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The effect of field-level clothianidin exposure on the prevalence and loads of pathogenic and non-pathogenic microbes in bumblebees (*B. terrestris*)

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Abstract

Neonicotinoids have been implicated in the declines of honeybees and wild bees based on their lethal and sublethal toxicity, identified in numerous laboratory studies. Recently, a field study with replicated and matched landscapes identified for the first time detrimental effects of a neonicotinoid on colonies of free-foraging bees. Bumblebee *(Bombus terrestris* L.*)* colonies that were exposed to oilseed rape flowers (*Brassica napus* L.) grown from clothianidin-coated seed, gained less weight and clony strength and produced fewer queens than control colonies. In this master thesis, the pathogenic and non-pathogenic microflorae of adults and pupae of these experimental *Bombus terrestris* colonies were examined using (Reverse transcripton-) quantitative polymerase chain reaction. Eight different RNA viruses were targeted in both pupae and adults. In addition the presence and loads of five eukaryotic pathogens and two non-pathogenic gut bacteria were examined in adults but not in pupae. The additional measurements of body mass suggested that clothianidin-exposed bees had a reduced body mass, while this effect was not observed in adults. Clothianidin-exposed adults were however smaller than control bees, as measured by their intertegular distance. Only one RNA virus (ABPV) was detected in pupae samples, while three viruses were detected in adult bees (ABPV, SBPV, SBV). In the adults we detected also the *Bombus*-specific pathogens *Nosema bombi* and *Crithidia bombi*. The latter was in general very prevalent, but found in significantly fewer control colonies and in smaller loads than clothianidin-exposed colonies. Both non-pathogenic bacteria, *Snodgrassella alvi* and *Gilliamella apicola,* were found in a majority of colonies. In contrast to *Crithidia bombi, Snodgrassella alvi* showed elevated loads and prevalence in clothianidin treated fields. We suspect an antagonistic relationship between the two microorganisms may be responsible for the opposite trends they showed towards the neonicotinoid. However, we could not establish such a relationship based on our data. We see a need to further study the interaction between pathogens, non-pathogenic bacteria and pesticides.

Keywords: Neonicotinoids, clothianidin, bumblebees, *B. terrestris*, bee pathogens, symbiotic gut bacteria

Author contributions

I, Dimitry Wintermantel, have written this master thesis and done the statistical analysis presented below. I have generated the data on microbial loads and prevalence of the experimental bumblebee colonies as well as data on physical parameters of adults (body mass & intertegular distance) and pupae (body mass, developmental stage, visible disease signs). Maj Rundlöf's research team (Rundlöf *et al.*, 2015) has generated complementary data on colony health parameters and bee collected plant residues. This research group has also been responsible for the sampling scheme and the set up of the field study from which the bees were taken. The assays used in this thesis were selected or designed by the main supervisor Joachim de Miranda.

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Abbreviations

ABPV	Acute bee paralysis virus
AmFV	*Apis mellifera* filamentous virus
bp	Number of base pairs in the amplicon of standards that are a PCR-product
BQCV	Black queen cell virus
CBPV	Chronic bee paralysis virus
CCD	Colony Collapse Disorder
cDNA	Complementary deoxyribonucleic acid
dil. fac	Dilution factor
DNA	Deoxyribonucleic acid
$[DNA_{average}]$	Average DNA concentration of all samples after automated purification/extraction
$[DNA_{sample}]$	Measured DNA concentration after automated purification/extraction
$[DNA_{standard}]$	Measured DNA concentration of a standard after purification
LD_{50}	Median lethal dosage
LSV1	Lake Sinai virus 1
LSV2	Lake Sinai virus 2
n_{bee}	Number of bees per sample that were pooled and processed together
RNA	Ribonucleic acid
RPL23	Ribosomal Protein L-23 (internal reference gene)
RPL23 $RNA_{average}$/bee	Average copy number of the reference RNA per bee of all samples
RPL23 RNA_{sample}/bee	Number of reference RNA copies per bee of the sample
RT-qPCR	Reverse Transcription quantitative polymerase chain reaction

SBPV	Slow bee paralysis virus
SBV	Sacbrood virus
SQ	Starting quantity
SQ_{mean}	Mean starting quantity of sample duplicates
V_{column}	Extract volume that has been transferred to a column of the Qiagen extraction robot
$V_{digested}$	Volume of resuspended pellet
V_{eluted}	Extract volume eluted by the extraction robot
$V_{pelleted}$	Volume of bee suspension that has been centrifuged to obtain a pellet for DNA extraction
$V_{reaction}$	Template volume per reaction
$V_{sampled}$	Extract volume that was sampled from a bee suspension for extraction of nucleic acid
V_{total}	Total volume of bee suspension

1 Introduction

1.1 The need for pesticides

The projected increase of both the human world population and the consumption of meat and dairy products in developing countries require a dramatic increase in agricultural food production (Godfray *et al.*, 2010; Popp *et al.*, 2013). Furthermore, climate change and the depletion of fossil fuels may promote the demand for biofuels, which in turn increases the demand for arable land (Fargione *et al.*, 2008; Godfray *et al.*, 2010; Popp *et al.*, 2013). However, already today approximately 38% of the world's land area is used agriculturally (World Bank, 2015). Production gains have to be achieved on largely existing agricultural lands, in order to restrict the destruction of habitats and subsequently the alarming rate of biodiversity loss (Rockström *et al.*, 2009; Phalan *et al.*, 2011). Pest control is possibly the most efficient way to increase crop productivity. Globally, pre-harvest yield losses due to pests are equally high as all (pre-) processing, transport, storage and plate waste losses combined (Popp *et al.*, 2013). The pre-harvest yield losses as compared to the maximum yield that can be technically attained on a certain site according to its abiotic growth conditions (the attainable yield) averages around 35% globally for different crops (Oerke, 2005; Popp *et al.*, 2013). If today no pest control were applied, 50% (wheat) to 80% (cotton) of the attainable yield of major crops would be lost (Oerke, 2005). Generally, potential yield losses due to weeds are approximately double those from either animal pests or pathogens. However, weeds can be controlled mechanically, while both pathogen and animal pest control depend broadly on synthetic chemicals.

An integrated pest management may include bio-pesticides, as well as synthetic pesticides. However, bio-pesticides have only a 2% share of the whole crop-protection market, even though the development of bio-pesticides is much less time consuming and expensive as synthetic pesticides (Popp *et al.*, 2013). Crops

that have been genetically modified to be resistant against pests are a relatively recent development that may reduce the demand for synthetic pesticides. However, engineered plants that express toxins to control pests also select strongly for pests that are resistant to these toxins. Generally, resistance of pests towards chemicals represent a large problem in pest management as they usually result in increasingly intensive use of increasingly ineffective pesticides until a substitute chemical has been found (Popp *et al.*, 2013). This particularly applies to insecticides (Thomas, 1999). An integrated pest management that includes biopesticides, diversity of crops, breeding of host-plant resistance, the protection of beneficial bacteria and the moderate use of target-specific rather than broad spectrum pesticides can decrease both the overall pesticide use and the development of resistances towards them (Thomas, 1999; Oerke, 2005; Popp *et al.*, 2013). Synthetic pesticides will however remain indispensable in the foreseeable future. Unfortunately, pesticides exhibit also toxicity to exposed non-target organisms. In the case of insecticides, this threatens insect pollinators, which has detrimental effects on both biodiversity and agriculture.

1.2 Importance of pollinators

It is estimated that 88% of the world's plant species (Ollerton et al. 2011) and three quarters of the most common crop species benefit from animal pollination (Cutler & Scott-Dupree, 2014). More than a third of the globally produced crop volumes depend on pollinators (Klein *et al.*, 2007) of which bees are the most important (Button & Elle, 2014). The European honeybee *Apis mellifera* is regarded as the most important crop pollinator (Aizen & Harder, 2009).

However wild pollinators are indispensable, too. An intercontinental study revealed that the maturation of flowers is more dependent on flower visitation by wild insect pollinators than by honeybees (Garibaldi *et al.*, 2013). Across various crop systems, wild insects provided better pollination services (i.e. more cross pollination) than honeybees. Honeybees are unable to fully substitute wild pollinators in the pollination of a broad range of crops, including even crops that are typically pollinated by large stocks of commercial honeybees like watermelon, almond or blueberry. For the latter, it was shown, that a combination of honeybees and bumblebees is required to maximize crop yields (Button & Elle, 2014).

Wild pollinators can compensate for the honeybee's inability to fly during bad weather conditions and avoidance of certain plant pollen (Javorek *et al.*, 2002). For instance, bumblebees can – in contrast to honeybees – access the pollen of flowers that require their anthers to be sonicated for pollen release. Therefore, bumblebees are commonly used in greenhouses for the pollination of tomatoes and peppers (Cameron *et al.*, 2011; Button & Elle, 2014). In addition, the long tongues

of many bumblebee species allow them to pollinate deep tubular flowers, that honeybees cannot access (Winter *et al.*, 2006). This illustrates the importance of keeping a diversity of different pollinators for different pollinating requirements in order to maximize agricultural yields and to minimize the rate of biodiversity loss.

1.3 The inadequacy of *Apis mellifera* as model organism in the risk assessment of pesticides on pollinators

The assessment of the risk of plant protection products to pollinators, required for the accreditation of pesticides, focuses in both the European Union (European and Mediterranean Plant Protection Organization, 2010) and the United States (United States Environmental Protection Agency (US EPA), 2014) on a single bee species, the European honeybee *apis mellifera*. Insect pollinators are, however, found in different taxonomic orders and bees alone comprise approximately 20000 different species, which differ in physiological, morphological and life history characteristics (Michener, 2007). These different characteristics may be responsible for differences in exposure and/or susceptibility to pesticides (Cutler & Scott-Dupree, 2014).

Colonies of the European honeybee comprise tens of thousands of individuals (Osborne, 2012), while the vast majority of bee species live a solitary lifestyle (Michener, 2007). Honeybees have developed a highly organized, eusocial communal life. The storage of honey allows large numbers of honeybees to survive flowerless periods and cold winter months. The overwintering of sterile females (workers) with the mated queen enables the queen to focus entirely on reproduction and to completely abstain from foraging. Foraging is considered the most hazardous task that bees perform (Goulson, 2003; Cremer *et al.*, 2007). Older and therefore more dispensable workers usually conduct this task (Cremer *et al.*, 2007). The redundancy of worker bees allows colonies to continuously replace a significant proportion of their worker force without noticeably affecting the performance of the colony as a unit (Bryden *et al.*, 2013). Honeybees are therefore more resilient than solitary or semi-social pollinators, as their social structure acts as an extra buffer against environmental hazards.

In addition honeybees may differ from other bees in their ability to detoxify themselves from environmental poisons. Indeed, it was shown that honeybees cleared the neonicotinoid imidacloprid at a faster rate than bumblebees (Cresswell *et al.*, 2014). Individual bumblebees consume also multiple times more nectar than honeybees and expose themselves therefore to higher pesticide doses. In comparison with bumblebees, honeybees may be less sensitive to dietary exposure of neonicotinoids (Cresswell *et al.*, 2012) and more sensitive to topical exposure (Sanchez-Bayo & Goka, 2014).

A recent field study showed detrimental effects of the insecticide clothianidin on the population parameters of both the solitary bee *Osmia bicornis* and the social bumblebee *Bombus terrestris*, while those for *Apis mellifera* colonies remained unaffected (Rundlöf *et al.*, 2015). This highlights the inadequacy of honeybees as a model organism for pollinators in general. Research on pesticide effects should therefore expand to pollinators other than honeybees.

1.4 Neonicotinoids

1.4.1 History of neonicotinoids

In the early 1990's, neonicotinoids (a class of insecticides) were introduced to the market. Due to their efficiency, as well as widespread resistance among insect pests to other insecticides, neonicotinoids have become the dominant insecticide class in the world, with a market share of 26% in 2010 (Casida & Durkin, 2013; Simon-Delso *et al.*, 2014). The three most frequently used neonicotinoids are imidaclorid, thiamethoxam and clothianidin. In 2009, their respective shares of the whole neonicotinoid market, worth US$ 2.63 billion, were 41.5%, 23.8% and 16.7% (Simon-Delso *et al.*, 2014). Thiamethoxam is readily metabolized to clothianidin in insects, plants (Nauen *et al.*, 2003) and mammals (Simon-Delso *et al.*, 2014).

1.4.2 Properties of neonicotinoids

Neonicotinoids promised to pose a lower risk to non-target organisms and the environment than the prevailing insecticides at the time, such as chlorinated hydrocarbons, organophosphates, methylcarbamates and pyrethroids (Tomizawa & Casida, 2011). Neonicotinoids can be applied at low doses due to their high toxicity to pest insects, their persistence and their systemic properties (Fishel, 2011; Van der Sluijs *et al.*, 2013). The latter describes the ability to translocate throughout all parts of the plant after entering the tissue (Van der Sluijs *et al.*, 2013; Simon-Delso *et al.*, 2014). This ensures a persistent protection from insecticidal feeding damages and transmission of plant viruses through insects (Simon-Delso *et al.*, 2014) and enables an extraordinary versatility in application methods (Jeschke et al. 2011). Neonicotinoids can for example be applied as foliar sprays, trunk injections or with irrigation water (Simon-Delso *et al.*, 2014). However, the most common usage is seed/soil treatment, which accounts for 60% of all neonicotinoid applications (Jeschke et al. 2011).

1.4.3 Mode of action

Neonicotinoids bind to nicotinic acetyl-choline receptors (nAChRs) in insects'

brains (Tomizawa & Casida, 2003), causing neuronal over-stimulation, paralysis and eventually death (Easton & Goulson, 2013). Selectivity is in part achieved through subsite specificity to the nAChRs (Tomizawa & Casida, 2003; Matsuda *et al.*, 2005). Receptor residues, that are present in insects but not in vertebrates, are exploited as binding targets (Matsuda *et al.*, 2005). Neonicotinoids show for example a high affinity for cationic amino acid residues in insect nAChRs, due to negatively charged regions in the neonicotinoids' cyano (-NC) or nitro ($-NO_2$) groups (Tomizawa & Casida, 2003). Differences in the detoxification contribute to the higher selectivity of neonicotinoids for insects than mammals compared to other common insecticide classes (Tomizawa & Casida, 2005).

1.4.4 Persistence in the environment

Ironically, the characteristics that contributed to the economic rise and environment-friendly image of neonicotinoids, are also the cause of environmental concerns. Neonicotinoids have the potential to accumulate in the environment, as implied by their relatively long half-lives in aerobic soil conditions (Van der Sluijs *et al.*, 2013). These vary widely from a few months to years (Krupke *et al.*, 2012). For example, the US EPA (2003) estimated the aerobic soil metabolism half-life of clothianidin to range from 148 to 1155 days. To be systemic, neonicotinoids have to be fairly water-soluble (Simon-Delso *et al.*, 2014). This causes a high leaching potential and consequently frequent contamination of ground and surface waters (Van der Sluijs *et al.*, 2013). Their persistence and water-solubility lead also to their frequent presence in wild flowers (Goulson *et al.*, 2015).

1.4.5 Non-target impact

Owing to their systemic nature, neonicotinoids also contaminate pollen, nectar and guttation droplets, which leads to neonicotinoids exposure of pollinators (Bonmatin *et al.*, 2014). Flying bees can also be in directly exposed to neonicotinoid dust during sowing of dressed seeds (Bonmatin *et al.*, 2014), which can cause immediate deaths of bees, since they get exposed to loads multiple times higher than the topical median lethal dose (LD_{50}: the dose that causes 50% mortality within 48 hours of contact exposure) for honeybees and bumblebees (Girolami *et al.*, 2013; Bonmatin *et al.*, 2014).

The implication of neoncotinoids in recent declines of bee populations have been the most heatedly debated environmental impact of neonicotinoids, which led to a moratorium of imidacloprid, thiamethoxam and clothianidin for applications on bee attractive plants in the EU (Commission Regulation (EU) No 485/2013). Neonicotinoids can be classified by the functional group they use for electron withdrawal, which is required for insecticidal activity. The three banned neonicotinoids belong to a group (imidacloprid, clothianidin, thiamethoxam, nitenpyram and di-

notefuran) that uses a nitro rather than a cyano group for electron acceptance, which seems to make those neonicotinoids markedly more toxic to bees than the ones that have a cyano group instead (Iwasa *et al.*, 2004; United States Environmental Protection Agency (US EPA), 2003).

1.5 Declines in bee populations

1.5.1 The European honeybee

The spread of the virus-transmitting ectoparasitic mite *Varroa destructor* from Asia around the globe has accelerated the disappearance of most feral honeybee colonies in Europe and the United States (Potts *et al.*, 2010). Since the introduction of *V. destructor* in the United States, the winter mortality rate of managed honeybees has also increased from 5-10% to 15-25% (Smith *et al.*, 2013) despite combatting the mite with pesticides (van Engelsdorp *et al.*, 2008).

In the winters of 2006/2007 and of 2007/2008 exceptionally high colony losses of more than 30% were noticed in the USA (van Engelsdorp *et al.*, 2008). The term Colony Collapse Disorder (CCD) was created to describe the disappearance of adult worker honeybees from their hives, leaving behind a colony consisting of mainly brood, a queen and a few young worker bees (vanEngelsdorp *et al.*, 2009).

A multitude of factors were investigated in the search for a major cause, but no overriding cause could be singled out. In the absence of a convincing single cause argument, the combination of pathogens, in particular the Israeli acute paralysis virus (Cox-Foster *et al.*, 2007), a weakened immune system and other environmental, genetic and management factors were presented as the most likely explanation for CCD (van Engelsdorp *et al.*, 2008; vanEngelsdorp *et al.*, 2009). The phenomenon has since slipped down the list of colony mortality causes in the USA, even while annual mortality keeps rising (vanEngelsdorp *et al.,* 2012).

In the last decades, the number of managed honeybee colonies in Central Europe (25% loss between 1985 and 2005) (Potts *et al.*, 2009) and the United States (46% loss between 1980 and 2008) has declined remarkably (van Engelsdorp *et al.*, 2008). The number of managed honeybee hives is largely subject to socio-economic developments (Smith *et al.*, 2013) and global stocks of domesticated honeybees have overall increased by approximately 45% between 1961 and 2008 (Aizen & Harder, 2009). Nevertheless such regional declines increase the dependence on wild bees.

1.5.2 Wild bees

Despite the lack of comprehensive standardized data on wild bee populations, there is accumulating evidence that these have also declined regionally (Potts *et*

al., 2010; Goulson *et al.*, 2015). Although for 57% of all bee species in Europe the threat of extinction could not be assessed, 9% were considered at risk (Cox & Temple, 2009). Population trends could only be assessed for 21% of European bee species, with 150 species declining, 13 species increasing and 244 species maintaining stable population size. In the Netherlands and the United Kingdom, the diversity of solitary bees has declined by approximately a third since 1980 (Biesmeijer *et al.*, 2006).

Bumblebee diversity has also decreased. During the last 50 years, four species have become extinct throughout Europe, in addition to at least 13 local extinctions across the continent (Goulson et al., 2008). Moreover, there are also indications of reduced bumblebee species diversity in China and North America (Goulson et al. 2015). In the latter formerly widespread and abundant species are becoming rare (Cameron et al., 2011). In Danish red clover fields noticeable reductions in the abundance and diversity of long-tongued bumblebees have been recorded (Dupont et al., 2011).

The combination of declining wild and managed bee populations and a rapid increase in the cultivation of crops that depend on animal pollination (300%), has been referred to as an impending "pollinator crisis" (Aizen & Harder, 2009).

1.6 Drivers of population declines

Multiple drivers for the decline in bee populations have been suggested including climate change, competition with non-native species, habitat destruction and pests (Potts *et al.*, 2010; Goulson *et al.*, 2015).

Land use change

Habitat loss is considered the most important cause of bee and biodiversity declines (Brown & Paxton, 2009). The subsequent fragmentation of habitats causes bees to be limited to habitats, which are to small to sustain a viable population. In addition inbreeding and a reduced genetic variability may occur as a result of isolation (Brown & Paxton, 2009). Fragmentation affects most heavily solitary, flower-specific and parasitic bees (Potts *et al.*, 2010). Paradoxically perhaps, while urbanization contributes to habitat fragmentation, it can also have a positive effect on bee abundance and diversity due to the variety of habitats and overlapping floral resources (Winfree *et al.*, 2007).

The agricultural intensification has caused a lack of suitable nesting and feeding sites. Farm machinery often destroys bumblebee nests (Goulson *et al.*, 2008). Leguminous crops, the favoured food source of long-tongued bumblebee species and historically a popular cover-crop for soil enrichment, are rarely grown these days due the advent of cheap synthetic fertilizers (Goulson *et al.*, 2008). Monocultures

force bees to a monotonous diet with an unclear effect (Goulson *et al.*, 2015). Monocultures are also responsible for a temporal or permanent absence of flowering plants in the affected (agricultural) landscapes.

Pesticides

Bees are exposed to a multitude of pesticides of which neonicotinoids have been most heavily implicated in the decline in bee diversity and abundance (Goulson *et al.*, 2015). It was shown for honeybees that they throughout their life feed on plant residues with mixtures of insecticides, fungicides, herbicides and miticides (Sanchez-Bayo & Goka, 2014). In particular insecticides belonging to the neonicotinoids and the pyrethroids were considered risky to bumblebees and honeybees based on their toxicity (topical LD_{50}), and the prevalence and doses of residues in plants. Ergosterol-inhibiting fungicides act synergistically to magnify the effect of these two classes of insecticides (Sanchez-Bayo & Goka, 2014). Herbicides have, as well as a direct effect on bees, also an indirect one by reducing the availability of weeds and wild flowers (Sanchez-Bayo & Goka, 2014).

Climate change and the introduction of alien species

An increased frequency of extreme weather events like storms, drought and floods, will probably have major impacts on many bee species. For instance, flooding may endanger bees that nest or hibernate below or on the ground (Goulson *et al.*, 2015). The most obvious effect of climate change is however an increase in temperature with an expected shift in species ranges (Brown & Paxton, 2009). It was already shown that the altitudinal ranges of montane bumblebee species in Spain decreased as the lower boundary of their range shifted further uphill than their upper one due to a regional air temperature increase (Ploquin *et al.*, 2013). It is expected that heat-sensitive bumblebees will shift their southern boundary further north (Goulson *et al.*, 2015). Generally, it remains unclear how the likely introduction of alien plant species in existing ecosystems will affect pollinators in general. It is suspected that the creation of novel communities will favour generalist pollinators rather than specialist pollinators (Schweiger *et al.*, 2010). Pests and other antagonistic species are likely to benefit from an increase of air temperature (Schweiger *et al.*, 2010). The importation of invasive species occurs not only due to changes in climatic conditions, but also because of deliberate or accidental transportation by humans. In recent years the latter was the case, when the yellow-legged hornet (*Vespa velutina*) was introduced to Europe. The native-Asian species is a predator of honeybees (Monceau *et al.*, 2014). In the past, the spread of invasive bee species with high loads of (foreign) pathogens or parasites contributed to declines in endemic bee abundance and diversity (Goulson *et al.*, 2015).

Pests

Bees naturally suffer from diverse bee diseases. Humans have exacerbated the problems caused by bee diseases by facilitating the transmission of non-native pathogens through the transport of honeybees and bumblebees over long distances (Goulson *et al.*, 2015). *V. destructor* is the best-known example of a non-native parasite, which has acted as a major driver for honeybee colony losses in North America and Europe, largely by efficiently vectoring several bee virus diseases (Rosenkranz *et al.*, 2010; Goulson *et al.*, 2015). Fortunately, the mite is restricted to the genus *Apis*, unlike other bee pathogens, including the viruses it transmits (Goulson *et al.*, 2015). *Nosema spp* are additional pathogens with a global distribution that are not restricted to one genus (Potts *et al.*, 2010).

