Free webinar

September 28, 2021
10am EDT, 7am PDT, 2pm BST
1pm EDT, 10am PDT, 6pm BST

Evaluationg Stormwater to Identify & Quantify Causal Toxins from Tire Degradants in Coho Salmon Mortality

For decades, scientists had been concerned about water quality impacts on Pacific Northwest coho salmon that returned from the Pacific Ocean to spawn in local streams and rivers. After rain events in the area, acute and widespread mortality of adult coho salmon in the streams occurred; this was subsequently called urban runoff mortality syndrome (URMS). The cause of this phenomena was unknown for many years with many regulated chemicals and pathogens ruled out as culprits.

This webinar will take you through the journey of researchers at the University of Washington successfully identifying the primary chemical cause of this mortality – 6PPD-quinone. 6PPD-quinone is an oxidation product of 6PPD, an industrial antioxidant compound commonly used in tires. Ed Kolodziej will go through how his team were the first to identify the emerging contaminant using effect-directed analysis workflows paired with a high-resolution LC-Q/TOF and software tools. He will also demonstrate the steps that the led to linking coho mortality to 6PPD and its degradation product 6PPD-quinone.

Following this, researchers at Vogon Laboratories will discuss developing a routine quantitative method on a liquid chromatograph coupled to a triple quadrupole mass spectrometer (LC/TQ) for analysis of 6PPD-quinone. This presentation describes a fast, direct-inject analytical method for the quantitation of 6PPD-quinone in surface water.

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Environmental Toxicology

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 nontarget 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 (3.6–13 µg/L) for 9 d. Comparison of the nominal with 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 those measured in the nectar (bioaccumulation factor = 0.2). Exposure to clothianidin did not affect mortality nor brood production, nor did it 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 with 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 with exposure. Still, clothianidin may elicit responses that affect feeding behavior of the pollinator *B. terrestris*, although our endpoints were not significantly affected. *Environ Toxicol Chem* 2021;40:2781–2790. © 2021 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

Keywords: Neonicotinoids; Bumblebees; Sublethal effects; *Bombus terrestris*; Accumulation; Feeding

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 due to several favorable characteristics, including their systemic properties (Stamm et al. 2016; Yong Li et al. 2018), a long half-life in soil (reducing the need for re-application; Goulson 2013; Yang Li et al. 2018), and an effective mode of action, namely, binding to the nicotinic acetylcholine receptors (nAChRs) in the nervous system of target organisms (Jeschke et al. 2013; Palmer et al. 2013). In 2018, outdoor use on crops of the 3 neonicotinoids imidacloprid, thiamethoxam, and clothianidin was permanently banned in the European Union because of their adverse effects on nontarget organisms, bee pollinators in particular (European Food Safety Authority 2018a, 2018b, 2018c). However, neonicotinoids are still in use and are present in the environment today; in England, emergency use of thiamethoxam has been allowed, to treat sugar beet seeds, and the imidacloprid used in veterinary flea products on companion animals has 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, because 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 than are honeybees (Arena and Sgolastra 2014; Gradish et al. 2019). However, more studies are needed on bumblebees to allow a proper interpolation between species.

This article includes online-only Supplemental Data.

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Sublethal effects are defined as behavioral 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), and 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, if 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). Because neonicotinoids have multiple effects at both the individual and the 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, that is, 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, whereas 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 (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, 2019a). 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 of these properties give the neonicotinoids the potential for bioaccumulation in bumblebees. So it can be expected that tissues with a high concentration of the nAChRs, such as the bee brain (Palmer et al. 2013), will accumulate more of the neonicotinoids, and thus quantifying the neonicotinoid substance in different body parts can indicate where neonicotinoids may have the strongest effect.

