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Distribution and effects of clothianidin in bumblebees

Within-Body Distributions and Feeding Effects of the Neonicotinoid Insecticide Clothianidin in Bumblebees (*Bombus terrestris*)

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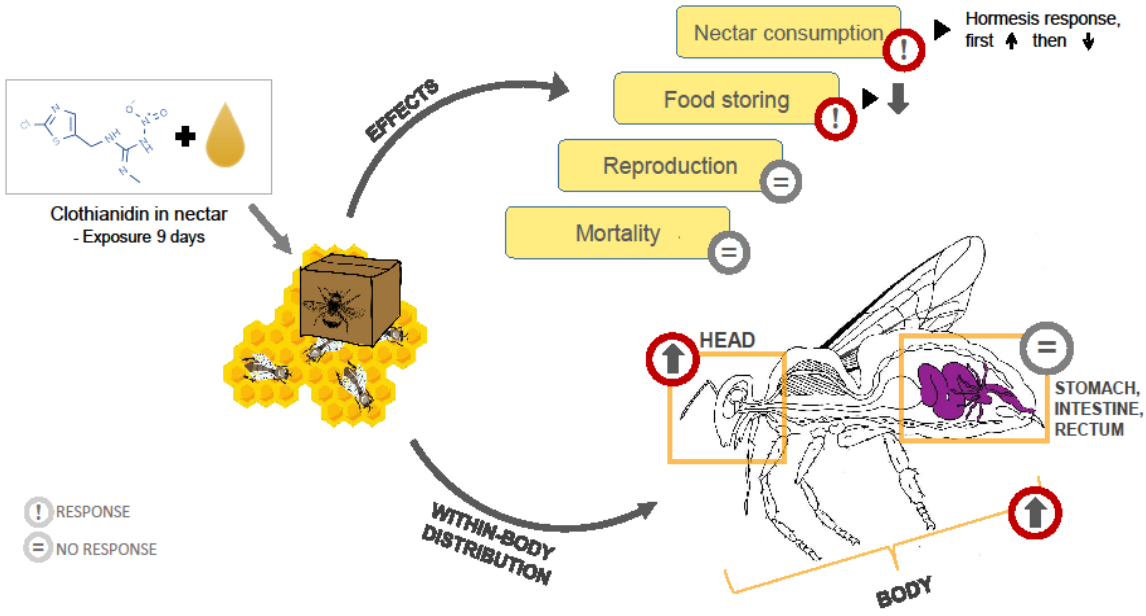
Abstract: Bumblebees can be exposed to neonicotinoid pesticides through nectar and pollen collected from treated crops, which can cause lethal and sublethal effects in these non-target pollinators. However, the body distribution of the compound after exposure to neonicotinoids in bumblebees is not well studied. Bumblebee colonies (*Bombus terrestris*, n = 20) were exposed to field-realistic concentrations of clothianidin through artificial nectar (3.6 µg/L – 13 µg/L) for nine days. Comparison of the nominal- to the measured exposure in nectar indicated good compliance, confirming the applicability of the method. When quantified, clothianidin showed a concentration-dependent occurrence in the head and body of workers (head: <0.2 –

2.17 µg/kg, body: <0.2 – 3.17 µg/kg), and in the body of queens (<0.2 – 2.49 µg/kg), although concentrations were below that measured in the nectar (Bioaccumulation factor = 0.2). Exposure to clothianidin did not affect mortality nor brood production, nor have a statistically significant effect on nectar consumption and size of food storage. However, visual inspection suggests higher nectar consumption of nectar with low clothianidin content compared to nectar with no or high clothianidin content. Our results show that dietary clothianidin is taken up in bumblebees, but does not bioaccumulate to elevated levels compared to exposure. Still, clothianidin may elicit responses that affect feeding behaviour of the pollinator *B. terrestris*, although our endpoints were not significantly affected.

Graphical Abstract



Keywords: Neonicotinoids, bumblebees, sublethal effects, *Bombus terrestris*, accumulation, feeding

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Introduction

Neonicotinoids were launched as a new group of insecticides in 1991 and quickly became a commercial success (Jeschke et al. 2013). Their success was much due to several favourable characteristics, including their systemic properties (Stamm et al. 2016; Yong Li et al. 2018), a long half-life in soil (reducing the need for reapplication; Goulson 2013; Yang Li et al. 2018), and an effective mode of action binding to the nicotinic acetylcholine receptors (nAChR) in the nervous system of target organisms (Jeschke et al. 2013; Palmer et al. 2013). Yet in 2018, outdoor use on crops of the three neonicotinoids, imidacloprid, thiamethoxam, and clothianidin, was permanently banned in the European Union (EU) due to their adverse effects on non-target organisms, bee pollinators in particular (EFSA (European Food Safety Authority) 2018a; EFSA (European Food Safety Authority) 2018b; EFSA (European Food Safety Authority) 2018c). However, neonicotinoids are still in use and present in the environment today, and emergency use of thiamethoxam has been allowed to treat sugar beet seeds in England, whereas imidacloprid used as veterinary flea products on companion animals have been found to contaminate English rivers (Perkins et al. 2021; Department for Environment Food & Rural Affairs 2021).

