Accumulation of the neonicotinoid insecticide clothianidin in bumblebees (*Bombus terrestris*)

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Abstract

Wild bees are declining on a local, regional, and global scale, which is due to the exposure of several stressors. Neonicotinoids, a group of neurotoxic insecticides, are one of these stressors, and are shown to cause lethal and sublethal effects in bees after exposure through pollen and nectar collected from treated crops. However, the accumulative potential of these neonicotinoids in pollinators has not been studied before, despite their high affinity for a neuroreceptor found in the insect brain and a long half-life in soil. Although bees are not exposed to high concentrations in the environment, accumulation can cause chronic poisoning, and affect both the individual and the colony. The present study, therefore, aimed to assess the accumulation of clothianidin, a neonicotinoid, in bumblebee workers and the queen, and assess whether this accumulation could cause change in mortality and sublethal effects on brood production, nectar consumption, and storage of food.

Bumblebee colonies (*Bombus terrestris*, N = 48) were exposed to field-realistic concentrations of clothianidin through the nectar, with concentrations ranging from 1 μg/L to 13 μg/L, in a chronic exposure regime lasting nine days. Five hives from three treatment levels (3.6 μg/L, 6.8 μg/L, and 13 μg/L) and the control (0 μg/L) were dissected, and their health was assessed. From each of these colonies, 10 bumblebee workers and the queen were dissected into three parts: the head, the stomach, intestine, and rectum (SIR), and the rest of the body. Each body compartment was then chemically analysed to assess the clothianidin content in the tissue.

Clothianidin showed a dose-response accumulation in the head and body of workers, and the body of the queen. It did not accumulate in the queen's head nor the SIR of workers and the queen. Exposure did not cause a change in mortality or brood production but showed a trend of a hormetic response in nectar consumption and a negative dose-response trend in the proportion of empty honeypots. Although no adverse effect could be measured in the present study, the accumulation of clothianidin in the head and body of bumblebees have the potential to cause chronic poisoning, where adverse effects are found after a prolonged period of exposure.

Abbreviations

ACh	Acetylcholine
AICc	Corrected Akakie information criterion
BAF	Bioaccumulation factor
CAS	Chemical Abstract Service
EFSA	European Food Safety Authority
ESI	Electro-spray ionisation
EU	European Union
GLM	Generalised linear models
HPLC-MS	High-performance liquid chromatography mass-spectrometry
IUPAC	International Union
LD_{50}	Lethal dose where 50% of experimental population is dead
LDD_{50}	Lethal daily dose where 50% of experimental population is dead
Max	Maximum
MeCN	Acetonitrile
MRM	Multiple reaction monitoring
MS/MS	Tandem mass spectrometry
N	Number of observations
NA	Not analysed
nAChR	Nicotinic acetylcholine receptor

NaCl	Sodium
NOEC	No observed effect concentration
NOEL	No observed effect level
NIVA	Norwegian Institute of Water Research
SIR	Stomach, intestine, and rectum
UiO	University of Oslo
UPLC	Ultra-performance liquid chromatography
w.w.	Wet weight

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1 Introduction

1.1 Farming and pollinators

Farming has been an essential part of human survival for nearly 10,000 years (Oerke, 2006). Through this long history, agrarian systems have been developed and renewed several times, and with each renewal, agriculture feed more and more people (Mazoyer & Roudart, 2006). The increasing demands of an exponentially growing human population have been a driver towards the intensification of agriculture, including an increasing and now extensive use of fertilisers and pesticides. (Mazoyer & Roudart, 2006). Pollinators have also had an important part in the history of agriculture, although often overlooked. Pollinators are animals that move pollen grains from the male part (stamen) of flowers to the female part (stigma) of flowers, enabling reproduction for the plant species. Globally, agriculture has become more dependent on pollinators due to increased production of pollinator-dependent crops (Aizen et al., 2008), and animal pollination has been economically valued at $253 billion-$577 billion for production that is directly linked to their pollination service (Potts et al., 2016).

In Norway, bumblebees and flies are considered the most important groups of pollinators (Norwegian Ministry of Agriculture and Food & Norwegian Ministry of Climate and Environment, 2018). Bees are entirely dependent on flowers for their survival, and nearly all bee species are pollinators (Michener, 2007). They have a significant role in the production of crops, and it is common for farmers to either rent or keep bees for the pollination of their crops (Potts et al., 2016; Norwegian Ministry of Agriculture and Food & Norwegian Ministry of Climate and Environment, 2018). Of the many thousands of known bee species, honeybees (Apis) are the most commonly used species in commercialised pollination, much due to their social lifestyle, large colony size, and perennial colonies (Michener, 2007). However, bumblebees are considered more effective pollinators than honeybees. Bumblebees are active earlier in the morning and later in the evening, carry more pollen on their bodies, and are more efficient in terms of pollen pick-up and pollen deposition (Willmer, 2011).

Trends during the last century show a local, regional, and global decrease in wild pollinators (Biesmeijer et al., 2006; Pauw, 2007; Williams et al., 2009; Cameron et al., 2011; Burkle et al., 2013; Morales et al., 2013; Nieto et al., 2014; Ollerton et al., 2014). Several factors are

involved in the negative trend for bumblebees, including habitat-change, like habitat loss and fragmentation (Williams & Osborne, 2009); pollution, especially the exposure to synthetic pesticides and fertilisers through nectar and pollen (Ollerton et al., 2014); biological factors, like viruses and pathogens (van der Steen, 2008; Cameron et al., 2011) or competition with introduced or invasive species (Norwegian Ministry of Agriculture and Food & Norwegian Ministry of Climate and Environment, 2018); and climate change (Kerr et al., 2015). Intensification of agriculture has the potential to inflict several of these factors.

Wild pollinators pollinate crops better than honeybees and enhance crop yield regardless of honeybee abundance (Garibaldi et al., 2013), as well as providing pollination to several important crops that honeybees do not pollinate, e.g. tomato, cocoa, fig, passion fruit, and oil palm (Klein et al., 2007). However, wild species are also more sensitive to pesticides and would in no doubt experience a negative impact due to the increased exposure to these chemicals (Arena & Sgolastra, 2014). Due to the impact wild pollinators have, both ecologically (Klein et al., 2007) and economically (Gallai et al., 2009), the effect agricultural management has on these species must be understood for the development of productive agricultural systems and healthy ecosystems.

1.2 Pesticides

Pesticides are chemicals developed to control and suppress organisms that are considered pests. They include groups of chemicals designed for specific pests, e.g. herbicides, insecticides, and fungicides, targeting weeds, insects, and fungi, respectively. Pesticides are applied to crops in large amounts globally, and the use of pesticide has since 1960 increased 15-20-fold (Oerke, 2006), totalling $6 billion annually in 2011 and 2012 (Atwood & Paisley-Jones, 2017). The efficiency of pest control in barley, cottonseed, maize, oilseed rape, potato, rice, soybean, cotton, sugar beet, tomatoes, and wheat averaged at 37.5% in 2001-03, reducing the potential losses due to pathogens, viruses, animal pests, and weeds substantially (Oerke, 2006). Of the many pesticide groups, herbicides had the largest market share globally in 2012, followed by insecticides accounting for 29% of the pesticide market (Atwood & Paisley-Jones, 2017). The main classes of insecticides include pyrethroids, methyl carbamates, organophosphates, chlorinated hydrocarbons, and neonicotinoids (Tomizawa &

Casida, 2011). As a class, insecticides have higher acute toxicity toward nontarget species compared to other classes of pesticides (Klaassen, 2013).

1.2.1 Neonicotinoids

Neonicotinoids are the newest group of insecticides. The first neonicotinoid insecticide, imidacloprid, was launched in 1991 and in the following 11 years, six more neonicotinoids were designed: acetamiprid, nitenpyram, thiamethoxam, thiacloprid, clothianidin, and dinotefuran (Elbert et al., 2008). Neonicotinoids can be divided into three groups based on their chemical structure, N-nitroguanidines (contains a nitro-group), N-cyano-amidines (contains a cyano-group), and nitromethylenes (nitenpyram) (Elbert et al., 2008). These differences in chemical structure have an impact on biological activity, photolytic stability, degradation in soil, and metabolism in plants (Jeschke et al., 2013). In 2011, neonicotinoids held 28% of the marked shares in the agrochemical insecticide marked and had revolutionised the insecticidal seed treatment marked, holding 70% of its marked shares (Jeschke et al., 2011; Jeschke et al., 2013).

There are several factors suggested to be the cause of the success of neonicotinoids:

1) Neonicotinoids are water soluble and are transported in the xylem tissue throughout the plant (Li et al., 2018b), which allow for a large variety of application methods (Elbert et al., 2008). Their systemic property allows them to reach all parts of the plant and therefore protect against pests utilising different parts of the plant (Stamm et al., 2016).
2) They have an effective mode of action with a broad spectrum of target pests (Elbert et al., 2008). Neonicotinoids are agonists of the nicotinic acetylcholine receptor (nAChR) found in high density in the insect brain and mimic the action of the neurotransmitter acetylcholine (ACh) (Jeschke et al., 2013). However, neonicotinoids are not cleaved from the receptor, like acetylcholine is, but stay bound over a prolonged period, which causes hyperexcitation followed by the exhaustion of the cell and paralysis (Palmer et al., 2013).
3) Neonicotinoids bind with high affinity to nicotinic receptors in the central nervous system of invertebrates (Palmer et al., 2013). Vertebrates do in general have a low amount of these receptors, which further allows for selective toxicity towards insects and other invertebrates (Tomizawa & Casida, 2005).

4) Neonicotinoids were, among other things, introduced as a solution to the expanding pest resistance to other insecticides, which include organophosphates, carbamates, and pyrethroids (Elbert et al., 2008).
5) Their long half-life in aerobic soil allows for long-term protection of crops and reduce the need for reapplication. As an example, clothianidin has a half-life of 90-6931 days (Goulson, 2013; Li et al., 2018c).

Banned in the EU

Three neonicotinoids, imidacloprid, thiamethoxam, and clothianidin, have been permanently banned in the European Union (EU). Growing evidence appearing in the early 2010s proposed that neonicotinoids have a negative impact on pollinators (Gill et al., 2012; Lu et al., 2012; Whitehorn et al., 2012). In response, the European Food Safety Authority (EFSA) released six risk assessments, two for each neonicotinoid, identifying knowledge gaps and confirming the risk the three neonicotinoids pose to bee pollinators (EFSA, 2013a, 2013b, 2013c, 2018a, 2018b; 2018c). All use and application of imidacloprid, thiamethoxam, and clothianidin are banned, except for use in permanent greenhouses. The ban has been controversial due to the conflicting evidence, some studies showing a marked effect in bee pollinators due to neonicotinoid exposure (Gill et al., 2012; Whitehorn et al., 2012) while other studies show no effect (Cutler et al., 2014; Osterman et al., 2019). These conflicting results highlight the fact that there is still a need for more research in this area.

The effects of neonicotinoids on bee pollinators

Neonicotinoids have been seen to have several adverse effects on bee pollinators, but the area is still a subject of much debate. Three of the main subjects being that many studies use doses which are above field-realistic concentrations (Carreck & Ratnieks, 2014) and the trend of many studies being centred around honeybees, as well as the small number of field realistic studies using long-term exposure (Potts et al., 2016).

The first risk assessments by EFSA were heavily criticised for their large data gaps, especially concerning the risk posed to other pollinators than honeybees and a lack of assessment of sublethal effects. Extrapolation from honeybees to other pollinators, like bumblebees, with the use of assessment factors, is problematic due to the large variety in behaviour and ecological adaptions found between species (Thompson & Hunt, 1999). There are 20,000 known bee

species globally, with a range of different levels of sociality, traits, and habitats (Potts et al., 2016; Ollerton, 2017). There are solitary bees, sub-social bees, quasi-social bees, semi-social bees, primitively eusocial bees, highly social bees, and parasitic bees (Willmer, 2011). These bees can also be either short-tongued, medium-tongued, or long-tongued, and can make nests in several different substrates, like in the ground or in threes (Willmer, 2011). This is only an indication of the large variation found among bees, and several of these different species show different sensitivity to pesticides(Arena & Sgolastra, 2014). Bumblebees are more sensitive than honeybees to neonicotinoids (Cresswell et al., 2012), and bumblebees and solitary bees show different sensitivity to neonicotinoids as well (Scott-Dupree et al., 2009). The second risk assessments by EFSA included honeybees, bumblebees, and solitary bees, and the process revealed once again large data gaps which are present when it comes to the potential risk neonicotinoids pose to non-honeybee species (EFSA, 2018a, 2018b, 2018c).

Sublethal effects are defined as behavioural and/or physiological effects appearing in individuals who have survived exposure to an environmental agent at a dose that gives no apparent mortality (Alkassab & Kirchner, 2017). Therefore, a dose which is considered sublethal is a dose below the LD_{50} (the lethal dose which kills 50% of the experimental population). However, bees have been observed to become more sensitive to neonicotinoids over time, where the LD_{50} decreases with prolonged exposure (Alkassab & Kirchner, 2016), making it difficult to determine the lowest concentration which does not have an impact. Sublethal effects can appear at different biological levels, and several have been identified in bees after exposure to neonicotinoids. These effects include impaired learning and memory (Stanley et al., 2015; Paus-Knudsen, 2017; Phelps et al., 2018), reduced consumption of food (Laycock et al., 2012; Cresswell et al., 2013; Thompson et al., 2015), reduction in forager efficiency (Gill et al., 2012; Feltham et al., 2014), reduced brood production (Gill et al., 2012; Laycock et al., 2012; Laycock & Cresswell, 2013), and reduced queen production (Whitehorn et al., 2012). Potential effects to the queen are of special interest, as the queen is essential for the initiation of both the hive and the colony (as detailed in section 2.1), and in an established colony she produces the workers, the drones, and the new queens (Willmer, 2011).

