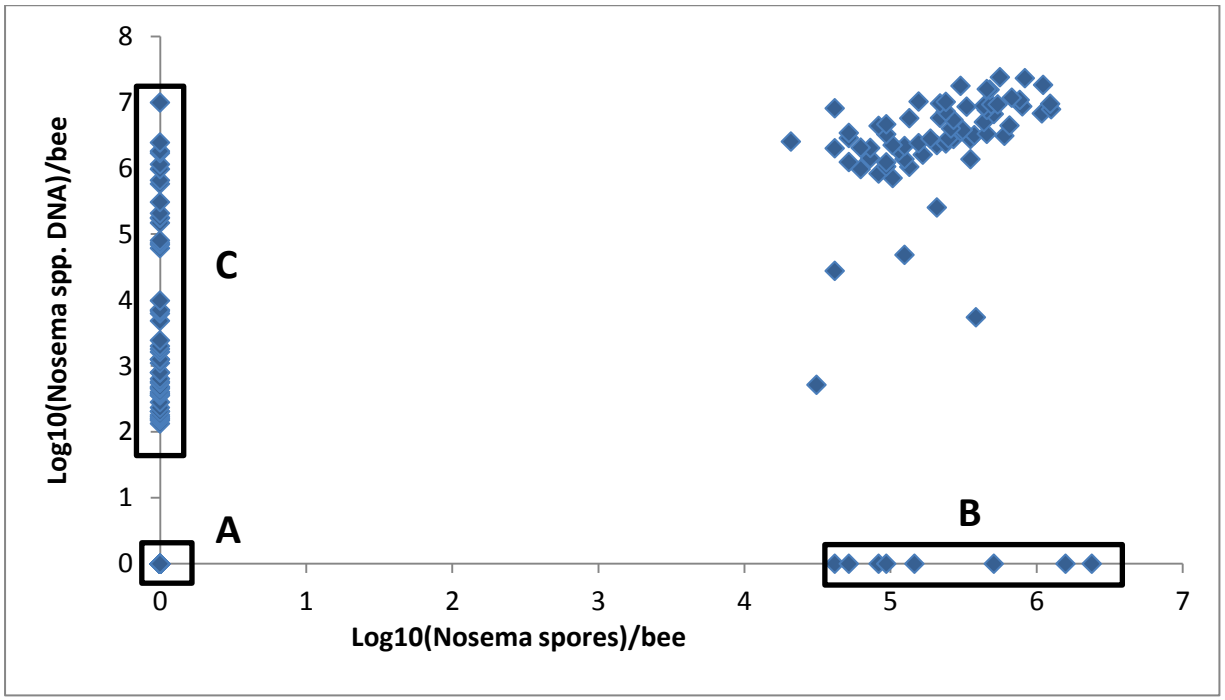

Figure 16. Comparison of two *Nosema spp.* detection methods

3.4 Viral diseases

Out of the eight viruses tested, only three (DWV, SBV and BQCV) were detected in this experiment. ABPV, CBPV, IAPV, KBV and SBPV were not present in the samples from individual colonies. The most prevalent virus was BQCV, followed closely by the SBV. All colonies (100%) were infested with the BQCV for both sampling dates. SBV was detected in 100% of all samples for the first sampling period, prior to the experiment and in 96 % of the samples for the second sampling occasion, after the experiment. The proportion of colonies infected with DWV increased from 4% to 36% between the first and the second sampling occasion.

3.4.1 Black queen cell virus

For BQCV there was a significant reduction in titre due to the season. No difference was seen between the treatment and control group and therefore site effect can be excluded. Furthermore the exposure to clothianidin had no effect on BQCV titres, since the interaction between time and treatment was not significant (Figure 17A; Table 6).

1.1.1 Sacbrood virus

The titres of SBV did not differ in time or between the treatment and control group. Furthermore the exposure to clothianidin had no influence on the SBV infection rate since the interaction between time and treatment was not significant (Figure 17B; Table 6).

Table 6. F values and levels of significance testing the infection rate of BQCV and SBV

	BQCV infection rate			SBV infection rate		
	df	**F**	**P**	**df**	**F**	**P**
Period	90	154.19	0.0001	92	0.39	0.53
Treatment	90	0.58	0.45	92	1.65	0.20
Period*treatment	90	0.00	0.99	92	0.10	0.76