The largest proportion of neonicotinoid studies concerns honeybees, as they are often used as the model insect pollinator in risk assessments, with the number of studies focusing on bumblebees and solitary bees increasing over the years (Franklin and Raine 2019). Bumblebees are important pollinators, both commercially and in the wild (Velthuis and Van Doorn 2006), and initial studies show that they are more

sensitive to pesticide exposure compared to honeybees (Arena and Sgolastra 2014; Gradish et al. 2019). However, more studies are needed on bumblebees to allow a proper interpolation between species.

Sublethal effects are defined as behavioural and/or physiological effects appearing in individuals who have survived exposure to an environmental agent at a concentration that gives no apparent mortality (Alkassab and Kirchner 2016). Several sublethal effects have been identified in *Bombus* and *Apis* bees after exposure to neonicotinoids, including impaired learning and memory (Stanley et al. 2015; Phelps et al. 2018), reduced consumption of food (Laycock et al. 2012; Cresswell et al. 2014; Thompson et al. 2015), reduced food storage (Scholer and Krischik 2014), reduction in foraging efficiency (Gill et al. 2012; Feltham et al. 2014), and reduced brood (Gill et al. 2012; Laycock et al. 2012; Laycock and Cresswell 2013) and queen production (Whitehorn et al. 2012). Furthermore, bumblebee queens are more sensitive to neonicotinoid exposure than workers (Mobley and Gegear 2018), thus, a critical window of exposure would be the time period when she initiates the colony (Willmer 2011). In bee colonies, substitute workers performing other tasks can take on the task of an impaired or dead bee. This type of buffering capacity among social bees (i.e. bees forming colonies) can allow for a functional colony even if there are adverse effects on an individual level, granted the mortality rate does not reach a certain point. However, sublethal effects can accumulate in the colony and ultimately cause colony mortality (Franklin and Raine 2019). As neonicotinoids have multiple effects on both individual- and colony level in bumblebees, it is of interest to target several responses simultaneously.

Bioaccumulation is the net enrichment of contaminants in organisms from their environment, of uptake versus elimination (Arnot and Gobas 2006). A chemical's

ability to accumulate in organisms is related to how it partitions between aqueous and organic phases, which is described by the octanol-water partition coefficient (K_{OW}; Arnot and Gobas 2006). A chemical with a low K_{OW} is hydrophilic, while a chemical with a high K_{OW} is hydrophobic, lipid soluble and more likely to accumulate. Neonicotinoids are in general considered not to bioaccumulate in animals due to low K_{OW}s (EFSA (European Food Safety Authority) 2008; Yong Li et al. 2018), despite studies showing accumulation in partridges, earthworms, and lizards (Lopez-Antia et al. 2015; Chevillot et al. 2017; Wang et al. 2018; Wang, Zhang, Zeng, et al. 2019). Neonicotinoids show incomplete clearance from the body after digestion in honeybees and bumblebees (Cresswell et al. 2014; Sánchez-Bayo et al. 2017), and have a prolonged and assumed irreversible binding to nAChR (Jeschke et al. 2013; Palmer et al. 2013; Yang Li et al. 2018), both properties giving the neonicotinoids the potential for bioaccumulation in bumblebees. Following, it can be expected that tissues with a high concentration of the receptors nAChRs, such as the bee brain (Palmer et al. 2013), will accumulate more of the neonicotinoids, thus quantifying the neonicotinoid substance in different body parts can be indicative of where neonicotinoids may have the strongest effect.

The aim of our study was to analyse how clothianidin distribute within the body of bumblebees, a non-target and non-*Apis* pollinator, after exposure to field-realistic concentrations of the neonicotinoid. We also addressed whether exposure to clothianidin caused effects on the hive mortality, brood production, nectar consumption, and storing of food. These aims were assessed through exposure of the bumble bee *Bombus terrestris* to clothianidin via artificial nectar solution, and by quantifying its distribution and accumulation in bumblebee body compartments, hive

mortality and reproduction, consumption of clothianidin-spiked nectar, and proportion of empty honeypots (i.e. consumption of stored, unexposed, nectar).

Materials and methods

Study species.The Buff-tailed bumblebee (*Bombus terrestris* L. (1758)) is a holometabolous insect with a life cycle consisting of four stages: egg, larvae, pupa, and adult (Willmer 2011). The species has haploid-diploid sex determination, where fertilised eggs develop into females and unfertilised eggs develop into males (Willmer 2011). *B. terrestris* are eusocial bees with castes made up of the queen, workers, and drones, and produce annual colonies, in which all workers are females, and males are only present late in the life cycle of the colony (Ødegaard et al. 2015). New queens which have not yet founded their own colony are called gynes

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 for Water Research (NIVA), from June 2018 to January 2019. The experiment was divided into three parts: exposure, dissection, and chemical analysis, and was blinded from the arrival of the colonies until the dissection. Twenty queenright colonies (presence of a fertile queen) of *Bombus terrestris* were obtained from Bombus natur AS (Bryne, Southern Norway) in standard plastic nest-boxes covered by a cardboard box. Underneath each nest-box, a plastic container holding 2 L of artificial nectar was placed that the bumblebees could access ad libitum through a sponge, and from which the bumblebees were exposed to clothianidin through nectar for nine days. The exposure period is based on a meta-analysis defining chronic as an exposure period of six days or more (Cresswell 2011). We further decided to expand the exposure period to nine days to ensure that all bumblebees had consumed clothianidin (Stanley and Raine 2016). The nectar bag was

provided by Bombus natur AS, completely emptied and cleaned before commercial syrup (called nectar from now on) and the treatment solution was added, and weighed before and after the exposure period to calculate the amount of nectar consumed. The hives were used as they were upon arrival, their condition was registered, and there were not standardised. During the experiment, each colony was fed an untreated pollen and nectar mixture every second day, and the hives were kept under a controlled environment of +28°C and 50% relative humidity. After the exposure period, the hives were frozen (-20°C) for at least two days before dissection.