1.3 Accumulation

None of the risk assessments of imidacloprid, thiamethoxam, or clothianidin performed by EFSA considered their accumulative potential in pollinators or other animals, as they in general are not thought to accumulate in animals. However, neonicotinoids have been found to accumulate in partridges, earthworms, and lizards (Lopez-Antia et al., 2015; Chevillot et al., 2017; Wang et al., 2018; Wang et al., 2019). Accumulation is the process where a substance is concentrated in the body of an organism as the rate of uptake is faster than the rates of metabolism and excretion. The three neonicotinoids do have properties that provide them with a potential for accumulation in bee species, i.e. a mode of action which involves a prolonged binding to receptors in the nervous system and a long half-life in soil (Goulson, 2013; Jeschke et al., 2013; Li et al., 2018c). With a long half-life, neonicotinoids can stay in the soil for a long time and leach into the surrounding terrestrial environment or the groundwater due to their water solubility. They can then be taken up by other plants than intended and thereby expose organisms more broadly and later in time (Botías et al., 2015). Both honeybees and bumblebees show incomplete clearance of imidacloprid with continuous exposure and the clearance rate has been observed to decrease over time (Cresswell et al., 2013; Sánchez-Bayo et al., 2017). Even if the exposure in nectar and pollen is low, over time and with continuous exposure due to the long half-life of neonicotinoids, a reduced clearance rate could cause an internal concentration of neonicotinoids that could be high enough to cause harmful impact.

Thiamethoxam can cause a structural change in the cells lining the midgut and the Malpighian tubules in honeybees, which are the primary areas for metabolism and uptake of ingested food and the excretion of molecules already taken up into the body (Catae et al., 2014). These structural changes could support an increased uptake in the gut and decreased the ability to excrete neonicotinoids that have already been taken up in the body. Bees could therefore have the potential to accumulate neonicotinoids in the head, because of a high amount of nAChRs, and in the stomach, intestine, and rectum, because of a change in their metabolic capabilities. Several studies have measured the neonicotinoid concentration in bees captured while foraging on neonicotinoid-treated crops, where the concentrations range from 0.15 μg/kg to 4.90 μg/kg in healthy bees and from 3.8 μg/kg to 13.3 μg/kg in dead or dying bees (Krupke et al., 2012; Osterman et al., 2019). The difference in concentrations measured in the foraging bees and the dead/dying bees is a strong indication that neonicotinoids have a harmful impact

to bee pollinators. However, these studies include a strong bias in their measurement as bees "healthy enough" to forage were more likely to be sampled, while the bees which stay in the hive or crawl on the ground due to poor health were not sampled (only dead/dying bees on the ground right outside of the hive were sampled). Moreover, the exposure to these bees was not measured, providing only a small part of the whole picture, as it is unknown whether it is high or low exposure concentration which have resulted in the concentration measured in the bee.

1.4 Aims and hypothesis

The main aim of this study was to analyse whether exposure to field-realistic concentrations of clothianidin accumulate in and has a toxic impact on bumblebees and whether this response follows a dose-response relationship. This was assessed through exposure to clothianidin via the nectar, and measurement of the accumulation in bumblebee body compartments, mortality- and reproduction rate, consumption of nectar, and proportion of empty honeypots. The study included three objectives and eight hypotheses:

Objective 1: Quantify the accumulation of clothianidin in bumblebees and see how it distributes in their bodies. Assessed by further developing a method first presented by Wiest et al. (2011).

Hypothesis 1.1: Clothianidin accumulates in the head of bumblebees.

Hypothesis 1.2: Clothianidin accumulates in the stomach, intestine, and rectum of bumblebees.

Hypothesis 1.3: Clothianidin accumulation is higher in the queen than her workers in all body compartments.

Objective 2: Quantify the lethal and sublethal impacts clothianidin has on colony dynamics.

Hypothesis 2.1: Exposure to clothianidin causes increased mortality.

Hypothesis 2.2: Exposure to clothianidin causes a decreased brood production.

Hypothesis 2.3: Nectar consumption decreases when bumblebees are exposed to clothianidin.

Hypothesis 2.4: Bumblebees store less food in the colony when exposed to clothianidin.

Objective 3: Evaluate the actual exposure to bumblebees by quantifying the actual clothianidin concentration in nectar and compare it to the nominal concentration.

Hypothesis 3.1: The nominal and measured exposures are the same.

2 Methods and materials

2.1 Study species: Bumblebees

The species used in this study is the Buff-tailed bumblebee (*Bombus terrestris* L. (1758), figure 1). Bumblebees (tribe *Bombini*, genus *Bombus*) are a part of the superfamily Apoidea, family Apidae, and subfamily Apinae. Other common tribes of Apinae are Orchid bees (*Euglossini*), Stingless bees (*Meliponini*), and Honeybees (*Apini*) (Willmer, 2011). There exist 250 bumblebee species worldwide belonging to 15 genera, where 35 species have been found in Norway (Ødegaard et al., 2015). Bumblebees are the most abundant in the northern hemisphere, and thrive in temperate, alpine, and arctic climate zones, but can also be found all the way down to moist lowland tropics (Guinea, Venezuela, Colombia; Williams, 1998). They are less common on the southern hemisphere, native only to the East Indies (the many islands of the Philippines and Indonesia) and South America, where they are found mostly in the mountains (Williams, 1994, 1998; Michener, 2007). This distribution pattern can to some degree, be explained by characteristics of bumblebees that make them adapted to colder climates, like big, stout, furry bodies and endothermic abilities (Willmer, 2011).

Figure 1. The study species *Bombus terrestris* foraging on a flower. Photo: Julie Sørlie Paus-Knudsen.

Bumblebees are holometabolous insects that undergo complete metamorphosis, and their life cycle consists of four stages: egg, larva, pupa, and adult. The queen mates with male bumblebees for a single period in her life and stores the sperm in her spermatheca. When producing eggs, she can determine the sex of the offspring by releasing sperm from the spermatheca or not. Bumblebees have haploid-diploid sex determination: fertilized eggs develop into females and unfertilized eggs develop into males. Both the queen and the workers can produce unfertilized eggs, but it is unknown how large the proportion of males is produced by the queen relative to the workers (Ødegaard et al., 2015).

B. terrestris are eusocial bees with castes made up of the queen, workers, and drones. The queen is morphologically similar to her workers, but larger (Ødegaard et al., 2015). There is also large diversity in the size of the workers, which may be linked to what type of tasks they perform in the hive, and is a response to the amount and quality of the food they received as larvae (Goulson et al., 2002). Some workers are foragers, collecting nectar or pollen or both, while other workers perform in-hive tasks, including tending to broods or cleaning the hive (Goulson et al., 2002).

B. terrestris produce annual colonies. A young queen mates with one or more male bumblebees in the autumn before going into a winter slumber. In spring, the fertilized queen wakes from her hibernation and establishes a hive, often in a hole in the ground or in a hollow tree (Ødegaard et al., 2015). She produces a small batch of eggs and tends to them by herself. Several eggs are placed in the same cell, where they develop into larvae. The larvae go through four development stages, and in the process, the cells divide into separate chambers, each chamber containing one larva (Cnaani et al., 1997; Michener, 2007). When mature, the larva spins a silk cocoon and pupate. These cocoons form irregular horizontal layers, and new cells are added on top of the old as the hive grows (Goulson et al., 2002; Michener, 2007). The first batch of workers hatches just in time for them to help the queen take care of a second larger batch of larvae.

B. terrestris has become a successful commercial pollinator used to pollinate, for example, tomatoes, pepper, eggplant, and raspberry (Willmer et al., 1994; Abak et al., 2000; Kwon & Saeed, 2003; Velthuis & van Doorn, 2006). The species is native to and widespread in Europe, stretching eastwards to Kazakhstan and Turkmenistan. *B. terrestris* has had a marked

entry into the Norwegian fauna over the last century, and 50 years ago, only a handful of observations had been registered (Løken, 1973). Today, they can be found in the area around the Oslofjord in the south and Nordland county in the north, moving along Eastern Norway and is most abundant in urban areas (Ødegaard et al., 2015). Their wide distribution, ability to produce large colonies, ability to adapt to artificial conditions, and mild temperament have made *B. terrestris* a suitable species for commercial rearing. As a consequence of this, the species has been introduced to China, Japan, Chile, Argentina, Mexico, South Korea, and New Zealand (Velthuis & van Doorn, 2006; Ødegaard et al., 2015). Due to the ease in handling and their importance as commercial crop pollinators and for wild plants, *B. terrestris* was chosen for the present study.

2.2 Study chemical: Clothianidin

Clothianidin is one of the three neonicotinoid insecticides currently banned by the European Union (EU). The IUPAC name is (E)-1-(2-chloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine (C6H8CIN5O2S), and the CAS name is [C(E)]-N-[(2-chloro-5-thiazolyl)methyl)-3-methyl-N'-nitroguanidine (CAS number 210880-92-5). Clothianidin is an important insecticide on its own but can also be found as one of the main metabolites of thiamethoxam (Nauen et al., 2003; Li et al., 2018a). It is therefore common to find concentrations of clothianidin in areas where thiamethoxam has been applied (Dively & Kamel, 2012; Pilling et al., 2013; EFSA, 2018a). and following, the occurrence of clothianidin in the environment is larger than what would be predicted by its application alone.

Based on the mode of action, clothianidin has the potential to be more toxic than imidacloprid and thiamethoxam, but experiments do not always show this. Clothianidin is a complete agonist of the nicotinic acetylcholine receptor (nAChR), while imidacloprid is an incomplete agonist (Brown et al., 2006), and thiamethoxam has been suggested to bind to another binding seat than clothianidin and imidacloprid altogether (Wellmann et al., 2004). Structural differences between neonicotinoids are suggested to cause a different affinity for different nAChR subtypes found in invertebrates, although this is an area in need of more research (Simon-Delso et al., 2015). The affinity for different subtypes can explain why some studies show that clothianidin is the least toxic among the three neonicotinoids (Stamm et al., 2016),

while other studies show that clothianidin is the most toxic (Scott-Dupree et al., 2009; Schneider et al., 2012; Laurino et al., 2013).

2.2.1 Pesticide risk assessment

The European Food Safety Authority (EFSA) define the lethal dose of a pesticide as the dose at which 50% of the population tested is dead (LD_{50}). In chronic studies, the lethal dose is defined as the daily dose where 50% of the tested population is dead after the set time period (LDD_{50}). The highest concentration giving no observed effect is called the "No Observed Effect Concentration" (NOEC) or "No Observed Effect Level" (NOEL). The results from the two risk assessments concerning clothianidin performed by EFSA are summarised in Table 1.

Table 1. LD_{50} (the dose at which 50% of the population tested is dead), LDD_{50} (the daily dose where 50% of the tested population is dead after the set time period of a chronic study), and NOEL (the highest level giving no observed effect) values for acute contact and oral toxicity, chronic oral toxicity, and larval toxicity for honeybees and bumblebees form clothianidin exposure given as μg active substance/bee. Concentrations are taken from EFSA's two risk assessments (EFSA, 2013a, 2018a).

Exposure type	**Endpoint and response**	**Honeybees**	**Bumblebees**
Acute contact toxicity	LD_{50} (μg a.s./bee)	0.03[b]	0.15[b]
Acute oral toxicity	LD_{50} (μg a.s./bee)	0.004[b]	0.002[b]
Acute contact toxicity	NOEL (μg a.s./bee)	0.0001[a]	-
Acute oral toxicity	NOEL (μg a.s./bee)	0.008[a]	-
Chronic oral toxicity	10-day LDD_{50} (μg a.s./bee per day)	0.0009[b]	0.00009[b,c]
Larval	NOEL (μg a.s./larva per developmental period)	0.005[b]	No endpoint available[a]

a: EFSA risk assessment 1 (2013).
b: EFSA risk assessment 2 (2018).
c: extrapolated from honeybees with an assessment factor of 10.

2.3 Experimental setup and design

The experiment was conducted at the Department of Biosciences at the University of Oslo (UiO), with subsequent chemical analysis at the Norwegian Institute of Water Research (NIVA) from June to January of 2018/19. The experiment was divided into three parts: exposure, dissection, and chemical analysis. The exposure part was performed in a climate room in the phytotron, the preparation of dilution series and dissection was carried out in the toxicology lab, and the chemical analysis was conducted at NIVA. The exposure part of the study was performed together with co-supervisor and PhD student Julie Sørlie Paus-Knudsen. During the experiment, the hives were kept under a controlled environment of +28°C and 50% relative humidity. After testing, the hives were frozen (-20°C) for at least two days.