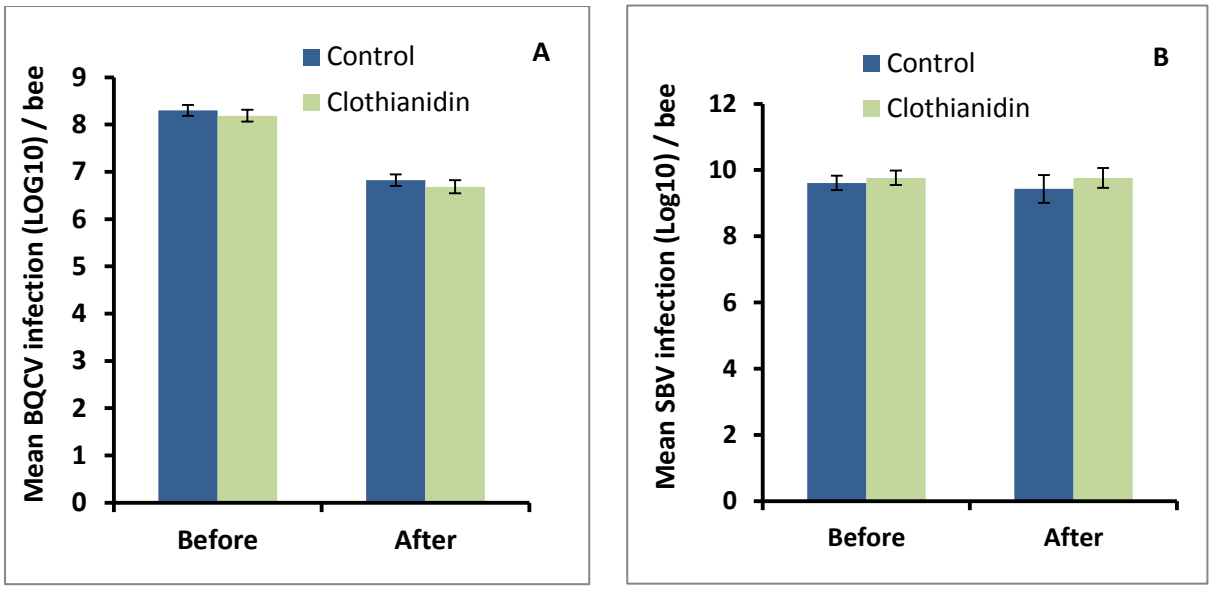

Figure 17. Mean BQCV infection (LOG10) per bee ± standard error (A) and mean SBV infection (LOG10) per bee ± standard error (B) before and after the experiment comparing control and treated colonies.

1.1.2 Deformed wing virus

The percentage of colonies infected with DWV was significantly higher for the second sampling date than for the first. Although a higher percentage of colonies were detected in the control group compared to the clothianidin exposed group, this difference between the treatment groups was not significant. Therefore a site effect is not significant. The exposure of the honeybee colonies to clothianidin seem not to influence the number of hives infected with DWV, as the interaction of treatment and period was not significant (Figure 18A; Table 7).

The titres of DWV in positives samples showed no difference between the time and the treatment groups. The exposure to clothianidin had no effect on the amount of DWV found in DWV positive samples, as the interaction of period and treatment was not significant (Figure 18B; Table 17).

Table 7. F values and levels of significance testing the prevalence of DWV

	DWV prevalence			DWV infection rate		
	df	F	P	df	F	P
Period	92	19.79	< 0.0001	2	0.00	0.96
Treatment	92	3.08	0.08	2	2.68	0.24
Period*treatment	92	0.29	0.59	2	0.40	0.59

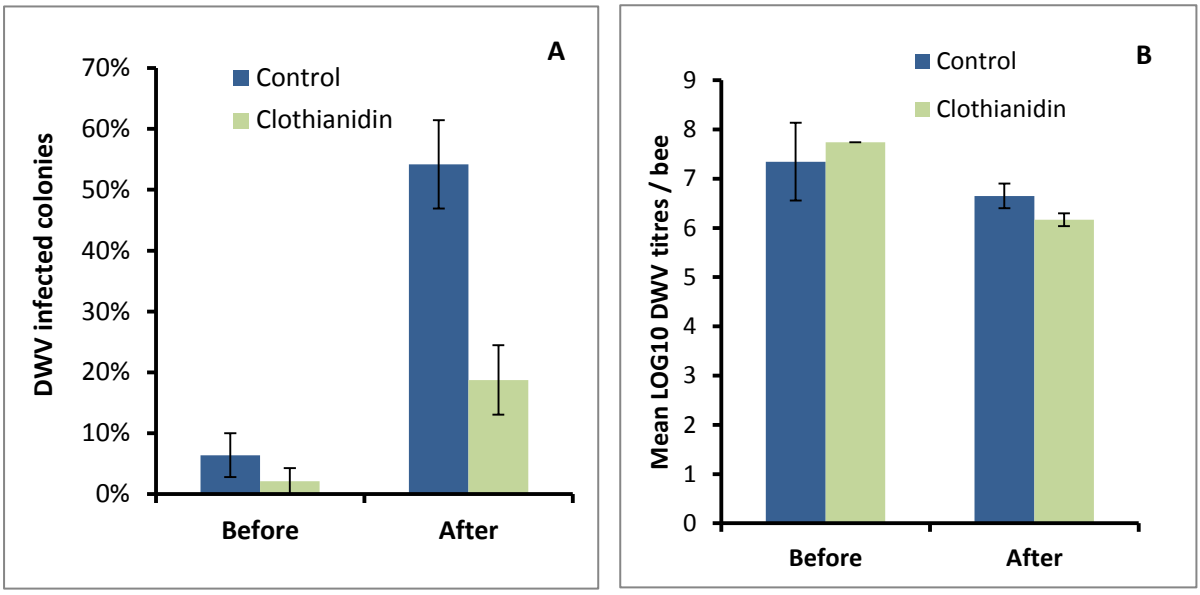

Figure 18. Colonies infected with DWV in percentage ± standard error (A) and mean (LOG10) DWV infestation rate in positive samples (B) ± standard error.

2. Discussion

2.1 Methods used

2.1.1 Field experimental setup

The overall experimental design and sampling-analysis strategy conceived, including several blinded and double-blinded procedures to minimize potential biases and conflicts of interest. Differences between groups of colonies were minimized a priori through colony equalization and control of the queen origin, and this was reflected in the very low variation between colonies at the start of the experiment in almost all parameters, not just the ones recorded here. In this study spring oilseed rape was used, which was sown in April-May. An alternative cultivation system is winter oilseed rape, which is sown the year before, between August and September, and flowers the following spring, in May-June. Although seed dressed with neonicotinoids protect crops for several months (Goulson 2013), spring-sown oilseed rape represents a different neonicotinoid exposure risk for honeybees than winter-sown soil seed rape. The usage of spring-sown or winter-sown oilseed rape could explain the lower insecticides concentrations found in other studies (Blacquière et al. 2012). Even so, the clothianidin concentrations detected in bees, pollen and nectar in this study, were all below the acute and chronic toxicity levels as determined by EFSA (Blacquière et al. 2012).

2.1.2 Laboratory work

Mites can be separated from bees in several ways and these are discussed and described in Dietemann et al. (2013). Using warm soupy water is considered to be environmental friendly and is relatively cheap. It is recommended to use 300 bees to estimate the infestation rate of the colony. Nevertheless, in this study only 100 bees were washed which could lead to errors (Dietemann et al. 2013). For further studies it could be considered to use larger samples from the colonies.