Treatment and preparation of dilution series. The experiment included four treatment levels: 0 µg/L (*control*), 3.6 µg/L (*low*), 6.8 µg/L (*medium*), and 13 µg/L (*high*) clothianidin, all in the range of field concentrations and with exposure treatment levels differing by a factor of 1.9 (Federoff and Barrett 2009; Rolke et al. 2016; see Table S1 in Supplementary Information).The original test range of treatments included concentrations 1 µg/L and 1.9 µg/L to obtain a better resolution at the lower range of exposure concentrations, but were later excluded from the study as chemical analysis returned clothianidin levels bordering the limit of detection (LOD) even in bumblebees from *low* exposure. The remaining 20 hives (n = 20) were randomly assigned a treatment level, giving five replicates per level.

The dilution series was performed in a dimmed room and stored in a dark environment to avoid rapid degradation of clothianidin dissolved in water due to light exposure (half-life in water under sunlight exposure is 13 h; Federoff and Barrett 2009; Yang Li et al. 2018). A stock solution was made by dissolving 20 mg pure clothianidin powder (PESTANAL™, analytical standard, 99.9% purity, Sigma-Aldrich) in distilled water, adding water until a concentration of 200 mg/L was reached. Each of the subsequent steps in the dilution series were made by adding

more distilled water. From the stock solution, an intermediate solution of 5 mg/L was made, and from the intermediate solution, each of the solution concentrations 360 µg/L, 680 µg/L, and 1300 µg/L were made to be further mixed with nectar in the nectar bags, diluting the solution concentrations to 3.6 µg/L, 6.8 µg/L, and 13 µg/L clothianidin. Before adding the solutions, the nectar was added distilled water to reduce the sugar content from 50% to 30%. The control was made from distilled water only and added to the nectar in a similar way as the exposure concentrations.

Dissection of hives and bumblebees. During dissection, the following units were identified and counted: adults, pupa, larvae, eggs, queens (original queen and gynes, full honeypots, half-filled honeypots, and empty honeypots. Each of the individuals were categorised as alive or dead at time of experiment termination, based on colour and physiological criteria during dissection (Table 1). At the same time as dissection of the hives was performed, approximately 1 mL of nectar was retrieved from the nectar bags of all hives for quantification of clothianidin concentration. Bumblebee workers and queens were dissected into three parts, (1) head, (2) stomach, intestine, and rectum (SIR), and (3) the rest of the body (Figure 1). Workers were distinguished from drones by the presence of a stinger at their tail, and all worker bees dissected were retrieved from the "alive" category. Some colonies also contained gynes, which is common when the colony has reached a tipping point, where the queen stops producing workers and starts producing drones and gynes (Bloch 1999). As there are no specific traits that differentiate the original queen from gynes, traits that indicate longevity, such as less hair or bald spots on the dorsal thorax, were used to identify the original queen. Samples from hive dissection were stored at -20˚C until sample preparation was performed. The dissection method used in our study is based on external characteristics, which can create some uncertainty concerning resolution of

the data, as the method does not detect differences on a high resolution. Our aim was to observe multiple parameters simultaneously, and therefore a trade-off was made between high resolution and targeting a broad spectrum of effects. The method used is quick, easy, and resilient enough to be able us to assess the responses we wished to observe.

Clothianidin analysis. The method used to quantify clothianidin was first established by Wiest and colleagues (2011) using a standard of 5 g of honeybee material. We modified the method to take into account the difference in size and weight of the body compartments ranging from smallest to largest: SIR<head<body. The chemical analysis was run on extracts from pooled samples using ten workers per colony to obtain clothianidin levels above the limit of detection. Distilled water and acetonitrile (MeCN; Sigma-Aldrich and VWR Chemicals) were added in a 1:3 relationship (water:acetonitrile). Samples weighing 1- 2 g were added 1 mL water and 3 mL acetonitrile, while samples weighing more than 2 g were added 2 mL water and 6 mL acetonitrile. If the material was not completely submerged, water and acetonitrile were added 1 mL every second time in a 1:1 relationship until submersion. Ten µL of internal standard, containing deuterated clothianidin, was added to each sample, and all samples except nectar were then homogenised individually. Later, acetonitrile was removed by the addition of 1 g of NaCl, shaking, centrifugation, and evaporation with heath (+60°C) under nitrogen. The remaining content was dissolved in 0.5 mL 10% acetonitrile in water. Each sample was analysed using high-performance liquid chromatography-mass spectrometry (HPLC-MS, see Supplementary Information for detailed information concerning instrument model and settings).

Data treatment. Statistical analyses were performed in R version 3.5.2 for Mac (R Core Team 2018). Normal error distribution was assessed using the Shapiro-Wilk test, and homoscedasticity was assessed using the Barlett's test.