Forty-eight queenright colonies (presence of a fertile queen) of *Bombus terrestris* were obtained from Bombus natur AS, located at Bryne, Southern Norway. The first colonies were delivered in June 2018, and the last colonies were delivered in October 2018. The colonies were delivered in standard plastic nest-boxes covered by a cardboard box, in which they remained throughout the whole experiment unless when training and testing in a flying arena. The flying arena was related to a behavioural experiment performed on the bumblebees by Julie, and the bees were allowed into the arena for 3 hours in total divided in two rounds, training and testing. There was a plastic container holding 2 L artificial nectar under the nest box. The bumblebees had access to the nectar *ad libitum* through a sponge at all times except for two days before the training and one day before the testing. The bumblebees were denied nectar in that period to encourage them to fly into the flying arena. In between training and testing, they were exposed to clothianidin thought the nectar for nine days. The nectar bag was weighed at the start of the exposure period and during dissection so that the amount of nectar consumed could be calculated. During the exposure, each colony was fed a pollen and nectar mixture every second day (both delivered separately with the colonies). The mixture was made by heating freeze-dried pollen and 50% sugar content nectar and mixing them with a hand blender until the pollen was dissolved and the mixture was homogenised. The mixture was stored cold at +4°C. The colonies were fed 6 g of the pollen-nectar mixture which was weighed out on a small plastic beaker and placed in the nest box. The schedule for feeding was created so that the colonies were not fed pollen when they were starved for nectar.

2.3.1 Treatment

The experiment included six treatment levels: 0 μg/L (control), 1 μg/L, 1.9 μg/L, 3.6 μg/L, 6.8 μg/L, and 13 μg/L clothianidin. These levels are all in the range of field-realistic concentrations, based on studies measuring residuals of clothianidin in plants and beehives (Table 2). Clothianidin can be found in both pollen and nectar in the field due to its hydrophilic and systemic properties, but in this study, bumblebees were only exposed through the nectar. Each treatment level was considered a group and each colony was considered a replicate. Forty-eight colonies (N = 48) were used in the experiment, giving 8 replicates per group. Each colony was randomly assigned a treatment level and exposed for nine days, starting on the day of training and ending one day before testing.

The experiment was blinded from the arrival of the colonies until the start of the dissection. A standard toxicology study requires a control and only three concentrations with five replicates per concentration (Holland-Letz & Kopp-Schneider, 2015). Therefore, dissection and chemical analysis were performed on three treatment levels as well as the control, using five replicates per level. To be able to pick out the colonies that would be used further in the study, the treatments given to each colony was revealed, and the experiment was no longer blinded from that point forward. There is little knowledge about what clothianidin concentrations to expect in bumblebees at certain exposure levels, and so hives in the treatment levels were dissected and analysed in a step-wise manner – a treatment level was analysed and then based on the concentrations found in those samples, the next treatment level was chosen. First, the highest treatment level (13 μg/L) and the control were analysed, then the middle treatment level (3.6 μg/L), and then finally the second highest treatment level (6.8 μg/L). The second highest treatment level was chosen because of a large number of values found below the limit of detection in the middle treatment level.

Table 2. Overview of field-realistic concentrations detected in plants relative to potential exposure routes, pollen loads, and nectar found in returning foragers.

Plant	**Compartment**	**Concentration (*μ*g/kg)**	**Country**	**Reference**
Canola	Nectar	2.24	Canada	Cutler and Scott-Dupree (2007)
	Pollen	2.59		
Oilseed rape	Nectar	2.6 ± 4.0 (max 10.1)	Poland	Pohorecka et al. (2012)
	Pollen load	0.6 ± 0.6		
Pumpkin	Nectar	0.1 – 4 (max 12.2) (metabolite)	USA	Dively and Kamel (2012)
Maize	Bee pollen	1 – 4 (metabolite)	France	Pilling et al. (2013)
Corn	Pollen	3.0	USA	Stewart et al. (2014)
Oilseed rape	Nectar (bumblebee)	5.4	Sweden	Rundlöf et al. (2015)
	Nectar (honeybee)	10.3 ± 1.8		
Winter oilseed rape	Pollen	1.7 ± 1.8 (max 3.5)	Germany	Rolke et al. (2016)
	Nectar	1.3 ± 0.9 (max 3.6)		
Corn	Pollen	1.8 ± 1.7	USA	Xu et al. (2016)
Oilseed rape	Pollen	13.9 ± 1.8 (max 23)	Sweden	Osterman et al. (2019)
	Nectar	10.3 ± 1.3 (max 16)		
	Pollen	6.1 ± 2 (max 16)		
	Nectar	4.9 ± 1.1 (max 9.8)		

2.3.2 Preparation of dilution series

Pure clothianidin powder was dissolved in distilled water, and the right concentrations were found by a subsequent dilution regime. To prevent the need for solvents, the clothianidin concentration in water was kept under 327 mg/L, the limit of precipitation for clothianidin (Federoff & Barrett, 2005), throughout the whole process. To obtain a blinded experiment, the dilution series was constructed so that the final step (the final solution to the nectar bag) was identical for all treatment levels, and thereby, the volume transferred to the nectar bag in the final step was dependent on the volume of nectar in the bag rather than the desired final concentration.

Clothianidin is rapidly photodegraded in water, having a half-life of 13 hours when exposed to continuous radiation from sunlight (Li et al., 2018a). To avoid degradation when preparing the dilution series, the laboratory procedures were performed in a dimmed room, and the final dilutions were stored in glass bottles wrapped in aluminium foil at +4°C. No significant clothianidin degradation has been found to occur in the dark (Federoff & Barrett, 2005). The nectar bag was not exposed to light as it was placed inside the cardboard box and under the plastic box containing the colony.

Stock solution

20 mg of pure clothianidin (Sigma-Aldrich, USA) was measured in a glass weighing boat on Mettler Toledo AG204 Analytical balance. The powder was transferred to a 100 mL volumetric glass flask and the remaining residues were flushed into the flask using distilled water from a 100 mL flask. Distilled water was added until the volume reached the line on the flask, giving a concentration of 200 μg/L. The solution was transferred to a 250 mL wide neck glass flask and stirred using a magnetic agitator and heater (Rotamix 550 MMH) at settings temperature 4 and speed 550-600. Clothianidin was verified to have dissolved completely into the water when no white powder was observed in the water.

Intermediate solution

1250 μL was transferred from the stock solution to a 50 mL volumetric glass flask using a 1000 μL micropipette (Eppendorf research plus). 48.75 mL distilled water was added until the volume reached the line on the flask, giving a concentration of 5 μg/L. The solution was transferred to a 100 mL wide neck glass flask and stirred using the magnetic agitator.

Final solution

The final solutions were obtained using the dilution regime depicted in table 3. A specific volume for the dose was transferred from the intermediate solution to a 50 mL volumetric glass flask using a 1000 μL or 5000 μL micropipette (Eppendorf research plus). Distilled water was added until the volume reached the line on the flask, giving the final concentration. The solution was transferred to a 100 mL wide neck glass flask and stirred using the magnetic agitator.

Table 3. The dilution regime used to obtain the final clothianidin solutions dissolved in distilled water.

Dose	**Volume intermediate solution (μL)**	**Volume distilled water (mL)**	**Final concentration (μg/L)**
1 μg/L	1000	49	100
1.9 μg/L	1900	48.1	190
3.6 μg/L	3600	46.4	360
6.8 μg/L	6800	43.2	680
13 μg/L	13000	37	1300
Control	0	50	0

Adding final solution to nectar bags

The nectar which accompanied the colonies from the distributor had a concentration of 50% sugar, while bumblebees prefer a sugar content of approximately 30% (Willmer, 2011). It also contained preservatives, which allow the nectar to stay fresh for a longer time. The nectar bag was retrieved from the cardboard box of the colony to be exposed. The quality of the nectar was checked (no bad smell or discolouration) before it was transferred to a large Erlenmeyer flask, and the nectar bag was cleaned and dried. 900 mL nectar and 585 mL distilled water were transferred to a 1500 mL Erlenmeyer flask using a measuring cylinder. 15 mL of the final concentration was added using a 5000 μL micropipette (Eppendorf research), which further diluted the treatment 100 times. The solution was mixed using a magnetic agitator and transferred back to the nectar bag.

2.4 Dissection of hives and bumblebees

Hive dissection

At the end of the exposure period, bumblebees in the hive were killed by freezing them in -20°C for at least 48 hours. During dissection, the following units were counted: number of adults, pupa, large larvae, small larvae, eggs, full honeypots, half-filled honeypots, and empty honeypots. The larvae were split into two categories, as they in their development go from sharing a cell with other larvae to developing their separate bulge (Michener, 2007). Small larvae were defined as the ones that shared a cell, while large larvae were defined as the ones that had their own bulge. Each of the individuals was divided into living or dead based on colour and physiological criteria (Table 4), which was the same method used in a previous study (Paus-Knudsen, 2017). In addition, the number of queens, whether pollen was present or not, and the weight of the nectar bag was noted. All individuals were stored in Eppendorf tubes based on their life stage and living or dead status. Finally, approximately 1 mL of nectar was retrieved from the nectar bag and transferred to a 15 mL Eppendorf tube using a micropipette (Eppendorf research). One hive without adult bumblebees is pictured in figure 2, taken during dissection of the hive.

Table 4. Criteria used when classifying individuals as dead or alive during dissection of hives. Dissection was performed after the hives had been frozen for at least two days.

Life stage	Alive	Dead
Adult	Normal shrinking due to freezing, positioned in the core hive	More than normal shrinking due to freezing, positioned along the corners of the box
Pupa	Colour: white/light yellow Other: moist	Colour: grey Other: shrunken and dried up
Larva	Colour: white/light yellow/brown Other: moist	Colour: dark brown/black Other: turgid/bloated or dried up
Egg	Colour: white Other: moist, containing solid substance	Colour: white/dark brown/black Other: if white – not containing solid substance

Figure 2. Hive made up of honeypots and cells containing eggs, larvae, and pupa, viewed from above. Picture was taken during the dissection of the hives, after most of the adult bumblebees had been taken out from the hive.

Bumblebee dissection

Bumblebee workers and the queen were dissected into three parts, (1) head, (2) stomach, intestine, and rectum, and (3) the rest of the body (Figure 3). Workers were distinguished from drones by the presence of a stinger at their tail. There was a large variation of worker size in the colonies, and so bees of intermediate and large size were picked because they were the easiest to handle during the dissection. In some bees, the entrails had dried up, and it was difficult to remove them from the exoskeleton as well as distinguishing them from the muscle tissue and binding tissue. The bees with moist entrails were therefore preferred. All worker bees dissected were retrieved from the "alive" category in the hive dissection.

Using thin tweezers or a scalpel, the head was first removed from the body. The stomach, intestine, and rectum were collected from the body by pulling them with tweezers either from the top or from the bottom of the abdomen. The crop and the poison sac were identified and stored together with the rest of the body as the different compartments were put in separate Eppendorf tubes (the body in 50 mL Eppendorf tubes and the other compartments in 15 mL Eppendorf tubes).

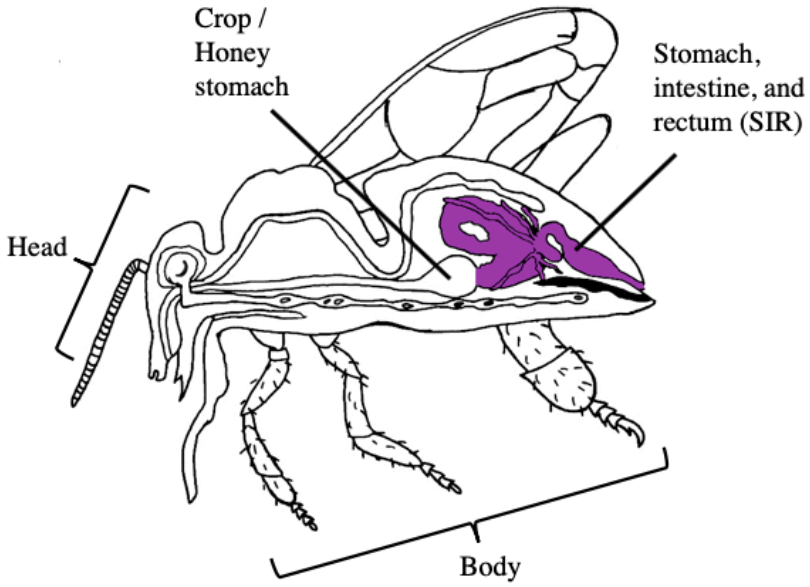

Figure 3. The bumblebee workers and the queen were dissected into three parts. The head, the stomach, intestine, and rectum (SIR), and the rest of the body. Figure made by the author.

Some of the colonies had more than one queen. There are no specific traits that differentiate the original queen of the colony to new queens after they have developed their colours (all new-born bumblebees are grey in colour). The best method available is to try to find signs that show that the queen has lived for a longer time. These signs could include less hair or bald spots on her dorsal thorax. When the original queen, or the one thought to be the original queen, had been identified, the same dissection procedure was performed on her as with the workers. It was difficult to properly differentiate between her reproductive organs and the digestive tract, and they were therefore combined.

Each sample was weighed. Eppendorf-tubes were weighed before and after the samples were stored in them, and the sample weight was calculated by subtracting the start-weight from the end-weight.

2.5 Clothianidin analysis

2.5.1 Preparation of samples

Preparation of samples was performed at the Norwegian Institute of Water Research (NIVA). The method used was first established by Wiest et al. (2011) and then further developed by Jan Thomas Rundberget, a chemist at NIVA. All sample preparations were conducted under a fume hood. To ensure clothianidin levels above the limit of detection, chemical analysis was run on pooled samples made up of ten workers per colony. This decision was based on a test-run of the developed method using bumblebees exposed to imidacloprid in a previous study (Paus-Knudsen, 2017). The results from the test-run will not be presented here or discussed further.