Samples were taken from the outer comb covered by bees for this study. However, it is recommended that older bees should be the target within a hive to detect Nosema ssp. infections. Therefore foraging bees from outside the hive should be collected (Fries et al. 2013).

Although the principal method to detect viruses in the apiary is the detection of symptoms, this method was not chosen. Though this has many advantages, limitation such as symptoms are not always present in all life stages or at all times and symptoms can very similar for many viruses it makes it hard to qualify and quantify them. Therefore a molecular method was chosen using RT-qPCR (de Miranda et al. 2013). The loads of virus can differ and is influenced by the task of the bee and therefore by the age (van der Steen 2012). But by sampling always in the same way, differences between colonies should be minimized in this experimental setup.

2.2 Results

Both pesticides and pollination by honeybees are essential if modern agriculture is to keep pace with the growing demand for food (Cutler & Scott-Dupree 2007). Although exposure of non-target organisms, in this case pollinators, to pesticides cannot be eliminated, the effects of exposure can be minimized by judicious use of the pesticides and integration of pollination and insect-control needs. Systemically delivered neonicotinoid insecticides are recommended as an environmentally friendlier alternative (Cutler & Scott-Dupree 2007). Even so, the honeybees in this field experiment were exposed significant sub-lethal doses of clothianidin as a result of their foraging activities in spring-sown oilseed rape fields sown with clothianidin-dressed seeds. Overall, no significant effect of this exposure was seen in the loads and prevalence of pathogens and parasites. Changes can be only explained by seasonal effects. These results will be discussed in detail below.

2.2.1 *Varroa destructor*

In this study an increasing infestation rate was found, as the season went along, which could be due to biological factors. After a colony becomes infested with *Varroa destrucor,* the mite population can increase rapidly within a few years. The population growth rate is dependent on many factors, such as mite mortality and reproduction rate, different features of the host, the honeybee, such as the amount of drone brood or the level of defence behaviour. In some studies a significant relation between the amount of brood and the population growth of the mites were found (Rosenkranz et al. 2010). Therefore, the infestation rate of V. destructor could increase during summer, as we saw it in this study. Furthermore, the increasing prevalence could be explained by an introduction of mites due to robbing behaviour of foraging honeybees within an apiary (Rosenkranz et al. 2010).

Dietemann et al. (2013) summarized different thresholds to prevent damages of V. destructor such as honey loss or winter mortality. The average levels of mites per 100 bees in this study were under all damage thresholds mentioned for the month April till September according to studies from Canada and the United States. Compared to the damage threshold for April from a Canadian study, 8 colonies were over the set limit of 2 mites per 100 bees. The lowest threshold level for August was 4 mites per 100 bees, which 2 colonies in this study reached (Dietemann et al. 2013).

In a study from 2011, a delayed development of honeybee larvae reared, was found in combes contaminated with a mixture of pesticides, including 5 (Wu et al. 2011). The extended period of larvae development could favour the condition for *V. destructor* mite development (van der Sluijs et al. 2013). A contamination with neonicotinoids could therefore increase the level of *V. destructor*.

However, in our study the exposure to clothianidin had no significant influence on the prevalence and infestation rates of *V. destructor* compared to control hives. Even though clothianidin was detected in pollen and honey, it does not imply that the combs were also contaminated as they were in the study by Wu et al. (2011). The exposure to clothianidin could have a long term effect and therefore the level of *V. destructor* infestation should be studied in the next years. However, the interaction of a high mite infestation and the exposure of honeybees to neonicotinoids would be interesting to analyse further.

2.2.2 *Nosema apis and Nosema ceranae*

The infection pattern of *N. apis* in temperate climates is characterized by low levels during the summer, when the infection is hardly detectable, followed by a small peak in the fall. While there is a slight increase during winter, the main infestation peak occurs in spring, as the winter bees are being replaced (Bailey 1955). This seasonal pattern could explain why no *N. apis* was detectable after the oilseed rape flowering period in summer at both clothianidin-treated and untreated fields. While the *N. apis* infestation rate differs depending on the season, the *N. ceranae* infestation does not follow such a strict seasonal patterns, with similar infestation rates throughout the year (Martín-Hernández et al. 2007). Although a reduction in the number of infested colonies was seen between the first and the second sampling, *N. ceranae* was still present in 14 % of all colonies at the end of July, whereas no *N. apis* was detected at all. However, since the *N. ceranae* infestation also decreased as the season went along. This fact suggests that there not only N. apis but also N. ceranae has seasonal changes. However, further studies are needed to confirm this.

It has been reported that in some areas such as Uruguay, the Balkan countries and Spain, *N. apis* has gradually been replaced with *N. ceranae* (Invernizzi et al. 2009; Stevanovic et al. 2011; Higes et al. 2009). However, in a nationwide study in Sweden between the years 2007 and 2011 it was found that *N. apis* was still the dominant Microsporidian infection in positive honeybees samples. In 2007 only one out of 319 *Nosema*-positive samples consisted of a pure *N. ceranae* infection and no trend was detected that *N. ceranae* infestation rate is increasing (Forsgren & Fries 2013). In our study, in the south of Sweden in 2013, the opposite was found with *N. ceranae* as the dominant species, especially towards the end of the season. Further studies are needed to verify if this is part of an ongoing trend of a replacement of *N. apis* with *N. ceranae* in the south of Sweden, and if this is representative for the rest of Sweden since the climate varies from temperate humid in the south to subpolar climate in the north. Since only N. ceranae was found after the experiment, there was little point in testing the effect of clothianidin on the *Nosema* species composition.

Overall, for both *Nosema* species combined, *Nosema* infection prevalence and rate in honeybees decreased during summer. However, in this experiment the exposure to clothianidin from seed dressing of oilseed rape seems not to affect the *Nosema spp.* prevalence or infestation rate. Although the prevalence is slightly higher in colonies sited at the control fields, this might be explained by a previous higher percentage of infected colonies.