The aim of our study was to analyze how clothianidin distributes within the body of bumblebees, a nontarget and non-*Apis* pollinator, after exposure to field-realistic concentrations of the neonicotinoid. We also addressed whether exposure to clothianidin caused effects on hive mortality, brood production, nectar consumption, and food storage. These aims were assessed by exposure of the bumble bee *Bombus terrestris* to clothianidin via an artificial nectar solution, and by quantification of 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 4 stages: egg, larvae, pupa, and adult (Willmer 2011). The species has haploid–diploid sex determination, whereby fertilized eggs develop into females and unfertilized eggs develop into males (Willmer 2011). They 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 that 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, with subsequent chemical analysis at the Norwegian Institute for Water Research, from June 2018 to January 2019. The experiment was divided into 3 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 *B. terrestris* were obtained from Bombus natur in standard plastic nest-boxes covered by a cardboard box. Underneath each nest-box, a plastic container was placed holding 2 L of artificial nectar that the bumblebees could access ad libitum through a sponge, and from which the bumblebees were exposed to clothianidin through nectar for 9 d. The exposure period was based on a meta-analysis defining chronic as an exposure period of 6 d or more (Cresswell 2011). We decided to expand the exposure period to 9 d to ensure that all bumblebees had consumed clothianidin (Stanley and Raine 2016). The nectar bag was provided by Bombus natur, completely emptied and cleaned before commercial syrup (called nectar from now on) and the treatment solution was added; the bag was weighed before and after the exposure period to calculate the amount of nectar consumed. The hives were used as they were on arrival, their condition was registered, and they were not standardized. 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 2 d before dissection.

Treatment and preparation of dilution series

The experiment included 4 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 the Supplemental Data).The original test range of treatments included concentrations at 1 and 1.9 μg/L to obtain a better resolution at the lower range of exposure concentrations, but were later excluded from the present study because chemical analysis showed clothianidin levels bordering on the limit of detection (LOD) even in bumblebees from the low exposure. The remaining 20 hives ($n = 20$) were randomly assigned a treatment level, with 5 replicates/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 was 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, 680, and 1300 μg/L) were made to be further mixed with nectar in the nectar bags, diluting the solution concentrations to 3.6, 6.8, and 13 μg/L clothianidin. Before adding the solutions, the nectar was added to distilled water to reduce the sugar content from 50 to 30%. The control was made from distilled water only and was 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, pupae, larvae, eggs, queens (original queen and gynes), full honeypots, half-filled honeypots, and empty honeypots. Each of the individuals was categorized as alive or dead at the time of experiment termination, based on color 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 3 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). Because 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, because the method does not detect differences at 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 allow us to assess the responses we wished to observe.

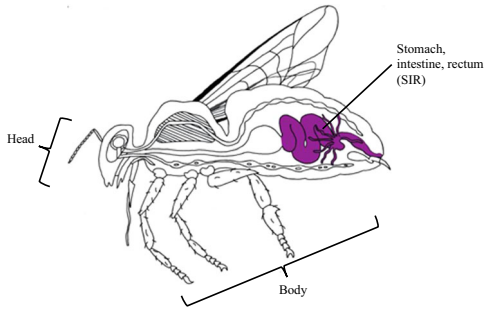

FIGURE 1: The bumblebee workers and queens were dissected into 3 parts: the head, the stomach, intestine, and rectum (SIR), and the rest of the body. The SIR are colored purple in the figure. Figure made by the author.

Clothianidin analysis

The method used to quantify clothianidin was first established by Wiest et al. (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 10 workers/colony to obtain clothianidin levels above the LOD.

TABLE 1: Criteria used when classifying individual bumblebees as dead or alive during dissection of hives[a]

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	Color: white/light yellow Other: moist	Color: gray Other: shrunken and dried up
Larva	Color: white/light yellow/light brown Other: moist	Color: dark brown/black Other: turgid/bloated or dried up
Egg	Color: white Other: moist, containing solid substance	Color: white/dark brown/black Other: if white—not containing solid substance

[a]Dissection was performed after the hives had been frozen for at least 2 d.