Distilled water and acetonitrile (MeCN) (Sigma-Aldrich and VWR Chemicals, US) were added to each sample with a micropipette (Biohit/Sartorius) in a 1:3 relationship (water:MeCN). A rule of thumb was set based on the weight of the samples: samples below 1 g were added 1 mL of water and 3 mL of MeCN, samples above 1 g were added 2 mL of water and 6 mL of MeCN. If the samples were not completely submerged by then, water and MeCN were added 1 mL every second time until satisfaction. 10 μL of internal standard, containing the deuterated parent compounds of several neonicotinoids including d3-clothianidin, was added to each sample with a micropipette (Biohit/Sartorius). All samples except nectar were homogenised using an Ultra-thurrax homogeniser (Pro Scientific PRO250), which was cleaned between each sample. The queen heads were first cut into smaller pieces using a scalpel before distilled water and MeCN were added, as they would not be homogenised efficiently as a whole. The samples were then stored in the dark at +4°C until further analysis, which was performed by the chemist.

Prior to High-performance liquid chromatography-mass spectrometry (HPLC-MS) analysis, each sample was added approximately 1 g of NaCl (salt) and shaken. The salt dissolved into the water, which then separated from the MeCN. By centrifuging the samples, distinct layers of water containing NaCl and MeCN could be found. The distinct layers allowed for the chemist to remove as much MeCN as possible. The MeCN fraction was evaporated until

almost dryness with heat (+60°C) under nitrogen. The remaining content was then dissolved in 0.5 mL 10% MeCN in water

2.5.2 High-performance liquid chromatography-mass spectrometry analysis

High-performance liquid chromatography-mass spectrometry (HPLC-MS) analysis was performed by Jan Thomas Rundberget at NIVA, and the procedure has been described by him in Appendix A.

2.6 Data treatment

2.6.1 Potential explanatory variables

Several potential explanatory variables were included in the statistical analysis when building the full model for explaining the variation in each response variable (Table 5). Due to seemingly large differences in age and some difference in the health of the colonies upon arrival to the University of Oslo (UiO), a health parameter was created to try to account for the variance. Each colony was given a health status based on whether they were above or below the mean of three parameters: proportion of empty honey pots, proportion of dead adults, and total number of broods produced (eggs, small larvae, large larvae, and pupa). Having a proportion of empty honey pots or number of broods produced below the mean was considered a failure while having a proportion of dead adults above the mean was considered a failure. Based on the number of failures received the colony was given one out of four statuses: good (0 failures), medium (1 failure), poor (2 failures), or very poor (3 failures).

Table 5. Overview of the explanatory variables used in the statistical analyses.

Accumulation and Colony data	
Treatment level	Numeric variable with four concentration levels: 0, 3.6, 6.8, and 13.
Days after delivery	Numeric variable with the number of days from the delivery of the colony to it was frozen.
Number of queens	Numeric variable with the number of queens found in the hive when it was dissected.
Health	Factor variable with four levels: good, medium, poor, and very poor[a].
Size	Numeric variable with the number of individuals counted in each colony.
Colony data only	
Concentration in head	Numeric variable with measured clothianidin concentrations found in the head of workers, with values ranging from 0.0 to 2.7 μg/kg w.w.
Concentration in body	Numeric variable with measured clothianidin concentrations found in the body of workers, with values ranging from 0.0 to 4.5 μg/kg w.w.

a: None of the colonies received 3 failures, and therefore the "very poor" level was not present in the statistical analysis.

2.6.2 Statistical analysis

Statistical analysis was performed in R version 3.5.2 for Mac (R Core Team 2018). Normal error distribution was assessed using the Shapiro-Wilk test, and homogeneity of variance was assessed using the Barlett's test. Clothianidin concentrations in tissues were analysed as concentrations in wet weight (μg/kg w.w.) Generalised linear models (GLMs) were generated to test whether there was an effect of the treatment (exposure to clothianidin) while also controlling for other variables (table 5). Following, Dunnett's test was used to test which treatment level(s) was statistically significantly different from the control.

Data below limit of detection

The chemical analysis of the different body compartments in workers and the queen resulted in a large number of values below the limit of detection (LOD), especially at the lowest exposure level. To include the measured doses as an explanatory variable, all the values below LOD had to be replaced as they in the raw data were denoted <LOD (table B1, Appendix B). Out of a total of six body compartments chemically analysed, both from workers and queens, only the head and body compartments from workers had more than 80% detections above LOD. Therefore, statistical analysis was performed only on these two compartments. For the non-detects, it was assumed that the actual values were close to LOD and not 0. Only including the values above LOD in the calculations would mean to assume that the values below LOD were 0, which would make the assessment conservative and break with the assumptions that have been made for these values previously. Therefore, random values between ½LOD and LOD were generated, which replaced each of the values below LOD. Inserting a value between ½LOD and LOD was assumed to make little impact on the calculations of the bioaccumulation factor. The control group received only values below LOD and were all given the value 0 as they were never exposed to clothianidin.

Not Analysed

One nectar sample in the highest treatment level came back as Not Analysed (NA). Ideally, this NA should be excluded from the statistical analysis, but it was included due to the small sample size. Therefore, the NA was replaced with the mean (10.2 μg/L) of the other values in the same treatment level.

Model selection

To identify the best model explaining each of the focal response variables, the model selection procedure "model.sel" from the R package MuMIn (Pohlert, 2018) was used. This procedure starts with a universal model (i.e. including all potential explanatory variables listed in table 5) and runs through all possible models containing subsets of the full variable set. Akaike's Information Criterion adjusted for sample size (AICc) was used as the model selection criterion.

Accumulation in body compartments

Generalised linear models (GLMs) were generated to assess whether concentration found in the head and body varied with clothianidin treatment level or any of the other explanatory variables. The concentrations measured in the head and body were used as individual response variables and fitted to models with a Gaussian error distribution.

Colony data

Generalised linear models (GLMs) were generated to assess whether the response variables from the colonies varied with clothianidin concentration or any of the other explanatory variables. The proportion of dead adults, dead pupa, dead large larvae, dead small larvae, dead eggs, and empty honeypots were used as individual response variables and fitted to models with a binomial error distribution. A logit link function was used due to non-normal distributed (proportion) errors. Each response variable was calculated as the number of failures (e.g. Dead adults or dead eggs) divided by the total number of observations in that category (total number of adults, total number of eggs). Consequently, the total number of observations were used as weights in the models.

Generalised linear models (GLMs) were generated to assess whether nectar consumption varied with clothianidin treatment level or any of the other explanatory variables. The models had a Gaussian error distribution. One of the colonies had a negative value for nectar consumed and was replaced with 0.001 so that it could be included in the statistical analysis, as R does not accept the value 0. Although the optimal solution would be to exclude the value, the colony was included due to the small sample size.

2.6.3 Calculation of bioaccumulation factors

The bioaccumulation factor (BAF) was calculated for workers at each treatment level using the following equation

$$BAF = \frac{[Chemical]_{organism}}{[Chemical]_{diet}} \quad \text{(Equation 1)}$$

$[Chemical]_{organism}$ meaning the concentration of the chemical measured in the organism, and $[Chemical]_{diet}$ meaning the concentration of the chemical measured in the diet. Application of this equation to the present study resulted in the following equations

$$BAF = \frac{[Clothianidin]_{bumblebee\ body\ compartment}}{[Clothianidin]_{nectar}} \quad \text{(Equation 2)}$$

$$BAF = \frac{[Clothianidin]_{bumblebee}}{[Clothianidin]_{nectar}} \quad \text{(Equation 3)}$$

Where $[Clothianidin]_{bumblebee\ body\ compartment}$ meant the mean of measured clothianidin concentration in the specific body compartment, $[Clothianidin]_{bumblebee}$ meant the mean of measured clothianidin concentration in all body compartments combined, and $[Clothianidin]_{nectar}$ meant the mean concentration of clothianidin measured in the nectar. $[Clothianidin]_{bumblebee}$ was calculated by adding together the means of the clothianidin concentrations measured in each body compartment. The stomach, intestine, and rectum compartment for workers was excluded from other statistical analysis due to nearly all samples being below the detection limit, but this compartment was included in the estimation of BAF due to the necessity to look at the whole organism (Arnot & Gobas, 2006). BAFs were not calculated for queen data due to the high amount of values below LOD in all body compartments.

3 Results

3.1 Nominal versus measured exposure in nectar

Comparison of the nominal exposure to the measured exposure in nectar indicated good compliance (Figure 4). The measured exposure (mean: 3.6 µg/L = 2.74 µg/L, 6.8 µg/L = 6.54 µg/L, and 13 µg/L = 10.18 µg/L) was on average 17% below the nominal exposure.

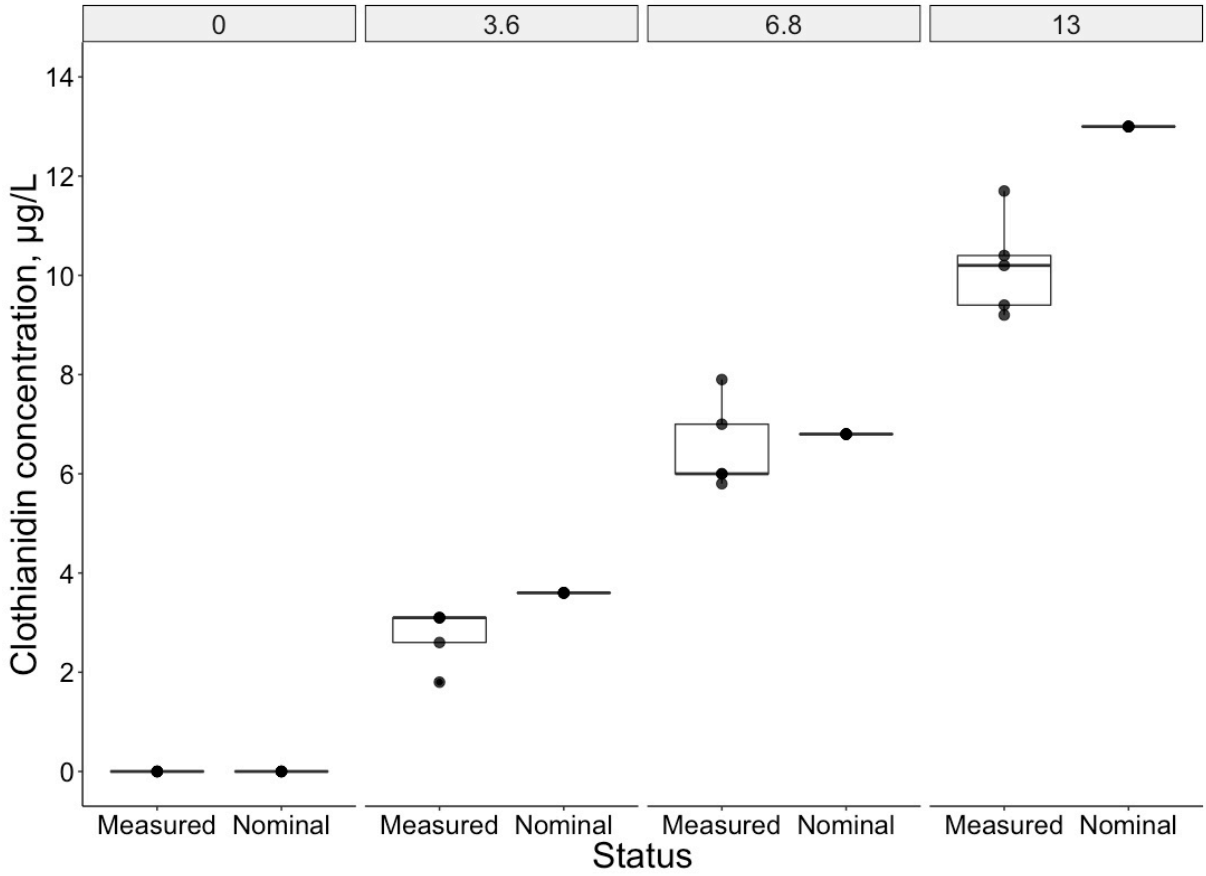

Figure 4. Comparison of clothianidin content (y-axis) between the measured and the nominal concentrations in the treatment levels (x-axis). The boxes show the variation of the dataset, with the bold black line specifying the median, the lower and upper lines of the box showing the first and third quartiles, and the whiskers show the largest and lowest "non-extreme" values. All values outside of this range are outliers. Each black dot refers to a separate colony.

3.2 Clothianidin accumulation in bumblebee workers

There was a positive relationship between the concentration measured in both the head and body compartments and treatment level, showing a clear dose-response relationship where the clothianidin concentration in the workers' head and body increased with experimental dose (Figure 5). The clothianidin concentrations in the two compartments increased in a similar manner to increased clothianidin exposure, but these concentrations did not exceed the clothianidin concentrations in the nectar.

For the head, the best model contained treatment level as the single explanatory variable (AICc = 31.3). The 6.8 μg/L and 13 μg/L levels were different from the control (Dunnett's test p-value = 0.003 and 0.0001), while the 3.6 μg/L level was not (Dunnett's test p-value = 0.39). However, the second and third model differed from the best model with <2 AICc, indicating that there was no actual difference in explanation power between the models. The second and third best models contained health, size, and treatment level (AICc = 31.5) and health and treatment level (AICc = 33.0) as explanatory variables, respectively.

For the body, the best model contained treatment level as the single explanatory variable (AICc = 49.3). The 6.8 μg/L and 13 μg/L levels were different from the control (Dunnett's test p-value: 6.8 μg/L = 0.001 and 13 μg/L = 0.00008), while the 3.6 μg/L was not (Dunnett's test p-value: 0.88). The second and third best models contained number of queens and treatment level (AICc = 51.8) and size and treatment level (AICc = 52.4) as explanatory variables, respectively.

There was no dose-response relationship between exposure to clothianidin and measurement in the stomach, intestine, and rectum. The highest concentrations were measured in the 6.8 μg/L level, and the 3.6 μg/L level contained only values below LOD.