Interaction of pressures on bee populations

The combination of stressors can have additive or even synergistic effects. As previously stated, the latter is the case for the combination of neonicotinoids with certain other pesticides. Despite having low toxicity by themselves, pyrethroids and piperonyl butoxide can increase neonicotinoid toxicity multiple hundred times (Goulson *et al.*, 2015). Starvation can increase pathogen virulence (Brown *et al.*, 2000). Bees store excess nutrition in their fat bodies in order to survive starvation periods and to activate energetically costly immune responses, such as the release of antimicrobial peptides (Moret & Schmid-Hempel, 2000; Foley *et al.*, 2012). It was also shown that pesticides can weaken the immune system of bees and subsequently increase pest infestation and virulence (James & Xu, 2012; Doublet *et al.*, 2014). Pesticides affect insect immune systems for example by inhibiting enzymes that are involved in detoxification or by reducing social grooming (James & Xu, 2012). Social grooming is done by social insects to remove pathogens or parasites from nest-mates (Neumann *et al.*, 2001; Cremer *et al.*, 2007).

1.7 Bumblebees

1.7.1 Life cycle

Bumblebees are either kleptoparasitic (‘cuckoo-bees’), which means they lay their eggs in the nests of other bumblebee species, or they are primitively eusocial (Michener, 2007). In primitively eusocial species, mated queens hibernate alone and found colonies after emerging in late winter or spring (Goulson, 2003). The queen forages until she has raised sufficient workers to feed her and her brood. Bumblebee queens hardly differ morphologically from workers except for a difference in size. In summer or autumn reproductives (drones and queens) are pro-

duced. Each newly emerged queen mates with one drone of another colony before they go into hibernation to complete the annual life cycle of bumblebees.

Bumblebee colonies comprise tens to hundreds of nest-mates (Rundlöf *et al.*, 2015).

1.7.2 Pathology

Viruses

Bumblebee pathogens include viruses, bacteria, protozoa and fungi (Goulson *et al.*, 2015). RNA viruses are considered the organisms that most easily adapt to new hosts because of their high mutation rate and short generation cycles (Pedersen *et al.*, 2005).

In a recent British study (McMahon *et al.*, 2015), Black queen cell virus (BQCV), deformed wing virus (DWV), Acute bee paralysis virus (ABPV), Slow bee paralysis virus (SBPV) and Sacbrood virus (SBV) have been detected in both *Apis mellifera* and *Bombus spp.* foragers. DWV and BQCV showed thereby significantly higher prevalence in *A. mellifera* than in *Bombus spp.,* while the opposite was true for ABPV and SBPV. In general, the prevalence of RNA viruses in bumblebees and honeybees is linked (McMahon *et al.*, 2015). This has been in particular shown for BQCV, ABPV (McMahon *et al.*, 2015) and DWV (Fürst *et al.*, 2014). *B. terrestris* and *A. mellifera* from the same site can share identical DWV strains, which indicates that viruses are being exchanged between honeybees and bumblebees (Fürst *et al.*, 2014).

Possible pathogen transmission routes between bee species are the use of the same floral resources or through direct contact, in particular during honey robbery by bumblebees from honeybee colonies (Genersch *et al.*, 2006). Higher DWV concentrations in workers than in males support the assumption that transmission is linked to foraging activity (Fürst *et al.*, 2014). Particularly in view of the theory that the haploid drones are inherently more susceptible than diploid females as it has been shown for honeybees (Retschnig *et al.*, 2014).

Although the direction of transmission remains unclear, it has been speculated that the extremely high DWV loads, which occur in honeybees infested with *V. destructor* have fostered the transfer of the RNA virus to wild bees (Fürst *et al.*, 2014).

Eukaryotic pathogens

Honeybees and bumblebees can also share pathogens other than viruses, such as the fungoid *Nosema ceranae* (Goulson *et al.*, 2015). *N. ceranae* originates in *Apis cerana,* but seems to be more virulent in bumblebees than in honeybees (Graystock et al. 2013). *Nosema* are microsporidians and as such they are intracel-

lular obligatory parasites (Otti & Schmid-Hempel, 2007). These parasites reproduce within their hosts and get then transmitted as spores in faeces.

The rearing of bumblebees for commercial purposes may facilitate the transmission of honeybee pathogens to bumblebees, as they are fed with pollen collected by honeybees (Velthuis & Doorn, 2006; Goulson *et al.*, 2015). Honeybees are also used to stimulate hibernating queens to emerge and initiate colonies for commercial purposes (Velthuis & Doorn, 2006). In addition, the high densities in managed bumblebee populations provide conditions for the fast spread of pathogens (Whitehorn *et al.*, 2013). The usage of bumblebees in greenhouses started infection waves of the bumblebee parasite *Chritidia* spp and *Nosema bombi* in wild pollinators around the greenhouses (Murray et al. 2013; Goulson et al. 2015). The outbreak of *Nosema bombi* in commercial bumblebee colonies in California has been linked to declines of North American *bombus* species (Cameron *et al.*, 2011). Identical ribosomal RNA sequences in European and North American *N. bombi* isolates support a recent introduction from Europe.

In addition to the native North American *B. impatiens*, the European *B. terrestris* is commonly used in the US for pollination in greenhouses (Winter *et al.*, 2006). In Europe, where the global trade of bumblebees for pollination in greenhouses began in the 1980s, more than a million *B. terrestris* colonies per year are reared commercially (Goulson *et al.*, 2015). A high proportion of supposedly parasite-free colonies contain pathogens, including the probably *Bombus*-specific *Apicystis bombi*, *Crithidia bombi* and *Nosema bombi* (Graystock et al. 2013).
The introduction of *B. terrestris* in South America precipitated the transmission of these parasites to native *Bombus* species and the subsequent local extinction of *B. dahlbomii* (Schmid-Hempel *et al.*, 2014).

1.7.3 Immune system

The spatial and genetic proximity of nest-mates, the relatively constant temperature and moisture content and the presence of food resources and brood make a bee hive prone to rapid within-colony pathogen/parasite spread (Richter *et al.*, 2012).

Individual immune system

Luckily, the bee's innate immune system is very effective at combating pathogens (Hoffmann, 1995). Insects show mainly two individual immune responses: The constitutive melanization-encapsulation response and the inducible expression of various microbial peptides (Moret & Schmid-Hempel, 2001). Hemocytes can phagocytize or encapsulate microbes (Chan *et al.*, 2009). This foreign target location initiates melanization, a process in which phenol-oxidases get activated to ultimately produce melanin, which covers the pathogen (González-Santoyo &

Córdoba-Aguilar, 2012). Intermediates in the synthesis of melanin are toxic to bacteria, viruses and fungi. This process is very quickly effective, so that only few persistent invaders need to be combated by antimicrobial peptides (Haine *et al.*, 2008). This may be the reason why development of resistances to these peptides is rare. Bees produce antimicrobial peptides mostly, but not exclusively in their fat bodies (Chan *et al.*, 2009).

Social immune system

In addition to their individual immune system, social insects have developed a social immune system. This is the ability to reduce the risk of pathogen spread within a colony through cooperation of nest-mates system (Cremer *et al.*, 2007).

Social bumblebees are considered less organized than honeybees. This is likely due to their smaller worker force and higher tendency to tolerate foreign objects in their nest, since they use various materials to build them (Hoffmann *et al.*, 2008). However individuals within a bumblebee colony are also given distinct tasks depending on their age and size (Goulson, 2003). For example older and larger bees are typically foraging or guarding the nest entrance to stop parasites or infected foreign bees from entering (Free, 1958; Blacher *et al.*, 2013), while younger ones are feeding the queen and the brood (Goulson, 2003). Bumblebees are also efficient at removing the eggs or larvae of parasitic beetles and will attack intruding beetles more often than honeybees do (Hoffmann *et al.*, 2008). Bumblebees avoid flowers with signs of pathogen contamination (Fouks & Lattorff, 2011) or recent predation (Abbott, 2006). Bumblebees that are parasitized by conopid flies may seek cold temperatures and choose to overnight outside the hive to retard the development of the parasite and reduce its transmission to nest-mates (Müller & Schmid-Hempel, 1993).

Linkage between the individual and the social immune system

The individual and the social immune system are linked, as is illustrated by the socially-induced up-regulation of the innate immune system (Moret & Schmid-Hempel, 2001; Richter *et al.*, 2012). Although bumblebees do not produce antibodies (Moret & Schmid-Hempel, 2001), they can transfer specific immunological memory between generations (Salmela *et al.*, 2015). Queens can prime offspring immunity via the egg-yolk precursor protein vitellogenin, which binds to bacteria and facilitates the transport of immune elicitors into developing eggs (Salmela *et al.*, 2015).

Potentially beneficial bacteria

Bumblebees acquire a distinct and species-poor microflora through feeding (Hauke Koch & Schmid-Hempel, 2011). Social bees share bacterial groups that

are absent in solitary bees and that could provide protection against pathogens. The prevalence of *Crithidia bombi* was shown to be negatively correlated to the abundance of "core bacteria" in bumblebee guts, including the Gammaproteobacteria *Gilliamella* and the Betaproteobacteria *Snodgrassella* (Koch & Schmid-Hempel, 2011; Koch & Schmid-Hempel, 2012; Cariveau *et al.*, 2014). The fact that this was also observed after transplanting gut microbiota between bumblebees suggests that these "core" bacteria actively contribute to the protection against *C. bombi* (Koch & Schmid-Hempel, 2012).

1.8 Effects of neonicotinoids on bumblebees

1.8.1 Laboratory experiments

Lethal doses

Acute toxicity is typically measured with a standardized test that measures the dose that kills 50% of test organisms within 48 hours (LD_{50}; US EPA, 2012). This allows for the easy comparison of the acute toxicity of different chemicals towards different organisms. The most common neonicotinoids exhibit a very high acute toxicity (in the range of ng bee^{-1}) to various bee species (Van der Sluijs *et al.*, 2013). However different neonicotinoids differ in their LD_{50} by a factor of more than 5000 (Pisa et al. 2014).

The topical LD_{50} for bumblebee exposure to clothianidin is 40 ng bee^{-1} (Sanchez-Bayo & Goka, 2014). The oral LD_{50} of imidaclorid is 30 ng bee^{-1}, which has the same topical LD_{50} as clothianidin (Sanchez-Bayo & Goka, 2014). An oral LD_{50} for clothianidin exposure of bumblebees is not available. Chronic exposure magnifies the toxicity to bees (Van der Sluijs *et al.*, 2013). Dosages that are considered sublethal can therefore lead to delayed mortality (Sanchez-Bayo & Goka, 2014).

Sublethal effects

Exposure of individual social bees to sublethal insecticide dosages may impair colony functioning even more than lethal doses. The most exposed bees of a colony are foraging workers. The premature death of a few worker bees does not necessarily affect colony functioning, as they can easily be replaced by nest-mates (Bryden *et al.*, 2013). However sublethal dosages may affect the development of colonies through the feeding of the insecticide to the brood (Osborne, 2012) and the induction of behavioural changes (Bryden *et al.*, 2013). In addition impaired foragers might be more likely to introduce pathogens into the colony (Di Prisco *et al.*, 2013).

Dietary exposure of bumblebees to neonicotinoids has been shown to affect brood production (Tasei *et al.*, 2000; Gill *et al.*, 2012; Laycock *et al.*, 2012; Laycock & Cresswell, 2013), foraging ability (Gill *et al.*, 2012; Laycock *et al.*, 2012; Gill & Raine, 2014; Elston *et al.*, 2013; Feltham *et al.*, 2014), worker longevity (Larson *et al.*, 2013; Fauser-Misslin *et al.*, 2014), flower choice (Gill & Raine, 2014), worker size (Gill & Raine, 2014) and the construction of honey pots (Gels *et al.*, 2002; Larson *et al.*, 2013; Elston *et al.*, 2013), brood chambers (Gels *et al.*, 2002) and nests (Elston *et al.*, 2013). The production of all three castes can be decreased by feeding on neonicotinoid-spiked nutrition: Queens (Whitehorn *et al.*, 2012; Larson *et al.*, 2013; Fauser-Misslin *et al.*, 2014), drones (Fauser-Misslin *et al.*, 2014) and workers (Gill *et al.*, 2012).

Differences in sublethal toxicity between common neonicotinoids

Most research on sublethal neonicotinoid effects has focused on imidiacloprid (Carreck & Ratnieks, 2014). However, the use of imidacloprid as a seed coating of oilseed rape has declined, while the use of clothianidin and its precursor thiamehoxam has vastly increased (Walters, 2013; Carreck & Ratnieks, 2014). Oilseed rape, which is typically seed-treated with neonicotinoids, is the most important mass flowering crop in large parts of Northern Europe and North America (Laycock & Cresswell, 2013).

Neonicotinoids differ in their sublethal toxicity as studies on brood production suggested. *B. terrestris* colonies that were fed with imidacloprid-spiked sugar water (0.7 ppm) and pollen (6 ppb) ad libitum exhibited 85% less queen production in comparison to control colonies (Whitehorn *et al.*, 2012).

Brood production of queenless *B. terrestris* microcolonies was reduced by 42% at a dose as low as 1.27 ppb imidacloprid in syrup (Laycock *et al.*, 2012), while no effect on such colonies was found when exposed to doses up to 11 ppb of thiamethoxam (Laycock *et al.*, 2014). Clothianidin doses as high as 36 ppb in pollen showed also no effect whatsoever on queenright *B. impatiens* colonies (Franklin *et al.*, 2004).

Sensitivity of brood to neonicotinoids

Ovary production appeared to be unaffected at field-realistic neonicotinoid dosages (Laycock *et al.*, 2012). Therefore, reductions in brood production were ascribed to nutritional limitations (Gill *et al.*, 2012; Laycock *et al.*, 2012; Feltham *et al.*, 2014) and a lack of brood care (Gill *et al.*, 2012). These result from, respectively, an impairment of the pollen foraging ability (Gill *et al.*, 2012; Larson *et al.*, 2013; Feltham *et al.*, 2014; Gill & Raine, 2014) and a larger proportion of the worker force that goes foraging (Gill *et al.*, 2012; Gill & Raine, 2014). Bumblebees exposed to imidacloprid collect pollen less often (Feltham *et al.*, 2014) and in small-

er bouts (Gill *et al.*, 2012; Feltham *et al.*, 2014; Gill & Raine, 2014). Even an increase in the number of foragers cannot compensate for this. The increased number of foraging worker bees may not only be a colony response, but also the result of a neuronal change, leading to a greater desire to forage (Gill & Raine, 2014). Neuroactive levels of neonicotinoids can be reached in bumblebee brains within three days through dietary exposure of 2.1 ppb imidacloprid or clothianidin (Moffat *et al.*, 2015). As a result, the mitochondria in neurons get depolarized: directly by clothianidin and indirectly by imidacloprid through an increase of sensitivity to a neurotransmitter. This is a mechanistic explanation of the observed reduced foraging ability.

However, the effects on pollen foraging efficiency may not be the only reason for the reduced brood production. The brood may inherently be more susceptible to neonicotinoids, as suggested by Fauser-Misslin et al. (2014) who exposed *B. terrestris* to a mixture of thiamethoxam (4 ppb) and clothianidin (1.5 ppb) via sugar water. They found consistently reduced sugar water consumption in neonicotinoid-exposed colonies, while pollen consumption was only reduced after one brood cycle. This indicates that pollen collection might also be decreased as a result of reduced rearing efforts.

However, young bees are not necessarily selectively affected by neonicotinoids. Gill & Raine (2014) found no effect of neonicotinoids on the age at the first foraging flight, but exposed foragers became less effective as they grew older, while control foragers improved with experience. It remains therefore unclear whether brood is more affected by neonicotinoid exposure than adults, despite less exposure.

Chronic sublethal effects

Laycock & Cresswell (2013) showed that at least partial recuperation of pollen consumption is possible within two weeks after imidacloprid exposure. Nevertheless, the fact that imidacloprid causes an ongoing increase in the proportion of foraging workers over the exposure time (Gill & Raine, 2014) and that impairment of foraging ability can persist for a minimum of four weeks (Feltham *et al.*, 2014) shows that neonicotinoid effects can be chronic. However, it remains unclear whether exposure at the larval stage or continuous imidacloprid exposure via stored honey was responsible for this.

Interactions between neonicotinoids and pathogens

The combination of pathogens and neonicotinoids can deteriorate bee health. For example, exposure to neonicotinoids and *Crithidia bombi* via sugar water reduces queen mother longevity, while this effect was not observed with either the parasite or the insecticides alone (Fauser-Misslin *et al.*, 2014). One possible explanation is

an increased virulence due to a lack of feeding (Brown *et al.*, 2000). As previously stated, bees' fat bodies serve as energy reserves for starvation periods or for priming immune responses (Moret & Schmid-Hempel, 2000; Foley *et al.*, 2012). *B. terrestris* infested with *C. bombi* were shown to invest more in their fat bodies and less in reproduction (Brown *et al.*, 2000). This may be to ensure functioning of the immune system, but could also be initiated by the parasite for its own benefit (Brown *et al.*, 2000).

The combination of parasite (*Nosema ceranae*) and imidacloprid can also increase honeybee mortality and decrease glucose oxidase activity, an enzyme used in the sterilization of food (Alaux *et al.*, 2010). The reduction of this activity may reduce the social immunity of bees just as the reduced tendency or workers to engage in brood care does. Newly emerged honeybees are more prone to *Nosema* infections, if being exposed to imidacloprid during the larval stage (Pettis *et al.*, 2012; Wu *et al.*, 2012).

Di Prisco et al. (2013) showed that clothianidin suppresses the transcription of an immune gene in honeybees, by enhancing the expression of an inhibitor. This stimulates the replication of the DWV. Unfortunately, there are doubts about the assays used to quantify these transcripts (J. R. de Miranda 2015, pers. comm., 24 June). There is further need to investigate how neonicotinoids affect the susceptibility of bumblebees to pathogens.

1.8.2 Potential weaknesses of laboratory studies

Frequency of exposure may not be representative for the real life

All of the above cited neonicotinoid effects in bumblebees have been obtained in laboratory studies with artificial feed or lack of choice between treated and untreated food sources. Under natural conditions bumblebees have access to different crops and they might sense the neonicotinoids and actively avoid them. However, two studies failed to determine such an effect when confining *B. impatiens* to imidacloprid-treated (Gels *et al.*, 2002), clothianidin-treated (Larson *et al.*, 2013) and untreated white clover. A recent study suggests that bees even prefer neonicotinoid-laced nectar over insecticide-free nectar, despite their inability to taste these insecticides (Kessler *et al.*, 2015). This preference, which was observed in both bumblebees and honeybees for imadiacloprid and thiametoxam, but not for clothiandin, may be due to the activation of a nicotinic acetyl-choline receptors (nAChRs) in the bees' brains.

Even when placed next to flowering oilseed rape (*Brassica napus* L.), bumblebees feed on a variety of other flowers (Carreck & Ratnieks, 2014). Unlike honeybees they were not observed to feed exclusively on oilseed rape, although it is a preferred crop for bumblebees (Stanley & Stout, 2014).

Doses may not be field-realistic

Carreck & Ratnieks (2014) noted that the neonicotinoid concentrations in some studies might not be representative for oilseed rape, although claimed otherwise. The lack of sufficient field data make it difficult to estimate the doses, which bumblebees are typically exposed to (Osborne, 2012). The EFSA (2012) reviewed neonicotinoid loads in oilseed rape residues in the field and found maximum clothianidin levels of 5.4 ppb in nectar and 4 ppb in pollen. The highest imidacloprid levels were 0.81 in nectar and 7.6 ppb in pollen. The residues with the highest imidacloprid levels were taken from honeybee hives in a North American study (EFSA 2012). These might however not be representative for a European setting as application rates and the timeframe between sowing and flowering differed from European recommendations (Carreck & Ratnieks, 2014). The studies reviewed by the EFSA (2012) showed a maximum of 2 ppb imidacloprid in oilseed rape pollen and no detectable imidacloprid in any nectar within the EU. In 40.5% of 185 honeybee-collected pollen samples in France, imidacloprid with an average load of 0.9 ppb and a maximum load of 5.7 ppb was found (Chauzat *et al.*, 2011). This pollen was not exclusively collected from oilseed rape but from different flowers including sunflower.

1.8.3 Field studies

Field studies on the effect of neonicotinoids on bumblebee colony health are relatively rare. Tasei et al. (2001) failed to detect any effects of imidacloprid seed dressing of sunflower on the homing behaviour of B. *terrestris* in a 9 day long field experiment, despite the observation that both the control and the treated group fed intensively on sunflower (98% of nectar, 25% of pollen). Subsequent monitoring of the colonies in the laboratory showed also no effect on the colony size, the queen production and the mating ability of emerging queens.

In a Canadian field study (Cutler & Scott-Dupree, 2014), *B. impatiens* colonies were placed next to corn grown from clothianidin and thiamethoxam treated seeds. Clothianidin was found in low levels in treated fields (0.1-0.8 ppb), while thiamethoxam was not detected. In comparison to colonies located near pesticide-free corn fields, the exposed colonies showed no difference in foraging activity, pollen loads or brood production, but did differ significantly in the number of workers. The authors regarded it as unlikely that this was a neonicotinoid effect as pollen analysis showed that only 0.6% of the collected pollen came from corn. Unlike oilseed rape, corn is not favoured by bumblebees nor does it produce nectar (Cutler & Scott-Dupree, 2014; Carreck & Ratnieks, 2014).

In 2013, the UK's Food & Environment Research Agency (FERA) published a report on a field study with clothianidin and imidacloprid treated oilseed rape. In this report, it was observed that the maximum colony mass, the number of larvae

and the foraging activity was reduced at the imidacloprid treated site, while at the clothianidin treated site the number of workers was reduced (Thompson *et al.*, 2013). Colonies at both neonicotinoid treated sites had also fewer nectar cups compared to the control site. However, the authors admitted several methodological weaknesses including the lack of replicated sites and an adequate reference group, as the professed control site was contaminated with thiamethoxam. They concluded that there is no significant connection between neonicotinoid exposure and colony development, based on residue analyses rather than site-based analyses. The European Food Safety Association declared this study as inconclusive due to its methodological weaknesses (EFSA 2013). Goulson (2015) conducted a simplified residue based re-analysis of the FERA study and came to the conclusion that higher neonicotinoid exposure led to poorer colony development, including reduced queen production.

Field experiments are required to verify the in the laboratory observed findings (Cutler & Scott-Dupree, 2014), but potential neonicotinoid effects can easily be masked by uncontrolled co-variates like climatic or landscape characteristics (EFSA 2013; Carreck & Ratnieks 2014).

While this bias cannot be excluded, Rundlöf et al. (2015) minimized it by setting up a field experiment with replicated control and treatment sites, which were paired according to geographical location, nearby landscape and land use. They identified that clothianidin seed coating of oilseed rape plants reduced the density of wild bees, nesting of the solitary bee *Osmia bicornis* and the colony growth and brood production of previously placed *Bombus terrestris* colonies, while honeybee colonies remained unaffected.

1.9 Aim

This study aims at assessing the impact of clothianidin seed dressing in oilseed rape (*Brassica napus)* on the pathogenic and non-pathogenic microbiota infestation of adult workers and premature *B. terrestris* workers and drones. In order to identify direct or indirect clothianidin effects on the immune system of *B. terrestris,* we are going to analyse the microbiota of experimental colonies from Rundlöf et al.' s field experiment (2015).