Distilled water and acetonitrile (MeCN; Sigma-Aldrich and VWR Chemicals) were added at a 1:3 relationship (water:-acetonitrile). Samples weighing 1 to 2 g were added to 1 mL water and 3 mL acetonitrile, and samples weighing >2 g were added to 2 mL water and 6 mL acetonitrile. If the material was not completely submerged, water and acetonitrile were added at 1 mL every second time at 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 homogenized individually. Later, acetonitrile was removed by adding 1 g of NaCl, shaking, centrifugation, and evaporation with heat (+60 °C) under nitrogen. The remaining content was dissolved in 0.5 mL 10% acetonitrile in water. Each sample was analyzed using high-performance liquid chromatography–mass spectrometry (see the Supplemental Data for detailed information concerning instrument model and settings).

Statistical analysis

Statistical analyses were performed in R Ver 3.5.2 for Mac. Normal error distribution was assessed using the Shapiro–Wilk test, and homoscedasticity was assessed using Barlett's test.

When the clothianidin concentrations were quantified, only the head and body of workers had >70% of the values above the LOD, when all treatment levels were combined, and 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 the LOD (0.2 µg/kg), to substitute for the remaining few data below the LOD (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 toward the larger values, which would also give a false structure. One nectar sample returned from chemical analyses as "not analyzed" and was replaced with the mean clothianidin concentration measured in the other nectar samples. The measured clothianidin concentrations in the nectar were compared with the nominal concentration and used to calculate the bioaccumulation factor (BAF) values.

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

Generalized 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 on arrival (looking for flies, mold, bad smell, etc.), we noted that the colonies showed some variation in age and size. The variation in size and age was not taken into account in the distribution of treatment levels among the colonies, because the assignment was randomized. We attempted to take the variation into account during the 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 3 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 categorization is described in detail in the Supplemental Data. The categorization 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 we tested for.

The proportions of dead adults, dead pupae, dead larvae, dead eggs, broods (pupae, 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 determine whether 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, whereby the model with the lowest AICc value was chosen as the best model (Burnham and Anderson 2002). If the ΔAICc differed between 2 or more models by <2, the models were considered to have the same explanatory power (Burnham and Anderson 2002).

The 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). Then a Tukey's multiple comparison test was used to test whether 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 vs 2.74 µg/L; 6.8 vs 6.54 µg/L; 13 vs 10.18 µg/L), which is below the 20% requirement for pollinator experiments (Organisation for Economic Co-operation and Development 2013). Although the measured clothianidin concentrations were lower than the nominal concentrations, the treatment levels did not overlap, and they provided a concentration gradient. In addition, the measured exposure was 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 the Supplemental Data 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 for workers' head, p values for treatment compared with control: 3.6 µg/L, $p = 0.67$; 6.8 µg/L, $p = 0.011$; 13 µg/L, $p = 0.0039$; Table 2 and Figure 2). Clothianidin did not bioaccumulate in the bumblebee workers, because the tissue concentrations did not exceed the clothianidin concentrations in the nectar (see Table S3 in the Supplemental Data), and the BAFs were <1 for all body compartments and exposure doses. The BAF_{HEAD} was 0.2 in all treatment levels, showing that the amount of clothianidin taken up increased with increasing concentrations, in a proportion consistent with that found in the diet. Moffat et al. (2015) found imidacloprid to accumulate in the brain of bumblebees at concentrations of 9.7 ± 0.8 nM after 3 d, having exposure of the bumblebees to 10 nM (2.1 ppb w/w) imidacloprid through their diet (sugar syrup). That exposure concentration was 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 for treatment compared with 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 the low and medium exposures. 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 analyzed 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 the medium and high exposure groups was not reflected in the concentrations measured in the body compartment, which were similar (Figure 2B), indicating that the concentrations measured were likely not due to spiked nectar in the crop, but rather to 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 of 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, because biotransformation has been suggested to be the main pathway for elimination of neonicotinoids in bees (Suchail et al. 2004a, 2004b). Furthermore, the BAF_{BODY} was 0.1