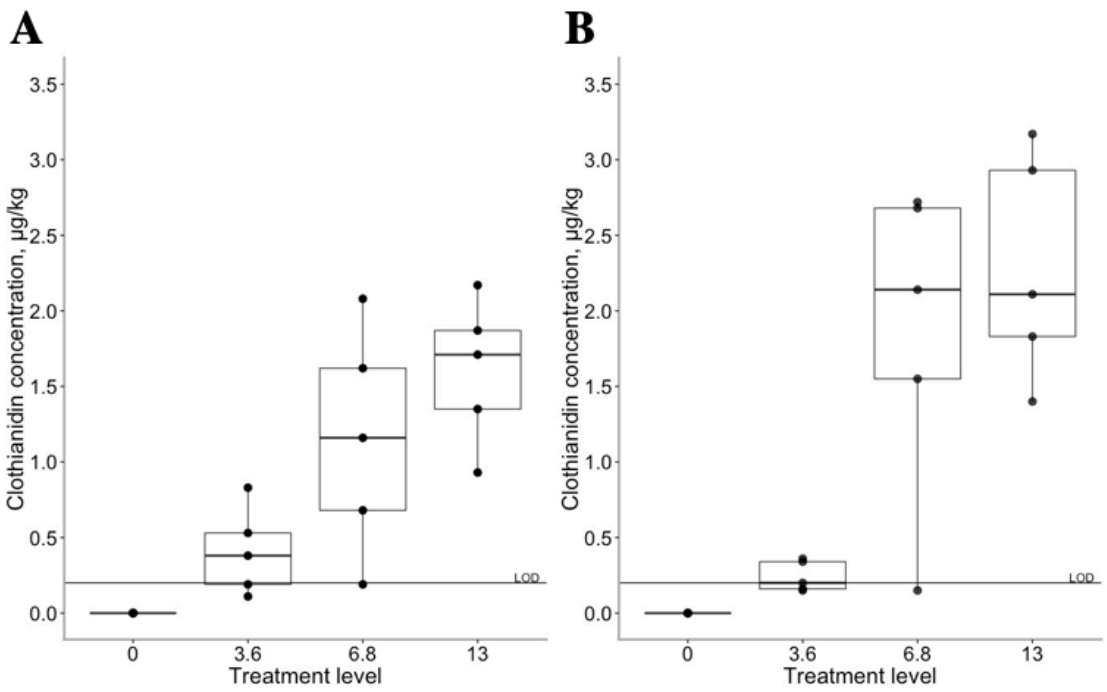

Figure 5. A) Relationship between the clothianidin concentration measured in the workers' head on the y-axis and the treatment levels on the x-axis. B) Relationship between the clothianidin concentration measured in the workers' body on the y-axis and the treatment levels on the x-axis. In both plots, the values below the limit of detection (LOD) have been replaced with randomly generated values between ½LOD and LOD. The boxes show the variation of the dataset, with the bold black line specifying the median, the lower and upper lines of the box showing the first and third quartiles, and the whiskers show the largest and lowest "non-extreme" values. All values outside of this range are outliers. Each black dot refers to a separate colony.

3.3 Clothianidin body distribution in bumblebee queens

In contrast to the workers, there was no detection of clothianidin in the queens' head except for two individuals. However, it should be noted that the LOD was higher in the queen heads (0.5 μg/kg), compared to all other compartments, both the workers and queens (0.2 μg/kg).

In the queen's body, accumulated clothianidin show a dose-response relationship (Figure 6). The 13 μg/L level had a large amount of variation in concentration compared to the other levels. Concentrations found in the body compartment of the queens were lower than concentrations found in the workers' body compartment (see figure 5B).

There was no trend of a dose-response relationship between exposure to clothianidin and measurement in the stomach, intestine, and rectum in bumblebee queens. There was a detection of clothianidin only in the 6.8 μg/L level.

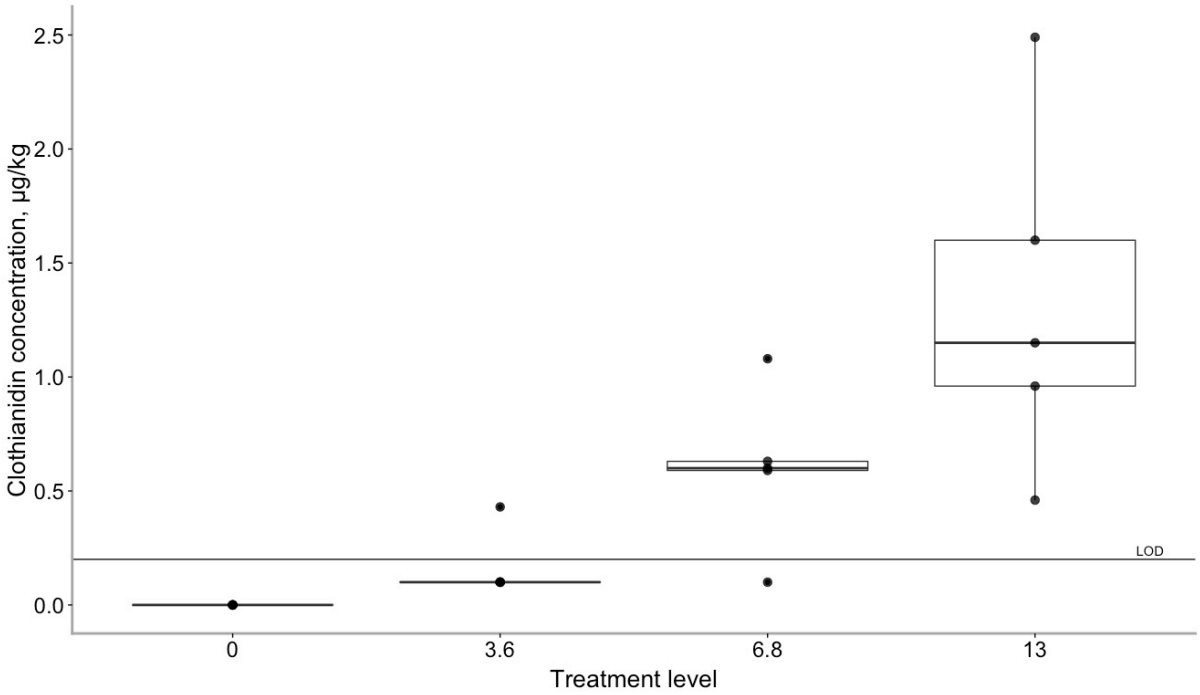

Figure 6. Relationship between the clothianidin concentration measured in the queens' body on the y-axis and the treatment levels on the x-axis. Values below LOD in the 3.6 μg/L level was replaced with ½LOD. The boxes show the variation of the dataset, with the bold black line specifying the median, the lower and upper lines of the box showing the first and third quartiles, and the whiskers show the largest and lowest "non-extreme" values. All values outside of this range are outliers. Each black dot refers to a separate colony.

3.4 Bioaccumulation factors

The Bioaccumulation factors (BAFs) from two body compartments and for the whole bumblebee is summarised in Table 6. The BAF for the control group was by default zero and has not been included here. The BAFs did not show a dose-response relationship. The BAF in the head was similar for all three treatment levels, while BAFs for the body and the whole bumblebee differed between the doses. As none of the clothianidin concentrations in the head and body compartments exceeded the clothianidin concentrations in the nectar, the BAFs were all below 1.

Table 6. A summary of the bioaccumulation factors calculated for the head, body, and whole bumblebee (total), based on measured clothianidin concentration in nectar. The control has not been included as it is by default zero (No clothianidin was detected in neither bumblebees nor the nectar in the control).

Treatment level	Head	Body	Total
3.6	0.2	0.1	0.3
6.8	0.2	0.3	0.7
13	0.2	0.2	0.5

3.5 Sublethal and lethal effects

3.5.1 Proportion of life stages in colony

The proportion of life stages showed no dose-response to exposure to clothianidin. The relative proportion of the life stages in the colonies showed that the control and the 13 μg/L level were the most similar (Figure 7), mostly due to their proportion of small and large larvae. They were also the most similar in colony size (mean number of individuals in colony: control: ~440, 13 μg/L: ~431) compared to the 3.6 μg/L and 6.8 μg/L levels (mean number of individuals in colony size: 3.6 μg/L: ~376, 6.8 μg/L: ~346), as seen in figure 8. There was a great variation in all the life stages, shown in the broad range (min – max) and the large standard deviations around the mean in Table B3 (Appendix B).

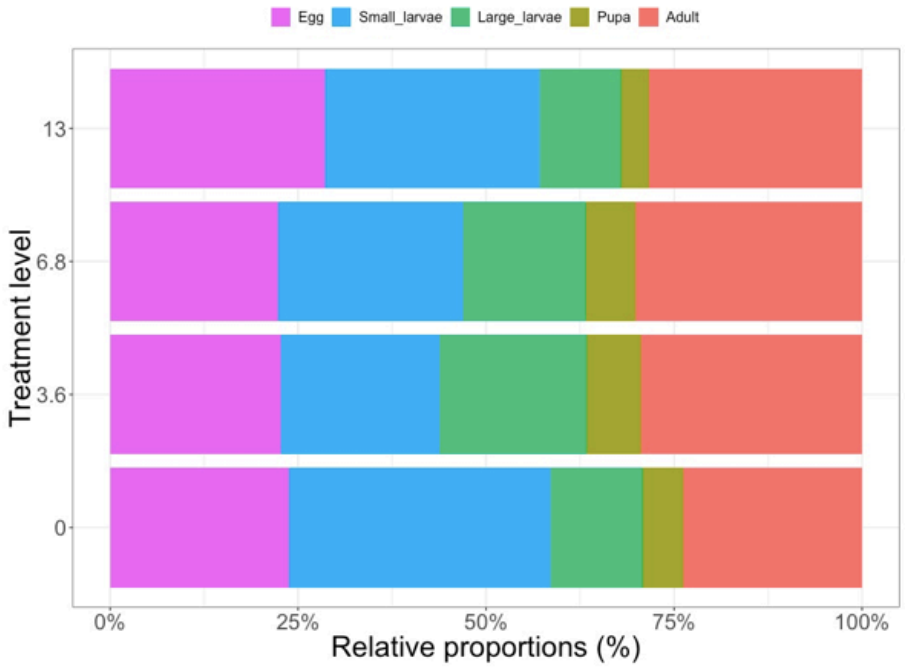

Figure 7. Relationship between the relative proportions of the bumblebee life stages as they are distributed in the colonies on the x-axis and the treatment levels on the y-axis.

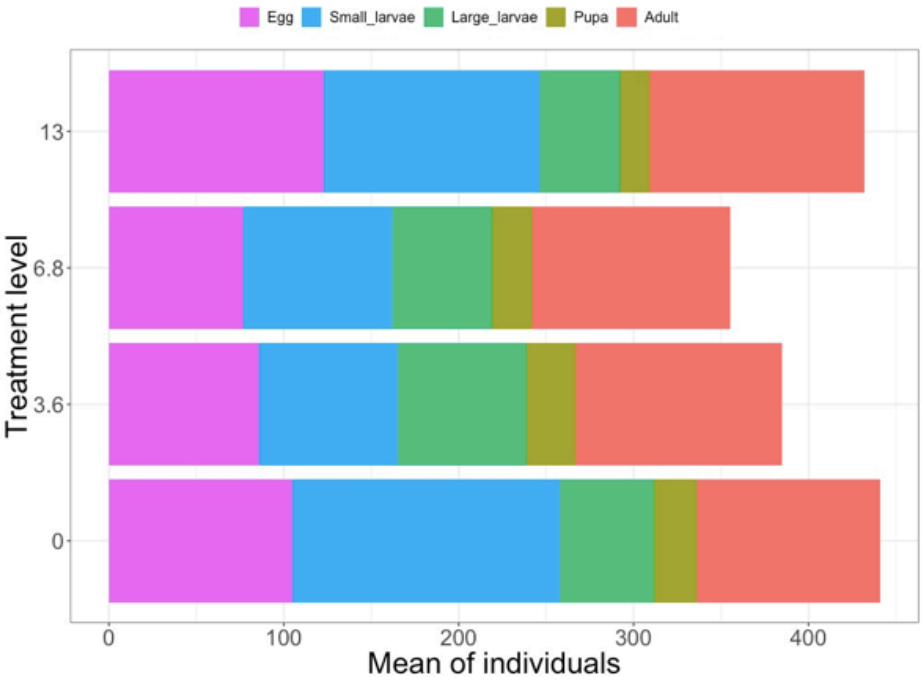

Figure 8. Relationship between the mean of individuals distributed by their life stages in the colonies on the x-axis and the treatment levels on the y-axis.

3.5.2 Brood production

Brood production was not affected by exposure to clothianidin. The best model included none of the explanatory variables (AICc = 19.0). The second and third best models contained number of queens (AICc = 21.4) and treatment level (AICc = 21.4) as the explanatory variables, respectively. The 3.6 μg/L level had the highest amount of variation in the proportion of produced broods.

3.5.3 Mortality of adults and broods

The proportion of dead individuals in the colonies showed no dose-response to clothianidin exposure (Figure 9). The control and the highest treatment level (13 μg/L) had the highest mortality rate of 35% and 34%. The control also had the highest mortality rate for eggs and small larvae, and the second highest mortality rate for pupa and adults (Table 7). There was no directional order in mortality rate in the other treatment levels. The 3.6 μg/L level had the second highest mortality rate for eggs and the highest for pupa, the 13 μg/L level had the second highest mortality rate for small larvae, and the highest for large larvae and adults. Surprisingly, the 6.8 μg/L level had the lowest mortality rate for all life stages.