When quantifying clothianidin concentrations, only the head and body of workers had more than 70% of the values above limit of detection, when combining all treatment levels, thus these were the only compartments included in the statistical analysis of within-body distribution. For the head and body of workers, random values were generated between 0.0 µg/kg and limit of detection (0.2 µg/kg), to substitute the remaining few data below limit of detection (Antweiler and Taylor 2008). The substitution allows inclusion of left-censored data without generating a false structure in the data. Exclusion of the censored data would create a skewed bias towards the larger values, which would also give a false structure. One nectar sample returned from chemical analyses as Not Analysed (NA) and was replaced with the mean clothianidin concentration measured in the other nectar samples. The measured clothianidin concentrations in the nectar was compared to the nominal concentration, and was used to calculate the BAF values.

The response variable *nectar consumed* was registered as negative in one colony from *medium* exposure, likely due to an error when weighing the nectar bag for this colony, and was excluded from the analysis.

Generalised linear models (GLMs) were generated to assess whether concentrations found in the head and body as well as nectar consumption were explained by any of the explanatory variables. Treatment, days after delivery from hive producer, number of queens (original queen and gynes) per hive, and size of the colony (i.e. number of individuals of all life stages present in the hive) were included as explanatory variables tin the statistical analyses.

During the general health check of the colonies upon arrival (looking for flies, mould, bad smell, etc.), we noted that the colonies showed some variation in age and size upon arrival. The variation in size and age was not taken into account in the distribution of treatment levels among the colonies, as the assignment was randomised. We attempted to take the variation into account during statistical analysis by giving each colony a status of good, medium, and bad condition, based on whether they were above or below the mean in the three categories proportion of empty honeypots, number of broods produced, and proportion of dead adults. Although the term "bad" was used to describe status, all colonies used were viable in terms of health. The categorisation is described in detail in Supplementary Information. The categorisation was included in the statistical analysis as a covariate. Through the analysis, we found that the variable did not have a statistically significant impact on the response variables which we tested for.

The proportion of dead adults, dead pupa, dead larvae, dead eggs, broods (pupa, larvae, and eggs, both dead and alive), and empty honeypots were used as individual response variables and fit to GLMs with a binomial error distribution and a logit link function due to non-normal distribution errors. Dunnett's test was used to test if the treatment levels were statistically significantly different from the control. To identify the best model explaining each of the focal response variables, the model selection procedure "model.sel" from the R package MuMIn (Pohlert 2016) was used. This procedure starts with a universal model (i.e. including all potential explanatory variables) and runs through all possible models containing subsets of the full variable set. Akaike's Information Criterion adjusted for sample size (AICc), which takes into account problems that can arise with lower sample sizes, was used as the model selection criterion, where the model with the lowest AICc value was chosen as the

best model (Burnham and Anderson 2002). If the ΔAICc differed between two or more models with <2, the models were considered to have the same explanatory power (Burnham and Anderson 2002).

The bioaccumulation factors (BAFs) for the head and body were calculated for workers at each treatment level by dividing the clothianidin concentration measured in the body compartment (µg/kg) by the clothianidin concentration measured in the nectar (µg/L). Following, a Tukey's multiple comparison test was used to test if there was a statistically significant difference between the treatment levels.

Results and discussion

Nominal versus measured clothianidin exposure in nectar. The measured clothianidin exposure was on average 17% below the nominal exposure (nominal vs mean measured: 3.6 µg/L vs 2.74 µg/L; 6.8 µg/L vs 6.54 µg/L; 13 µg/L vs 10.18 µg/L), which is below the 20% requirement for pollinator experiments (OECD 2013). Although the measured clothianidin concentrations are lower than the nominal concentrations, the treatment levels do not overlap and they provide a concentration gradient. In addition, the measured exposure is still in the range of field-realistic concentrations and is therefore highly relevant.

Within-body distribution of clothianidin in bumblebee workers. The clothianidin concentrations in the head and body compartments increased with treatment level following a clear exposure concentration-response relationship (Figure 2; see Table S3 in Supplementary information for an overview of the measurements). For the head, the clothianidin concentrations were best explained by treatment level as the single explanatory variable (; Dunnett's test workers' head, p-values when treatment compared to control: 3.6 µg/L $p = 0.67$, 6.8 µg/L $p = 0.011$, 13 µg/L $p = 0.0039$; Table 2, Figure 2). Clothianidin did not bioaccumulate in the bumblebee workers, as

the tissue concentrations did not exceed the clothianidin concentrations in the nectar (see Table S3 in Supplementary Information), and the BAFs were below 1 for all body compartments and exposure doses. The BAF_{HEAD} was 0.2 in all treatment levels, showing the amount of clothianidin taken up increase with increasing concentrations, in a consistent proportion of what is found in the diet. Moffat and colleagues (Moffat et al. 2015) found imidacloprid to accumulate in the brain of bumblebees at concentrations of 9.7 ± 0.8 nM after three days, having exposed the bumblebees to 10 nM (2.1 ppb w/w) imidacloprid through their diet (sugar syrup). The exposure concentration is below what was used in our study and included a very low number of samples. Nevertheless, their study shows that neonicotinoids can accumulate in the brain of bumblebees at concentrations similar to the exposure found in their diet. Treatment level was also the single explanatory variable for clothianidin concentrations in the body of workers (Dunnett's test workers' body, p-values when treatment compared to control: 3.6 µg/L p = 0.99, 6.8 µg/L p = 0.0024, 13 µg/L p = 0.0023). The highest clothianidin concentrations found in the bumblebees were in the body compartment, with a steep increase between *low* and *medium exposure*. One explanation may be that clothianidin is not taken up into the body, but rather dissolves in the crop, a nectar-collecting organ used by bees when they are out foraging (Willmer 2011). Nearly all the dissected bumblebees had a crop filled with nectar, which was analysed chemically for clothianidin content together with the body compartment. The presence of spiked nectar in the crop has been proposed as the explanation for elevated levels of neonicotinoids detected in the body in a previous study (Cresswell et al. 2014). However, the concentration-dependent difference in clothianidin concentrations in nectar in *medium* and *high* is not reflected in the concentrations measured in the body compartment, which were similar (Figure 2b),