In a laboratory experiment, N. ceranae and thiacloprid increased the mortality of individual of adult honeybees (Doublet et al. 2014). It should be tested, if the infestation of the colonies with N. ceranae and the exposure to clothianidin had an effect on the colony strength.

2.2.3 Different methods used for Nosema

Even though 69 % of all samples got the same results for both methods, the microscope spore count and the qPCR DNA analysis, there are still the remaining 31 % where either one or the other method failed to detect Nosema. The less frequent discrepancy between the methods was where spores of Nosema spp. were counted but no DNA was found by the qPCR could have two possible explanations: Either spores of yeast were mistakenly counted or there was some problem with the DNA samples that prevented detection. The more frequent difference between the two methods was when no spores were seen, but Nosema DNA could be detected by qPCR. One explanation could be that the level of infestation was too low for spores to be detected.

However, in an experimental design as it was used here, in which controls are included and one method is used throughout, it should not affect the comparison as the same errors occur in both groups.

2.2.4 Viruses

Black queen cell virus (BQCV) is thought to be associated with *Nosema spp*. infestations. The infestation with Nosema, which infects the midgut tissues of adult bees, makes them more vulnerable for the BQCV. This virus follows a seasonal pattern with a strong peak in the spring (de Miranda et al. 2012), similar to pattern of *N. apis* in temperate climates (Bailey 1955). In our study higher BQCV titres were detected during the first sampling than at the end of July. An interaction has been found between BQCV and the pesticide thiacloprid on the larval mortality which was noticeable at a level of $1.4 * 10^9$ BQCV. However, in adult bees the same level of infestation had no effect on the honeybee mortality which might reflect the different tolerance levels of the two stages

(Doublet et al. 2014). In further studies it could be interesting to determine the infection rate of larvae as well as adult bees and synergistic effects between clothianidin and BQCV during this experiment on the mortality of adult bees and larvae remain to be studied.

While Sacbrood Virus can cause colony losses of *Apis ceranae*, it is minor and unimportant disease for Apis mellifera (de Miranda et al. 2012). However, SBV is seen as the most common honeybee virus infection in some areas (Riebiere et al. 2007) and in these experiments it was present in nearly all colonies before and after the experiment. Therefore, no comparison of the effect of clothianidin exposure on SBV prevalence could be made. In this survey high SBV titres, up to 10^{12} and 10^{13} genome copies per adult honeybee, were detected. Even though clothianidin exposure did not seem to affect the prevalence and the SBV titres, there could be an interaction between them. There was also no seasonal effect seen, although SBV outbreaks most commonly occurrence in spring or early summer (Riebiere et al. 2007). The brood development should be further analysed in relation to the SBV infection.

In a study in France, 97 % of all examined apiaries were infected with the Deformed Wing Virus (DWV) (Tentcheva et al. 2004). Although an individual colony investigation was made in this experiment, the proportion of infected colonies is definitely lower than in the French survey. DWV has a worldwide distribution and is closely linked to the infestation by the mite *Varroa destructor.* While DWV can be detected in nearly 100% of adult bees in the colonies with a high *V. destructor* infestation rate, treatments against the mite can lower that (de Miranda et al. 2012). This relation and the low infestation rate of colonies with *V. destructor* might explain the rather low prevalence of DWV infection of colonies in this study. The prevalence of the DWV was significantly higher for the second sampling time than for the first sampling time, similar to the V. destructor prevalence and infestation rate. The percentage of DWV infected colonies is nearly three times higher for the control colonies than for the colonies exposed to clothianidin, both before and after the experiment, but with no significant impact of clothianidin exposure on DWV prevalence, despite the large differences seen at the end of the experiment. It might be that already infected colonies transmitted the virus to neighbouring colonies within an apiary, or that drifting bees from infected colonies were included in the adult samples, since there was a noticeable clustering of DWV positive colonies in just a few apiaries, especially for the clothianidin exposed colonies. Thus the difference could be explained.

2.3 Social immunity versus individual immunity

Previous studies found an association, under laboratory conditions, between neonicotinoid exposure and a decreased immune defence which was linked to an increase in DWV titres (Di Prisco et al. 2013). However, no difference in the prevalence or in the amount of parasites and pathogens were found in this experiment, conducted at field-scale. These apparently contrasting results can be reconciled, since the effects seen in the laboratory experiments were measured within hours of exposure, whereas the field experiment exposure was measured in months before samples were taken for analysis. Another colony-level study showed that fluvalinate, a commonly used synthetic acaricide, has a temporal effect on bee immunity, increasing DWV titres slightly, but then the colony adapts and DWV titres decrease again (Locke et al. 2012). Therefore it could be concluded, that clothianidin may affect the immune system of individual honeybees soon after exposure, but that at colony level over long periods of time such effects can no longer be detected. However, immune response expression responsible for the immunity of individual bees were not been tested yet and this conclusion cannot be drawn. It might be possible, that the social immunity, which plays a major role in the defence mechanism of a honeybee colony (Cremer et al. 2007) can compensate for alterations.

2.4 Sustainable use of pesticides

It has been estimated that by 2050 the word population will be around 9 billion people. The demand for food will increase as well as for crops for bioenergy or other purposes (FAO 2009). To secure the food requirements for a growing human world population, it is essential to have pollination and pesticides co-exist cooperatively in modern agriculture (Cutler & Scott-Dupree 2007). Non-target organisms, such as for instance pollinators, are exposed through many different pathways to insecticides and might be affected by them (Brittain & Potts 2011). While the usage of pesticides cannot be avoided, the risks and benefits of pesticide usage can be evaluated. Some classes of pesticide might be more harmful to the environment than others. The effects of pesticides on the environment might not be eliminated, but they can be minimized. For instance, foliar applications of pesticides during the flowering season expose bees to acute levels of high concentrations of pesticides, systemic foliar applied pesticides can also be trans located to pollen and nectar (Alix & Miles 2012) while seed or soil treatment with systemic insecticides is considered to be more ecological friendly (Cutler & Scott-Dupree 2007). Neonicotinoids offer many benefits for pest control because it is highly toxic for insects but not for vertebrates (Goulson 2013).