We hypothesize that both adults and pupae show higher pathogen prevalence (proportion of infested colonies) and loads (concentrations), when exposed to clothianidin at field level.

In addition we investigate whether this exposure affects levels of the potentially beneficial “core” bacterial species *Gilliamella apicola* and *Snodgrassella alvi*.

2 Materials and methods

2.1 Study design

The 16 experimental oilseed rape fields, located in southern Sweden (Fig. 1), were paired according geographical proximity and land use (Rundlöf *et al.*, 2015). One field in each pair was sown with clothianidin-treated oilseed rape seed while its pair was sown with untreated oilseed rape seed. In a shaded area close to the border of each field, six honeybee (*Apis mellifera*) colonies, six bumblebee (*Bombus terrestris*) colonies and eighty-seven solitary bee nesting tubes with twenty-seven *Osmia bicornis* cocoons were placed. Two ventilated boxes were placed; each containing three commercially produced *Bombus terrestris* colonies.

One selection criterion for the fields was the absence of other oilseed rape fields within a 2 km radius. However for two experimental fields, relatively small secondary oilseed rape fields were within flight distance at 0.9 km (6.5 ha) and 1 km (4.4 ha).

For each field pair, one field was randomly assigned to be sown with seeds treated with Elado (25 mL kg^{-1}) and the fungicide thiram. The control fields were sown with seeds being treated only with thiram. As recommended for spring sown oilseed rape hybrids, 150 seeds per square meter were sown. This corresponds to seeding rates of 7.5 kg ha^{-1} for seeds treated with only thriam and 7.7 kg ha^{-1} for seeds treated with thriam and Elado. Elado (Bayer) contains two active ingredients: Clothianidin (400 g L^{-1}) and β-cyfluthrin (80 g L^{-1}). The latter is not systemic and could therefore not be detected in residue analyses of honeybee-collected pollen and nectar.

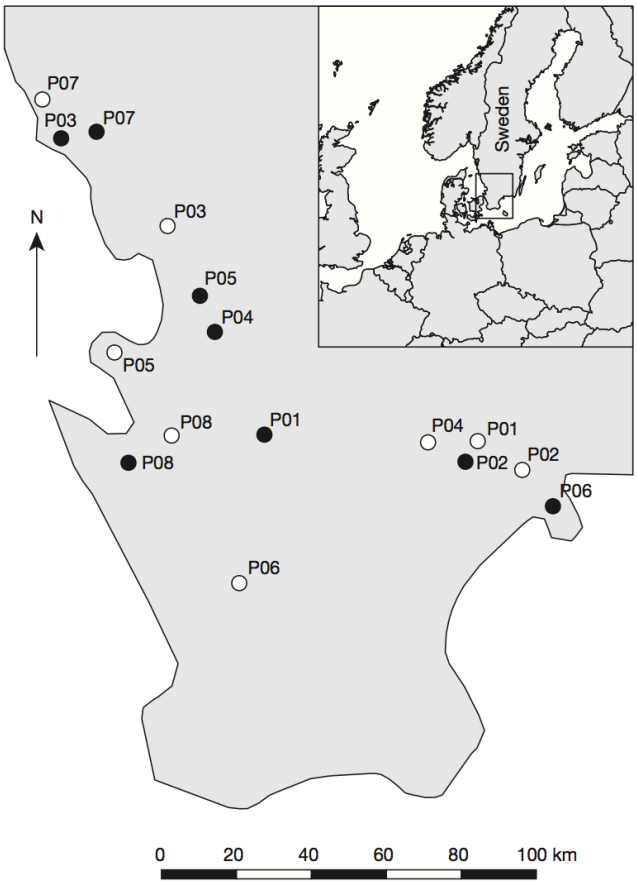

Figure 1. Paired study design of the experiment from which the bumblebee samples were derived. Open circles represent the location of clothianidin-treated oilseed rape field pairs; filled circles represent untreated control field pairs. Pairing was based on the land-use within a 2 km radius around the fields and geographical vicinity (Rundlöf *et al.*, 2015).

Clothianidin was found in honeybee-collected pollen (mean = 10.3 ± 1.8 (s.e.m.) ppb) and nectar collected by honeybees (mean = 10.3 ± 1.3 ng L^{-1}) and bumblebees (5.4 ± 1.4 ng L^{-1}) at the treated fields, while it was only found in marginal concentrations in honeybee nectar (0.1 ± 0.1 ng L^{-1}) from control fields and not at all in bumblebee-collected nectar and honeybee-collected pollen. Pollen analysis showed that pollen collected by *B. terrestris* was mainly from oilseed rape (80.1 ± 5.0 %). Counter to the instructions, one farmer applied Biscaya in a control field. Biscaya contains the neonicotinoid thiacloprid, which has a considerably lower acute toxicity for honeybees than clothianidin as indicated by a more than 5000 times higher LD_{50} (Pisa et al. 2014). Neither Rundlöf *et al.* (2015) nor we observed any qualitative effect on the results of excluding the field pair, in which Biscaya (0.3 L h^{-1}) was applied in a control field six days before the placement of the bumblebee colonies.

2.2 Bumblebee colonies

The 96 *B. terrestris* colonies (NATUPOL beehives) were acquired from Koppert Biological Systems and placed at the 16 experimental fields between the 14 and the 28 June 2013. Placement of the colonies was conducted to coincide with the onset of flowering in the experimental field. In five of the field-pairs, this occurred at the same day for both fields within the pair and in one field pair within 2 days. Two field pairs showed a more asynchronous oilseed rape phenology, resulting in an 8-day difference in placement between the two fields.

At the time of placement, each of the colonies was ca. 10 weeks old and contained ca. 50 workers, one queen and both pupae and larvae. It is unclear whether the colonies were infested with pathogens, as this was not examined prior to placement.

All 12 colonies of those field-pairs with synchronous flowering phenology were freeze-killed at -20 °C, when emergence of new queens in one of the colonies was noticed. For those field pairs with asynchronous oilseed rape flowering phenology, the colonies were left an equal number of days in the fields and were therefore not all terminated at the same date. All colonies were removed from the fields between the 7 July and the 5 August 2013 after 23-38 days of field exposure.

Out of the three colonies that were placed in each box, two had been assessed by Rundlöf *et al.* (2015) for certain colony development parameters; number of queens, workers, pollen/nectar cells as well as the mass of the nest, cocoons and larvae. In this thesis, the microflorae of these 64 *B. terrestris* colonies were analysed. In a previous master thesis, the honeybee colonies used in the same field experiment were examined for their pathogens (Goss, 2014).

2.3 Physical measurements

Cocoons were separated into queen and worker/drone brood by the width of the cocoon. The cut-off width (12 mm) was the lowest value between the peaks of a bimodal distribution of the cocoon width (Rundlöf *et al.*, 2015). Pupae from the smaller cocoons were then separated into males and females mainly based on the presence of male genitalia. The developmental stage was rated into 6 categories based on eye colour (white = 1, pink = 2, brown = 3), body colour (white = 1~3, brown = 4, black= 5~6) and the presence of wings (6). It was noted whether the pupae appeared healthy or diseased by the absence/presence of signs of necrosis or deformation/damage. Both pupae and adult workers were weighed individually. The intertegular distance (ITD), which is the distance between the insertion points of the wings (Cane, 1987) and a standard measure of body size, was measured for adult workers using a digital caliper. It was noted whether the adult workers missed any body parts. The body mass and ITD values obtained from bees with

missing body parts were excluded from the statistical analysis of biological measurements.

2.4 Sample homogenization

Ten adult bees of each colony were pooled together. The same was intended with drone and worker pupae. However two colonies contained no cocoons whatsoever (both were from treated fields). From all the other colonies at least a pupae sample of either sex was taken and in four cases samples of both drone and worker pupae could be collected. This resulted in 44 drone and 22 worker pupae samples. Five worker pupae samples (four from treated fields, one from untreated field) contained seven to nine individuals, rather than ten.

The pooled samples were stored in a polyethylene mesh-bag (BioReba) and then mashed with a pestle. The use of bags in the sample homogenization ensured that pestles were not in direct contact with the bees, which prevents cross-contamination (de Miranda *et al.*, 2013). After mashing, 1 mL nuclease free (Milli-Q) water per bee was added and mixed manually until the suspension looked homogenous. The mesh within the polyethylene bag facilitated the creation and retrieval of a homogenous aqueous suspension. Four separate 1 mL aliquots of the suspension were taken, for microscopic examination, DNA extraction, RNA extraction and as reserve.

2.5 RNA extraction

100 µL of the bee suspension was mixed with 350 µL of Buffer RLT containing 1 % (by volume) of β-mercaptoethanol. The QiaCube and the RNeasy® Plant Mini Kit (Qiagen) were used for automated purification of the total RNA. The purified RNA was eluted in 50 µL nuclease-free water. The RNA concentrations were determined via UV/Vis spectroscopy using NanoDrop 1000. In one pupae sample (from a control field) a very low RNA concentration was yielded. It was assumed that this might have been due to clogging of the Qiagen column. In order to improve the extraction efficiency, the extraction was repeated with a lower volume of primary extract (25 µL). The extracted RNA from all samples was then stored at -80 °C until further analysis.

2.6 DNA extraction

The protocol for DNA extraction was derived from a protocol for extraction of DNA from *Nosema* spores (Fries *et al.*, 2013). 1 mL of the suspension was centrifuged (5 min, 13000 × g) and the supernatant was discarded. In order to destroy

Nosema spore walls, the pellet was repeatedly frozen by holding it into liquid nitrogen and crushed with a sterile teflon pestle. The pulverized pellet was then mixed with 400 µL Lysis Buffer AP1 and 4 µL RNase A (Qiagen, DNeasy Plant Mini Kit.). The mixture was then incubated and shaken for 10 min at 65 °C. Afterwards, 130 µL Neutralization Buffer P3 (3.0 M potassium acetate pH 5.5) was mixed with the lysate, which was then incubated for 5 min on ice. After centrifugation for 5 min at 14000 rpm, 500 µL of the supernatant was used for automated DNA extraction by QiaCube (Qiagen) using the DNAeasy® Plant Mini Kit following the standard protocol for purification of total DNA from plant cells and tissue. DNA concentrations were measured using NanoDrop 1000. For 25 (13 control, 12 treated) samples with particular low DNA concentration, the whole extraction process was repeated. In the repeated extraction a lower volume of extract was transferred to the Qiagen column to avoid clogging. The extracted DNA of all samples was then stored at -20 °C until further analysis.

2.7 Assays

2.7.1 Detection and quantification of target DNA / RNA

Quantitative polymerase chain reaction (qPCR) was used to quantify pathogen nucleic acid levels. For RNA targets, the qPCR was preceded by a Reverse Transcription step (RT-qPCR). Only the adult samples were analysed for pathogens with DNA genomes, while both adults and pupae were analysed for RNA viruses. Prior to running the assays, DNA samples were diluted to 2 ng μL^{-1} and RNA samples were diluted to 5 ng μL^{-1}. Samples were run in duplicate on two separate 96-well plates together with non-template controls, negative extraction controls (a mixture of buffers used for DNA/RNA extraction) and a series of seven to eight 10-fold dilutions of a positive control (standard) with a known concentration. These standards consisted of a plasmid clone of the target sequence, except for *Nosema bombi, Crithidia bombi, Snodgrassella alvi, Gilliamella apicola* and the RNA reference gene sequence Bt-RPL23, where PCR products were used as positive controls. These PCR products were purified using the E.N.Z.A.® Cycle Pure Kit and the Cycle Pure Spin Protocol (Omega Bio-Tek) and quantified via NanoDrop 1000. The size of each PCR product was confirmed using the Microchip Electrophoresis System MCE®-200 MultiNA (Shimadzu) and the purified PCR-Products were sequenced to verify the target sequence. The copy number per reaction of those standards was calculated as follows:

$$standard\ DNA/_{Rx} = \frac{[DNA_{standard}] * V_{reaction} * 6.022 * 10^{23}\ mol^{-1}}{dil.fac. * (157.9\ g\ mol^{-1} + 607.4\ g\ mol^{-1} * bp)}$$

$[DNA_{standard}]$ = Measured DNA concentration of a standard after purification
$V_{reaction}$ = Template volume per reaction
dil. fac = Dilution factor
bp = Number of base pairs in the amplicon of standards that are a PCR-product

The iScript™ One-Step RT-PCR Kit with SYBR® Green (Bio- Rad) was used for quantification of RNA viruses and the RNA reference gene. The master mix of the RNA assays contained 10 µL of iScript SYBR® Green RT PCR reaction mix, 6.8 µL of nuclease free water and 0.4 µL of each of the primers (forward and reverse) (Table 1) as well as 0.4 µL reverse transcriptase solution. The iScript SYBR® Green RT PCR reaction mix contains iTaq DNA polymerase, which converts RNA into complementary deoxyribonucleic acid (cDNA) and a dye that binds to any double-stranded DNA in the reaction mix. 18 µL of master mix was aliquoted to each well, to which 2 µL nucleic acid template was added. For the non-template controls 2µL of nuclease-free water was mixed with the master mix.

Table 1. *Forward (F) and reverse (R) primers used in RNA assays with literature reference to previous usage of these*

Target	Primers	Sequence '5-'3	Reference:
ABPV	ABPV-F6548 (F)	TCATACCTGCCGATCAAG	(Locke *et al.*, 2012)
	KIABPV-B6707 (R)	CTGAATAATACTGTGCGTATC	
BQCV	BQCV-qF7893 (F)	AGTGGCGGAGATGTATGC	(Locke et al., 2012)
	BQCV-qB8150 (R)	GGAGGTGAAGTGGCTATATC	
RPL23	Bt-RPL23 (F)	GGGAAAACCTGAACTTAGGAAAA	(Niu *et al.*, 2014)
	Bt-RPL23 (R)	ACCCTTTCATTTCTCCCTTGTTA	
CBPV	CBPV1-qF1818 (F)	CAACCTGCCTCAACACAG	(Locke *et al.*, 2012)
	CBPV1-qB2077 (R)	AATCTGGCAAGGTTGACTGG	
DWV	DWV-F8668 (F)	TTCATTAAAGCCACCTGGAACATC	(Locke *et al.*, 2012)
	DWV-B8757 (R)	TTTCCTCATTAACTGTGTCG	
LSV1	qLSV1-F2569 (F)	AGAGGTTGCACGGCAGCATG	(Runckel *et al.*, 2011)
	qLSV1-R2743 (R)	GGGACGCAGCACGATGCTCA	
LSV2	qLSV2-F1722 (F)	CGTGCTGAGGCCACGGTTGT	(Runckel *et al.*, 2011)
	qLSV2-R1947 (R)	GCGGTGTCGATCTCGCGGAC	
RNA250	RNA250 (F)	TGGTGCCTGGGCGGTAAAG	(Mondet *et al.*, 2014)
	RNA250 (R)	TGCGGGGACTCACTGGCTG	
SBPV	SBPV-F3177 (F)	GCGCTTTAGTTCAATTGCC	(De Miranda *et al.*, 2010)
	SBPV-B3363 (R)	ATTATAGGACGTGAAAATATAC	
SBV	SBV-qF3164 (F)	GCTCTAACCTCGCATCAAC	(Locke *et al.*, 2012)
	SBV-qB3461 (R)	TTGGAACTACGCATTCTCTG	

The DNA master mix contained 0.8 µL of the forward and reverse primer (Table 2), 6.4 µL of nuclease-free water and 10 µL of SsoFast™ EvaGreen® Supermix

(Bio-Rad). The latter consists of a dye that binds to double-stranded DNA (EvaGreen), DNA polymerases and a protein that stabilizes polymerase-template complexes.

Table *2*. *Forward (F) and reverse (R) primers used in DNA assays with literature reference to previous usage of these*

Target	Primers	Sequence '5-'3	Reference:
AmFV	AmFV-BroN (F)	TTATTAACACCGCAGGCTTC	(Hartmann et al., 2015)
	AmFV-BroN (R)	CATGGTGGCCAAGTCTTGCT	
C. bombi	Cbombi-GADH-F2 (F)	CAAGAGCTCGCCGGGT	This work
	Cbombi-GADH-R2 (R)	GGACGCGTTCGACACC	
N. apis	Napis-qF3 (F)	TAGTATATTTGAATATTTGTTTACAATGG	This work
	NosUniv-qR3 (R)	CGCTATGATCGCTTGCC	
N. ceranae	Nceranae-qF3 (F)	GTATGTTTGAATAATTATTTATTTTATTG	This work
	NosUniv-qR3 (R)	CGCTATGATCGCTTGCC	
N. bombi	Nbombi-qF3 (F)	TAGTATGTTTGAATATTTATTTATTACGA	This work
	NosUniv-qR3 ®	CGCTATGATCGCTTGCC	
G. apicola	Gilliam 16S (F)	GTAACATGAGTGCTTGCACT	This work
	Gilliam 16S (R)	CGCATGGCCCGAAGG	
S. alvi	Snodgras 16S (F)	ACGGAGAGCTTGCTCTC	This work
	Snodgras 16S (R)	AAATAACGCGAGGTCTTTCGA	

The RT-qPCR of the RNA assays starts with 10 min of cDNA synthesis at 50 °C. Afterwards the temperature was increased to 95 °C for 5 min to inactivate the reverse transcriptase. Afterwards, 40 amplification cycles were run. Each cycle consisted of 10 s at 95 °C for denaturation and 10 s at 58 °C for annealing of the primers and extension of the target sequence. After each cycle, the fluorescence emitted by the dye upon binding to double-stranded DNA was measured, quantifying the total amount of double-stranded DNA in the reaction mixture. The cycle number at which this fluorescence signal surpassed a certain detection threshold (Cq) was determined by the CFX Connect™ software running the thermocycler (Bio-Rad). The CFX Manager Software also constructs a standard curve based on (the logarithm of) the known starting quantities of the standards and their threshold cycle values (Fig. 2). The starting quantity (SQ) of the samples, which is the amount of target DNA/cDNA that was present in the reaction tube before amplification, is then inferred from this standard curve and the background fluorescence of the non-template-controls (Fig. 2).

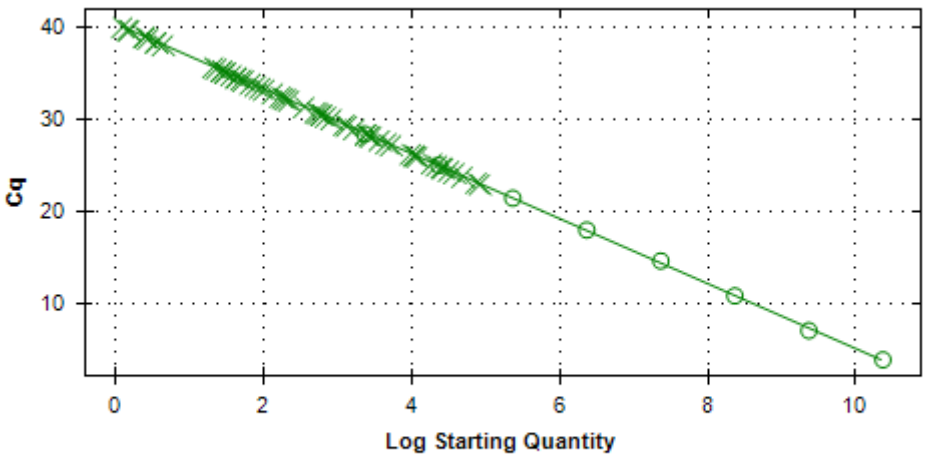

Figure 2. Standard curve of a *Crithidia bombi* assay. Standards are illustrated as circles and samples as crosses. The line represents the regression between the logarithmic starting quantities of the standards and their quantification cycle (Cq).

2.7.2 Melt curve analysis

Directly after completion of the 40 amplification cycles the temperature was held for an additional minute at 95 °C and another one at 60 °C. Then, the temperature was stepwise (0.5 °C every 5 s) increased from 65 °C to 95 °C, while fluorescence was measured. The CFX Manager Software (Version 2.1, Bio-Rad) generates from this, melt curves and determines melt peaks, which occur at the temperature where the negative first derivative of the fluorescence intensity is maximal (Fig. 3). Melting curves were evaluated manually in order to separate out non-specific amplifications, which differ from the target cDNA/DNA fragment. These were deleted from the data set.

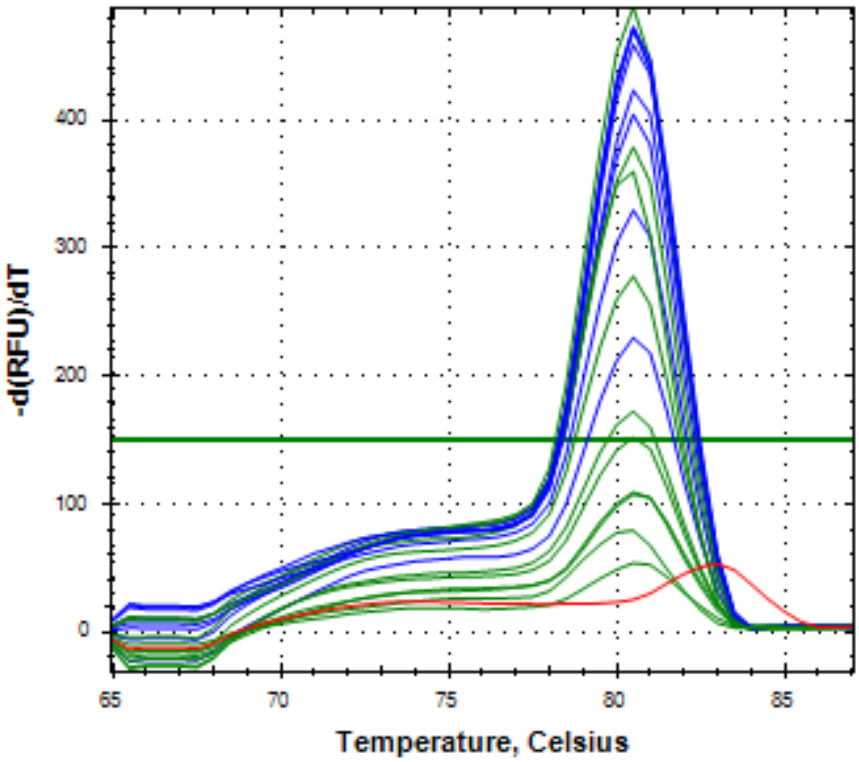

Figure 3. The graph shows a section of melt peaks of an Acute bee paralysis virus (ABPV) assay. Standards are represented in blue, positive samples in green and a negative sample is shown in red.

Each assay was run in duplicate, such that the means of the individual SQ values (SQ_{mean}) were used in the statistical analyses. Both duplicates have to yield a positive quantitative value and pass the Melting Curve test for non-specific products to be included as a positive result in the data set. Failure of one or both assays in these tests results in a negative result (non-detection) for the sample.

The qPCR protocol for the DNA assays was identical to the RT-qPCR protocol for the RNA assays described above, except that the reverse transcription step was omitted and that the annealing-extension temperature was 60 °C rather than 58 °C.