indicating that the concentrations measured was likely not due to spiked nectar in the crop, but the clothianidin taken up into the body. The internal clothianidin concentrations in the body compartment measured after *medium* exposure was 10 times higher than after *low* exposure, which exceeded the difference in nominal exposure concentrations (factor 1.9 between treatments). This difference suggests a metabolic threshold between the *low* and *medium* exposure concentration. Exposure to neonicotinoids can cause downregulation of genes involved in biotransformation of pesticides (Li et al. 2019), which can have harmful effects in bumblebees, as biotransformation has been suggested to be the main pathway for elimination of neonicotinoids in bees (Suchail, Debrauwer, et al. 2004; Suchail, De Sousa, et al. 2004). Furthermore, the BAF_{BODY} was 0.1 for *low*, 0.3 for *medium*, and 0.2 for *high* exposure, with a significant difference between *low* and *medium*, but not significant difference between *low* and *high*, or *medium* and *high* (Tukey's multiple comparison test p value: *low* vs. *medium* 0.028; *low* vs. *high* 0.154; *medium* vs. *high* 0.590). Due to the high number of clothianidin concentrations in the stomach, intestine, and rectum (SIR) below the limit of detection (53% concentrations below LOD), no statistical relationship could be analysed between treatment levels. Graphic presentation showed no strong relationship either (see Supplementary Information). Studies depicting the accumulation of neonicotinoids in animals remain scarce, and accumulation in bees even more scarce. Neonicotinoids are not expected to bioaccumulate due to their low KOWs (EFSA (European Food Safety Authority) 2008; Yong Li et al. 2018). Yet, neonicotinoids are found to enrich body tissues of lizards, partridges, and earthworms after prolonged exposure, although, in most of these studies, the exposure concentration is not considered field-realistic (Lopez-Antia et al. 2015; Chevillot et al. 2017; Wang et al. 2018; Wang, Zhang, Zeng, et al.

2019; Wang, Zhang, Li, et al. 2019). Further, clothianidin, as a metabolite of thiamethoxam, has been found to enrich the brain of honeybees after they consumed food treated with the parent compound (Tackenberg et al. 2020). Also in this study, the exposure concentration was much higher than what is found in the environment. In all of these accumulation studies, the accumulation tissue concentrations were below that of the exposure concentrations and none calculated BAFs. In the case of farmland lizards and honeybees, biotransformation and excretion is the main process of elimination of neonicotinoids, both of which are quite effective (Suchail, De Sousa, et al. 2004; Suchail, Debrauwer, et al. 2004; Wang, Zhang, Li, et al. 2019)

Within-body distribution of clothianidin in bumblebee queens. In contrast to the workers, clothianidin was not detected in the queens' head except for two individuals (1.13 µg/kg from *low* and 0.87 µg/kg from *high*). The limit of detection was higher for the queen heads (0.5 µg/kg) compared to the other body compartments (0.2 µg/kg). However, even with the higher limit of detection, we still observe a marked difference between workers and queens, as the clothianidin concentrations measured in the workers head ranged from below the limit of detection of 0.2 µg/kg to 2.17 µg/kg. The difference in concentrations could be due to challenging homogenisation of samples. Acetonitrile extracts clothianidin from the tissue, and it is therefore critical that the samples are homogenised properly for complete extraction. It was easier to homogenise the worker heads because we could pool ten to obtain sufficient mass per hive, while for the queen only one head was homogenized per sample. If the queen heads were not homogenised properly after cutting them into tiny pieces using a scalpel or crushing them using a mortar and pestle, the extraction of clothianidin by acetonitrile may have been incomplete if not all parts of the tissue have been reached. This challenge may also explain the difference in limit of detection. Others whom

have used the same extraction method have arrived at different limits of detection for different matrices, but have not tried to find potential explanations for why that is (Lambert et al. 2013). However, another explanation for the difference in clothianidin concentrations measured in the heads of workers and the queens could be differences in either behaviour or physiology between the workers and queens, as the anatomy of the two is similar, other than the size.

In the queens' body, accumulated clothianidin showed a concentration-response relationship, and with lower concentrations measured in the queens' body were lower than the concentrations found in the workers' body (Figure 2). When controlling for body weight, workers of *B. impatiens* have a higher total daily intake than queens, which would result in a higher enrichment of clothianidin to the body tissues of workers (Mobley and Gegear 2018). A daily total intake which cause toxic effects in queens, is not sufficient to cause toxic effects in workers, which suggest a lower internal concentration of clothianidin is needed in queens to elicit toxic reactions (Mobley and Gegear 2018). On the other hand, honeybee queens are more tolerant to acaricide exposure than workers, suggesting that the internal concentration needed to elicit effects in queens versus workers may be pesticide specific (Dahlgren et al. 2012). There was no relationship between clothianidin concentration in SIR and treatment level in queens.