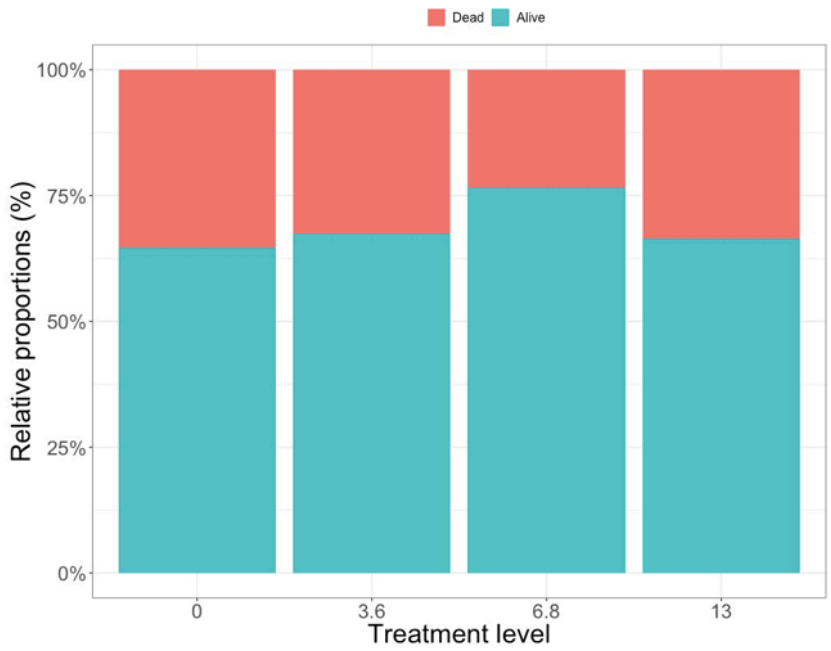

Figure 9. Relationship between the relative proportion of living and dead in all life stages on the y-axis and treatment levels on the x-axis.

Table 7. The relative proportions of dead individuals of each life stage in each treatment level.

Treatment level	Dead adults (%)	Dead pupa (%)	Dead large larvae (%)	Dead small larvae (%)	Dead eggs (%)
Control	19.2	70.3	40.3	55.6	56.1
3.6 µg/L	13.9	70.8	43.3	42.1	20.6
6.8 µg/L	11.3	41.7	37.3	39.2	9.0
13 µg/L	27.2	46.8	52.3	53.9	12.0

Mortality of adults and broods

There was no relationship between the proportion of dead adults and dead broods and exposure to clothianidin. The best model explaining the mortality variation in each life stage, except for pupa, contained none of the proposed explanatory variables. For pupa, the best model contained number of queens. However, for pupa, large larvae, and small larvae, the best models had a difference in AICc smaller than 2, indicating that there was no actual difference in explaining power between the models. For all other life stages except in pupa and eggs, number of queens was the explanatory variable in the second best model. There was no clear trend found in the data set. Due to no relationship with treatment level, LC_{50} values were not calculated.

3.6 Nectar consumption and food storage

Nectar consumption

There was a relationship between the amount of nectar consumed and treatment level (Figure 10). The best model contained both concentration in worker head and treatment level as explanatory variables (AICc = 239.2). Despite treatment level being an important explanatory variable, none of the levels were different from the control (Dunnett's test p-value: 3.6 µg/L = 0.69, 6.8 µg/L = 1.0, 13 µg/L 0.96). However, the difference in AICc between the two best models was smaller than 2, indicating that there was no actual difference in explanation power between the two models. The second best model contained concentration in worker head, number of queens, and treatment level (AICc = 240.8), while the third best model contained concentration in worker head only (AICc = 241.8). There seemed to be a trend

present where the bumblebees consumed more nectar at exposure to low concentrations of clothianidin (3.6 μg/L mean: 248 g, 6.8 μg/L mean: 240 g) and then consumed less at no and high exposure to clothianidin (control mean: 200 g, 13 μg/L mean: 211 g).

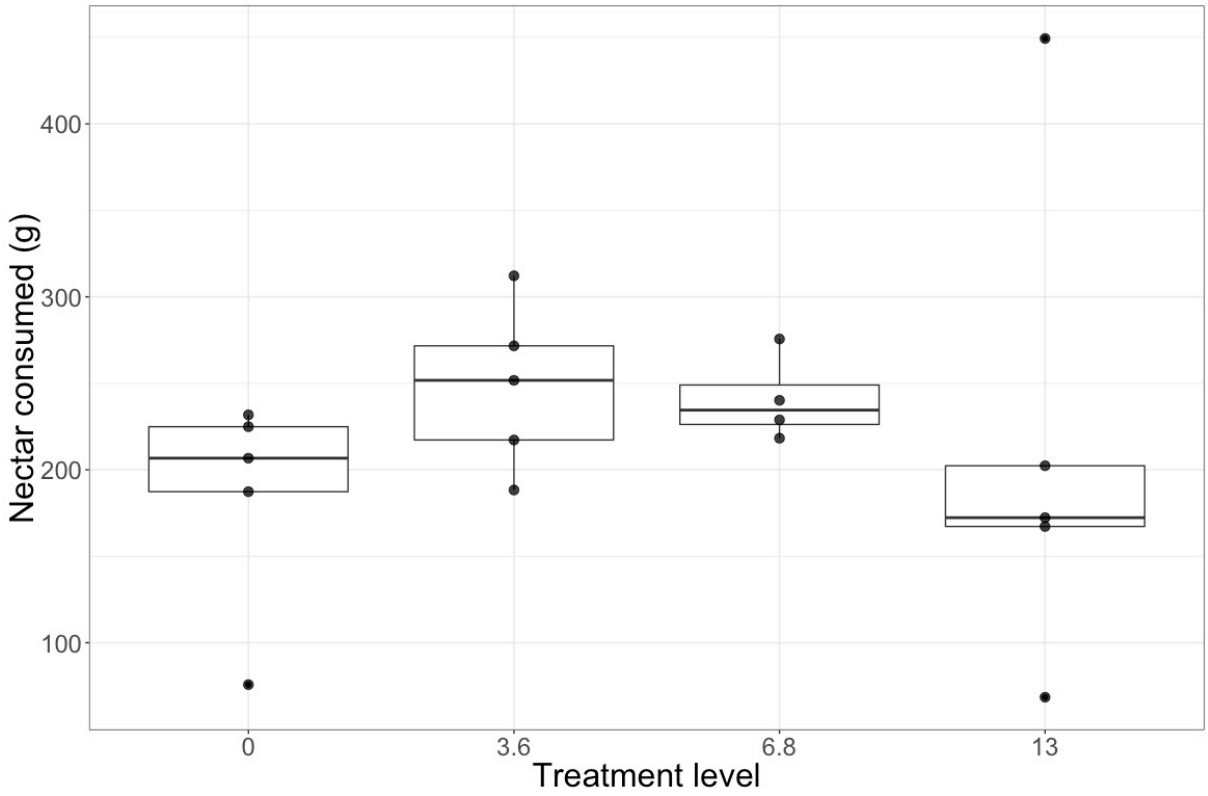

Figure 10. Relationship between the amount of nectar consumed on the y-axis and the treatment levels on the x-axis. One colony was removed from the 6.8 μg/L level due to it having a negative value. The boxes show the variation of the dataset, with the bold black line specifying the median, the lower and upper lines of the box showing the first and third quartiles, and the whiskers show the largest and lowest "non-extreme" values. All values outside of this range are outliers. Each black dot refers to a separate colony.

Food storage in honeypots

There was no statistical relationship between the proportion of empty honeypots and treatment level or any of the other explanatory variables (Figure 11). The best model contained none of the explanatory variables (AICc = 28.8), while the second and third best models contained treatment level (AICc = 29.9) and concentration in worker body (AICc = 30.3), respectively. The difference in AICc between these models is <2, suggesting that none of the models explains the data better than the others. There seemed to be a trend. From the control to the highest treatment level, there is a trend of a decrease in the proportion of empty honeypots. Dunnett's test showed no difference between the levels and control (Dunnett's test

p-value: 3.6 μg/L = 0.97, 6.8 μg/L = 0.37, 13 μg/L = 0.66), but this may be due to the large variance found in the 13 μg/L level.

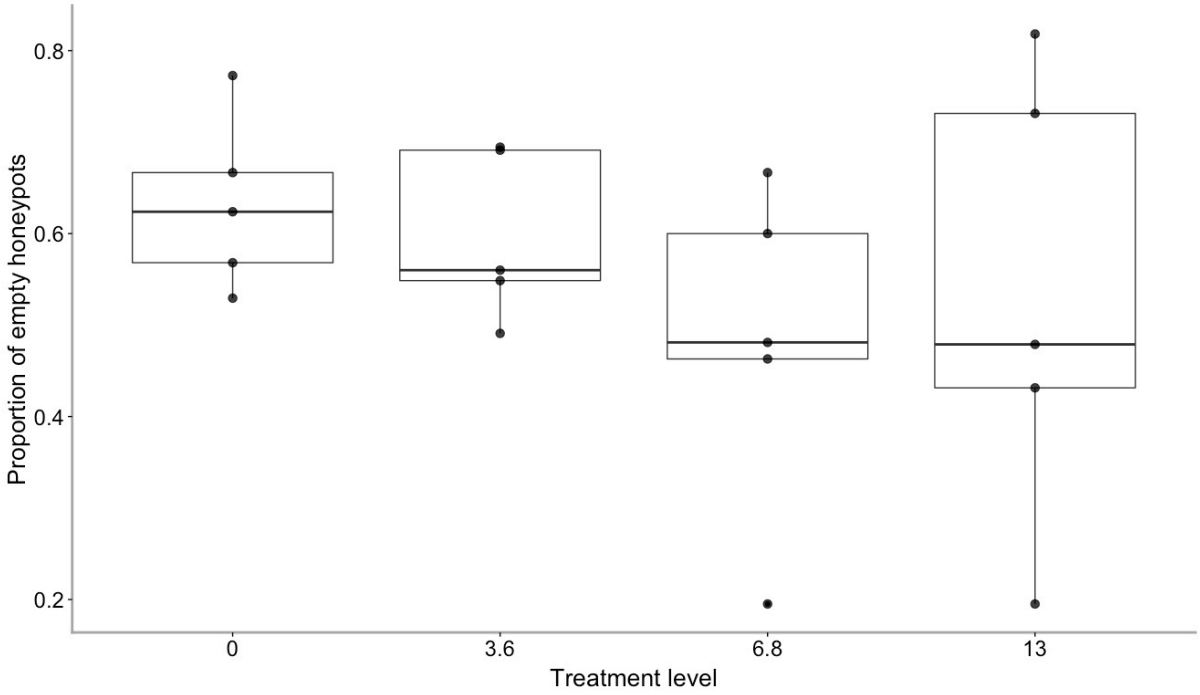

Figure 11. Relationship between the proportion of empty honeypots on the y-axis and the treatment levels on the x-axis. The boxes show the variation of the dataset, with the bold black line specifying the median, the lower and upper lines of the box showing the first and third quartiles, and the whiskers show the largest and lowest "non-extreme" values. All values outside of this range are outliers. Each black dot refers to a separate colony.

4 Discussion

4.1 Nominal versus measured exposure

In line with the expectations (H3.1), the nominal and measured exposure showed overall good compliance. The small average difference of 17% lower concentration in measured exposure could be due to an error in the performance of the dilution series, minimal degradation during the preparation of samples, or accuracy of the High-performance liquid chromatography-mass spectrometry (HPLC-MS) machine. The good compliance between the nominal and measured exposure prove that the analytical method worked and show that the precautions used in the present study to avoid degradation, including working in dimmed rooms and storing the dilution series and samples in a dark and cold environment, have worked.

It is important to know the actual exposure in dose-response experiments. By measuring the actual exposure used in the experiment, calculations based on the exposure become more accurate. The measured exposure was used in the calculations of the bioaccumulation factors (BAFs) in the present study, and since the measured exposure was lower than the nominal exposure, the calculated BAFs became higher. As many of these calculations (e.g. hazard quotients, risk quotients, BAFs) are used in regulations at local, regional, and international levels, it is important that these calculations are accurate. If several of the calculations are inaccurate, providing ratios that are too low or too high, they may cause severe damage.

4.2 Clothianidin accumulation in bumblebee workers

Clothianidin accumulated in the head of bumblebee workers, which is in line with the expectations (H1.1). The BAFs for the head were the same for all treatment levels, indicating that the same proportion of the clothianidin in the diet was taken up and transported to the brain. Binding of neonicotinoids to the nicotinic acetylcholine receptors (nAChRs) in the bee brain has been connected to sublethal effects like reduced cognitive function and memory, showing a response at similar exposure levels as the present study (Stanley et al., 2015; Paus-Knudsen, 2017). The relatively small proportion of clothianidin taken up into the head suggests that only a very small internal dose is needed to elicit a sublethal response. Due to increased sensitivity to neonicotinoids over time, it is likely that bumblebees will perform

worse with prolonged exposure as more and more nAChRs become occupied (Alkassab & Kirchner, 2016). Some studies show that bees can recover after the exposure has ended (Cresswell et al., 2012), suggesting that the accumulation is time- and exposure dependent. However, other studies show that brood production in honeybees is not able to recover completely after ended exposure, and exposure during larvae stages can cause impaired olfactory behaviour in adults (Yang et al., 2012; Laycock & Cresswell, 2013). There is to the author's knowledge no studies reporting neonicotinoid concentrations measured in bee heads, making it difficult to compare the findings of the present study to others.