2.8 Estimation of target DNA/RNA amount per bee

Based on the previously obtained SQ_{mean} values, the amount of target nucleotide per bee was estimated. The number of RNA copies per bee was calculated as follows:

$$target\ RNA/_{bee} = \frac{SQ_{mean} * dil.fac.}{V_{reaction} *\ cDNA\ efficiency} * \frac{V_{eluted}}{V_{sampled}} * \frac{V_{total}}{n_{bee}}$$

$V_{reaction}$ = Template volume per reaction
V_{eluted} = Extract volume eluted by the Qiagen extraction robot
$V_{sampled}$ = Extract volume that was sampled from a bee suspension for extraction of nucleic acid
V_{total} = Total volume of bee suspension
n_{bee} = Number of bees in this suspension
dil. fac = Dilution factor

The cDNA efficiency represents the proportion of target RNA copies that was converted to cDNA before amplification. To estimate this individually for each sample, an assay was run in duplicate that contained, as well as the 2 µL of sample RNA, a known amount of a synthetic RNA (Ambion® RNA-250) plus corresponding primers in the master mix. The ratio of the copy number of RNA250 estimated by the RT-qPCR assay to the known number of copies RNA250 put into the reaction constitutes the cDNA conversion efficiency.

The number of DNA copies per bee was determined as described by the following equation:

$$target\ DNA/_{bee} = \frac{SQ_{mean} * dil.fac.}{V_{reaction}} * \frac{V_{eluted}}{V_{column}} * \frac{V_{digested}}{V_{pelleted}} * \frac{V_{total}}{n_{bee}}$$

$V_{digested}$ = Volume of in buffers re-suspended pellet
$V_{pelleted}$ = Volume of bee suspension that has been centrifuged to obtain a pellet for DNA extraction
V_{column} = Extract volume that was transferred to a column of the Qiagen extraction robot.

2.9 Normalization of target DNA/RNA amount per bee

The mRNA data was normalized with a constitutively expressed internal reference gene (Ribosomal Protein L23; RPL23). This is recommended for accurate determination of the relative expression of the target mRNA (Bustin *et al.*, 2010). The normalization corrects for differences in RNA degradation between the samples and inhibitors of the PCR reaction. The target RNA amount per bee was normalized by the concentration of the internal reference gene RPL23 as shown below:

$$normalized\ target\ RNA/_{bee} = target\ RNA/_{bee} * \frac{RPL23\ RNA_{average}/_{bee}}{RPL23\ RNA_{sample}/_{bee}}$$

RPL23 $RNA_{average}$/bee = Average copy number of the reference RNA per bee of all samples

RPL23 RNA_{sample}/bee = Number of reference RNA copies per bee of the sample.

DNA concentrations were normalized by the DNA concentration of the sample after purification in the Qiagen ([DNA_{sample}]) and the average concentration of all DNA samples ([DNA $conc_{average}$]):

$$normalized\ target\ DNA/_{bee} = target\ DNA/_{bee} * \frac{[DNA_{average}]}{[DNA_{sample}]}$$

This normalization step compensated for differences in DNA yield after purification due to the amount of bumblebee DNA in suspension and differences in extraction efficiency.

2.10 Statistical analyses

All statistical analyses were conducted using R version 3.1.2 (R Core Team, 2014). Null hypotheses were rejected based on p-values compared to a significance level of 5%.

2.10.1 Physical parameters

Adults

To test the effect of clothianidin exposure on the physical parameters body mass and intertegular distance a linear mixed effects model was used, which was implemented in R by the use of the lmer function of the lme4 package (Bates *et al.*, 2014). Linear mixed effects models are analyses of variance (ANOVA) that include random effects in addition to fixed effects (Hagenbuch, 2010). Random effects in this context are defined as having levels that are not of primary interest, but are considered a random sample of a larger population. Its terms represent the general variability of the levels rather than giving information on how population means of each level differ from each other, as it is the case for fixed effects (Seltman, 2012). Fixed effects are of primary interest and are estimated in the lmer function by maximizing the restricted log-likelihood (Bates *et al.*, 2014). The inclusion of random effects can control for non-independence of samples from the same experimental unit without inflating the Type I error rate by the specification of fixed effects that are not of importance for the research question (Hagenbuch,

2010). The model was chosen to reflect the hierarchical structure of the study design with the hives being nested in boxes, boxes being nested in fields and fields being nested in field pairs. All these were included as random effects in the model, while treatment (clothianidin or control) was included as a fixed effect. The model can be mathematically expressed as follows:

$$y = \mu + \alpha_i + d_i + f_{l(i)} + g_{m(il)} + h_{n(ilm)} + e_{ilmn}$$

With the following variables:

y = Response variable
μ = Intercept
α = Treatment
d = Field pair
f = Field
g = Box
h = Hive
e = Residuals

and the following indices for the levels of each variable:

i = Field pair (1-8)
l = Field (1-16)
m = Box (1-32)
n = Hive (1-64)

Due to deviations from normality, the body mass values for adults were transformed by taking the square root prior to fitting an lmer model. The model with intertegular distance showed normally distributed residuals; therefore no transformation was required. Confidence intervals of lmer estimates were determined based on least squares means using the lsmeans function of the lmerTest package (Kuznetsova *et al.*, 2015). Differences in least squares means were used to create confidence intervals for the difference between estimates. This was done, using the difflsmeans function of the same R package.

An ANCOVA was conducted to test how the combination of body mass and treatment affects the intertegular distance of adult bumblebees. Three different linear models were compared. The models describe the intertegular distance by

1. An interaction of treatment and body mass effects
2. Effects of both treatment and body mass, but without interaction
3. A body mass effect

An ANOVA of the fist two models was conducted to determine whether treatment changes the effect body mass (potentially) has on the intertegular distance of adult

bumblebees. Possible differences in a body mass-independent treatment effect were analysed by means of an ANOVA of the latter two models. An analogue ANCOVA was used to test whether ITD, treatment and an interaction of both influence the square root transformed body mass values of the adults.

Pupae

Effects on the body mass of pupae could not be examined with the model that was used for the adults, because both the observations and residuals were not normally distributed. Therefore a linear model based on ranks was used to study the effects on the body mass of individual pupae. The explanatory variables in this case were treatment (clothianidin and control), developmental stage (1-6) and sex (male and female).

The prevalence of physical disease signs was only evaluated for the first three developmental stages when the pupae's bodies are still white and signs of necrosis are easily visible. A log-logistic generalized linear mixed-effects model was used to analyse effects on visible disease signs. The glmer function of the lme4 packages was exploited for this purpose (Bates *et al.*, 2014). The model included sex and developmental stage as fixed effects in addition to the explanatory variables used in the linear mixed effects model shown above.

2.10.2 Microorganism prevalence

Microorganism presence was not determined for individual bees but only for a pooled sample of ten bees. Therefore the sample size of the microbial data was much smaller than for the physical data. Small sample sizes can introduce bias in multilevel (nested) and mixed effects models (Harrell *et al.*, 1996; Bell *et al.*, 2010; Maas & Hox, 2005). Estimates of variables with binary response cannot be accurately determined if the less frequent outcome is only 10 (or 20 times) larger than the number of regression coefficients (Harrell *et al.*, 1996). Therefore a two-sided z-test (or Pearson's Chi-squared test) rather than a generalized linear mixed-effects model was used to determine the effects of clothianidin exposure on microorganism prevalence in bumblebees. The test was used to identify confidence intervals for the difference between the clothianidin-exposed and the control colonies. Confidence intervals of the proportion of one group (either clothianidin or control) were derived from a binomial test. Pearson's Chi-Square test may be inaccurate when the expected values (control proportions) are small (McDonald, 2014). Therefore we used the Fisher's exact test to determine whether relevantly different p-values were obtained when testing for a treatment effect on the proportion of infected colonies. This was not the case for any of the microorganisms that we detected, therefore only the Pearson's Chi-Squared p-values are reported.

2.10.3 Effects of microorganism prevalence on colony development parameters

Rundlöf *et al.* (2015) found a significant treatment effect on queen production and suggested that pathogen prevalence may have added noise to the data, since this had not yet been determined. The effect of treatment and microorganism prevalence on the colony development parameters, queen production (the number of newly hatched queens), size of the worker force (number of worker bees in the colony) and colony strength (number of adult bees) were tested. For that purpose one-way ANOVAs with either treatment or the prevalence of a specific microorganism as predictors were conducted. Interactions between microorganism prevalence and treatment were determined by means of two way ANOVAs.

2.10.4 Interaction between *Snodgrassella alvi* and pathogen prevalence

A negative interaction between the abundance of Betaproteobacteria and *Crithidia bombi* infection rate and load has been suggested (Koch & Schmid-Hempel, 2011). In addition it was claimed that *Snodgrassella alvi* can stimulate the immune system of bees and that this can lead to a reduction in virus loads (Katsnelson, 2015). Therefore the relation between the prevalence of the Betaproteobacterium *Snodgrassella alvi* and all detected pathogens was studied using log-logistic models. The models explained pathogen prevalence by *S. alvi* prevalence.

2.10.5 Microorganism loads

Effects on the microorganism loads were only tested for the bacteria *Gilliamella apicola* and *Snodgrassella alvi* and the protozoan *Crithidia bombi,* because all other microorganisms were detected too infrequently to allow for analysis of the quantitative data, due to excessive inflation of the data-set by zero (non-detection) values. Wilcoxon signed-rank tests were performed to examine treatment effects on the loads of these three microorganisms.

3 Results

3.1 Physical parameters

3.1.1 Adults

A linear mixed-effects model showed that adult body mass values did not significantly differ between bumblebees of the control group and those of the clothianidin group ($p = 0.23$). However, the intertegular distance was significantly larger for the control bees than for clothianidin-exposed bees (Fig 4, $p = 0.026$). The estimated difference was 0.26 mm (95%-Confidence interval: 0.03 mm – 0.49 mm).

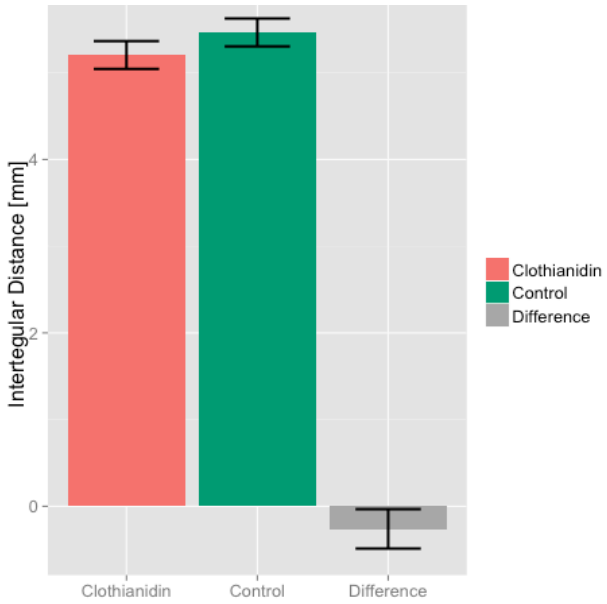

Figure 4. Estimates and confidence intervals for the mean intertegular distance of the clothianidin-exposed bumblebees (red), bees of the control group (green) and the difference between the two groups (grey; Clothianidin – Control; p-value = 0.026)

As expected, the intertegular distance (ITD) of adult bumblebees increased with their body mass, since both are measures of bumblebee size. The degree with which it did this differed between the control and clothianidin-exposed group ($p = 0.0002$). Clothianidin exposed bees showed a lower increase in ITD with increasing body mass than bees of the control fields did (Fig 5). The difference in increase was 2.0×10^{-3} mm×mg^{-1} (confidence interval: 9.7×10^{-4} – 3.1×10^{-3} mm×mg^{-1}). The linear regressions between intertegular distance and body mass differed also significantly in their intercepts between the control and the clothianidin-exposed bees ($p = 0.0046$). The intercept of the control group was smaller. This shows that light clothianidin-exposed bees tended to be larger in their intertegular distance than control bees. Among heavy bees the reverse was true (Fig. 5). ITD and body mass were not perfectly proportional to each, as suggested by an R-squared-value of only 0.52, neither were the square root transformed body mass values and the ITD values (R-squared = 0.49). The square-root transformed body mass values could be explained by a single regression with ITD as explanatory variable as the treatment did not significantly affect the intercept (p-value = 0.31) nor the slope (p-value = 0.18).

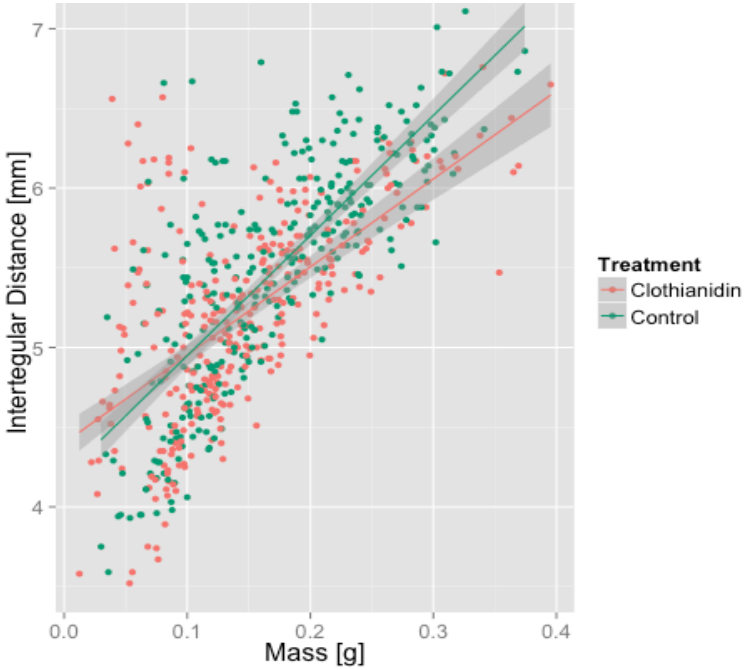

Figure 5. Linear regressions between the intertegular distance and body mass of individual bumblebees of both treated (red) and untreated (green) fields. 95%-confidence intervals are illustrated in grey. Regression coefficients: Control-intercept = 4.19; Control-slope = 7.54; Clothianidin-intercept: 4.40; Clothianidin-slope = 5.53

3.1.2 Pupae

The body mass of the pupae was highly significantly affected by their developmental stage, their sex and the treatment ($p < 0.0001$). On average, pupae from control fields were heavier than the ones from treated fields and males were heavier than females. There was a gradual decrease of body mass with an increase of developmental stage with the exception of stage 4 ($p = 0.19$). This is also the only developmental stage that did not differ significantly from stage 1 in pupal body mass. Pupae were heaviest at stage 1.

Pupae at the first stage showed also significantly more frequently physical signs of disease than pupae at developmental stage 2 ($p = 0.0002$) or stage 3 ($p < 0.0001$). The later stages were excluded from this analysis as signs of necrosis signs are difficult to see on darkened pupae bodies. There was no significant difference in the frequency of necrosis between the two sexes ($p = 0.084$) or between the control and treated groups ($p = 0.92$).

3.2 Microorganism prevalence

3.2.1 Adults

Symbiotic bacteria

The symbiotic gut bacteria *Gilliamella apicola* and *Snodgrassella alvi* were present in a majority of the colonies. *Gilliamella apicola* was detected in 91% of both the control and the treated group (Fig. 6). *Snodgrassella alvi* was also detected in 91% of the clothianidin group colonies, but in only 66% of the control group colonies (Fig. 7). The prevalence of *S. alvi* differed significantly between the two groups (Chi-squared-test, $p = 0.035$). The difference in absolute terms was 25% (confidence interval: 3% - 47%)

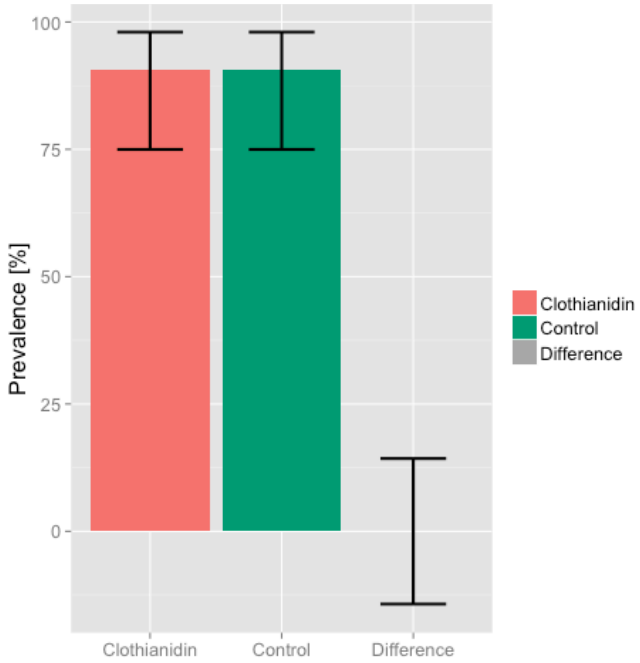

Figure 6. Proportion of colonies with *Gilliamella apicola* infected adults for the clothianidin-exposed (red) and control (green) groups and the difference between the two groups (Clothianidin – Control; p-value = 1). The error bars represent the 95%-confidence intervals.

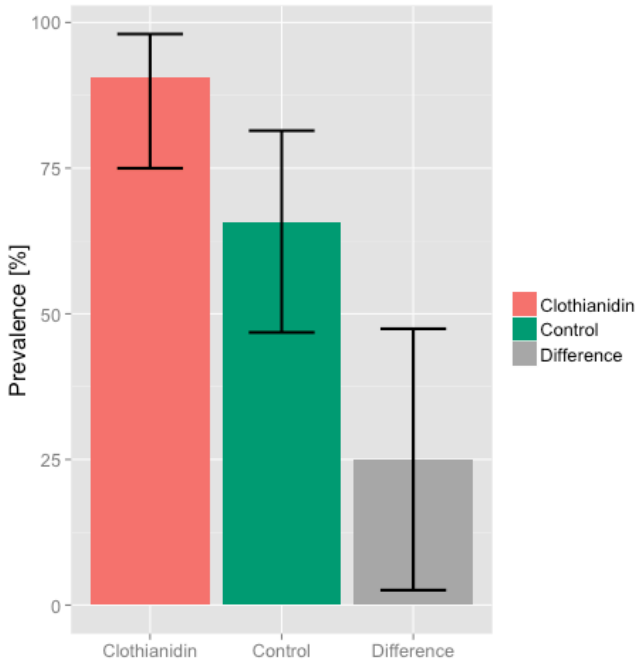

Figure 7. Proportion of colonies with *Snodgrassella alvi* infected adults for the clothianidin-exposed (red) and control (green) groups and the difference between the two groups (grey; Clothianidin – Control; p-value = 0.035). The error bars represent the 95%-confidence intervals.

Eukaryotic pathogens

The *Nosema* species *N. apis* and *N. ceranae* were not detected in any of the colonies. *N. bombi* however was detected in both the control (13%) and the treated group (3%; Fig. 9). The difference was not significant (Chi-squared-test, p-value = 0.35). *Crithidia bombi* was significantly more often found in control colonies than in colonies placed in clothianidin-treated fields (Chi-squared-test, p-value = 0.046). The difference between the groups amounted to 22% in absolute terms (Confidence interval: 1% - 43%). *C. bombi* was the most prevalent pathogen as it was detected in 72% of the clothianidin-exposed group and 94% of the control colonies (Fig. 8).

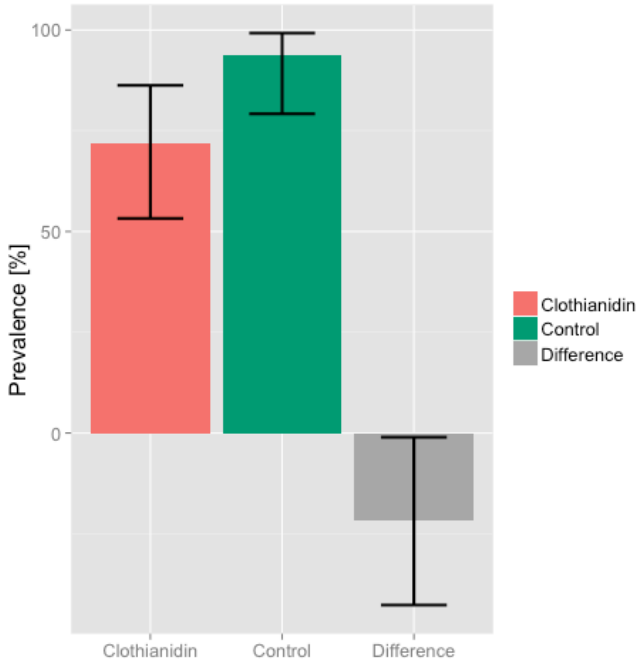

Figure 8. Proportion of colonies with *Crithidia bombi* infected adults for the clothianidin-exposed (red) and control (green) groups and the difference between the two groups (grey; Clothianidin – Control; p-value = 0.046). The error bars represent the 95%-confidence intervals.

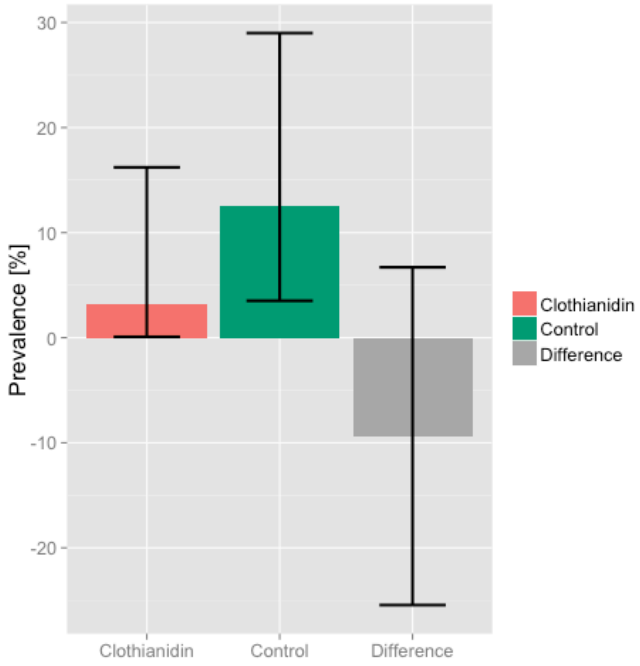

Figure 9. Proportion of colonies with *Nosema bombi* infected adults for the clothianidin-exposed (red) and control (green) groups and the difference between the two groups (grey; Clothianidin – Control; p-value = 0.35). The error bars represent the 95%-confidence intervals.

Viruses

Apis mellifera filamentous virus (AmFV), Black queen cell virus (BQCV), chronic bee paralysis virus (CBPV), deformed wing virus (DWV), lake Sinai virus 1 (LSV-1) and lake Sinai virus 2 (LSV-2) were not detected in any of the colonies. The Sacbrood virus (SBV) showed the highest prevalence among all viruses with 22% infected colonies in the treated group and 9% in the control group. Acute bee paralysis virus (ABPV) was found in adult bees of 3% of the clothianidin-exposed colonies and 13% of the control colonies. Slow bee paralysis virus (SBPV) was found in about 6% of the control colonies and twice as often in the treated group. However for none of the viruses were the differences between the treatment groups significant (p-values > 0.3).

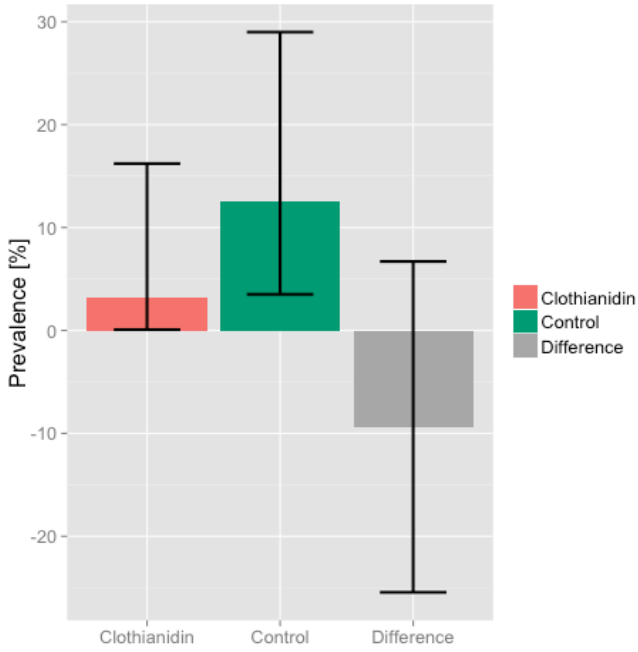

Figure 10. Proportion of colonies with Acute bee paralysis virus (ABPV) infected adults for the clothianidin-exposed (red) and control (green) groups and the difference between the two groups (grey; Clothianidin – Control; p-value = 0.35). The error bars represent the 95%-confidence intervals.