Effects of clothianidin exposure on reproduction and mortality assessed in the hive.

There was no effect of clothianidin exposure on reproduction, using the proportion of brood life stages identified during hive dissection as a proxy for brood production (Supplementary information Figure S2). Statistical analysis resulted in three models which best explained the data were: the null model (AICc = 21.7), size of the colony (AICc = 22.3), and number of queens (original queens and gynes) present in the

colony (AICc = 23.1). However, size of the colony and number of queens (original queens and gynes) present in the colony are not significant (size p = 0.367, number of queens p = 0.6297). Exposure to neonicotinoids can cause a reduction in the production of proteins involved in reproduction and length of lifespan in bees, and reduction in sperm quality and sperm amount stored in the spermathecal in honeybee queens, leading 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 (Cresswell et al. 2012; Catae et al. 2014; Ødegaard et al. 2015). Reduction in brood production might be a delayed response occurring after a longer exposure period, as the studies finding reduced brood production after exposure observed this decrease after 14 days (Gill et al. 2012; Laycock and Cresswell 2013). In comparison, our study was terminated after 9 days. Clothianidin exposure did not affect mortality in the bumblebees. *Control* and *high* had the highest mortality of 35% and 34%, respectively, while *medium* had the lowest mortality for each individual life stage, and an overall mortality of 24%. *Control* and *high* also had the largest colony size (mean ± sd number of individuals in the colony: *control*: 440 ± 105 individuals, *low*: 377 ± 177 individuals, *medium*: 350 ± 169 individuals, *high*: 431 ± 85 individuals). The colony size can be an indicator for its age, where larger colonies are also older (Bloch 1999), and may contain older workers whom have reached the end of their natural lifespan. In addition to containing the largest colonies, *control* and *high* also had the highest mortality for adult bumblebees (percentage dead adults: *control*: 19%, *low*: 14%, *medium*: 11%, *high*: 27%), indicating that these colonies were the oldest. Inspection of mortality in individual life stages found that the number of queens (original queen and gynes) present in the colony was included as an explanatory variable in the best and the second best model

for pupa and larva, although the variable was not statistically significant (Table 2; see model transcripts in Supplementary Information).

Mortality among *Bombus* and non-*Bombus* bee species due to chronic neonicotinoid exposure is found at concentrations starting from 20 µg/kg and higher (Alkassab and Kirchner 2016; Wood et al. 2020). Lower and field-realistic concentrations, which often does not lead to increased mortality, lead to sublethal responses like impaired learning and memory (Stanley et al. 2015; Phelps et al. 2018), and reduced consumption of food (Laycock et al. 2012; Cresswell et al. 2014; Thompson et al. 2015). Although, increased mortality due to chronic exposure has been observed at exposure concentrations as low as 10 ppb (Mobley and Gegear 2018). As our selected clothianidin concentrations did not affect colony mortality, our study reflects sublethal exposure.

Foraging behaviour: Nectar consumption and food storage. Clothianidin exposure did not affect nectar consumption in bumblebees nor the proportion of empty honetpots (i.e. the null model was the best model for both response variables). However, visual representation of the bees' nectar consumption indicates a hormesis trend, where they consumed more nectar when exposed to low concentrations of clothianidin and less nectar when exposed to no or high concentration of clothianidin (Figure 3). Hormesis is defined by its inverted U-shape form, where exposure to low concentrations of an environmental agent cause beneficial or stimulatory effects, while exposure to high concentrations cause adverse effects (Curtis D. Klaassen 2013). Reduced consumption of nectar at higher concentrations of neonicotinoids is hypothesised to be due to the collapse of the detoxification system, which handles neonicotinoids at lower concentrations (Cresswell et al. 2012), which handles neonicotinoids at lower concentrations, but is suppressed by increased neonicotinoid

exposure. Toxic responses to neonicotinoids include downregulation of genes involved in metabolism and damage to cells lining the digestive tract (Catae et al. 2014; Li et al. 2019).

Our study differs from several previous studies assessing nectar consumption in that our bumblebees could choose between nectar from the nectar bag and food stored in honeypots, instead of only being allowed to consume from feeders (Cresswell et al. 2012; Kessler et al. 2015; Thompson et al. 2015). Our results show some of the challenges in understanding the relationship between clothianidin exposure, nectar consumption, storing of food, and accumulation of clothianidin.

Our study is the first, to our knowledge, to quantify the accumulative potential of a neonicotinoid insecticide to this detail in several bumblebee body compartments simultaneously. We have shown that clothianidin is present in the head and body of bumblebee workers, as well as in the body of queens, after exposure through the nectar. The concentrations measured in the body compartments did not accumulate to exceed the nectar concentrations, and the bioaccumulation factors were similar between exposures. Also, the concentrations measured in queens were lower than in workers, suggesting a difference in sensitivity. The clothianidin exposure did not affect mortality or reproduction nor have a statistically significant effect on nectar consumption or size of food storage. However, visual interpretation of our results indicate that the bumblebees consumed more nectar of low clothianidin concentration than nectar of no or high clothianidin concentration. Our results show that only a small proportion of the exposure concentration in the food is taken up and therefore does not bioaccumulate in bees. There are some indications that this small portion may lead to sublethal responses in nectar consumption, however, more research is needed to link internal concentrations to external responses in bumblebees.