Clothianidin did also accumulate in the body of bumblebees, suggesting that bumblebees may not be able to sufficiently metabolise clothianidin above a certain concentration. Exposure to neonicotinoids cause a downregulation of genes involved in metabolism of pesticides (Li et al., 2019), which can have harmful effects on bumblebees as metabolism has been suggested to be strongly involved in their main pathway of excretion of neonicotinoids (Suchail et al., 2004a; Suchail et al., 2004b). The clearance rate of imidacloprid in bumblebees decrease and the residues of imidacloprid from the daily dose increase with exposure time (Cresswell et al., 2013; Sánchez-Bayo et al., 2017). Both suggest a decreased metabolism in bee pollinators after imidacloprid exposure.

In the present study, there was a steep increase in the clothianidin concentrations in the body compartment between the 3.6 μg/L level and the 6.8 μg/L level, suggesting the presence of a threshold. In the 3.6 μg/L level, three out of five values in the body compartment were below the level of detection (LOD). In the subsequent treatment level (6.8 μg/L), only one value was below the LOD, while the other values were comparable to the ones in the highest treatment level (13 μg/L). The difference in values above the LOD between the two treatment levels suggests that the bumblebees were able to metabolise and eliminate clothianidin at the lower treatment level (3.6 μg/L), but that the exposure at the next treatment level (6.8 μg/L) exceeded a response threshold and significantly slowed down the metabolism. Another explanation might be that clothianidin is not taken up into the body at all, but is dissolved in nectar residing in the crop, a food-collecting organ (Figure 3). Bee foragers collect nectar in the crop, where it is processed to only a very small degree (Willmer, 2011). In the present study, nearly all of the bumblebee workers had a crop filled with nectar, which was chemically analysed together with the body compartment. The presence of nectar in the crop

has been proposed as the explanation for elevated levels of neonicotinoids detected in the body in a previous study as well (Cresswell et al., 2013). However, as the measured concentrations of clothianidin in the nectar showed a dose-dependent difference between the 6.8 μg/L level and the 13 μg/L level, it is assumed that detection of nectar in the crop would also show a difference in concentration. As already mentioned, this was not the case, which increases the probability that it is clothianidin taken up into the body that has been detected in the body compartment. Although the BAF varied between treatment levels in the body compartments, the variation was very low and did not follow a dose-response relationship, and could be due to chance.

Clothianidin has previously been observed to accumulate in the rectum of honeybees after exposure to thiamethoxam (Coulon et al., 2018), and thiamethoxam can cause detrimental effects to the cell in the midgut of bees (Catae et al., 2014). The speculations of an accumulation of clothianidin in the stomach, intestine, and rectum (SIR) were based on these two studies, assuming that clothianidin had the same effect on the cells of the midgut as thiamethoxam. However, the results in the present study contradicted these expectations (H1.2). Interestingly, the highest number of detections of clothianidin in the SIR was in the 6.8 μg/L level, and the median concentrations of the 6.8 μg/L and 13 μg/L levels in the body compartment were comparable. Thus, the BAFs did not follow a dose-response curve and were the highest in the 6.8 μg/L level, both for the body compartment and the whole bumblebee.

4.3 Clothianidin accumulation in bumblebee queens

There is to the knowledge of the author, no studies looking at the accumulation of neonicotinoids in bumblebee queens, and this study is therefore the first. In contrast to expectations (H1.3), the queen did not have a higher accumulation in any of the body compartments compared to the workers. The queen is in general similar to her workers, although some differences exist. She is the longest-living individual in the colony, and she never leaves the hive after the first batch of workers has hatched (Willmer, 2011). As she is a larger individual, due to workers terminating larval development early, it is likely that she needs to consume more food compared to workers (Cnaani & Hefetz, 2001). However, the difference in clothianidin concentrations measured in the queen and the workers may indicate

that there are larger differences between them. Especially the lack of clothianidin in the queens' head is of particular interest. The queen could be able to metabolise clothianidin at a higher rate than the workers or she may not have an uptake of clothianidin to the head at all. The chemical analysis of the queens' head did have a higher LOD (0.5 μg/kg) than the other body compartments (0.2 μg/kg), and the real values in the head could be ranging from 0.2 μg/kg to 0.5 μg/kg. If that is true, the queen would be more comparable to the workers, but a large gap in detected concentrations would still remain and mark a difference between the two castes. The difference in detection of clothianidin in the head could also be due to weaknesses in sample preparations. An important step before the chemical analysis is for the acetonitrile (MeCN) to properly reach all parts of the tissues in the head, and therefore homogenisation of the body compartment is important here. Propper homogenisation of the queen's head proved to be difficult as it was difficult to achieve the same homogenisation for it as for the workers' heads. The low detection rate of clothianidin in the queen's head could, therefore, be due to MeCN not reaching all parts of the head and not because of a lack of clothianidin in the head. Prior knowledge indicates that clothianidin should be there, especially since there was an accumulation of clothianidin in the workers' head.

The queens showed a dose-response relationship between clothianidin concentrations measured in the body and the level of exposure, although the concentrations measured were lower than the ones measured in the workers. The detection of clothianidin in the body compartment shows that the queens consumed nectar containing clothianidin, and so the lack of clothianidin detected in the head could not be due to the queens consuming food from honeypots only. Queens could potentially have rid themselves of clothianidin though egg production. However, the SIR, which also included the reproductive organs of the queens, had a very low detection rate, where nearly all values were below the limit of detection. If the queens cleared themselves of clothianidin through egg laying, their rate of excretion through this method would have to be relatively high for there to be such low detections of clothianidin in this body compartment. Queens are more sensitive to neonicotinoids than workers, showing increased mortality at a lower nectar consumption rate (Mobley & Gegear, 2018). The mortality rate for queens was not surveyed in the present study, but the lack of clothianidin measured in the queens' body may suggest that this increased susceptibility is not so straight-forward.

4.4 Brood production

In contrast to expectations (H2.2), brood production was not affected by exposure to clothianidin. Exposure to imidacloprid and clothianidin cause a reduction in vitellogenin and hexamerin 70b production, and a reduction in sperm quality and sperm amount stored in the spermatheca in honeybee queens, which can lead to reduced reproduction and longevity (Williams et al., 2015; Chaimanee et al., 2016). Despite this evidence for a potential underlying mechanism, changes in brood production are not always observed, supporting the findings of the present study (Cutler & Scott-Dupree, 2007; Laycock et al., 2012; Laycock & Cresswell, 2013; Osterman et al., 2019).

In some of the studies which observed a decrease in brood production, a decrease was only observed after a prolonged period of exposure which exceeded the time period of the present study (Gill et al., 2012). This could indicate that a detrimental change in reproduction, for example, that the queen is not able to reproduce viable eggs, could be a delayed response. It takes approximately 22 days from an egg is laid until it hatches as an adult bumblebee (Gill et al., 2012), and so the delayed impact would only be observed approximately three weeks after the original queen died. Paus-Knudsen (2017) observed an increase in brood production in bumblebees with increased exposure to imidacloprid and suggested it to be an immediate response to the increased mortality in broods. A change in mortality due to increased clothianidin exposure, which will be further discussed later, did not occur in the present study and it would therefore not be needed for the colonies to overcompensate for dead broods.

4.5 Mortality in adults and broods

In contrast to the expectations (H2.1), mortality did not increase due to exposure to clothianidin. The mortality rate for all life stages combined was highest in the control and the highest treatment level (13 μg/L). These were also the treatment levels with the largest average colony populations. The workers remained in the hive for the whole duration of the experiment except for the three hours they were allowed into the flying arena. With a large population, the space in the hive could have become cramped, or the workers may have been too few to be able to feed the larvae and eggs sufficiently. The size may also be an indicator for the age of the colony, where larger colonies are also older (Bloch, 1999). If the colonies

were older, there might have been a large number of older workers who had reached the end of their natural lifespan and, in combination, caused an increased mortality rate.

Pupa had the highest mortality rate of 70% in both the control and the 3.6 μg/L treatment level. This was the only life stage where the number of queens best explained the response. Five hives had more than one queen present in the hive, and they were all, by chance, assigned to the control level or the lowest treatment level (3.6 μg/L). In nature, the life of the colony reaches a "switching point" during the late summer, where it stops producing workers and starts to mass produce first drones and then queens (Bloch, 1999). When the queen starts to lay unfertilised eggs, the sexual organs in some workers develop and they can lay unfertilized eggs (Bloch, 1999). Brood production from workers has the potential to cause aggression between workers and workers, and workers and the queen, which often cause both the workers and the queen to kill the unhatched broods (Alaux et al., 2004). Due to the presence of more than one queen in several hives of the control and the lowest treatment level (3.6 μg/L), it could be related to the high mortality rate observed here, especially for the pupa. Noticeably, in hive no. 1 and 6, which had 32 and 38 queens respectively, all the pupas were dead.

The experiment performed in this study was designed to assess the immediate effects of chronic exposure to clothianidin, but it should be noted that exposure to neonicotinoids could have a time-delayed effect. A study performed by Gill et al. (2012) showed a delayed effect by imidacloprid exposure which correlated with the time it takes for workers to develop from eggs to adults (22 days) and the effects were only prominent after two weeks. In the present study, it took nine days from the start of the exposure until termination. Following the findings from Gill et al. (2012) and other studies (Decourtye et al., 2004; Li et al., 2019), the time period in the present study could be too short to show the time-delayed effects of neonicotinoid exposure and accumulation. On the other hand, other studies have observed increased mortality to bees exposed to neonicotinoids during the same time period as used in the present study (Alkassab & Kirchner, 2016; Sánchez-Bayo et al., 2017).

4.6 Nectar consumption and food storage

In the present study, there was no statistical difference in consumption of nectar among the treatment levels, which contradicted the expectations (H2.3) However, the bumblebees showed a hormesis trend of consuming more food when exposed to low concentrations of clothianidin and consuming less food with no or high exposure to clothianidin, which indicates a relationship with clothianidin treatment. Hormesis is illustrated by a biphasic dose-response curve, where a positive response is observed at low dose stimulation of an environmental agent and then a subsequent negative response at higher exposure (Calabrese et al., 2007). The results of the present study are comparable to the feeding response found in individual bumblebees (Thompson et al., 2015) but are contradictory to a previous study by Paus-Knudsen (2017) which used a nearly identical experimental set-up and showed a clear trend of reduced nectar consumption with increasing imidacloprid concentration. Bumblebees prefer nectar spiked with neonicotinoids but consume less than if only offered "clean" nectar (Kessler et al., 2015). In the study by Paus-Knudsen (2017), nectar consumption was reduced only at exposure to 10 μg/L or higher, which could be comparable to the highest treatment level in the present study (13 μg/L).

The present study and the study by Paus-Knudsen (2017) could show two different parts of the whole picture, the detoxification hypothesis. The detoxification hypothesis can be illustrated by a twice-inflected dose-response curve and is based on the assumption of an inducible detoxification system (Cresswell et al., 2012). At low levels of exposure, the consumption of neonicotinoids has a toxic impact, but at some point, with increasing exposure, the detoxification system is induced, and the feeding rate returns to normal. When the level of exposure increases further, the detoxification system is overwhelmed, and the toxic effects reassume to an extent proportional to the exposure level (Cresswell et al., 2012). Nectar consumption after the detoxification system has collapsed could be further suppressed by the toxic effects of neonicotinoid exposure, which include downregulation of genes involved in metabolism and damage to cells lining the digestive tract (Catae et al., 2014; Li et al., 2019). In the study by Cresswell et al. (2012), the exposure level which seemed to induce the detoxification system was approximately 1 μg/L, and a decrease in nectar consumption was observed at 8 μg/L. The exposure level suggested to induce the detoxification system is below the treatment levels used in the present study, but the level of exposure where a decrease in nectar consumption was observed is comparable to where a decrease was

observed in the present study. However, both the studies by Paus-Knudsen (2017) and Cresswell et al. (2012) exposed bees to imidacloprid, while the present study used clothianidin. Bumblebees show small differences in their response to exposure to imidacloprid, thiamethoxam, and clothianidin, where they consume more of nectar spiked with thiamethoxam and imidacloprid than clothianidin (Kessler et al., 2015). These differences make the comparison less than optimal.

The proportion of empty honeypots decreased with increasing clothianidin exposure, meaning that a larger portion of the honeypots was on average full in the hives of high clothianidin exposure, and further contradicts the expectations (H2.4). The present study differs from several previous studies assessing nectar consumption in that bumblebees could choose between the nectar from the nectar bag and food stored in honeypots instead of only being allowed to consume from a feeder (Cresswell et al., 2012; Kessler et al., 2015; Thompson et al., 2015). This choice makes the assessment of nectar consumption more challenging but also makes it more field realistic. In the present study, the control colonies had lower consumption of nectar but seemed to have the highest proportion of empty honeypots, which could mean that the bumblebees had no preference between the nectar from the nectar bag and the food stored in the honeypots and therefore consumed from both indiscriminately. The colonies of the highest treatment level (13 μg/L) showed a very interesting response, where they both consumed less compared to the colonies from the other treatment levels, but they also had the hives with the lowest proportion of empty honeypots, meaning that they overall consumed less than the colonies of the other treatment levels. The highest treatment level did also have some interesting outliers, both in nectar consumption and the proportion of empty honeypots, which pulled the data in opposite directions. However, except for hive no. 20, which contained the largest proportion of empty honeypots and the colony with the lowest nectar consumption, none of the other outliers were affiliated with more than one extreme value.