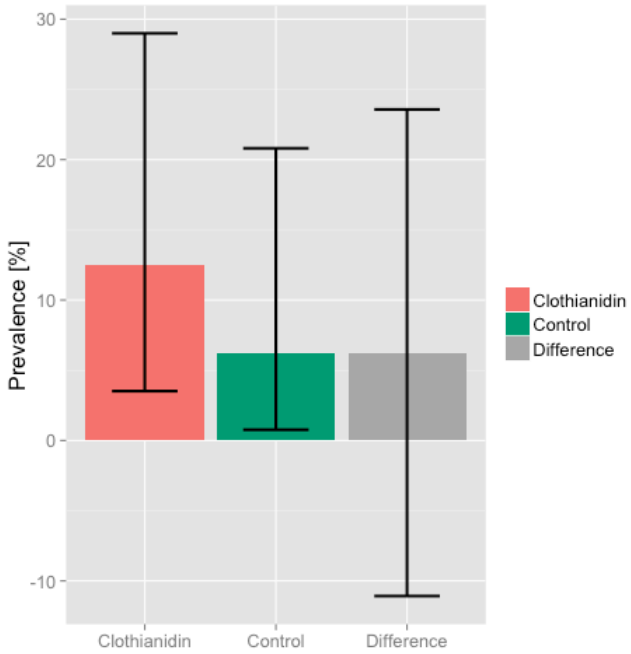

Figure 11. Proportion of colonies with Slow bee paralysis virus (SBPV) infection adults for the clothianidin-exposed (red) and control (green) groups and the difference between the two groups (grey; Clothianidin – Control; p-value = 0.67). The error bars represent the 95%-confidence intervals.

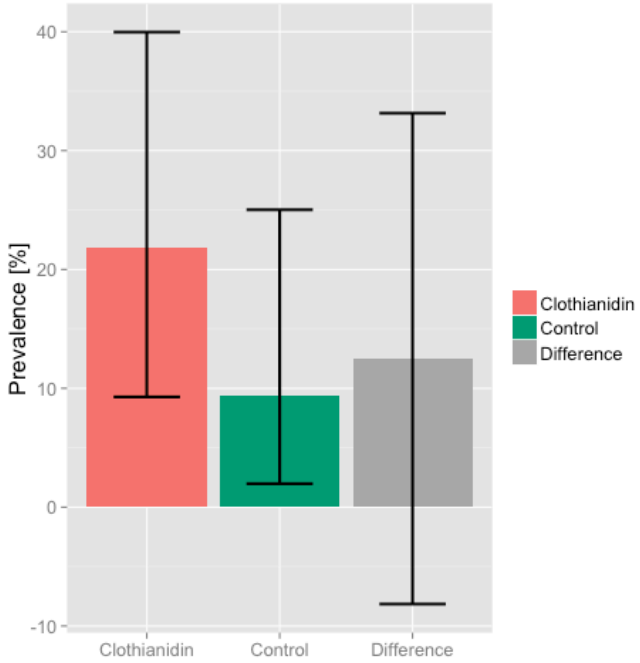

Figure 12. Proportion of colonies with Sacbrood virus (SBV) infected adults for the clothianidin-exposed (red) and control (green) groups and the difference between the two groups (grey; Clothianidin – Control; p-value = 0.30). The error bars represent the 95%-confidence intervals.

3.2.2 Pupae

ABPV was the only virus that was found in the examined bumblebee pupae, but only in two colonies from the control group (Fig. 13). Both colonies that contained ABPV-infected pupae had also ABPV-infected adults, while there were an additional 3 Control colonies that had only ABPV-infected adults.

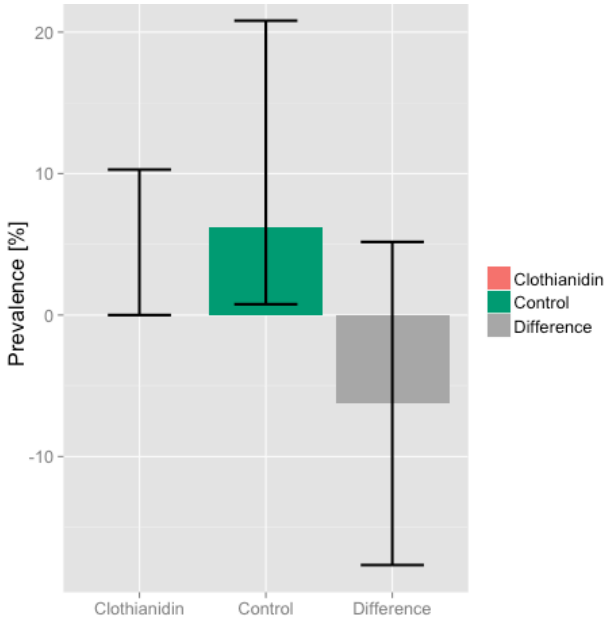

Figure 13. Proportion of colonies with Acute bee paralysis virus (ABPV) infected pupae for the clothianidin-exposed (red) and control (green) groups and the difference between the two groups (grey; Clothianidin – Control; p-value = 0.35). The error bars represent the 95%-confidence intervals.

3.2.3 Effects of microorganism prevalence and treatment on colony development parameters

The colony strength, the size of the worker force and the queen production were subjected to two-way ANOVAs having two levels of treatment (clothianidin and control) and two levels of microorganism prevalence (present, absent). These suggested two interactive effects of treatment and microorganism prevalence. The combination of clothianidin exposure and the absence of SBV was predictive for decreased queen production (p-value = 0.03).

Table 3. *Significant predictors (α = 0.5) of reductions in the colony strength, the number of workers and the queen production as shown by p-values*

Explanatory variable	Colony strength	Number of workers	Queen production
Clothianidin-Exposure	0.0041		0.0017
Nosema bombi-Presence		0.047	
Clothianidin : *Snodgrassella alvi* -absence		0.040	
Clothianidin : SBV-absence			0.032

One-way ANOVAs suggested that SBV presence alone had no significant effect on queen production (p-value = 0.87), but treatment alone did (p-value = 0.002). Colonies that were exposed to clothianidin and in which *Snodgrassella alvi* was absent showed a reduced size of the worker force (p-value = 0.04). Neither the microorganism alone (p-value = 0.28), nor the treatment (p-value = 0.38) was associated with significant effects on the size of the worker force.

3.2.4 Interaction between *Snodgrassella alvi* and pathogens

Snodgrassella alvi prevalence was a significantly negatively correlated with *Nosema bombi* prevalence (p = 0.046). However a log-logistic model that included treatment (clothianidin and control), *Snodgrassella alvi* prevalence and an interaction between the two as explanatory variables resulted in no significant effects (p = 1.00). *Snodgrassella alvi* prevalence could not significantly predict the prevalence of any of the other pathogens: *Crithidia bombi* (p = 0.37), ABPV (p = 0.96), SBPV (p = 0.80), SBV (p = 1.00). *Crithidia bombi* showed a weak trend towards a negative relation between *S. alvi* and *C. bombi*.

3.3 Microorganism loads

3.3.1 Treatment effect on microorganism loads

For most microorganisms, quantitative analyses of the amounts (loads) could no validly be analysed, due to the high frequency of non-detection and therefore the presence of too many zero values in the data and a highly distorted data distribution. Only *Snodgrassella alvi, Gillimaella apicola* and *Crithidia bombi* had a sufficient high detection frequency to allow quantitative analysis of the microorganism loads.

The median normalized loads of both symbiotic bacteria were higher in the clothianidin treated colonies than in the control group (Fig. 14). The difference between the two treatment groups was significant for *Snodgrassella alvi* (Wilcoxon-signed test, p-value = 0.037) but not for *Gilliamella apicola* (Wilcoxon-signed test, p-value = 0.29). The mean normalized loads were almost two orders of magnitude higher for *G. apicola* (2.1×10^6) than for *S. alvi* (7.4×10^4). The mean normalized load of *Crithidia bombi* (5.9×10^6) was even slightly higher than the one of *G. apicola*. In contrast to the two bacteria, *C. bombi* showed lower normalized loads in the clothianidin-treated colonies than in the control colonies (Fig. 15). The difference between the two groups was significant (p-value = 0.007).

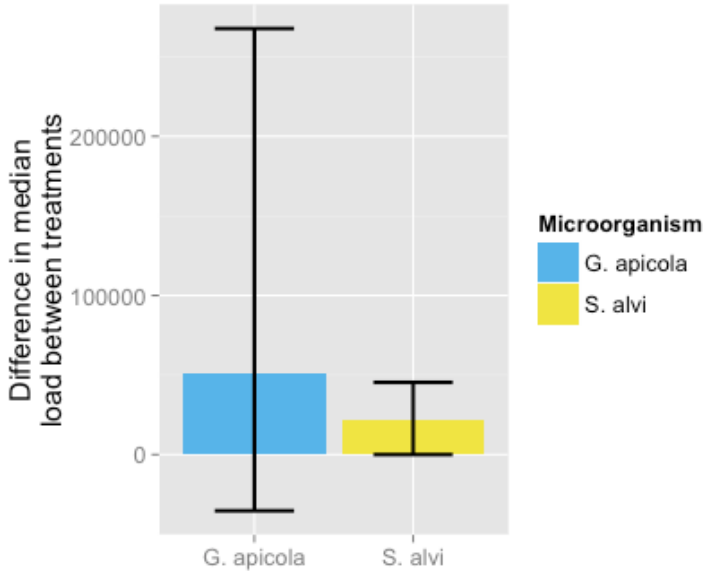

Figure 14. Comparison of the differences in median normalized loads between the clothianidin-exposed colonies (+) and the control colonies (-) of *Gilliamella apicola* (blue) *and Snodgrassella alvi* (yellow). Normalization was done by multiplying with the sample's DNA concentration after extraction and dividing by the average DNA concentration of all samples. The error bars represent 95%-confidence intervals (*Gilliamlla apicola*: p-value = 0.29; *Snodgrassella alvi:* p-value = 0.037).

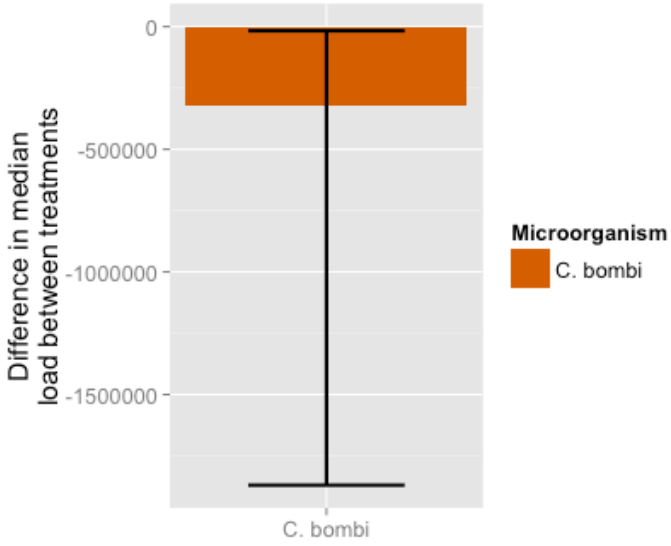

Figure 15. Illustration of the difference in median normalized loads between the clothianidin-exposed colonies (+) and the control colonies (-) of *Crithidia bombi* (p-value = 0.007). Normalization was done by multiplying with the sample's DNA concentration after extraction and dividing by the average DNA concentration of all samples.

4 Discussion

4.1 Methods

4.1.1 Field experiment

Replication of clothianidin-treated and control fields

The neonicotinoid class of insecticides has been heavily implicated with declines in bee populations, largely because of detrimental effects that were found in laboratory experiments (Blacquière *et al.*, 2012; Whitehorn *et al.*, 2012). It was only recently that a field experiment could unambiguously show neonicotinoid effects on colonies with free-foraging bees (Rundlöf *et al.*, 2015). The provision of bumblebees from colonies of this experiment represented a unique chance to study the effect of field exposure of neonicotinoids on the microflora of bumblebees.

In field studies there are many covariates that cannot be controlled technically, including climatic conditions, pesticide exposure from the surroundings and flower choice of the subjects (EFSA, 2013). The pairing of replicating landscapes reduced the variability of these covariates between the experimental groups (control and clothianidin-exposed). The radius of the examined surroundings (2 km) seems appropriate, since *Bombus terrestris* typically forage within a radius of less than 1.75 km (Walther-Hellwig & Frankl, 2000; Osborne *et al.*, 2008), although they can also forage in a distance of up to 4 km from their hives (Walther-Hellwig & Frankl, 2000). Palynological analyses confirmed that the bumblebee colonies of the present experiment fed primarily on oilseed rape pollen (Rundlöf *et al.*, 2015). Analyses of honeybee and bumblebee collected plant residues confirmed the significantly different clothianidin exposure of bees placed at treated and untreated oilseed rape fields.

Lack of determination of temporal development of microbe levels

Rundlöf *et al.* (2015) aimed however not primarily at identifying effects on the microflorae of the experimental colonies. Therefore, the prevalence and loads of pests and beneficial bacteria were not determined prior to the experiment, as was done for the honeybee colonies, or better still, at various intervals during exposure. Hence, it could not be identified how the microflorae of the colonies changed over time, nor could be ensured that the control and treatment colonies had the same levels of the examined species prior to the placement in the oilseed rape fields. Recording changes with increasing exposure time would have been beneficial for the scope of this study. However, sampling bees causes disturbance of the colonies, that may affect colony functioning, and likely more so for bumbleee colonies than for honeybee colonies due to the smaller colony size. Sampling feces rather than bees prior and during placement would entail less sacrifice of bees and be more neutral to colony development. In particular the quantitative data obtained through faeces would however be difficult to compare to the data obtained from whole bees, nor would there be any way of knowing the (cumulative) age of the faeces. Faeces must be frozen soon after defecation to avoid degradation of nucleic acids (Evans *et al.*, 2013), which may not always be feasible. In addition mRNA may be degraded within the host and therefore not be detectable in the faeces. It may also be difficult to ensure that the faeces come from an equal number of bees as for example *Nosema* infected bees may produce larger volumes of faeces within the hive (Fries *et al.*, 2013). This may also lead to an overestimation of loads of *Nosema* species and associated viruses (e.g. BQCV).

Considering these drawbacks of using faeces as samples, we think it would have been best to collect a few cocoons and adult workers from each hive before placement of the colonies in the fields and once during the flowering period. This would not have discriminated any colony as they started with an approximately equal number of workers (50), and an equal number of samples would have been taken. This could have been complemented by the analysis of queen faeces to test whether differences in brood production are linked to differences in the microflorae of the queens.

Sample selection

The colonies were left in the oilseed rape fields between 23 and 38 days. The development time of *B. terrestris* workers varies with the time of the year, mostly due to differences in temperature and feeding rates (Duchateau & Velthuis, 1988). It usually takes around three weeks, but up to four weeks for workers to develop from egg to adult (Duchateau & Velthuis, 1988; Goulson, 2003). We can therefore assume that most of the pupae sampled here were exposed throughout their lifetime to pollen collected from the adjacent oilseed rape fields. We were unable to

determine the age of adults and could therefore not estimate to what extent the adults had been exposed during their brood stage to the oilseed rape pollen collected from the treated or untreated fields. *B. terrestris* foragers live around 3 weeks (ZONDA). "Nest-bees" live a less dangerous and therefore longer life (Duchateau & Velthuis, 1988; Goulson, 2003) of up to 12 weeks (ZONDA). Due to the shorter lifespan and the fact that very young bees usually do not forage, we could assume that all foragers are approximately of the same age and that they are therefore exposed to the oilseed rape at a similar proportion of their life time. In contrast "nest-bees" may have been exposed to the oilseed rape at very different stages of their life. Since bees were collected from the hives after freeze-killing the colonies, it was not possible to distinguish between "nest-bees" and foragers. "Nest-bees" and foragers are also differently exposed to pesticides and pathogens. For example foragers are likely exposed more to airborne pesticides and they consume more nectar due to their larger body mass and energy use (Goulson, 2003). However, "nest-bees" that feed the brood regurgitated large amounts of food, in particular pollen (Pendrel & Plowright, 1981) and expose themselves in such a way to pesticides. Foraging brings bees inevitably in contact with the environment outside the hive, which poses the risk of the uptake of pathogens that are not found in the bee hive (Cremer *et al.*, 2007). However "nest-bees" are constantly in contact with potential disease vectors, their nest-mates (Cremer *et al.*, 2007; Erler *et al.*, 2012). Marking foragers could have enabled the selection of e.g. foragers as samples or the inclusion of task (caste) as an additional factor, as well as distinguish between bumblebees born in the nest and those drifting from other nests. Whether the possible gain in sample homogeneity would have been worth the extra effort required is not clear. The marking of foragers would have also allowed for the monitoring of changes in the proportion of workers that engage in foraging (Gill & Raine, 2014). This might have helped to identify mechanisms by which neonicotinoids affect bees.

A sample of ten bees per colony is reasonably representative of a total colony size of about 400 bees. With ten bees there is a 50 % chance to detect any given microorganism if only 6.6 % of the bees of a colony are infested (Pirk *et al.*, 2013). Bees of a colony interact heavily and likely transmit microbes easily to their nest mates (Cremer *et al.*, 2007). A share of 20 % infected bees, results already in a detection probability of 90 % as the probability of detection increases exponentially with a linear decrease in the proportion of uninfected bees (Pirk *et al.*, 2013). The stated values above are for an assumed detection efficiency of 100 %. The use of eight replicates of control and clothianidin-exposed colonies (i.e. field-pairs) should level out differences in the sample composition of foragers and "nest-bees", if the colonies do not vary in their composition themselves (i.e. due to a treatment effect).

4.1.2 Laboratory analyses

Detection and quantification of microbes using pPRC

The amounts of all microorganisms were quantified using quantitative Polymerase Chain Reaction (qPCR). This is an established method for the detection and quantification of microbes found in bee bodies, including RNA viruses (de Miranda *et al.*, 2013), bacteria (Forsgren *et al.*, 2013) and *Nosema* species (Fries *et al.*, 2013). The method uses laser optics to detect a florescence signal that increases with the quantity of target DNA sequence. Based on the cycle number when the signal becomes detectable, the initial quantity of the target sequence can very accurately determined (Evans *et al.*, 2013). There are two different types of markers that can be used in qPCR: Non-discriminatory reporters like SYBR Green that bind to any double stranded DNA and specific markers such as TaqMan that are designed to bind only to the target sequence. SYBR Green markers are inherently more universally applicable and do not require complex adaptations to new targets as TaqMan reporters do (de Miranda *et al.*, 2013). The use of SYBR Green markers allowed us to use the same marker for all the assays and design new primers for our purposes (Table 1&2). In comparison to TaqMan, SYBR Green is less prone to false negative findings and more susceptible to false positive findings (Evans *et al.*, 2013). We minimized the risk of false detections by stringent exclusion of observations with melting curves that differed significantly in shape and/or melt peak temperature from those of the positive controls. In addition we ran all assay plates in duplicate and counted the sample as positive only if both plates detected the target microorganism, and the target was confirmed by melt curve analysis. This conservative approach revealed apparent differences in sensitivity between the plates for several RNA assays (ABPV, SBPV, SBV), leading to the elimination of a number of data points. Those detections occurred typically within the last few amplification cycles, which are particularly susceptible to false-positive detection errors (Evans *et al.*, 2013; de Miranda *et al.*, 2013). Running all samples on the same plate ensured that samples were not discriminated by plate-to-plate variability. The fact that the non-template controls of all assays were negative suggests that the reagents used in the experiments were free of contamination (Bustin *et al.*, 2010).

Reverse transcription of target RNA

In order to quantify RNA, it has to be converted to complementary DNA (cDNA) prior to the qPCR. The reverse transcription (i.e. the synthesis of cDNA) and the qPCR can be done in two separate reactions (Two-Step RT-qPCR) or in a single reaction tube (One-Step RT-qPCR). We conducted a One-Step RT-qPCR, which is less prone to contamination than the two step equivalent (de Miranda *et al.*, 2013).

A Two-Step RT-qPCR however allows for better control of differences in cDNA efficiency between reactions. Reverse transcription is generally the most variable step in a RT-qPCR, as the synthesis of cDNA is affected by the reaction conditions, the presence of inhibitors and the quantity of nucleic acid (de Miranda *et al.*, 2013). We therefore controlled for sample-specific differences in cDNA efficiency by determining the cDNA efficiencies of all samples using an RT-qPCR assay for a synthetic RNA (RNA-250) included in reaction buffer in precisely known amounts, and compared this to the starting quantities of RNA-250 estimated for each sample by the RT-qPCR. We controlled statistically for those differences. In addition we controlled for differences in RNA degradation between the samples and inhibitors of the PCR reaction by the use of an internal reference gene. The chosen reference gene (Ribosomal Protein L23; RPL23) has been shown to be comparatively stable between Israeli acute paralysis infected and uninfected *Bombus terrestris* samples (Niu *et al.*, 2014). The RPL23 assay was the last RNA assay that was conducted. The fact that the reference gene was found at similar levels in all RNA samples shows that the sample processing and RNA extraction protocols were uniformly conducted and with minimal differential RNA degradation between the samples and therefore unlikely to be cause of the numerous negative results from the RNA pathogen assays. This indicates that the negative results represent true absence of these pathogens in the samples.

DNA extraction

The quantified DNA was normalized by the samples' DNA concentrations after extraction and purification in the Qiagen extraction robot. This allowed us to diverge slightly from the extraction protocol for 25 DNA samples (13 control, 12 treated). For those samples, a second extraction was conducted involving a smaller volume of extract transferred to the Qiagen columns, because the initial extraction resulted in low quantities of extracted DNA. Suboptimal DNA yields are often due to excess sample loading of the extraction columns (Qiagen information leaflet), such that often lowering the amount of applied extract results in higher DNA yields. As we statistically control for the amount of extracted DNA and diluted the DNA to uniform concentrations prior to qPCR, we think this divergence from the protocol does not reduce the comparability of the results in any way. The used protocol has been designed for the detection of *Nosema* species (Fries *et al.*, 2013). It involves an initial pelleting of the bee suspension, in order to crush Nosema walls in a subsequent step. The compression of bee material may have hampered the following lysis and therefore caused clogging of the Qiagen DNeasy columns, resulting in low yields (Qiagen, 2012). The fact that for most samples a higher yield was achieved when a smaller volume of the extract was transferred to a DNeasy column supports the assumption that clogging occurred. The low con-

centrations of purified DNA may have slightly decreased the sensitivity of the subsequent qPCRs. Therefore we recommend paying special attention to complete lysis in future experiments. To ensure this the ratio of lysis buffer to substrate may be increased (Qiagen, 2012). In addition DNA yields may be increased and differences in extraction efficiency may be decreased by using a more standardized semi-automated sample homogenization method, as sample homogenization is considered the most variable step in sample processing (Evans *et al.*, 2013; de Miranda *et al.*, 2013). Bead-mill homogenizers are known to homogenize bee samples more uniformly than the manual use of pestles (de Miranda *et al.*, 2013).