In the future, it would be interesting to observe whether the actual exposure changes over time in the nectar, for example, by measuring the nectar at more than one time point. Thus, characterising whether there is a change in concentration over time and if that can be reflected in the internal concentrations found in the bumblebees. Comparing the internal concentration with other sublethal effects, could widen the scope of what internal neonicotinoid concentration cause what external sublethal effect.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx.

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Data availability statement—Data, associated metadata, and calculation tools are available from the corresponding author (katrine.borga@ibv.uio.no).

Author contributions statement—A.N. and K.B. developed the project ideas and study design, and acquired funds. J. S. P-K. designed the experimental set-up, M.R.AA and J.S.P-K. conducted the experiment, M.R.AA prepared the samples for chemical analysis. J.T.R. performed chemical analysis. M.R.AA generated figures and performed data analysis. M.R.AA was the main author, and J.S.P-K, A.N, J.T.R, and K.B. were involved in interpretation of results and writing the manuscript.

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Figure legends

Figure 1. The bumblebee workers and queens were dissected into three parts: the head, the stomach, intestine, and rectum (SIR), and the rest of the body. The stomach, intestine, and rectum are coloured purple in the figure. Figure made by the author.

Figure 2. Relationship between the clothianidin concentrations measured in bumblebee compartments and the treatment levels (con = 0.0 μg/L, low = 3.6 μg/L, med = 6.8 μg/L, high = 13 μg/L nectar). From the left, the three plots show the concentrations measured in the (a) workers' head, (b) workers' body, and (c) queens' body. The values below limit of detection (LOD) were replaced with randomly generated values between 0.0 and 0.2 (LOD). The boxes show the variation in 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. Significant differences between the treatments and the control are identified with * ($p<0.05$) or ** ($p< 0.01$), according to Dunnett's test.

Figure 3. (a): relationship between the amount of nectar consumed and the treatment levels (con = 0.0 μg/L, low = 3.6 μg/L, med = 6.8 μg/L, high = 13 μg/L in nectar). (b): relationship between the proportion of empty honey pots and the treatment levels. For both plots, the boxes show the variation of the dataset. The bold black line specifies the median, the lower and upper lines of the box show 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. There were no significant differences between

the treatments and the controll according to Dunnett's test.

Table 1. Criteria used when classifying individual bumblebees 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/light 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

Table 2. Overview of models from statistical analysis mentioned in the results and discussion. Statistical analysis of mortality was performed using different life stages (see Materials and Methods). Life stages was not differentiated in the final model, but rather presented and discussed as one, as the life stages did not differ in mortality. Model outputs are found in Supplementary Information. The significance level was $p < 0.05$. The null model is the statistical model in which none of the explanatory variables were included.

Response variable	**Model**	**Significant/Non-significant/Null model**
Clothianidin conc. In workers' head	Head ~ Treatment	Significant
Clothianidin conc. In workers' body	Body ~ Treatment	Significant
Reproduction	Totalbroods/Totalpopulation ~ 1	Null model
	Totalbroods/Totalpopulation ~ Size	Non-significant
	Totalbroods/Totalpopulation ~ Number of queens	Non-significant
Nectar consumption	Nectar consumed ~ 1	Null model
Proportion empty honeypots	Empty honeypots ~ 1	Null model

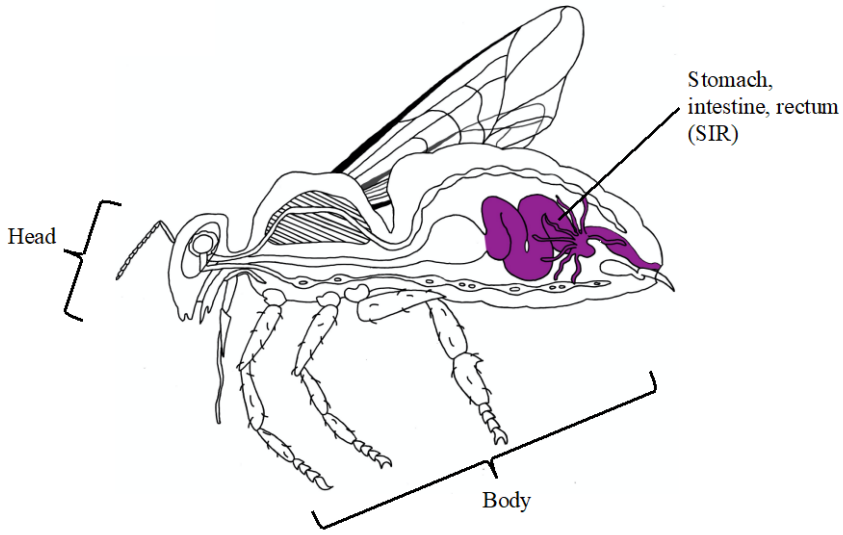

Figure 1.