As described previously the demand for food has to be satisfied. Instead of increasing the yield of agriculture, the actually existing food should be used in a more efficient way. Within Europe 90

million tons of food is wasted per year. Globally, one third of all food for human consumption is not eaten (European Commission 2014). A more sustainable farming would provide agricultural products as well as ecosystem services which are both essential to human life (Tilman et al. 2002).

As mentioned before neonicotinoids are seen as an environmental friendlier alternative to other pesticides. This fact can be questioned due to its systemic and persistent properties, which are advantages for the plant protection, but can be also seen as drawbacks (Alix & Miles 2012; European Commission 2006). Neonicotinoids are water soluble and can therefore leach from the soils and enter the water cycle (Goulson 2013). Clothianidin is not mineralized in anaerobic conditions and very slowly mineralized in aerobic conditions (European Commission 2006). Estimates for the half-life of neonicotinoids vary between studies. For instance, the half-life of clothianidin has been reported as being anywhere between 148 and 6931 days, and reasons for such large differences in estimates are not yet fully understood (Goulson 2013). However, there is a risk of accumulation of active neonicotinoids in the soil, as well as relocation because of their solubility properties. Effects of such accumulation and relocation are not yet known. One notorious example of unexpected and delayed impacts of a pesticide is DDT and other mid-20th pesticides. The accumulation of these in the food chain lead to reduced reproduction success of many birds, particular those at the top of the food chain (for instance bald eagles; Grier 1982). With repeated application and a possible accumulated concentration in the soil, more arable land is expected to contain detectable levels of neonicotinoids in the future. Additionally, neonicotinoids have been found in samples from streams and groundwater (Goulson 2013). Using the pre-cautionary principle we should be careful in dealing with substances that are long-lasting and might accumulate in the nature.

In this study it is shown that honeybees are exposed to sub-lethal doses of clothianidin while foraging under field realistic conditions. While such exposure might have little influence on honeybee colonies, which can be considered relatively robust super organisms with multiple mechanisms for dealing with environmental stresses, it might have different effects on solitary bee species or bumblebees. Not all pollinators are equally sensitive to environmental disturbances, such as pesticides, and therefore pesticide exposure might alter the species composition (Brittain & Potts 2011). It has been shown that imidacloprid reduced the colony growth and the queen reproduction of *Bombus terrestris* (Whitehorn et al. 2012). For stability of the ecosystem it is vital to maintain adequate pollination. It is important to preserve the relationships and the interaction between plants and pollinators, which might be affected by pesticides (Brittain & Potts 2011). Further studies should be carried out to determine the effect of clothianidin for wild pollinators under field conditions.

In the European Union it is already acknowledged that there is a possible risk to use the neonicotinoids clothianidin, imidacloprid and thiamethoxam and their application is restricted, while research is conducted to measure their possible effects on honeybees and other wild pollinators. The study reported here is one of those.

3. Conclusion

The unexpected high losses of honeybee colonies are thought to be caused by multiple stress factors. Although neonicotinoids were found to influence the loads and prevalence of pathogens and parasites in honeybee colonies, a seed dressing with clothianidin of spring oilseed rape showed no significant effect within this field experiment. The colony as a "super organism" might buffer the effect on individual bees demonstrated in some of the laboratory experiments. The interactions between different stress agents are very complex and remain to be studied. Within this study only the influence of clothianidin on parasites and pathogens was studied but not the synergic interaction. It is still an open question as if already high present loads and prevalence of pathogens and parasites in combination with neonicotinoids are affecting the health of honeybees and the survival of the colonies.

Pollination is indeed a vital element for the conservation of biodiversity and the main pollinator for food production. However, the role of other pollinating insects such as bumble bees and solitary bees are often under evaluated and limited information on the influence of neonicotinoids on their lifecycle is available. This should be considered in further analyses. Furthermore long term influences of neonicotinoids and their residues are still to be studied.

As part of the analyses a possible shift from *N. apis* infection to *N. ceranae* infection was seen. It should be further analysed if this in an ongoing trend and which consequences this could have. *N. ceranae* was found to be invasive in other regions.

Regarding the methodology of Nosema spp. quantification, we found disparities between molecular and microscopically analyses. Therefore it is should be further discussed which methods should be used and preferred. Although in experiment with treatment and control sites it is only important to be consistent during the process.

Acknowledgment

A lot of people contributed in many different ways to make this thesis possible. I am very glad for all the help, support, ideas and motivation I received. TACK!

Without my group of supervisors, Joachim de Miranda, Ingemar Fries and Peter Rosenkranz, this thesis would have been not possible. But the biggest gratitude is going to my main supervisor Joachim de Miranda. You were a big help with all the bureaucratic problems and I am very thankful for the discussions and for the introduction to the field of Microbiology. During many meetings you helped me a lot to move along with my thesis. Your comments and ideas helped me during the writing procedure. Ingemar, I would like to thank you for giving me the opportunity to work in this group. It was a great experience where I learned a lot of new things, especially in the field of Microbiology. Furthermore I thank Peter for being my co-supervisor.

I am very glad that I was able to be part of a bigger research project and I would like to thank Maj Rundlöf for answering all my questions regarding the background to the experiment. Without the great setup of this field experiment, my thesis would be not as meaningful as it is. I thanks also all the landowners which offered their sides for this study.

I would like to thank Emilia Semberg, for instructions and assistance during the implementation of the laboratory work. With her expertise in laboratory work it was possible for me to learn much from her. Furthermore, I would like to thank Sara Janbrink for her help with the laboratory work. My thanks also go to Riccardo Bommarco and Johannes Forkman, who discussed and advised me with the statistical analyses.