Although each microbial species may have its own obtimal DNA extraction protocol, the fact that all non-*Nosema* species were detected in the DNA assays suggests that the extraction protocol we used was reasonably suitable for the detection of all species that we targeted.

4.2 Results

4.2.1 Physical parameters

Clothianidin-exposed pupae weighted significantly less than pupae of the control colonies. For the adults the difference in body mass between exposed and control bees was not significant. However clothianidin-exposed adults were smaller as measured by their intertegular distance (ITD) than bees from the control fields. The difference in ITD between the two treatment groups (clothianidin and control) seemed furthermore to be most prevalent among heavy bees. The maximum ITD seemed also to differ between the two groups. Both a generally reduced ITD and a reduced pupae body mass can be ascribed to reduced feeding. The size of bumblebees is directly proportional to the amount of food they get during their brood stages (Goulson, 2003). The size of emerged adults and the body mass of pupae is therefore determined by the sister's efforts to rear them (Goulson, 2003).

Neonicotinoid-exposure of bumblebees has been associated with a negligence of brood (Gill *et al.*, 2012) and an impairment of foraging ability (Gill *et al.*, 2012; Laycock *et al.*, 2012; Feltham *et al.*, 2014). In particular, pollen was collected in smaller amounts (Gill *et al.*, 2012; Feltham *et al.*, 2014; Gill & Raine, 2014) and at a lower frequency (Feltham *et al.*, 2014). Reduced brood-care was ascribed to a larger proportion of workers that go forage, either to compensate for a reduced colony-level foraging efficiency or due to a neuronal change leading to a greater desire to forage (Gill & Raine, 2014). An addictive effect of neonicotinoids in nectar on bumblebees has also been suggested (Kessler *et al.*, 2015).

An increased tendency of neonicotinoid-exposed workers to forage and to feed themselves may explain why no significant weight difference between the bees of

the clothianidin-treated and control fields were observed among adults. Even an impairment of foraging behaviour without a neurologically induced tendency to feed themselves rather than their young could describe those findings. Malnourished adults may simply put preference on covering their own nutritional demands over feeding their brood.

Bumblebees rear their young in different sizes for different tasks. Foragers tend to be larger than workers that mostly do in-hive tasks (Goulson, 2003). It is possible that if food is not available in sufficient amounts, bees avoid rearing very large pupae. This would explain why the maximum ITD of clothianidin-exposed colonies was reduced. Even if all pupae are equally food-deprived the phenomenon could be ascribed to a lack of feeding. The apparent lack of a difference in minimum ITD, despite a difference in maximum ITD, may be because pupae that don't receive a minimum of food are just not viable. This in combination with a recuperation of the reduced weight gain may also explain why the ITD of clothianidin-exposed bees was particularly reduced in heavy bees. This holds in particular true if clothianidin exposed foragers do not feed themselves less than their counterparts from the control fields, but bring less food to their colonies due to a reduced foraging ability. A selective effect on heavy bees may also be ascribed to different median ages of "nest-bees" and foragers. Workers that do the hazardous task of foraging die typically earlier than bees working inside the hive (Duchateau & Velthuis, 1988; Goulson, 2003). This means the heavier foragers may have more likely been exposed during their brood stages to the insecticide than "nest-bees". Malnutrition caused by an impaired foraging behaviour is only one possible explanation for the observed differences in physical parameters between clothianidin-exposed and unexposed bees. Another possible explanation could also be reduced digestion efficiency in clothianidin-exposed bees or even effects unrelated to nutrition.

4.2.2 Pathogen prevalence

Three of the nine targeted honeybee viruses were detected:

- Sacbrood virus (SBV)
- Slow bee paralysis virus (SBPV)
- Acute bee paralysis virus (ABPV)

The highest prevalence was found for SBV (16%). This virus has been detected in *B. terrestris* before, but at a lower prevalence (McMahon *et al.*, 2015) as well as in other bumblebee species (Manley *et al.*, 2015; McMahon *et al.*, 2015). The prevalence of ABPV and SBPV in the present study are comparable to other studies (Manley *et al.*, 2015; McMahon *et al.*, 2015). ABPV was the only RNA virus that was also detected in the pupae. This may illustrate the functioning of the social immune system, that aims at minimizing pathogen contact of brood and queen

(Cremer *et al.*, 2007). Surprisingly Deformed wing virus (DWV) and Black queen cell virus (BQCV) were not detected in any of the samples, although they are typically more prevalent in adult bees than the viruses that were detected (Evison *et al.*, 2012; Fürst *et al.*, 2014; Manley *et al.*, 2015). On the other hand it is not surprising that the virus prevalence in our study differs from broad national wild bumblebee surveys of other countries, in which the colonies are spatially separated and of different origin. Furthermore, the prevalence of these honeybee viruses in bumblebees is often linked to high titres in local honeybee colonies (Singh *et al.*, 2010; Fürst *et al.*, 2014), while the honeybee colonies used in these experiments were largely free of DWV, ABPV and SBPV (Goss, 2014). All the colonies we used in our experiment were commercial colonies from the same supplier. Commercial colonies have been shown to differ in pathogen prevalence from wild colonies (Colla *et al.*, 2006; Otterstatter & Thomson, 2008). Mostly, commercial bumblebees showed elevated levels of pathogens such as *Crithidia bombi* and *Nosema bombi* (Colla *et al.*, 2006; Graystock, Yates, Evison *et al.*, 2013). Both pathogens were detected in this experiment and the prevalence of *Crithidia bombi* appeared exceptionally high with 72% in the clothianidin-exposed group and 94% in the control group. As the pathogen prevalence was not determined prior to placement in the fields, we cannot judge whether the colonies were already infested at the time of the purchase (Graystock, Yates, Evison *et al.*, 2013), they acquired the trypanosome parasite due to the relatively high density at the fields (Otterstatter & Thomson, 2008), a high prevalence among wild bees or a low genetic diversity of the commercial colonies (Whitehorn *et al.*, 2011).

4.2.3 Effects of clothianidin exposure on microorganism levels

The proportion of *Crithidia bombi* infected colonies differed significantly between the control and the clothianidin-exposed group, with a higher prevalence in control fields. A potential reason for this is a direct effect of the insecticide on the microsporidian parasite itself or on vectors of the parasite. *Crithidia bombi* has been shown to be transmitted between bees by a shared flower use (Durrer & Schmid-Hempel, 1994). Infectious cells can then be transported into colonies and be ingested by nest-mates. This main infection pathway of the parasite in bumblebees is very dependent on the number of vectors available, in particular the colony size (Erler *et al.*, 2012). *C. bombi* infection may therefore be reduced in the clothianidin-exposed colonies, because of a reduction in abundance of wild bumblebees or colony strength caused by the insecticide. This explanation is supported by the findings that in the clothianidin-exposed fields the wild bee density (Rundlöf *et al.*, 2015) and the colony strength were decreased.

A reduced number of vectors should however also affect the prevalence and loads of other *Bombus*-infecting species. The finding that the prevalence of none

of the viruses was affected by the treatment may be due to an increase in virus replication (Di Prisco *et al.*, 2013) in clothianidin-exposed bees that could have counterbalanced a reduced number of vectors. In addition, the prevalence of *Nosema* and (most) virus species was rather low. A higher virus and *Nosema* pressure may have resulted in more variation.

It is also possible that the combined stress of insecticide and parasite prevented infected colonies to return to their hives, while infected bees that were not additionally immune-challenged by the clothianidin treatment could return. Increased *Crithidia bombi* virulence in neonicotinoid-exposed bumblebees has been previously shown (Fauser-Misslin *et al.*, 2014). However we did not observe a significant difference in the number of workers between the clothianidin-exposed and the control group. Nevertheless, a potential effect on the homing behaviour of *C. bombi* and clothianidin challenged bumblebees cannot be excluded. The fact that the total number of bees was lower in clothianidin-exposed colonies than in control colonies, despite no significant difference in the size of the worker force, suggests that clothianidin-exposed bees may have still been compensating losses of workers. This may have prevented many clothianidin-exposed colonies to switch from the production of workers to reproductives (Rundlöf *et al.*, 2015). *Crithidia bombi* prevalence alone or in combination with treatment could not predict the number or workers. This again does not exclude a potential effect on the return rate of pesticide-parasite challenged bumblebees, as infected bees have to return to their hives for their colonies to be classified as *C. bombi* infected.

A treatment effect either on *C. bombi* itself or its vectors as well as a reduced detection due to a failure to return of pesticide-parasite challenged bees serve not only as explanations for the decrease in prevalence, but also in loads. An additional explanation for the increased *Crithidia bombi* loads and prevalence in the clothianidin-exposed colonies may be that the insecticide harms an antagonist of the parasite. In other studies *Crithidia bombi* loads and prevalence have been negatively associated with “core” gut bacteria of bumblebees (Koch & Schmid-Hempel, 2011; Koch & Schmid-Hempel, 2012; Cariveau *et al.*, 2014). Particularly, Betaproteobacteria, which include *Snodgrassella* species have been suggested to protect bumblebees from *Crithidia bombi* infections (Koch & Schmid-Hempel, 2011). Indeed we found that in contrast to *Crithidia bombi, Snodgrassella alvi* was more prevalent in in clothianidin-treated fields than control fields.

This suggests three scenarios: First, clothianidin benefits *Snodgrassella alvi* (e.g. through combatting any antagonist), which allows in turn *C. bombi* to flourish in the clothianidin-treated fields. Second, increased *Crithidia bombi* loads and prevalence in control fields drive *Snodgrassella alvi* reductions by a direct effect of the parasite on the bacterium. Third, increased *Crithidia bombi* levels in control fields put a pressure on bees that selects for bees with *Snodgrassella alvi* present.

The first two scenarios should result in a negative correlation between the protozoan and the bacterium, while the last scenario suggests that *C. bombi* infection would be positively associated with *S. alvi* infection. We failed to detect any significant effect, which may support the coincidence of different mechanisms. The weak negative association between the two microorganisms may indicate that any or both of the first two scenarios played a role and that this may have been masked by counteracting effects as for example the third scenario. This however remains speculative.

4.2.4 Effects on colony development parameters

The clothianidin-exposure was associated with a reduction in colony mass gain, queen production (Rundlöf *et al.*, 2015) and colony strength. A trend towards a reduction in the size of the worker force was also observed. The difference between the clothianidin-exposed and the control colonies was however not significant. The interaction of clothianidin-exposure and *Snodgrassella alvi* absence was however associated with a significantly reduced number of workers in a colony.

This suggests a protective role of *Snodgrassella alvi*. The gut bacterium *Snodgrassella alvi* may be able to counteract fitness losses caused by the insecticide. Bacteria living in the digestive tract of bees have shown to contribute to their hosts' nutrition in several ways. Gut bacteria can enhance digestion and the ability to live on suboptimal diets and provide vitamins (Dillon & Dillon, 2004). If *S. alvi* helps meeting the bumblebees' nutritional demands, this could counteract malnutrition of the colony caused by clothianidin-exposure. An improved nutrition caused by *S. alvi* would however not explain its negative effect on *Crithidia bombi* (H. Koch & Schmid-Hempel, 2011), since the parasite was shown to reach higher loads in well-fed bumblebees than in under-nourished ones (Sadd, 2011).

The ingestion of non-pathogenic bacteria has been previously associated with an activation of the immune system of honeybees (Evans & Lopez, 2004). According to a popular science article (Katsnelson, 2015), such a role was attributed to *Snodgrassella alvi* on the 2014 meeting of the International Union for the Study of Social Insects. The bacterium was apparently associated with a reduction of virus loads in honeybees, however the results appear not to be published in detail yet. We detected a negative correlation between *Snodgrassella alvi* loads and SBV prevalence. This may support a protective role of the bacterium against viruses. The fact that *N. bombi* prevalence tended to be increased in bees with high *Snodgrassella alvi* loads, suggests that *S. alvi* may harm specifically viruses and *Crithidia* (Koch & Schmid-Hempel, 2011) and not just increase the general fitness of bumblebees. The negative correlation between the prevalence of *Nosema bombi* and *Snodgrassella alvi* may support the assumption that *S. alvi* can enhance the immune system. However, the association between *S. alvi* and *N. bombi* is likely

an artefact of the treatment as a model that included both the prevalence of the bacterium and the treatment suggested. The same holds true for the negative relation between the queen production and an interaction of clothianidin-exposure and SBV presence. This suggested the fact that treatment alone showed a even more evident negative association with the production of queens.

Caution has to be applied when interpreting correlations between different microorganisms or between microorganisms and an insecticide treatment. They are not necessarily causative. Particularly field studies are prone to the effects of uncontrolled covariates that may cause such relations (EFSA, 2013). In addition we should bear in mind that even significant findings can just occur by pure chance. The false discovery rate (probability of a false positive finding) is typically multiple times higher the significance level (α) even if the experiment has been conducted correctly and all pre-requisites of the chosen significance test are met (Colquhoun, 2014; Nuzzo, 2014). The false discovery rate increases with a decrease in the prevalence of true positives and of statistical power. The latter depends on the sample size and the (potential) magnitude of the effect one aims at detecting. We believe that the sample size we chose was representative and that the power of our tests are higher than what seems to be prevalent in some scientific areas (Colquhoun, 2014). Nevertheless we are aware that marginal significant findings like those form the microbial data have a high probability (likely more than 30 %) of being false positives and are therefore not to be regarded as discoveries yet, but rather as calls for further analysis (Colquhoun, 2014; Nuzzo, 2014).

5 Conclusion

Numerous sublethal effects of neonicotinoids on bees have been determined in laboratory experiments (Blacquière *et al.*, 2012; Van der Sluijs *et al.*, 2013). Recently, a field study (Rundlöf *et al.*, 2015) with replicated and matched landscapes identified for the first time neonicotinoid effects on colonies of free-foraging bees. The authors of this study provided us with 64 experimental bumblebee colonies of both control and clothianidin-treated fields. This gave us the opportunity to investigate the effect of the neonicotinoid clothianidin, applied as a seed coating in oilseed rape (*Brassica napus* L.) fields, on the non-pathogenic and pathogenic microbiota of bumblebee (*Bombus terrestris* L.*)* colonies.

We also investigate the effect of the clothianidin-treatment on physical parameters and found that adult bumblebees that were exposed to the insecticide had a smaller intertegular distance (standard measure of size) and that their pupae weighted less. We ascribed this to the previously identified impairment of the foraging ability (Gill *et al.*, 2012; Laycock *et al.*, 2012; Feltham *et al.*, 2014) of adult bees and a subsequent undernourishment of the colony and in particular the brood. This experiment was not designed to study the mechanism by which clothianidin affects physical parameters of bees. Providing pupae with neonicotinoid-spiked and insecticide-free pollen and nectar droplets could show if neonicotinoids directly alter the development of bees. In addition hormonal changes and other physiological parameters may be recorded.

Previous studies suggested an impairment of the bees' social and individual immune system by neonicotinoids with resulting higher pathogen infestation and virulence (Alaux *et al.*, 2010; Wu *et al.*, 2012; Pettis *et al.*, 2012; Di Prisco *et al.*, 2013). We did not observe such an effect. This indicates that the by Rundlöf *et al.* (2015) recorded impairment of colony development and queen production was likely not due to pathogen infections, but through a direct clothianidin effect. It also asks for further analysis of pesticide parasite interactions in bees. The by Di Prisco *et al.* (2013) identified immune gene expression in honeybees still needs to

be confirmed and neonicotinoid effects on immune genes in other pollinators remain to be studied.

In this thesis, we observed reduced loads and prevalence of the bumblebee parasite *Crithidia* bombi and elevated levels of the likely mutualistic bacterium *Snodgrassella alvi* in the clothianidin-treated fields. *Snodgrassella alvi* is the most prevalent Betaproteobacterium in honeybees and bumblebees (Koch & Schmid-Hempel, 2012; Moran *et al.*, 2012). Betaproteobacteria have been negatively associated with *Crithidia bombi* (Koch & Schmid-Hempel, 2011; Koch & Schmid-Hempel, 2012; Cariveau *et al.*, 2014). It has been suggested that the bacteria actively fight the trypanosome parasite, as well as viruses (Koch & Schmid-Hempel, 2011; Katsnelson, 2015).

We suspect that the opposite trends that the two microorganisms showed towards the clothianidin treatment may be due to an antagonistic relationship between the gut bacterium and the gut parasite. However, we failed to detect a direct linkage between the prevalence and loads of these microorganisms. There is a need to further study the mechanisms of interaction between the co-adapted non-pathogenic microflora of social bees and their pathogens. In order to protect bees efficiently, their immune system and their ecological needs have to be understood. An establishment of a beneficial role of these gut microbiota should be reflected in the management of bees and their habitats. This means for example beekeepers could actively promote growth of these bacteria for example by adequate food supplements and the choice of pesticide should take negative effects on these microbes into account. In this experiment we observed however a positive relation between clothianidin and *Snodgrassella alvi*. It is conceivable that the insecticide harmed an antagonist of *Snodgrassella alvi,* which promoted the growth of the gut bacterium. Such pesticide-bacterium interactions have been observed (Hussain *et al.*, 2009). Pesticide effects on microbes may not only harm pollinators, but also soil microorganisms that provide ecosystem services such as nutrient cycling (Topp, 2003) and carbon sequestration (Power, 2010). Pesticide effects on non-pathogenic microbiota, particular in soil, have been studied (Hussain *et al.*, 2009; Imfeld & Vuilleumier, 2012). However it appears that this has hardly been done for neonicotinoids, despite their excessive application in soil and their persistence in soil and water (Van der Sluijs *et al.*, 2013). The identification of negative neonicotinoid effects on bees, that occurred post-accreditation of the pesticide, highlight the need to thoroughly study effects on non-target organisms that are vital for the maintenance of ecosystem services, before launching a pesticide on the market.

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Appendices

7.1 Appendix 1: Physical parameters

7.1.1 Adults

Table A1. *Mean body mass of adult bees of all colonies and the standard error of the mean*

Treatment	Pair	Field	Box	Hive	Mass (mean) [g]	Mass (s.e.m.) [g]
Control	P01	VR07	A	1	0.171	0.019
Control	P01	VR07	A	3	0.215	0.022
Control	P01	VR07	B	1	0.227	0.016
Control	P01	VR07	B	3	0.231	0.012
Clothianidin	P01	VR17	A	1	0.121	0.011
Clothianidin	P01	VR17	A	3	0.139	0.013

Clothianidin	P01	VR17	B	1	0.083	0.014
Clothianidin	P01	VR17	B	3	0.142	0.014
Control	P02	VR02	A	1	0.162	0.018
Control	P02	VR02	A	3	0.157	0.030
Control	P02	VR02	B	1	0.112	0.021
Control	P02	VR02	B	3	0.109	0.020
Clothianidin	P02	VR03	A	1	0.038	0.006
Clothianidin	P02	VR03	A	3	0.125	0.010
Clothianidin	P02	VR03	B	1	0.123	0.011
Clothianidin	P02	VR03	B	3	0.132	0.018
Control	P03	VR14	A	1	0.146	0.013
Control	P03	VR14	A	3	0.168	0.016
Control	P03	VR14	B	1	0.101	0.011
Control	P03	VR14	B	3	0.152	0.019
Clothianidin	P03	VR18	A	1	0.132	0.019
Clothianidin	P03	VR18	A	3	0.120	0.011
Clothianidin	P03	VR18	B	1	0.057	0.004
Clothianidin	P03	VR18	B	3	0.206	0.019
Control	P04	VR09	A	1	0.191	0.036
Control	P04	VR09	A	3	0.129	0.015
Control	P04	VR09	B	1	0.155	0.020
Control	P04	VR09	B	3	0.160	0.024
Clothianidin	P04	VR23	A	1	0.160	0.013
Clothianidin	P04	VR23	A	3	0.157	0.015
Clothianidin	P04	VR23	B	1	0.101	0.014
Clothianidin	P04	VR23	B	3	0.247	0.026
Clothianidin	P05	VR12	A	1	0.151	0.016
Clothianidin	P05	VR12	A	3	0.120	0.009
Clothianidin	P05	VR12	B	1	0.120	0.012
Clothianidin	P05	VR12	B	3	0.128	0.010
Control	P05	VR13	A	1	0.145	0.017
Control	P05	VR13	A	3	0.125	0.012
Control	P05	VR13	B	1	0.093	0.015
Control	P05	VR13	B	3	0.188	0.017
Clothianidin	P10	VR04	A	1	0.190	0.024
Clothianidin	P10	VR04	A	3	0.177	0.028
Clothianidin	P10	VR04	B	1	0.139	0.018
Clothianidin	P10	VR04	B	3	0.173	0.016
Control	P10	VR16	A	1	0.081	0.012
Control	P10	VR16	A	3	0.119	0.020
Control	P10	VR16	B	1	0.118	0.014
Control	P10	VR16	B	3	0.175	0.016

Clothianidin	P11	VR05	A	1	0.072	0.011
Clothianidin	P11	VR05	A	3	0.146	0.017
Clothianidin	P11	VR05	B	1	0.151	0.012
Clothianidin	P11	VR05	B	3	0.191	0.032
Control	P11	VR06	A	1	0.259	0.014
Control	P11	VR06	A	3	0.271	0.015
Control	P11	VR06	B	1	0.164	0.021
Control	P11	VR06	B	3	0.243	0.014
Clothianidin	P12	VR20	A	2	0.242	0.019
Clothianidin	P12	VR20	A	3	0.242	0.019
Clothianidin	P12	VR20	B	1	0.144	0.019
Clothianidin	P12	VR20	B	3	0.192	0.030
Control	P12	VR21	A	1	0.187	0.014
Control	P12	VR21	A	3	0.193	0.026
Control	P12	VR21	B	2	0.213	0.015
Control	P12	VR21	B	3	0.187	0.015

Table A2. *Mean intertegular distance of adult bees of all colonies and the standard error of the mean*

Treatment	Pair	Field	Box	Hive	ITD (mean) [mm]	ITD (s.e.m) [mm]
Control	P01	VR07	A	1	5.19	0.21
Control	P01	VR07	A	3	5.59	0.23
Control	P01	VR07	B	1	5.90	0.16
Control	P01	VR07	B	3	5.76	0.07
Clothianidin	P01	VR17	A	1	4.79	0.14
Clothianidin	P01	VR17	A	3	4.96	0.20
Clothianidin	P01	VR17	B	1	5.52	0.15
Clothianidin	P01	VR17	B	3	4.91	0.19
Control	P02	VR02	A	1	5.82	0.18
Control	P02	VR02	A	3	5.36	0.31
Control	P02	VR02	B	1	5.12	0.20
Control	P02	VR02	B	3	4.65	0.27
Clothianidin	P02	VR03	A	1	4.67	0.21
Clothianidin	P02	VR03	A	3	5.27	0.11
Clothianidin	P02	VR03	B	1	5.25	0.13
Clothianidin	P02	VR03	B	3	5.36	0.23
Control	P03	VR14	A	1	5.72	0.24
Control	P03	VR14	A	3	5.43	0.18
Control	P03	VR14	B	1	4.71	0.15
Control	P03	VR14	B	3	5.00	0.24
Clothianidin	P03	VR18	A	1	4.82	0.24