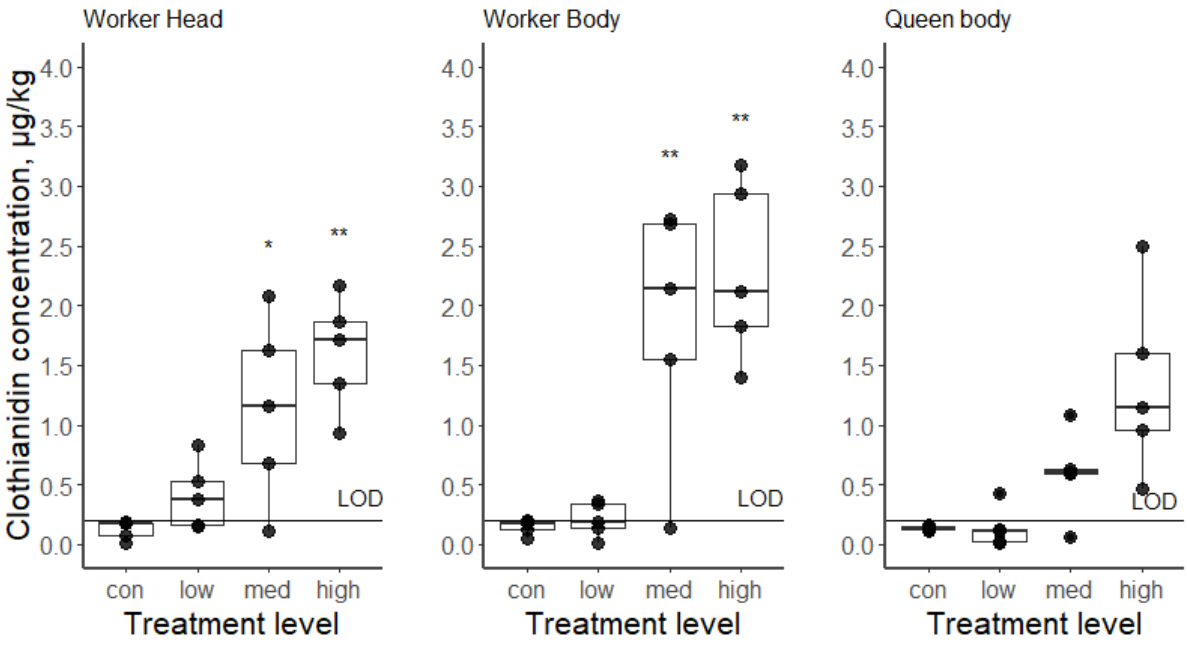

Figure 2.

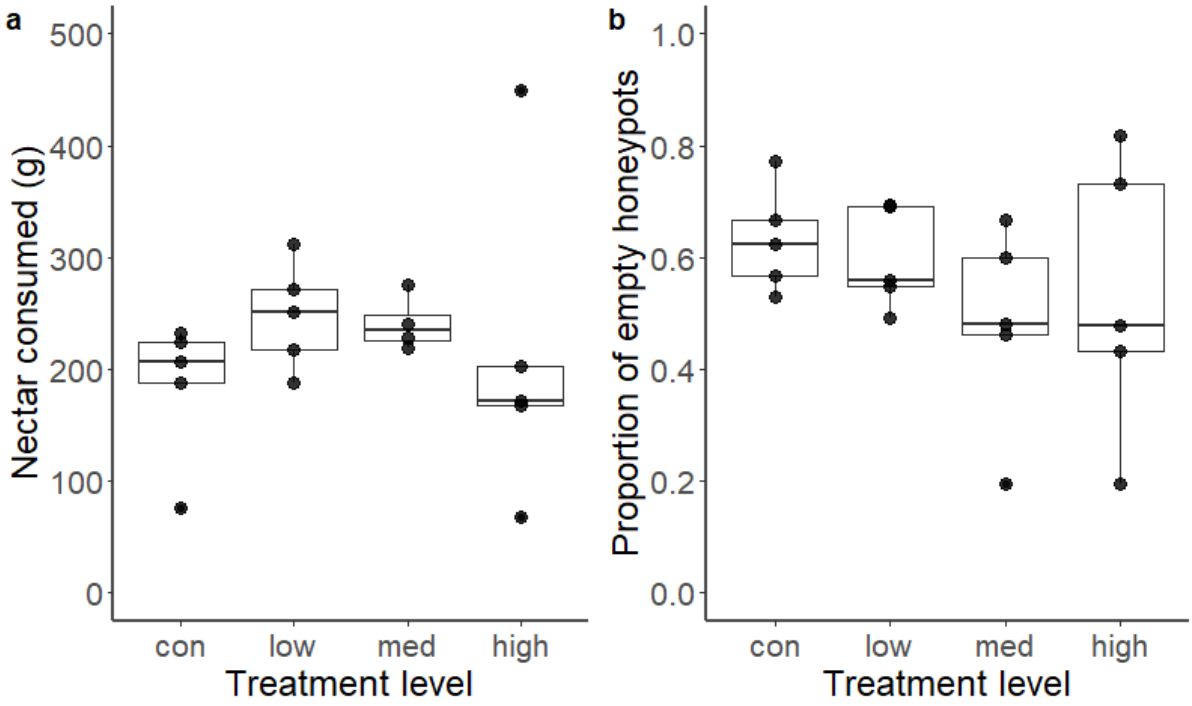

Figure 3.