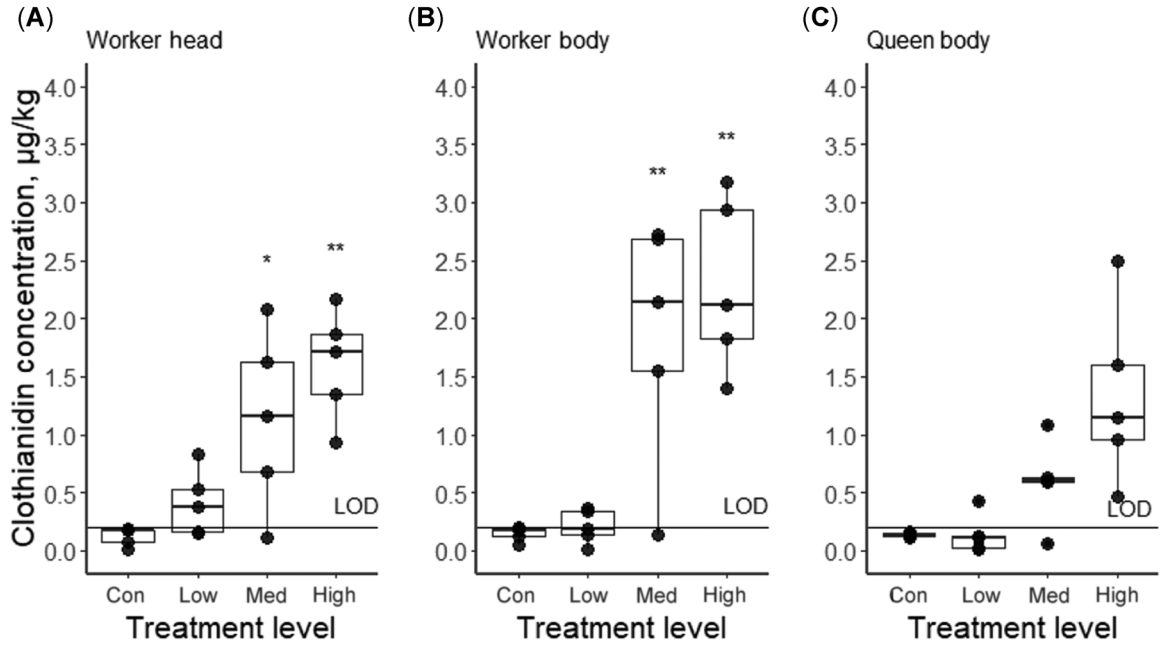

FIGURE 2: Relationship between the clothianidin concentrations measured in bumblebee compartments and the treatment levels (con = 0.0 µg/L, low = 3.6 µg/L, med = 6.8 µg/L, high = 13 µg/L nectar). From the left, the 3 plots show the concentrations measured in the (**A**) workers' head, (**B**) workers' 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 showing the largest and lowest "nonextreme" 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.

TABLE 2: Overview of models from the statistical analysis described in the *Results and Discussion* section[a]

Response variable	Model	Significant/nonsignificant/null model
Clothianidin conc. in workers' head	Head = treatment	Significant
Clothianidin conc. in workers' body	Body = treatment	Significant
Reproduction	Total broods/total population = 1	Null model
	Total broods/total population = size	Nonsignificant
	Total broods/total population = no. of queens	Nonsignificant
Nectar consumption	Nectar consumed = 1	Null model
Proportion empty honeypots	Empty honeypots = 1	Null model

[a]Statistical analysis of mortality was performed using different life stages (see *Materials and Methods*). Life stages were not differentiated in the final model, but are rather presented and discussed as one, because the life stages did not differ in mortality. Model outputs are found in the Supplemental Data. The significance level was $p < 0.05$. The null model is the statistical model in which none of the explanatory variables were included.

for low, 0.3 for medium, and 0.2 for high exposure, with a significant difference between low and medium, but no 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 SIR below the LOD (53% concentrations below the LOD), no statistical relationship could be analyzed between treatment levels. Graphic presentation showed no strong relationship either (see the Supplemental Data).