Clothianidin	P03	VR18	A	3	4.76	0.19
Clothianidin	P03	VR18	B	1	5.61	0.26
Clothianidin	P03	VR18	B	3	5.48	0.17
Control	P04	VR09	A	1	5.70	0.35
Control	P04	VR09	A	3	5.66	0.13
Control	P04	VR09	B	1	5.32	0.26
Control	P04	VR09	B	3	5.47	0.21
Clothianidin	P04	VR23	A	1	5.24	0.16
Clothianidin	P04	VR23	A	3	5.11	0.17
Clothianidin	P04	VR23	B	1	5.77	0.12
Clothianidin	P04	VR23	B	3	5.94	0.18
Clothianidin	P05	VR12	A	1	5.10	0.17
Clothianidin	P05	VR12	A	3	4.75	0.14
Clothianidin	P05	VR12	B	1	5.14	0.18
Clothianidin	P05	VR12	B	3	5.31	0.14
Control	P05	VR13	A	1	5.04	0.17
Control	P05	VR13	A	3	5.15	0.17
Control	P05	VR13	B	1	5.45	0.27
Control	P05	VR13	B	3	5.78	0.22
Clothianidin	P10	VR04	A	1	5.32	0.16
Clothianidin	P10	VR04	A	3	4.80	0.19
Clothianidin	P10	VR04	B	1	5.00	0.19
Clothianidin	P10	VR04	B	3	5.29	0.18
Control	P10	VR16	A	1	5.77	0.14
Control	P10	VR16	A	3	5.04	0.19
Control	P10	VR16	B	1	5.34	0.23
Control	P10	VR16	B	3	5.59	0.12
Clothianidin	P11	VR05	A	1	4.94	0.16
Clothianidin	P11	VR05	A	3	5.11	0.18
Clothianidin	P11	VR05	B	1	5.14	0.14
Clothianidin	P11	VR05	B	3	5.08	0.32
Control	P11	VR06	A	1	6.09	0.13
Control	P11	VR06	A	3	6.31	0.16
Control	P11	VR06	B	1	4.99	0.20
Control	P11	VR06	B	3	6.13	0.16
Clothianidin	P12	VR20	A	2	5.78	0.09
Clothianidin	P12	VR20	A	3	5.65	0.17
Clothianidin	P12	VR20	B	1	5.46	0.13
Clothianidin	P12	VR20	B	3	5.24	0.23
Control	P12	VR21	A	1	5.46	0.13
Control	P12	VR21	A	3	5.46	0.21
Control	P12	VR21	B	2	5.48	0.13

Control	P12	VR21	B	3		5.53	0.17

7.1.2 Pupae

Table A3. *Mean body mass of pupae of all samples including the standard error of the mean*

Treatment	Pair	Field	Box	Hive	Sex	Quantity	Mass (mean) [g]	Mass (s.e.m.) [g]
Control	P01	VR07	A	1	male	10	0.375	0.011
Control	P01	VR07	A	3	male	10	0.326	0.038
Control	P01	VR07	B	1	male	10	0.351	0.019
Control	P01	VR07	B	3	male	10	0.429	0.018
Clothianidin	P01	VR17	A	1	male	10	0.242	0.018
Clothianidin	P01	VR17	A	3	female	9	0.128	0.025
Clothianidin	P01	VR17	B	3	female	10	0.138	0.016
Clothianidin	P01	VR17	B	3	male	10	0.233	0.011
Control	P02	VR02	A	1	male	10	0.359	0.021
Control	P02	VR02	A	3	male	10	0.332	0.011
Control	P02	VR02	B	1	male	10	0.321	0.021
Control	P02	VR02	B	3	male	10	0.380	0.009
Clothianidin	P02	VR03	A	1	female	7	0.433	0.059
Clothianidin	P02	VR03	A	3	female	10	0.208	0.016
Clothianidin	P02	VR03	B	1	female	10	0.066	0.014
Clothianidin	P02	VR03	B	3	male	10	0.356	0.016
Control	P03	VR14	A	1	male	10	0.383	0.013
Control	P03	VR14	A	3	male	10	0.395	0.010
Control	P03	VR14	B	1	female	7	0.696	0.018
Control	P03	VR14	B	3	male	10	0.419	0.010
Clothianidin	P03	VR18	A	1	female	10	0.249	0.030
Clothianidin	P03	VR18	A	1	male	10	0.263	0.023
Clothianidin	P03	VR18	A	3	male	10	0.281	0.009
Clothianidin	P03	VR18	B	1	female	10	0.163	0.013
Clothianidin	P03	VR18	B	3	female	10	0.258	0.027
Control	P04	VR09	A	1	male	10	0.421	0.013
Control	P04	VR09	A	3	male	10	0.366	0.025
Control	P04	VR09	B	1	male	10	0.409	0.011
Control	P04	VR09	B	3	male	10	0.414	0.013
Clothianidin	P04	VR23	A	1	female	10	0.276	0.027
Clothianidin	P04	VR23	A	3	male	10	0.218	0.014
Clothianidin	P04	VR23	B	1	female	10	0.077	0.007
Clothianidin	P04	VR23	B	3	male	10	0.355	0.019
Clothianidin	P05	VR12	A	1	male	20	0.341	0.008

Clothianidin	P05	VR12	A	3	male	10	0.273	0.017
Clothianidin	P05	VR12	B	1	female	7	0.104	0.012
Clothianidin	P05	VR12	B	3	female	10	0.239	0.012
Control	P05	VR13	A	1	female	10	0.288	0.012
Control	P05	VR13	A	3	male	10	0.380	0.012
Control	P05	VR13	B	1	female	10	0.075	0.012
Control	P05	VR13	B	3	male	10	0.419	0.037
Clothianidin	P10	VR04	A	3	male	10	0.372	0.012
Clothianidin	P10	VR04	B	1	female	8	0.224	0.014
Clothianidin	P10	VR04	B	1	male	10	0.285	0.019
Clothianidin	P10	VR04	B	3	male	10	0.422	0.016
Control	P10	VR16	A	1	female	10	0.154	0.018
Control	P10	VR16	A	3	male	10	0.399	0.015
Control	P10	VR16	B	1	male	10	0.338	0.011
Control	P10	VR16	B	3	male	10	0.360	0.010
Clothianidin	P11	VR05	A	1	female	10	0.180	0.010
Clothianidin	P11	VR05	A	3	female	10	0.139	0.009
Clothianidin	P11	VR05	A	3	male	10	0.218	0.035
Clothianidin	P11	VR05	B	1	female	10	0.225	0.013
Clothianidin	P11	VR05	B	3	male	10	0.287	0.008
Control	P11	VR06	A	1	male	10	0.441	0.019
Control	P11	VR06	A	3	male	10	0.445	0.016
Control	P11	VR06	B	1	male	10	0.323	0.019
Control	P11	VR06	B	3	male	10	0.399	0.008
Clothianidin	P12	VR20	A	2	female	10	0.149	0.007
Clothianidin	P12	VR20	A	3	male	10	0.329	0.013
Clothianidin	P12	VR20	B	1	male	10	0.276	0.020
Clothianidin	P12	VR20	B	3	female	10	0.248	0.016
Control	P12	VR21	A	1	male	10	0.348	0.014
Control	P12	VR21	A	3	male	10	0.376	0.013
Control	P12	VR21	B	2	male	10	0.356	0.021
Control	P12	VR21	B	3	male	10	0.396	0.016

Table A4. *Total and visibly diseased quantity of pupae of all samples*

Treatment	Pair	Field	Box	Hive	Sex	Quantity	Diseased
Control	P01	VR07	A	1	male	10	4
Control	P01	VR07	A	3	male	10	3
Control	P01	VR07	B	1	male	10	5
Control	P01	VR07	B	3	male	10	8
Clothianidin	P01	VR17	A	1	male	10	3
Clothianidin	P01	VR17	A	3	female	9	4

Clothianidin	P01	VR17	B	3	female	10	3
Clothianidin	P01	VR17	B	3	male	10	3
Control	P02	VR02	A	1	male	10	3
Control	P02	VR02	A	3	male	10	3
Control	P02	VR02	B	1	male	10	0
Control	P02	VR02	B	3	male	10	0
Clothianidin	P02	VR03	A	1	female	7	2
Clothianidin	P02	VR03	A	3	female	10	1
Clothianidin	P02	VR03	B	1	female	10	1
Clothianidin	P02	VR03	B	3	male	10	10
Control	P03	VR14	A	1	male	10	1
Control	P03	VR14	A	3	male	10	3
Control	P03	VR14	B	1	female	7	2
Control	P03	VR14	B	3	male	10	6
Clothianidin	P03	VR18	A	1	female	10	0
Clothianidin	P03	VR18	A	1	male	10	0
Clothianidin	P03	VR18	A	3	male	10	1
Clothianidin	P03	VR18	B	1	female	10	1
Clothianidin	P03	VR18	B	3	female	10	1
Control	P04	VR09	A	1	male	10	1
Control	P04	VR09	A	3	male	10	3
Control	P04	VR09	B	1	male	10	3
Control	P04	VR09	B	3	male	10	1
Clothianidin	P04	VR23	A	1	female	10	5
Clothianidin	P04	VR23	A	3	male	10	4
Clothianidin	P04	VR23	B	1	female	10	0
Clothianidin	P04	VR23	B	3	male	10	10
Clothianidin	P05	VR12	A	1	male	20	0
Clothianidin	P05	VR12	A	3	male	10	4
Clothianidin	P05	VR12	B	1	female	7	0
Clothianidin	P05	VR12	B	3	female	10	3
Control	P05	VR13	A	1	female	10	0
Control	P05	VR13	A	3	male	10	0
Control	P05	VR13	B	1	female	10	3
Control	P05	VR13	B	3	male	10	7
Clothianidin	P10	VR04	A	3	male	10	9
Clothianidin	P10	VR04	B	1	female	8	3
Clothianidin	P10	VR04	B	1	male	10	6
Clothianidin	P10	VR04	B	3	male	10	3
Control	P10	VR16	A	1	female	10	0
Control	P10	VR16	A	3	male	10	5
Control	P10	VR16	B	1	male	10	6
Control	P10	VR16	B	3	male	10	4

Clothianidin	P11	VR05	A	1	female	10	1
Clothianidin	P11	VR05	A	3	female	10	9
Clothianidin	P11	VR05	A	3	male	10	3
Clothianidin	P11	VR05	B	1	female	10	8
Clothianidin	P11	VR05	B	3	male	10	1
Control	P11	VR06	A	1	male	10	6
Control	P11	VR06	A	3	male	10	9
Control	P11	VR06	B	1	male	10	4
Control	P11	VR06	B	3	male	10	6
Clothianidin	P12	VR20	A	2	female	10	3
Clothianidin	P12	VR20	A	3	male	10	1
Clothianidin	P12	VR20	B	1	male	10	7
Clothianidin	P12	VR20	B	3	female	10	3
Control	P12	VR21	A	1	male	10	8
Control	P12	VR21	A	3	male	10	5
Control	P12	VR21	B	2	male	10	2
Control	P12	VR21	B	3	male	10	9

7.2 Microorganism loads

7.2.1 Adults

Table A5. *Mean starting quantities (SQ) of Acute bee paralysis virus (ABPV) per reaction and normalized amount of ABPV RNA per bee of all colonies*

Treatment	Pair	Field	Box	Hive	ABPV SQ (mean)	Normalized ABPV/bee
Control	P01	VR07	A	1	not detected	not detected
Control	P01	VR07	A	3	not detected	not detected
Control	P01	VR07	B	1	not detected	not detected
Control	P01	VR07	B	3	not detected	not detected
Clothianidin	P01	VR17	A	1	not detected	not detected
Clothianidin	P01	VR17	A	3	not detected	not detected
Clothianidin	P01	VR17	B	1	not detected	not detected
Clothianidin	P01	VR17	B	3	not detected	not detected
Control	P02	VR02	A	1	not detected	not detected
Control	P02	VR02	A	3	not detected	not detected
Control	P02	VR02	B	1	not detected	not detected

Control	P02	VR02	B	3	not detected	not detected
Clothianidin	P02	VR03	A	1	not detected	not detected
Clothianidin	P02	VR03	A	3	not detected	not detected
Clothianidin	P02	VR03	B	1	not detected	not detected
Clothianidin	P02	VR03	B	3	not detected	not detected
Control	P03	VR14	A	1	not detected	not detected
Control	P03	VR14	A	3	not detected	not detected
Control	P03	VR14	B	1	not detected	not detected
Control	P03	VR14	B	3	not detected	not detected
Clothianidin	P03	VR18	A	1	not detected	not detected
Clothianidin	P03	VR18	A	3	not detected	not detected
Clothianidin	P03	VR18	B	1	not detected	not detected
Clothianidin	P03	VR18	B	3	not detected	not detected
Control	P04	VR09	A	1	not detected	not detected
Control	P04	VR09	A	3	not detected	not detected
Control	P04	VR09	B	1	not detected	not detected
Control	P04	VR09	B	3	not detected	not detected
Clothianidin	P04	VR23	A	1	not detected	not detected
Clothianidin	P04	VR23	A	3	not detected	not detected
Clothianidin	P04	VR23	B	1	not detected	not detected
Clothianidin	P04	VR23	B	3	not detected	not detected
Clothianidin	P05	VR12	A	1	not detected	not detected
Clothianidin	P05	VR12	A	3	not detected	not detected
Clothianidin	P05	VR12	B	1	not detected	not detected
Clothianidin	P05	VR12	B	3	not detected	not detected
Control	P05	VR13	A	1	not detected	not detected
Control	P05	VR13	A	3	not detected	not detected
Control	P05	VR13	B	1	not detected	not detected
Control	P05	VR13	B	3	not detected	not detected
Clothianidin	P10	VR04	A	1	not detected	not detected
Clothianidin	P10	VR04	A	3	1.95E+01	3.02E+06
Clothianidin	P10	VR04	B	1	not detected	not detected
Clothianidin	P10	VR04	B	3	not detected	not detected
Control	P10	VR16	A	1	3.93E+05	1.14E+11
Control	P10	VR16	A	3	3.31E+01	2.65E+06
Control	P10	VR16	B	1	2.42E+02	2.76E+07
Control	P10	VR16	B	3	1.18E+01	1.84E+07
Clothianidin	P11	VR05	A	1	not detected	not detected
Clothianidin	P11	VR05	A	3	not detected	not detected
Clothianidin	P11	VR05	B	1	not detected	not detected
Clothianidin	P11	VR05	B	3	not detected	not detected
Control	P11	VR06	A	1	not detected	not detected

Control	P11	VR06	A	3	not detected	not detected
Control	P11	VR06	B	1	not detected	not detected
Control	P11	VR06	B	3	not detected	not detected
Clothianidin	P12	VR20	A	2	not detected	not detected
Clothianidin	P12	VR20	A	3	not detected	not detected
Clothianidin	P12	VR20	B	1	not detected	not detected
Clothianidin	P12	VR20	B	3	not detected	not detected
Control	P12	VR21	A	1	not detected	not detected
Control	P12	VR21	A	3	not detected	not detected
Control	P12	VR21	B	2	not detected	not detected
Control	P12	VR21	B	3	not detected	not detected

Table A6. *Mean starting quantities (SQ) of Slow bee Paralysis virus (SBPV) per reaction and normalized amount of SBPV RNA per bee of all colonies*

Treatment	Pair	Field	Box	Hive	SBPV SQ (mean)	Normalized SBPV/bee
Control	P01	VR07	A	1	not detected	not detected
Control	P01	VR07	A	3	not detected	not detected
Control	P01	VR07	B	1	not detected	not detected
Control	P01	VR07	B	3	not detected	not detected
Clothianidin	P01	VR17	A	1	not detected	not detected
Clothianidin	P01	VR17	A	3	not detected	not detected
Clothianidin	P01	VR17	B	1	not detected	not detected
Clothianidin	P01	VR17	B	3	not detected	not detected
Control	P02	VR02	A	1	not detected	not detected
Control	P02	VR02	A	3	not detected	not detected
Control	P02	VR02	B	1	not detected	not detected
Control	P02	VR02	B	3	not detected	not detected
Clothianidin	P02	VR03	A	1	not detected	not detected
Clothianidin	P02	VR03	A	3	not detected	not detected
Clothianidin	P02	VR03	B	1	not detected	not detected
Clothianidin	P02	VR03	B	3	not detected	not detected
Control	P03	VR14	A	1	not detected	not detected
Control	P03	VR14	A	3	not detected	not detected
Control	P03	VR14	B	1	not detected	not detected
Control	P03	VR14	B	3	not detected	not detected
Clothianidin	P03	VR18	A	1	not detected	not detected
Clothianidin	P03	VR18	A	3	not detected	not detected
Clothianidin	P03	VR18	B	1	not detected	not detected
Clothianidin	P03	VR18	B	3	not detected	not detected
Control	P04	VR09	A	1	1.48E+00	2.28E+05
Control	P04	VR09	A	3	not detected	not detected

Control	P04	VR09	B	1	not detected	not detected
Control	P04	VR09	B	3	not detected	not detected
Clothianidin	P04	VR23	A	1	not detected	not detected
Clothianidin	P04	VR23	A	3	not detected	not detected
Clothianidin	P04	VR23	B	1	not detected	not detected
Clothianidin	P04	VR23	B	3	1.21E+02	3.51E+07
Clothianidin	P05	VR12	A	1	not detected	not detected
Clothianidin	P05	VR12	A	3	not detected	not detected
Clothianidin	P05	VR12	B	1	not detected	not detected
Clothianidin	P05	VR12	B	3	not detected	not detected
Control	P05	VR13	A	1	not detected	not detected
Control	P05	VR13	A	3	not detected	not detected
Control	P05	VR13	B	1	not detected	not detected
Control	P05	VR13	B	3	not detected	not detected
Clothianidin	P10	VR04	A	1	not detected	not detected
Clothianidin	P10	VR04	A	3	5.20E+00	4.16E+05
Clothianidin	P10	VR04	B	1	not detected	not detected
Clothianidin	P10	VR04	B	3	2.80E+01	3.19E+06
Control	P10	VR16	A	1	not detected	not detected
Control	P10	VR16	A	3	not detected	not detected
Control	P10	VR16	B	1	not detected	not detected
Control	P10	VR16	B	3	not detected	not detected
Clothianidin	P11	VR05	A	1	not detected	not detected
Clothianidin	P11	VR05	A	3	not detected	not detected
Clothianidin	P11	VR05	B	1	not detected	not detected
Clothianidin	P11	VR05	B	3	not detected	not detected
Control	P11	VR06	A	1	not detected	not detected
Control	P11	VR06	A	3	5.67E+00	8.82E+06
Control	P11	VR06	B	1	not detected	not detected
Control	P11	VR06	B	3	not detected	not detected
Clothianidin	P12	VR20	A	2	not detected	not detected
Clothianidin	P12	VR20	A	3	7.44E+00	2.04E+06
Clothianidin	P12	VR20	B	1	not detected	not detected
Clothianidin	P12	VR20	B	3	not detected	not detected
Control	P12	VR21	A	1	not detected	not detected
Control	P12	VR21	A	3	not detected	not detected
Control	P12	VR21	B	2	not detected	not detected
Control	P12	VR21	B	3	not detected	not detected

Table A7. *Mean starting quantities (SQ) of Sacbrood virus (SBV) per reaction and normalized amount of SBV RNA per bee of all colonies*

Treatment	Pair	Field	Box	Hive	SBV SQ (mean)	Normalized SBV/bee
Control	P01	VR07	A	1	not detected	not detected
Control	P01	VR07	A	3	not detected	not detected
Control	P01	VR07	B	1	not detected	not detected
Control	P01	VR07	B	3	not detected	not detected
Clothianidin	P01	VR17	A	1	not detected	not detected
Clothianidin	P01	VR17	A	3	not detected	not detected
Clothianidin	P01	VR17	B	1	not detected	not detected
Clothianidin	P01	VR17	B	3	not detected	not detected
Control	P02	VR02	A	1	not detected	not detected
Control	P02	VR02	A	3	not detected	not detected
Control	P02	VR02	B	1	not detected	not detected
Control	P02	VR02	B	3	not detected	not detected
Clothianidin	P02	VR03	A	1	not detected	not detected
Clothianidin	P02	VR03	A	3	7.93E+00	1.23E+06
Clothianidin	P02	VR03	B	1	not detected	not detected
Clothianidin	P02	VR03	B	3	not detected	not detected
Control	P03	VR14	A	1	not detected	not detected
Control	P03	VR14	A	3	3.30E+01	3.76E+06
Control	P03	VR14	B	1	not detected	not detected
Control	P03	VR14	B	3	7.70E+00	2.23E+06
Clothianidin	P03	VR18	A	1	not detected	not detected
Clothianidin	P03	VR18	A	3	1.51E+02	1.20E+07
Clothianidin	P03	VR18	B	1	not detected	not detected
Clothianidin	P03	VR18	B	3	not detected	not detected
Control	P04	VR09	A	1	not detected	not detected
Control	P04	VR09	A	3	not detected	not detected
Control	P04	VR09	B	1	not detected	not detected
Control	P04	VR09	B	3	not detected	not detected
Clothianidin	P04	VR23	A	1	2.76E+01	7.59E+06
Clothianidin	P04	VR23	A	3	6.57E+00	1.02E+07
Clothianidin	P04	VR23	B	1	not detected	not detected
Clothianidin	P04	VR23	B	3	not detected	not detected
Clothianidin	P05	VR12	A	1	not detected	not detected
Clothianidin	P05	VR12	A	3	9.50E+00	1.20E+06
Clothianidin	P05	VR12	B	1	not detected	not detected
Clothianidin	P05	VR12	B	3	6.41E+00	1.87E+06
Control	P05	VR13	A	1	not detected	not detected
Control	P05	VR13	A	3	not detected	not detected
Control	P05	VR13	B	1	not detected	not detected
Control	P05	VR13	B	3	not detected	not detected

Clothianidin	P10	VR04	A	1	not detected	not detected
Clothianidin	P10	VR04	A	3	not detected	not detected
Clothianidin	P10	VR04	B	1	not detected	not detected
Clothianidin	P10	VR04	B	3	not detected	not detected
Control	P10	VR16	A	1	not detected	not detected
Control	P10	VR16	A	3	not detected	not detected
Control	P10	VR16	B	1	not detected	not detected
Control	P10	VR16	B	3	not detected	not detected
Clothianidin	P11	VR05	A	1	not detected	not detected
Clothianidin	P11	VR05	A	3	not detected	not detected
Clothianidin	P11	VR05	B	1	8.72E+01	1.69E+07
Clothianidin	P11	VR05	B	3	not detected	not detected
Control	P11	VR06	A	1	3.89E+00	6.43E+05
Control	P11	VR06	A	3	not detected	not detected
Control	P11	VR06	B	1	not detected	not detected
Control	P11	VR06	B	3	not detected	not detected
Clothianidin	P12	VR20	A	2	not detected	not detected
Clothianidin	P12	VR20	A	3	not detected	not detected
Clothianidin	P12	VR20	B	1	not detected	not detected
Clothianidin	P12	VR20	B	3	not detected	not detected
Control	P12	VR21	A	1	not detected	not detected
Control	P12	VR21	A	3	not detected	not detected
Control	P12	VR21	B	2	not detected	not detected
Control	P12	VR21	B	3	not detected	not detected

Table A8. *Mean starting quantities (SQ) of Crithidia bombi per reaction and normalized amount of C. bombi DNA per bee of all colonies*