A big thanks goes also to Martina Lamprecht for being my opponent.

For being a constant source of motivation, suggestions and for all kinds of help I would like to thank my friends and family.

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Appendices

Appendix 1: Primers used for RT-qPCR

Table 8: Name and sequence of primers used for the RNA analasis

Virus	Primer	Sequence 5‘-3‘
DWV	DWV-F8668	TTCATTAAAGCCACCTGGAACATC
	DWV-B8757	TTTCCTCATTAACTGTGTCGTTGA
ABPV	ABPV-F6548	TCATACCTGCCGATCAAG
	KIABPV-B6707	CTGAATAATACTGTGCGTATC
KBV	KBV-F6639	CCATACCTGCTGATAACC
	KIABPV-B6707	CTGAATAATACTGTGCGTATC
IAPV	IAPV-F6627	CCATGCCTGGCGATTCAC
	KIABPV-B6707	CTGAATAATACTGTGCGTATC
BQCV	BQCV-qF7893	AGTGGCGGAGATGTATGC
	BQCV-qB8150	GGAGGTGAAGTGGCTATATC
SBV	SBV-qF3164	TTGGAACTACGCATTCTCTG
	SBV-qB3461	GCTCTAACCTCGCATCAAC
SBPV	SPV-F3177	GCGCTTTAGTTCAATTGCC
	SPV-B3363	ATTATAGGACGTGAAAATATAC
CBPV	CBPV1-qF1818	CAACCTGCCTCAACACAG
	CBPV1-qB2077	AATCTGGCAAGGTTGACTGG
RP49	RP49-qF	AAGTTCATTCGTCACCAGAG
	RP49-qB	CTTCCAGTTCCTTGACATTATG
N. ceranae	forward	TATTGTAGAGAGGTGGGAGATT
	reverse	GTCGCTATGATCGCTTGCC
N. apis	forward	CTAGTATATTTGAATATTGTTTACAATGG
	reverse	GTCGCTATGATCGCTTGCC

Appendix 2: Code for SAS

```
Data cloth;
input
KupID Lokalpar $  LokalID $   Treatment $ Period $ Varroa Nosema  Ceranae
DWV   SBV BQCV DWVbin LOGNOS NOSbin LOGSBV LOGBQCV;
cards;
```

/ V. destructor analyses for infestation rate per 100 bees/

```
proc means data=cloth;
class treatment period;
var Varroa;
run;

proc means stderr data=cloth;
class treatment period;
var Varroa;
run;

proc glimmix data=cloth;
class treatment period KupID LokalID Lokalpar;
model Varroa= treatment period treatment*period / dist=poisson;
random Lokalpar LokalID*Lokalpar KupID*LokalID*Lokalpar;
run;
```

/ Percentage of colonies infested with *V. destructor*/

```
proc means data=cloth;
class treatment period;
var varrbin;
run;

proc means stderr data=cloth;
class treatment period;
var varrbin;
run;

proc glimmix data=cloth;
class treatment period KupID LokalID Lokalpar;
model varrbin= treatment period treatment*period / dist=binary;
random Lokalpar LokalID*Lokalpar KupID*LokalID*Lokalpar;
run;
```

/ Nosema spp. prevalence analyses /

```
proc means data=cloth;
class treatment period;
var NOSbin;
run;

proc means stderr data=cloth;
class treatment period;
var NOSbin;
run;
```

```
proc glimmix data=cloth;
class treatment period KupID LokalID Lokalpar;
model NOSbin= treatment period treatment*period / dist=binary;
random Lokalpar LokalID*Lokalpar KupID*LokalID*Lokalpar;
run;
```

/ Nosema spp. infestation rate analyses /

```
data Nosema;
Input KupID $ Lokalpar $ LokalID $ Treatment $ Period $ Nosema LOGNOS;
Cards;

proc means data=Nosema;
class treatment period;
var LOGNOS;
run;
proc means stderr data=Nosema;
class treatment period;
var LOGNOS;
run;
proc mixed data=Nosema;
class treatment period KupID LokalID Lokalpar;
model LOGNOS= treatment period treatment*period;
random Lokalpar LokalID*Lokalpar KupID*LokalID*Lokalpar;
run;
```

/ BQCV analyses /

```
proc means data=cloth;
class treatment period;
var LOGBQCV;
run;

proc means stderr data=cloth;
class treatment period;
var LOGBQCV;
run;

proc mixed data=cloth;
class treatment period KupID LokalID Lokalpar;
model LOGBQCV= treatment period treatment*period;
random Lokalpar LokalID*Lokalpar KupID*LokalID*Lokalpar;
run;
```

/ SBV analyses /

```
proc means data=cloth;
class treatment period;
var LOGSBV;
run;

proc means stderr data=cloth;
class treatment period;
var LOGSBV;
run;
```

```
proc mixed data=cloth;
class treatment period KupID LokalID Lokalpar;
model LOGSBV= treatment period treatment*period;
random Lokalpar LokalID*Lokalpar KupID*LokalID*Lokalpar;
run;
```

/ DWV prevalence analyses /

```
proc means data=cloth;
class treatment period;
var DWVbin;
run;

proc means stderr data=cloth;
class treatment period;
var DWVbin;
run;

proc glimmix data=cloth;
class treatment period KupID LokalID Lokalpar;
model DWVbin= treatment period treatment*period / dist=binary;
random Lokalpar LokalID*Lokalpar KupID*LokalID*Lokalpar;
run;
```

/ DWV infection rate analyses /

```
data DWV;
input KupID Lokalpar $ LokalID $ Treatment $ Period $ DWVLOG DWV;
cards;

proc mixed data=DWV;
class treatment period KupID LokalID Lokalpar;
model DWVLOG = treatment period treatment*period;
random Lokalpar LokalID*Lokalpar KupID*LokalID*Lokalpar;
run;
proc means data=DWV;
class treatment period;
var DWVLOG;
run;

proc means stderr data=DWV;
class treatment period;
var DWVLOG;
run;
```