Studies depicting the accumulation of neonicotinoids in animals remain scarce, and accumulation in bees even scarcer. Neonicotinoids are not expected to bioaccumulate due to their low K_{OW}s (European Food Safety Authority 2008; Yong Li et al. 2018). However, neonicotinoids have been found in body tissues of lizards, partridges, and earthworms after prolonged exposure, although in most of these studies, the exposure concentration cannot be not considered field realistic (Lopez-Antia et al. 2015; Chevillot et al. 2017; Wang et al. 2018, 2019a, 2019b). Furthermore, clothianidin, as a metabolite of thiamethoxam, has been found in the brain of honeybees after they consumed food treated with the parent compound (Tackenberg et al. 2020). In that study also, 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 of the authors calculated the 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 et al. 2004a, 2004b; Wang et al. 2019b).

Within-body distribution of clothianidin in bumblebee queens

In contrast to the workers, clothianidin was not detected in the queens' heads except for 2 individuals (1.13 µg/kg from the low exposure group and 0.87 µg/kg from the high). The LOD was higher for the queens' heads (0.5 µg/kg) compared with the other body compartments (0.2 µg/kg). However, even with the higher LOD, we still observedheads except for 2 individuals a marked difference between workers and queens, because the clothianidin concentrations measured in the workers' heads ranged from below the LOD of 0.2 to 2.17 µg/kg. The difference in concentrations could be due to challenging issues in the homogenization of samples. Acetonitrile extracts clothianidin from the tissue, and it is therefore critical that the samples be homogenized properly for complete extraction. It was easier to homogenize the workers' heads because we could pool 10 to obtain sufficient mass/hive, whereas for the queen, only one head was homogenized/sample. If the queens' heads were not homogenized 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 all parts of the tissue were not reached. This challenge may also explain the difference in the LOD. Others who have used the same extraction method have arrived at different LODs for different matrices, but have not tried to find potential explanations (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 behavior or physiology between the workers and queens, because the anatomy of the 2 is similar, other than for size.

In the queens' bodies, accumulated clothianidin showed a concentration–response relationship; concentrations measured in the queens' bodies were lower than the concentrations found in the workers' bodies (Figure 2). When body weight was controlled for, *B. impatiens* workers had a higher total daily intake than queens, which would result in a higher accumulation of clothianidin in the body tissues of workers (Mobley and Gegear 2018). A daily total intake that causes toxic effects in queens is not sufficient to cause toxic effects in workers, which suggests that 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 of 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 (Supplemental Data, Figure S2). Statistical analysis resulted in 3 models that best explained the data: the null model (AICc = 21.7), the size of the colony (AICc = 22.3), and the 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 were 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 life span in bees, and reduction in sperm quality and sperm amount stored in the spermatheca 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, because the studies finding reduced brood production after exposure observed this decrease after 14 d (Gill et al. 2012; Laycock and Cresswell 2013). In comparison, our study was terminated after 9 d.

Clothianidin exposure did not affect mortality in the bumblebees. The control and high groups had the highest mortalities, 35 and 34%, respectively, whereas the medium group had the lowest mortality for each individual life stage, and an overall mortality of 24%. The control and high groups also had the largest colony size (mean ± standard deviation number of individuals in the colony: control, 440 ± 105; low, 377 ± 177; medium, 350 ± 169; and high, 431 ± 85 individuals). Colony size can be an indicator of its age, with larger colonies being older (Bloch 1999), and a colony may contain older workers who have reached the end of their natural life span. In addition to containing the largest colonies, the control and high groups also had the highest mortalities for adult bumblebees (percentage dead adults: control, 19%; low, 14%; medium, 11%; and 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 pupae and larvae, although the variable was not statistically significant (Table 2; see model transcripts in the Supplemental Data).