Based on the discussion above, the low consumption of nectar from the nectar bag could be expected, although, the low proportion of empty honeypots could not. The bumblebees needed food to survive, and so it would be expected that they would consume the food from the honeypots as they prefer that over nectar spiked with neonicotinoids of high concentration (Kessler et al., 2015), and they could have actually done that. Bees respond to a depletion of the food storage by recruiting more workers to fill the honeypots again (Dornhaus & Chittka,

2005). As they bumblebees of the highest treatment level (13 μg/L) eat up their food storages, they may have filled the empty honeypots with nectar from the nectar bag. This correlates well with the comparable concentrations of clothianidin in the body compartment in workers in the two highest treatment levels (6.8 μg/L and 13 μg/L) as the bumblebees of the highest treatment level (13 μg/L) would have consumed smaller doses of clothianidin as they started to deplete their food storage. However, this does not explain the difference in concentrations measured in the head of the bumblebees at the two highest treatment levels. It seems like there is a complicated relationship between increasing exposure of clothianidin, nectar consumption, storing of food, and accumulation of clothianidin, which has not been adequately captured in the present study.

5 Conclusion

The overall aim of this study was to assess whether field-realistic concentrations of clothianidin accumulate and have a toxic impact on bumblebees, and whether they have a dose-response relationship with increasing exposure. Clothianidin accumulated in the head of workers and in the body of workers and the queen, where both showed a dose-dependent accumulation. It did not accumulate in the queen's head, which may have been due to weaknesses in the sample preparations, nor did it accumulate in the stomach, intestine, and rectum of workers and the queen. No changes were observed in mortality and brood production, but exposure caused a trend of hormesis in nectar consumption and a negative dose-response in the proportion of empty honeypots.

The findings of the present study show that even though no detrimental effects could be measured, exposure to clothianidin can cause changes in bumblebees, which may elicit responses in the future. Responses due to accumulation could be time-delayed as it can take time for the pesticide to build-up in body tissues until a concentration is reached where a response can be observed. However, other studies have found lethal and sublethal responses in bumblebees and other bees when exposing them to neonicotinoids in the same concentration range as the present study. The findings of the present study underline the importance of looking at different parameters in exposure studies, both sublethal effects and potential underlying mechanisms, like accumulation.

It is worth noting that this was a laboratory study which allowed for the control of several factors. Wild bees are exposed to several stressors at the same time. It can, therefore, be problematic to extrapolate directly from a laboratory study to the environment were many factors remain unknown. However, laboratory studies are suitable mediums for observing changes and responses in organisms which could be challenging to observe in field studies, especially in parameters that have never been tested before. The present study is to the knowledge of the author the first to quantify the accumulative potential of a neonicotinoid insecticide to this detail in bumblebees and sheds light on a large data gap in the potential risk neonicotinoids pose to bees and other pollinators.

6 Future studies

Due to the lack of studies looking into the accumulation of neonicotinoids in wild bees, there are several directions to go in in future studies. First, studies with exposure lasting longer could reveal more of how neonicotinoids accumulate in bumblebees. For example, how much has accumulated in bumblebees after 22 days, approximately the time it takes from an egg is laid until it hatches (Gill et al., 2012). A prolonged exposure could also increase the chances of observing lethal and sublethal responses, e.g. mortality, brood production, and nectar consumption. Secondly, clothianidin could show a different accumulation in broods than in adult bees. Larvae exposed to neonicotinoids show detrimental effects in their behaviour as adults (Yang et al., 2012), which could be due to accumulation. Comparing accumulation in different life stages could therefore be an important next step.

It is uncertain whether clothianidin measured in the body of the bumblebees was due to clothianidin accumulation or due to clothianidin dissolved in the nectar in the crop. One solution could be to perform chemical analysis on the crop and the body separately. Doing that would not only answer this question but could also show whether clothianidin is degraded in the crop (although it should in theory not (Willmer, 2011)). If clothianidin is not degraded, it could be very interesting to measure the concentration of clothianidin in nectar stored in honeypots. The insides of hives are usually dark which could mean that clothianidin stored in honeypots are not exposed to light and can potentially expose bumblebees for a long time and maybe cause time-delayed effects.

Several of the studies cited in the thesis used imidacloprid or thiamethoxam in their experiments. Clothianidin, thiamethoxam, and imidacloprid do share many of the same properties and are expected to behave in a similar manner. However, some subtle differences do exist among them, making it a bit problematic to extrapolate effects observed from one of them to the others. This underlines the need for more toxicological studies using clothianidin in particular.

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Appendix A

High-performance liquid chromatography-mass spectrometry (HPLC-MS) analysis was performed by Jan Thomas Rundberget, and the procedure is described by him below.

Liquid chromatography was performed on an Acquity HSS C18 column (1.8μm, 100 × 2.1 mm) (Waters, Milford, MA, USA), using a Waters Acquity UPLC module. Separation was achieved using linear gradient elution at 0.5 mL/min starting with MeCN–water (5:95, water containing 0.1% formic acid rising to 100% MeCN over 9 min. Isocratic elution with 100% MeCN was maintained for 2 min before the eluent was switched back to 5% MeCN. The UPLC system was coupled to a Quattro Premier XE tandem mass spectrometer operating with an ESI interface (Waters Micromass, Manchester, UK). Typical ESI parameters were a spray voltage of 3.5 kV, desolvation temperature at 400 °C, source temperature at 120 °C and cone gas and desolvation gas at 50 and 800 L/h of N_2, respectively. The mass spectrometer was operated in MS/MS mode with argon as collision cell gas at 1.5×10^{-3} Torr. Ionization and MS/MS collision energy settings (typically 25 eV) were optimized while continuously infusing (syringe pump) 20 ng/mL of clothianidin, at a flow rate of 5 μL/min. Screening of clothianidin was performed with multiple reaction monitoring (MRM) in positive ionization mode; clothianidin 250>132, 250>169 and deuterated 253>132, 253>172. The LOD (limit of detection, author remark) of 0.5 ng/g clothianidin was estimated as three times the signal to noise (S/N) using spiked control samples (all body compartments except for the queen's head had a LOD of 0.2 ng/g).

Appendix B

Table B1. Raw data from worker and queen tissues measured for clothianidin concentration. Each row represents one colony. All values denoted <0.2 or <0.5 are values below limit of detection. SIR stands for Stomach, Intestine, and Rectum.

	Worker			Queen			
Treatment level	**Head**	**SIR**	**Body**	**Head**	**SIR**	**Body**	**Nectar**
0	<0.2	<0.2	<0.2	<0.5	<0.2	<0.2	<0.2
0	<0.2	<0.2	<0.2	<0.5	<0.2	<0.2	<0.2
0	<0.2	<0.2	<0.2	<0.5	<0.2	<0.2	<0.2
0	<0.2	<0.2	<0.2	<0.5	<0.2	<0.2	<0.2
0	<0.2	<0.2	<0.2	<0.5	<0.2	<0.2	<0.2
3.6	<0.2	<0.2	<0.2	<0.5	<0.2	<0.2	1.8
3.6	0.53	<0.2	<0.2	<0.5	<0.2	0.43	2.6
3.6	0.83	<0.2	<0.2	<0.5	<0.2	<0.2	3.1
3.6	<0.2	<0.2	0.34	<0.5	<0.2	<0.2	3.1
3.6	0.38	<0.2	0.36	1.13	<0.2	<0.2	3.1
6.8	<0.2	<0.2	<0.2	<0.5	<0.2	<0.2	7.0
6.8	2.08	2.01	2.68	<0.5	<0.2	0.59	7.8
6.8	1.16	1.76	2.14	<0.5	0.86	1.08	5.7
6.8	1.62	2.24	2.72	<0.5	0.6	0.63	6.0
6.8	0.68	1.35	1.55	<0.5	0.81	0.6	5.9
13	2.17	0.85	3.17	<0.5	<0.2	0.46	NA
13	1.87	<0.2	2.93	<0.5	<0.2	2.49	11.7
13	1.71	<0.2	2.11	<0.5	<0.2	1.60	9.2
13	1.35	1.17	1.40	<0.5	<0.2	0.69	10.4
13	0.93	1.24	1.83	0.87	<0.2	1.15	9.4

LOD: Queens head = 0.5 μg/kg, All = 0.2 μg/kg.

Table B2. Raw data from the dissection of the hives. Each row represents one hive.

Treatment level	Full honey-pots	Half-filled honey-pots	Empty honey-pots	Alive adults	Dead adults	Alive pupa	Dead pupa	Alive large larvae	Dead large larvae
0	22	35	75	113	42	0	34	1	4
0	7	13	68	114	32	6	29	37	20
0	13	28	68	74	8	14	5	50	5
0	34	6	45	81	11	7	1	45	39
0	7	7	28	41	8	8	14	28	41
3.6	26	53	96	123	11	0	63	13	24
3.6	5	17	50	106	24	16	11	32	4
3.6	8	13	47	120	18	12	10	44	1
3.6	19	9	27	70	14	8	8	79	62
3.6	12	10	28	59	9	3	3	41	69
6.8	26	15	38	65	10	0	0	0	4
6.8	1	21	44	95	16	38	0	42	1
6.8	17	12	25	62	5	10	10	28	2
6.8	43	23	16	105	15	1	4	41	83
6.8	7	21	42	136	13	17	34	67	16
13	14	22	98	119	99	1	5	9	1
13	22	15	34	110	21	29	4	24	3
13	15	14	22	77	19	12	10	40	39
13	43	23	16	81	23	2	4	17	56
13	3	3	27	51	10	10	7	20	22

Table B2, continued. Raw data from the dissection of the hives. Each row represents one hive.

Treatment level	Alive small larvae	Dead small larvae	Alive eggs	Dead eggs	Weight nectar bag before (g)	Weight nectar bag after (g)	Nectar consumed	Weight hive in nest box (g)	Nr of queens
0	3	5	97	6	1843	1618	224	519	32
0	72	76	169	14	1945	1713	231	480	8
0	59	144	96	28	1954	1747	206	382	1
0	99	110	80	7	1961	1774	187	413	1
0	107	92	24	4	1977	1901	75	349	1
3.6	7	24	104	10	1885	1632	251	582	38
3.6	35	5	58	3	1938	1721	217	479	2
3.6	8	6	53	0	1948	1677	271	521	14
3.6	90	79	83	75	1962	1773	188	402	1
3.6	89	53	41	0	1986	1673	312	385	1
6.8	48	55	32	16	1864	1935	-71	373	1
6.8	39	11	38	11	1931	1691	240	420	1
6.8	30	6	19	0	1995	1777	218	448	1
6.8	72	59	137	5	1954	1678	275	430	1
6.8	70	36	125	3	1974	1745	228	422	1
13	24	12	18	12	1919	1470	449	419	1
13	36	45	211	23	1964	1762	202	466	1
13	93	74	47	8	1956	1784	172	414	1
13	6	48	166	20	1964	1787	167	423	1
13	124	153	100	11	1961	1892	68	343	1

Table B3. Range (Min – Max), median, and mean ± standard deviation (SD) of the different life stages of bumblebees categorised as living or dead.

	Control		**3.6 µg/L**		**6.8 µg/L**		**13 µg/L**	
	Min – Max (Median)	**Mean ± SD**	**Min – Max (Median)**	**Mean ± SD**	**Min – Max (Median)**	**Mean ± SD**	**Min – Max (Median)**	**Mean ± SD**
Living adults	41 – 114 (81)	84.6 ± 30.4	59 – 123 (106)	95.6 ± 29.4	62 – 136 (95)	92.6 ± 30.6	51 – 119 (81)	87.6 ± 27.3
Dead adults	8 – 42 (11)	20.2 ± 15.8	9 – 24 (14)	15.2 ± 6	5 – 16 (13)	11.8 ± 4.4	10 – 99 (21)	34.4 ± 36.4
Living pupa	0 – 14 (7)	7 ± 5	0 – 16 (8)	7.8 ± 6.5	0 – 38 (10)	13.2 ± 15.5	1 – 29 (10)	10.8 ± 11.3
Dead pupa	1 – 34 (14)	16.6 ± 14.5	3 – 63 (10)	19 ± 24.8	0 – 34 (4)	9.6 ± 14.2	4 – 10 (5)	6 ± 2.6
Living large larvae	1 – 50 (37)	32.2 ± 19.3	13 – 79 (41)	41.8 ± 24.1	0 – 67 (41)	35.6 ± 24.4	9 – 40 (20)	22 ± 11.5
Dead large larvae	4 – 41 (20)	21.8 ± 17.8	1 – 69 (24)	32 ± 32	1 – 83 (4)	21.2 ± 35.1	1 – 56 (22)	24.2 ± 23.6
Living small larvae	3 – 107 (72)	68 ± 41.2	7 – 90 (35)	45.8 ± 41.5	30 – 72 (48)	51.8 ± 18.7	6 – 124 (36)	56.6 ± 49.8
Dead small larvae	5 – 144 (92)	85.4 ± 51.6	5 – 79 (24)	33.4 ± 32.1	6 – 59 (36)	33.4 ± 24.4	12 – 153 (48)	66.4 ± 53.2
Living egg	24 – 169 (96)	93.2 ± 51.8	41 – 104 (58)	67.8 ± 25.4	19 – 137 (38)	70.2 ± 56.1	18 – 211 (100)	108.4 ± 80.4
Dead egg	4 – 28 (7)	11.8 ± 9.8	0 – 75 (3)	17.6 ± 32.4	0 – 16 (5)	7 ± 6.4	8 – 23 (12)	14.8 ± 6.4