/ DWV positive analyses /

```
PROC UNIVARIATE NORMAL PLOT data=cloth;
var LOGDWV;
HISTOGRAM LOGDWV/normal (color=red w=5);
TITLE 'PROC UNIVARIATE LOGDWV';
FOOTNOTE 'Evaluate distribution of variables';
run;
```

```
proc means data=cloth;
class treatment period;
var LOGDWV;
run;

proc means stderr data=cloth;
class treatment period;
var LOGDWV;
run;

proc glimmix data=cloth;
class treatment period KupID LokalID Lokalpar;
model LOGDWV= treatment period treatment*period / dist=poisson;
random Lokalpar LokalID*Lokalpar KupID*LokalID*Lokalpar;
run;
```

Appendices 3: SAS output for all tests

Appendix 3.1: Loads of *Varroa destructor*

Analysis Variable : Varroa							
Treatment	**Period**	**N Obs**	**N**	**Mean**	**Std Dev**	**Minimum**	**Maximum**
A	**After**	**48**	48	0.6666667	0.7809796	0	3.0000000
	Before	**46**	46	0.2608696	0.6810052	0	4.0000000
B	**After**	**48**	48	0.6458333	1.1758127	0	6.0000000
	Before	**48**	48	0.4375000	0.9203665	0	4.0000000

Analysis Variable : Varroa			
Treatment	**Period**	**N Obs**	**Std Error**
A	**After**	**48**	0.1127247
	Before	**46**	0.1004087
B	**After**	**48**	0.1697139
	Before	**48**	0.1328435

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Treatment	1	92	0.34	0.5591
Period	1	92	9.10	0.0033
Treatment*Period	1	92	1.57	0.2137

Appendix 3.2: Prevalence of *Varroa destructor*

Analysis Variable : varrbin							
Treatment	**Period**	**N Obs**	**N**	**Mean**	**Std Dev**	**Minimum**	**Maximum**
A	**After**	**48**	48	0.5000000	0.5052912	0	1.0000000
	Before	**48**	48	0.1875000	0.3944428	0	1.0000000
B	**After**	**48**	48	0.3750000	0.4892461	0	1.0000000
	Before	**48**	48	0.2291667	0.4247444	0	1.0000000

Analysis Variable : varrbin			
Treatment	**Period**	**N Obs**	**Std Error**
A	**After**	**48**	0.0729325
	Before	**48**	0.0569329
B	**After**	**48**	0.0706166
	Before	**48**	0.0613066

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Treatment	1	94	0.14	0.7082
Period	1	94	11.22	0.0012
Treatment*Period	1	94	1.38	0.2435

Appendix 3.3: Prevalence of Nosema spp.

Analysis Variable : NOSbin							
Treatment	Period	N Obs	N	Mean	Std Dev	Minimum	Maximum
A	After	48	48	0.1875000	0.3944428	0	1.0000000
	Before	46	45	0.6888889	0.4681794	0	1.0000000
B	After	48	48	0.0833333	0.2793102	0	1.0000000
	Before	48	47	0.6382979	0.4856879	0	1.0000000

Analysis Variable : NOSbin			
Treatment	Period	N Obs	Std Error
A	After	48	0.0569329
	Before	46	0.0697921
B	After	48	0.0403150
	Before	48	0.0708449

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Treatment	1	90	2.07	0.1538
Period	1	90	45.45	<.0001
Treatment*Period	1	90	0.81	0.3692

Appendix 3.4: Infestation rate of Nosema spp.

Analysis Variable : LOGNOS							
Treatment	Period	N Obs	N	Mean	Std Dev	Minimum	Maximum
A	After	9	9	5.3300000	0.6607950	4.5000000	6.3800000
	Before	34	34	5.3485294	0.4622549	4.3200000	6.1000000
B	After	4	4	4.8075000	0.1652019	4.6200000	4.9700000
	Before	30	30	5.3550000	0.3575467	4.7200000	6.0900000

Analysis Variable : LOGNOS			
Treatment	Period	N Obs	Std Error
A	After	9	0.2202650
	Before	34	0.0792761
B	After	4	0.0826009
	Before	30	0.0652788

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Treatment	1	6	3.01	0.1332
Period	1	6	4.57	0.0763
Treatment*Period	1	6	3.21	0.1233

Appendix 3.5: Infestation with BQCV

Analysis Variable : LOGBQCV							
Treatment	Period	N Obs	N	Mean	Std Dev	Minimum	Maximum
A	After	48	48	6.8252083	0.8601583	5.1900000	9.1800000
	Before	46	45	8.3006667	0.7699244	5.8900000	9.6500000
B	After	48	48	6.6835417	0.9624580	5.3700000	9.4700000
	Before	48	47	8.1889362	0.8470107	5.6200000	9.6600000

Analysis Variable : LOGBQCV			
Treatment	Period	N Obs	Std Error
A	After	48	0.1241532
	Before	46	0.1147736
B	After	48	0.1389189
	Before	48	0.1235492

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Treatment	1	90	0.58	0.4469
Period	1	90	154.19	<.0001
Treatment*Period	1	90	0.00	0.9925

Appendix 3.6: Infestation with SBV

Analysis Variable : LogSBV							
Treatment	Period	N Obs	N	Mean	Std Dev	Minimum	Maximum
A	After	48	48	9.4320833	2.9099309	0	13.3700000
	Before	48	48	9.4127083	2.0077231	0	12.7900000
B	After	48	48	9.7622917	2.0950684	0	12.7100000
	Before	48	48	9.5622917	2.0375979	0	12.0900000

Analysis Variable : LOGSBV			
Treatment	Period	N Obs	Std Error
A	After	48	0.3059931
	Before	48	0.3658084
B	After	48	0.1939077
	Before	48	0.3816275

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Treatment	1	92	1.65	0.2018
Period	1	92	0.39	0.5342
Treatment*Period	1	92	0.10	0.7532