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 do 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). However, increased mortality due to chronic exposure has been observed at exposure concentrations as low as 10 ppb (Mobley and Gegear 2018). Because our selected clothianidin concentrations did not affect colony mortality, we conclude that our study reflects sublethal exposure.

Foraging behavior: Nectar consumption and food storage

Clothianidin exposure did not affect nectar consumption in bumblebees nor did it affect the proportion of empty honeypots (i.e., the null model was the best model for both response variables). However, visual representation of the bees' nectar consumption indicated a hormesis trend, whereby they consumed more nectar when exposed to low concentrations of clothianidin and less nectar when exposed to no or high concentrations of clothianidin (Figure 3). Hormesis is defined by its inverted U-shape form, whereby exposure to low concentrations of an environmental agent causes beneficial or stimulatory effects, whereas exposure to high concentrations causes adverse effects (Curtis and Klaassen 2013). Reduced consumption of nectar at higher concentrations of neonicotinoids is hypothesized to be due to collapse of the detoxification system (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 relationships among clothianidin exposure, nectar consumption, storing of food, and accumulation of clothianidin.

To our knowledge, our study is the first to quantify the accumulative potential of a neonicotinoid insecticide in such 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 enough to exceed the nectar concentrations, and the BAFs were similar between exposures. Also, the concentrations measured in queens were lower than those in workers, suggesting a difference in sensitivity. The clothianidin exposure did not affect mortality or reproduction, nor did it have a statistically significant effect on nectar consumption or size of food storage. However, visual interpretation of our results indicates 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, but more research is needed to link internal concentrations to external responses in bumblebees.

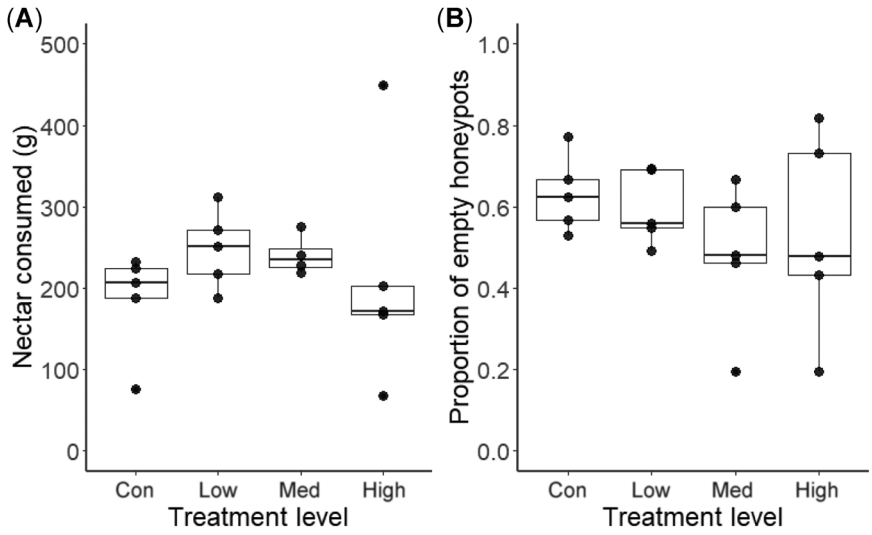

FIGURE 3: (**A**) Relationship between the amount of nectar consumed and the treatment levels (con = 0.0 µg/L, low = 3.6 µg/L, med = 6.8 µg/L, high = 13 µg/L in nectar). (**B**) Relationship between the proportion of empty honey pots and the treatment levels. For both plots, the boxes show the variation 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 "nonextreme" 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 control according to Dunnett's test.

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 and thus characterizing whether there is a change in concentration over time and whether such a change can be reflected in the internal concentrations found in the bumblebees. Comparing the internal concentration with other sublethal effects could widen the scope of which internal neonicotinoid concentration causes which external sublethal effect.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at https://doi.org/10.1002/etc.5154.

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

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