Treatment	Pair	Field	Box	Hive	*C. bombi* SQ (mean)	Normalized C. bombi/bee
Control	P01	VR07	A	1	1.28E+02	4.94E+04
Control	P01	VR07	A	3	not detected	not detected
Control	P01	VR07	B	1	1.85E+03	7.12E+05
Control	P01	VR07	B	3	6.14E+04	2.37E+07
Clothianidin	P01	VR17	A	1	not detected	not detected
Clothianidin	P01	VR17	A	3	not detected	not detected
Clothianidin	P01	VR17	B	1	7.48E+01	2.88E+04
Clothianidin	P01	VR17	B	3	3.95E+00	1.52E+03
Control	P02	VR02	A	1	2.36E+03	4.55E+06
Control	P02	VR02	A	3	5.72E+03	2.20E+06
Control	P02	VR02	B	1	7.75E+02	1.49E+06
Control	P02	VR02	B	3	8.22E+04	3.17E+07
Clothianidin	P02	VR03	A	1	not detected	not detected
Clothianidin	P02	VR03	A	3	4.19E+02	8.07E+05
Clothianidin	P02	VR03	B	1	6.59E+01	2.54E+04

Clothianidin	P02	VR03	B	3	1.39E+04	5.36E+06
Control	P03	VR14	A	1	4.18E+01	1.61E+04
Control	P03	VR14	A	3	6.68E+03	1.29E+07
Control	P03	VR14	B	1	4.87E+01	9.38E+04
Control	P03	VR14	B	3	3.52E+04	6.79E+07
Clothianidin	P03	VR18	A	1	4.60E+03	8.87E+06
Clothianidin	P03	VR18	A	3	1.35E+03	5.22E+05
Clothianidin	P03	VR18	B	1	not detected	not detected
Clothianidin	P03	VR18	B	3	2.69E+03	5.17E+06
Control	P04	VR09	A	1	3.95E+01	7.62E+04
Control	P04	VR09	A	3	1.99E+02	7.65E+04
Control	P04	VR09	B	1	5.60E+01	2.16E+04
Control	P04	VR09	B	3	8.56E+01	1.65E+05
Clothianidin	P04	VR23	A	1	8.26E+04	3.18E+07
Clothianidin	P04	VR23	A	3	2.47E+02	9.52E+04
Clothianidin	P04	VR23	B	1	2.02E+02	3.89E+05
Clothianidin	P04	VR23	B	3	3.07E+04	1.18E+07
Clothianidin	P05	VR12	A	1	1.25E+04	4.83E+06
Clothianidin	P05	VR12	A	3	1.13E+02	4.35E+04
Clothianidin	P05	VR12	B	1	2.28E+01	8.80E+03
Clothianidin	P05	VR12	B	3	1.09E+01	2.10E+04
Control	P05	VR13	A	1	1.26E+04	2.43E+07
Control	P05	VR13	A	3	7.17E+02	2.76E+05
Control	P05	VR13	B	1	3.50E+02	1.35E+05
Control	P05	VR13	B	3	2.68E+04	1.03E+07
Clothianidin	P10	VR04	A	1	not detected	not detected
Clothianidin	P10	VR04	A	3	not detected	not detected
Clothianidin	P10	VR04	B	1	not detected	not detected
Clothianidin	P10	VR04	B	3	1.65E+02	3.18E+05
Control	P10	VR16	A	1	3.48E+03	6.70E+06
Control	P10	VR16	A	3	7.34E+02	1.41E+06
Control	P10	VR16	B	1	1.23E+03	2.37E+06
Control	P10	VR16	B	3	1.36E+03	5.22E+05
Clothianidin	P11	VR05	A	1	3.56E+01	6.86E+04
Clothianidin	P11	VR05	A	3	1.79E+01	3.44E+04
Clothianidin	P11	VR05	B	1	4.09E+00	1.57E+03
Clothianidin	P11	VR05	B	3	5.38E+00	2.40E+03
Control	P11	VR06	A	1	2.77E+01	1.07E+04
Control	P11	VR06	A	3	8.79E+00	1.69E+04
Control	P11	VR06	B	1	not detected	not detected
Control	P11	VR06	B	3	1.95E+04	3.75E+07
Clothianidin	P12	VR20	A	2	not detected	not detected

Clothianidin	P12	VR20	A	3	1.13E+02	4.36E+04
Clothianidin	P12	VR20	B	1	4.13E+04	1.59E+07
Clothianidin	P12	VR20	B	3	not detected	not detected
Control	P12	VR21	A	1	2.57E+03	9.90E+05
Control	P12	VR21	A	3	2.25E+04	4.34E+07
Control	P12	VR21	B	2	9.96E+03	3.84E+06
Control	P12	VR21	B	3	2.65E+03	1.02E+06

Table A9. *Mean starting quantities (SQ) of Nosema bombi per reaction and normalized amount of N. bombi DNA per bee of all colonies*

Treatment	Pair	Field	Box	Hive	*N. bombi* SQ (mean)	Normalized N. bombi/bee
Control	P01	VR07	A	1	not detected	not detected
Control	P01	VR07	A	3	not detected	not detected
Control	P01	VR07	B	1	not detected	not detected
Control	P01	VR07	B	3	not detected	not detected
Clothianidin	P01	VR17	A	1	not detected	not detected
Clothianidin	P01	VR17	A	3	not detected	not detected
Clothianidin	P01	VR17	B	1	not detected	not detected
Clothianidin	P01	VR17	B	3	not detected	not detected
Control	P02	VR02	A	1	6.09E+02	2.35E+05
Control	P02	VR02	A	3	1.06E+03	4.07E+05
Control	P02	VR02	B	1	not detected	not detected
Control	P02	VR02	B	3	3.60E+03	1.39E+06
Clothianidin	P02	VR03	A	1	not detected	not detected
Clothianidin	P02	VR03	A	3	6.05E+04	2.33E+07
Clothianidin	P02	VR03	B	1	not detected	not detected
Clothianidin	P02	VR03	B	3	not detected	not detected
Control	P03	VR14	A	1	not detected	not detected
Control	P03	VR14	A	3	not detected	not detected
Control	P03	VR14	B	1	6.05E+03	2.33E+06
Control	P03	VR14	B	3	not detected	not detected
Clothianidin	P03	VR18	A	1	not detected	not detected
Clothianidin	P03	VR18	A	3	not detected	not detected
Clothianidin	P03	VR18	B	1	not detected	not detected
Clothianidin	P03	VR18	B	3	not detected	not detected
Control	P04	VR09	A	1	not detected	not detected
Control	P04	VR09	A	3	not detected	not detected
Control	P04	VR09	B	1	not detected	not detected
Control	P04	VR09	B	3	not detected	not detected
Clothianidin	P04	VR23	A	1	not detected	not detected
Clothianidin	P04	VR23	A	3	not detected	not detected
Clothianidin	P04	VR23	B	1	not detected	not detected
Clothianidin	P04	VR23	B	3	not detected	not detected

Clothianidin	P05	VR12	A	1	not detected	not detected
Clothianidin	P05	VR12	A	3	not detected	not detected
Clothianidin	P05	VR12	B	1	not detected	not detected
Clothianidin	P05	VR12	B	3	not detected	not detected
Control	P05	VR13	A	1	not detected	not detected
Control	P05	VR13	A	3	not detected	not detected
Control	P05	VR13	B	1	not detected	not detected
Control	P05	VR13	B	3	not detected	not detected
Clothianidin	P10	VR04	A	1	not detected	not detected
Clothianidin	P10	VR04	A	3	not detected	not detected
Clothianidin	P10	VR04	B	1	not detected	not detected
Clothianidin	P10	VR04	B	3	not detected	not detected
Control	P10	VR16	A	1	not detected	not detected
Control	P10	VR16	A	3	not detected	not detected
Control	P10	VR16	B	1	not detected	not detected
Control	P10	VR16	B	3	not detected	not detected
Clothianidin	P11	VR05	A	1	not detected	not detected
Clothianidin	P11	VR05	A	3	not detected	not detected
Clothianidin	P11	VR05	B	1	not detected	not detected
Clothianidin	P11	VR05	B	3	not detected	not detected
Control	P11	VR06	A	1	not detected	not detected
Control	P11	VR06	A	3	not detected	not detected
Control	P11	VR06	B	1	not detected	not detected
Control	P11	VR06	B	3	not detected	not detected
Clothianidin	P12	VR20	A	2	not detected	not detected
Clothianidin	P12	VR20	A	3	not detected	not detected
Clothianidin	P12	VR20	B	1	not detected	not detected
Clothianidin	P12	VR20	B	3	not detected	not detected
Control	P12	VR21	A	1	not detected	not detected
Control	P12	VR21	A	3	not detected	not detected
Control	P12	VR21	B	2	not detected	not detected
Control	P12	VR21	B	3	not detected	not detected

Table A10. *Mean starting quantities (SQ) of Gilliamella apicola per reaction and normalized amount of G. apicola DNA per bee of all colonies*

Treatment	Pair	Field	Box	Hive	G. apicola SQ (mean)	Normalized G. apicola/bee
Control	P01	VR07	A	1	2.69E+02	1.04E+05
Control	P01	VR07	A	3	not detected	not detected
Control	P01	VR07	B	1	not detected	not detected
Control	P01	VR07	B	3	4.03E+02	1.55E+05
Clothianidin	P01	VR17	A	1	1.09E+03	4.20E+05
Clothianidin	P01	VR17	A	3	1.33E+02	5.11E+04
Clothianidin	P01	VR17	B	1	9.86E+01	3.80E+04

Clothianidin	P01	VR17	B	3	5.19E+02	1.00E+06
Control	P02	VR02	A	1	2.04E+02	3.94E+05
Control	P02	VR02	A	3	1.27E+02	4.91E+04
Control	P02	VR02	B	1	not detected	not detected
Control	P02	VR02	B	3	6.44E+02	2.48E+05
Clothianidin	P02	VR03	A	1	4.29E+01	1.65E+04
Clothianidin	P02	VR03	A	3	6.81E+01	2.62E+04
Clothianidin	P02	VR03	B	1	7.78E+01	1.50E+05
Clothianidin	P02	VR03	B	3	4.34E+02	1.67E+05
Control	P03	VR14	A	1	1.84E+02	3.54E+05
Control	P03	VR14	A	3	1.43E+02	2.76E+05
Control	P03	VR14	B	1	3.52E+02	6.79E+05
Control	P03	VR14	B	3	2.27E+02	4.37E+05
Clothianidin	P03	VR18	A	1	1.32E+02	5.09E+04
Clothianidin	P03	VR18	A	3	3.50E+04	6.75E+07
Clothianidin	P03	VR18	B	1	not detected	not detected
Clothianidin	P03	VR18	B	3	1.52E+02	2.94E+05
Control	P04	VR09	A	1	7.04E+01	2.71E+04
Control	P04	VR09	A	3	1.30E+02	2.51E+05
Control	P04	VR09	B	1	1.67E+02	6.45E+04
Control	P04	VR09	B	3	3.05E+02	5.87E+05
Clothianidin	P04	VR23	A	1	1.05E+02	4.06E+04
Clothianidin	P04	VR23	A	3	1.55E+02	5.99E+04
Clothianidin	P04	VR23	B	1	not detected	not detected
Clothianidin	P04	VR23	B	3	1.28E+02	4.95E+04
Clothianidin	P05	VR12	A	1	2.57E+03	9.89E+05
Clothianidin	P05	VR12	A	3	5.20E+03	2.00E+06
Clothianidin	P05	VR12	B	1	1.00E+04	3.87E+06
Clothianidin	P05	VR12	B	3	2.07E+04	3.98E+07
Control	P05	VR13	A	1	4.43E+01	8.54E+04
Control	P05	VR13	A	3	6.93E+01	2.67E+04
Control	P05	VR13	B	1	2.95E+02	1.14E+05
Control	P05	VR13	B	3	2.39E+02	9.20E+04
Clothianidin	P10	VR04	A	1	6.14E+01	1.18E+05
Clothianidin	P10	VR04	A	3	2.40E+02	4.63E+05
Clothianidin	P10	VR04	B	1	1.73E+02	3.34E+05
Clothianidin	P10	VR04	B	3	1.66E+02	3.19E+05
Control	P10	VR16	A	1	6.41E+01	1.24E+05
Control	P10	VR16	A	3	1.63E+02	3.14E+05
Control	P10	VR16	B	1	7.38E+01	2.84E+04
Control	P10	VR16	B	3	6.93E+02	2.67E+05
Clothianidin	P11	VR05	A	1	not detected	not detected

Clothianidin	P11	VR05	A	3	2.11E+02	8.14E+04
Clothianidin	P11	VR05	B	1	1.16E+02	5.17E+04
Clothianidin	P11	VR05	B	3	2.69E+02	5.18E+05
Control	P11	VR06	A	1	2.30E+02	4.44E+05
Control	P11	VR06	A	3	2.78E+02	1.07E+05
Control	P11	VR06	B	1	1.22E+02	4.70E+04
Control	P11	VR06	B	3	2.11E+02	8.12E+04
Clothianidin	P12	VR20	A	2	3.88E+02	1.49E+05
Clothianidin	P12	VR20	A	3	1.67E+03	3.23E+06
Clothianidin	P12	VR20	B	1	1.10E+03	4.23E+05
Clothianidin	P12	VR20	B	3	2.99E+02	5.77E+05
Control	P12	VR21	A	1	2.49E+02	4.79E+05
Control	P12	VR21	A	3	5.46E+02	2.10E+05
Control	P12	VR21	B	2	2.47E+02	4.76E+05
Control	P12	VR21	B	3	1.52E+02	5.85E+04

Table A11. *Mean starting quantities (SQ) of Snodgrassella alvi per reaction and normalized amount of S. alvi DNA per bee of all colonies*

Treatment	Pair	Field	Box	Hive	*S. alvi* SQ (mean)	Normalized *S. alvi*/bee
Control	P01	VR07	A	1	3.58E+01	1.38E+04
Control	P01	VR07	A	3	not detected	not detected
Control	P01	VR07	B	1	not detected	not detected
Control	P01	VR07	B	3	not detected	not detected
Clothianidin	P01	VR17	A	1	9.38E+01	3.61E+04
Clothianidin	P01	VR17	A	3	1.22E+02	4.71E+04
Clothianidin	P01	VR17	B	1	1.30E+02	4.99E+04
Clothianidin	P01	VR17	B	3	6.52E+02	2.51E+05
Control	P02	VR02	A	1	not detected	not detected
Control	P02	VR02	A	3	not detected	not detected
Control	P02	VR02	B	1	2.33E+01	4.49E+04
Control	P02	VR02	B	3	not detected	not detected
Clothianidin	P02	VR03	A	1	4.21E+01	8.11E+04
Clothianidin	P02	VR03	A	3	1.00E+02	3.87E+04
Clothianidin	P02	VR03	B	1	4.68E+01	1.80E+04
Clothianidin	P02	VR03	B	3	3.66E+01	1.41E+04
Control	P03	VR14	A	1	1.20E+02	2.31E+05
Control	P03	VR14	A	3	7.03E+01	1.35E+05
Control	P03	VR14	B	1	1.84E+02	7.08E+04
Control	P03	VR14	B	3	1.23E+02	4.74E+04
Clothianidin	P03	VR18	A	1	7.85E+01	1.51E+05
Clothianidin	P03	VR18	A	3	1.67E+02	3.22E+05
Clothianidin	P03	VR18	B	1	2.36E+01	4.55E+04
Clothianidin	P03	VR18	B	3	5.20E+01	2.00E+04

Control	P04	VR09	A	1	not detected	not detected
Control	P04	VR09	A	3	4.91E+01	9.46E+04
Control	P04	VR09	B	1	not detected	not detected
Control	P04	VR09	B	3	not detected	not detected
Clothianidin	P04	VR23	A	1	1.44E+02	2.78E+05
Clothianidin	P04	VR23	A	3	6.01E+01	2.31E+04
Clothianidin	P04	VR23	B	1	2.62E+01	5.04E+04
Clothianidin	P04	VR23	B	3	3.46E+01	1.33E+04
Clothianidin	P05	VR12	A	1	1.25E+02	4.82E+04
Clothianidin	P05	VR12	A	3	2.49E+02	4.80E+05
Clothianidin	P05	VR12	B	1	not detected	not detected
Clothianidin	P05	VR12	B	3	4.31E+01	8.30E+04
Control	P05	VR13	A	1	6.60E+01	2.54E+04
Control	P05	VR13	A	3	1.64E+01	6.33E+03
Control	P05	VR13	B	1	1.83E+02	7.04E+04
Control	P05	VR13	B	3	not detected	not detected
Clothianidin	P10	VR04	A	1	not detected	not detected
Clothianidin	P10	VR04	A	3	1.01E+02	3.87E+04
Clothianidin	P10	VR04	B	1	1.38E+02	5.33E+04
Clothianidin	P10	VR04	B	3	3.63E+01	1.40E+04
Control	P10	VR16	A	1	2.87E+01	1.11E+04
Control	P10	VR16	A	3	3.34E+02	1.29E+05
Control	P10	VR16	B	1	not detected	not detected
Control	P10	VR16	B	3	7.23E+01	1.39E+05
Clothianidin	P11	VR05	A	1	8.48E+01	1.63E+05
Clothianidin	P11	VR05	A	3	not detected	not detected
Clothianidin	P11	VR05	B	1	5.07E+01	9.76E+04
Clothianidin	P11	VR05	B	3	6.03E+01	1.16E+05
Control	P11	VR06	A	1	1.32E+02	2.54E+05
Control	P11	VR06	A	3	1.27E+02	2.46E+05
Control	P11	VR06	B	1	7.41E+01	2.86E+04
Control	P11	VR06	B	3	1.49E+02	5.75E+04
Clothianidin	P12	VR20	A	2	1.09E+02	2.10E+05
Clothianidin	P12	VR20	A	3	1.02E+02	3.93E+04
Clothianidin	P12	VR20	B	1	6.81E+01	3.04E+04
Clothianidin	P12	VR20	B	3	1.89E+01	3.65E+04
Control	P12	VR21	A	1	1.85E+01	3.57E+04
Control	P12	VR21	A	3	3.19E+02	1.23E+05
Control	P12	VR21	B	2	7.43E+01	2.86E+04
Control	P12	VR21	B	3	3.39E+01	1.30E+04

7.2.2 Pupae

Table A12. *Mean starting quantities (SQ) of Acute bee paralysis virus (ABPV) per reaction and normalized amount of S. alvi DNA per bee of pupae samples*

Treatment	Pair	Field	Box	Hive	Sex	ABPV SQ (mean)	Normalized ABPV/bee
Control	P01	VR07	A	1	male	not detected	not detected
Control	P01	VR07	A	3	male	not detected	not detected
Control	P01	VR07	B	1	male	not detected	not detected
Control	P01	VR07	B	3	male	not detected	not detected
Clothianidin	P01	VR17	A	1	male	not detected	not detected
Clothianidin	P01	VR17	A	3	female	not detected	not detected
Clothianidin	P01	VR17	B	3	female	not detected	not detected
Clothianidin	P01	VR17	B	3	male	not detected	not detected
Control	P02	VR02	A	1	male	not detected	not detected
Control	P02	VR02	A	3	male	not detected	not detected
Control	P02	VR02	B	1	male	not detected	not detected
Control	P02	VR02	B	3	male	not detected	not detected
Clothianidin	P02	VR03	A	1	female	not detected	not detected
Clothianidin	P02	VR03	A	3	female	not detected	not detected
Clothianidin	P02	VR03	B	1	female	not detected	not detected
Clothianidin	P02	VR03	B	3	male	not detected	not detected
Control	P03	VR14	A	1	male	not detected	not detected
Control	P03	VR14	A	3	male	not detected	not detected
Control	P03	VR14	B	1	female	not detected	not detected
Control	P03	VR14	B	3	male	not detected	not detected
Clothianidin	P03	VR18	A	1	female	not detected	not detected
Clothianidin	P03	VR18	A	1	male	not detected	not detected
Clothianidin	P03	VR18	A	3	male	not detected	not detected
Clothianidin	P03	VR18	B	1	female	not detected	not detected
Clothianidin	P03	VR18	B	3	female	not detected	not detected
Control	P04	VR09	A	1	male	not detected	not detected
Control	P04	VR09	A	3	male	not detected	not detected
Control	P04	VR09	B	1	male	not detected	not detected
Control	P04	VR09	B	3	male	not detected	not detected
Clothianidin	P04	VR23	A	1	female	not detected	not detected
Clothianidin	P04	VR23	A	3	male	not detected	not detected
Clothianidin	P04	VR23	B	1	female	not detected	not detected
Clothianidin	P04	VR23	B	3	male	not detected	not detected
Clothianidin	P05	VR12	A	1	male	not detected	not detected
Clothianidin	P05	VR12	A	3	male	not detected	not detected
Clothianidin	P05	VR12	B	1	female	not detected	not detected
Clothianidin	P05	VR12	B	3	female	not detected	not detected
Control	P05	VR13	A	1	female	not detected	not detected

Control	P05	VR13	A	3	male	not detected	not detected
Control	P05	VR13	B	1	female	not detected	not detected
Control	P05	VR13	B	3	male	not detected	not detected
Clothianidin	P10	VR04	A	3	male	not detected	not detected
Clothianidin	P10	VR04	B	1	female	not detected	not detected
Clothianidin	P10	VR04	B	1	male	not detected	not detected
Clothianidin	P10	VR04	B	3	male	not detected	not detected
Control	P10	VR16	A	1	female	not detected	not detected
Control	P10	VR16	A	3	male	not detected	not detected
Control	P10	VR16	B	1	male	1.50E+01	3.51E+06
Control	P10	VR16	B	3	male	3.25E+01	3.99E+06
Clothianidin	P11	VR05	A	1	female	not detected	not detected
Clothianidin	P11	VR05	A	3	female	not detected	not detected
Clothianidin	P11	VR05	A	3	male	not detected	not detected
Clothianidin	P11	VR05	B	1	female	not detected	not detected
Clothianidin	P11	VR05	B	3	male	not detected	not detected
Control	P11	VR06	A	1	male	not detected	not detected
Control	P11	VR06	A	3	male	not detected	not detected
Control	P11	VR06	B	1	male	not detected	not detected
Control	P11	VR06	B	3	male	not detected	not detected
Clothianidin	P12	VR20	A	2	female	not detected	not detected
Clothianidin	P12	VR20	A	3	male	not detected	not detected
Clothianidin	P12	VR20	B	1	male	not detected	not detected
Clothianidin	P12	VR20	B	3	female	not detected	not detected
Control	P12	VR21	A	1	male	not detected	not detected
Control	P12	VR21	A	3	male	not detected	not detected
Control	P12	VR21	B	2	male	not detected	not detected
Control	P12	VR21	B	3	male	not detected	not detected

7.3 Standard curve parameters

7.3.1 Adults

Table A13. *Reaction efficiency (E) and correlation coefficient (R^2) of the adult DNA/RNA assays with target detections. A reaction efficiency of 100% corresponds to a duplication of the amount of DNA per cycle.*

Plate	1		2	
Parameter	E	R^2	E	R^2
ABPV	78.0%	0.999	89.8%	0.999
SBPV	73.8%	0.997	81.1%	0.992
SBV	81.2%	0.998	78.3%	0.997

RNA250	96.3%	0.987	100.5%	0.993
RPL23	83.8%	0.997	87.8%	0.996
C. bombi	92.7%	1.000	95.2%	1.000
N. bombi	70.0%	0.981	60.8%	0.985
G. apicola	87.5%	1.000	82.6%	1.000
S. alvi	87.4%	0.994	81.6%	0.999

7.3.2 Pupae

Table A14. *Reaction efficiency (E) and correlation coefficient (R^2) of the pupae DNA/RNA assays with target detections. A reaction efficiency of 100% corresponds to a duplication of the amount of DNA per cycle.*

Plate	1		2	
Parameter	E	R	E	R
ABPV	79.9%	1	78.0%	0.999
RNA250	116.7%	0.992	91.9%	0.995
RPL23	82.1%	0.996	83.6%	0.993