Appendix 3.7: Prevalence of DWV

Analysis Variable : DWVbin							
Treatment	**Period**	**N Obs**	**N**	**Mean**	**Std Dev**	**Minimum**	**Maximum**
A	**After**	**48**	48	0.5416667	0.5035336	0	1.0000000
	Before	**48**	47	0.0638298	0.2470922	0	1.0000000
B	**After**	**48**	48	0.1875000	0.3944428	0	1.0000000
	Before	**48**	47	0.0212766	0.1458650	0	1.0000000

Analysis Variable : DWVbin			
Treatment	**Period**	**N Obs**	**Std Error**
A	**After**	**48**	0.0726788
	Before	**48**	0.0360421
B	**After**	**48**	0.0569329
	Before	**48**	0.0212766

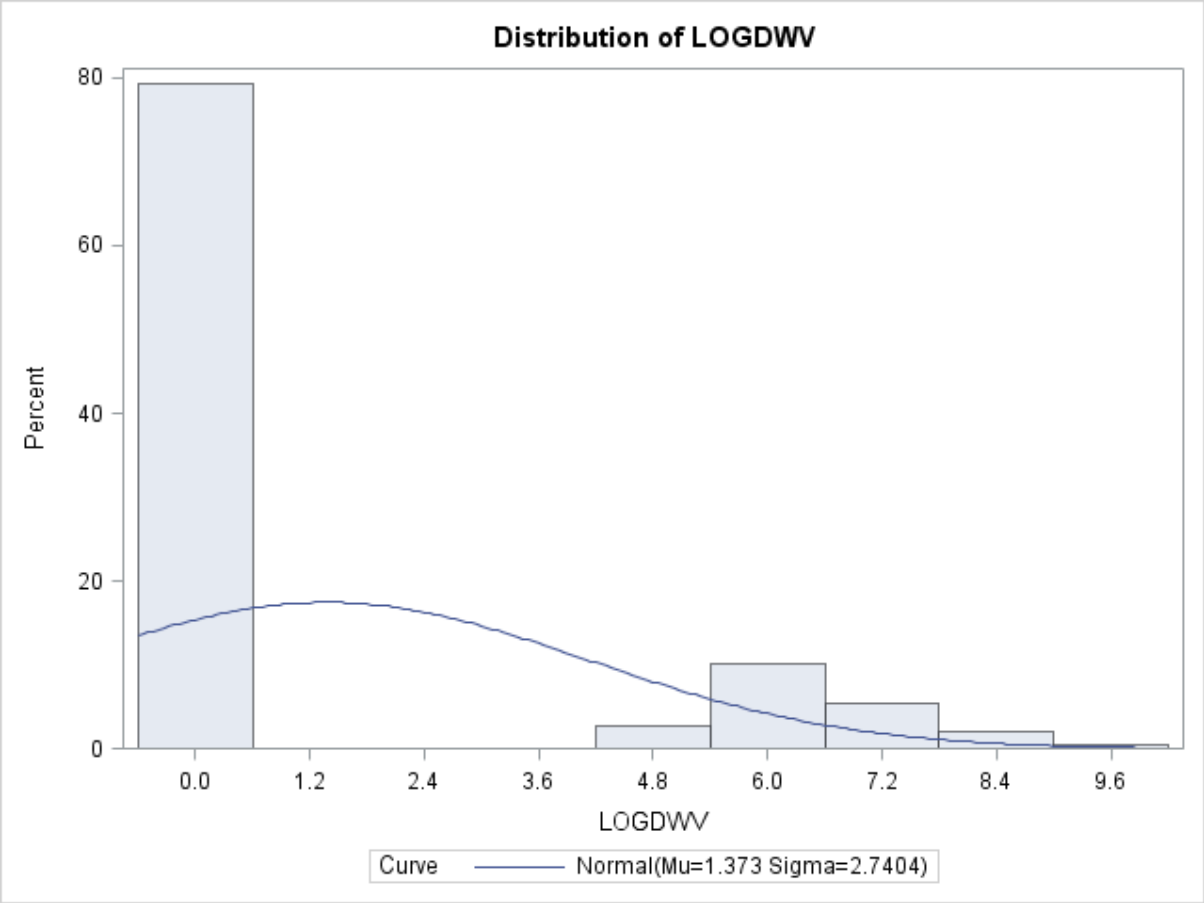
Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Treatment	1	92	3.08	0.0828
Period	1	92	19.79	<.0001
Treatment*Period	1	92	0.29	0.5893

Analysis Variable : DWVLOG							
Treatment	**Period**	**N Obs**	**N**	**Mean**	**Std Dev**	**Minimum**	**Maximum**
A	**After**	**26**	26	6.6500000	1.2712985	5.0000000	9.8000000
	Before	**3**	3	7.3433333	1.3682958	5.8100000	8.4400000
B	**After**	**9**	9	6.1666667	0.3840573	5.4000000	6.8000000
	Before	**1**	1	7.7400000	.	7.7400000	7.7400000

Analysis Variable : DWVLOG			
Treatment	**Period**	**N Obs**	**Std Error**
A	**After**	**26**	0.2493222
	Before	**3**	0.7899859
B	**After**	**9**	0.1280191
	Before	**1**	.

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Treatment	1	2	0.00	0.9558
Period	1	2	2.68	0.2436
Treatment*Period	1	2	0.40	0.5904

Appendix 3.8: Distribution of LOGDWV



Goodness-of-Fit Tests for Normal Distribution				
Test		**Statistic**	**p Value**	
Kolmogorov-Smirnov	**D**	0.4843740	**Pr > D**	<0.010
Cramer-von Mises	**W-Sq**	9.1799002	**Pr > W-Sq**	<0.005
Anderson-Darling	**A-Sq**	45.0153534	**Pr > A-Sq**	<0.005

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Treatment	1	92	4.77	0.0315
Period	1	92	81.77	<.0001
Treatment*Period	1	92	0.04	0.8395