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Acute contact with imidacloprid in soil affects the nesting and survival success of a solitary wild bee, *Osmia lignaria* (Hymenoptera: Megachilidae)

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HIGHLIGHTS

- Many wild bee species utilize soil as nesting material or substrate.
- Neonicotinoid pesticides such as imidacloprid can persist in soil.
- We expose mason bees (*Osmia lignaria*) to soil containing imidacloprid residues.
- Exposure to imidacloprid in soil impaired nesting behavior among females.
- Mortality was relative to the degree of moisture in the soil.

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ABSTRACT

We assessed impacts of direct acute contact with imidacloprid-treated soil on nesting behavior and mortality of the blue orchard mason bee (*Osmia lignaria* Say), which is a native solitary cavity-nesting species that collects mud for nest partitions. Laboratory-reared *O. lignaria* females were exposed to three concentrations of imidacloprid (0, 50, 390 and 780 ppb), in wet (30% moisture) soil for 20 min and released in large flight cages, where impacts on nesting activity and nest cell production were evaluated. Mortality was tested in another experiment using exposure at the same concentrations with two differing soil moisture levels (20% and 40%). Nesting activity was reduced by 42% for females exposed at 390 ppb and by 66% for females exposed at 780 ppb. Females treated at 780 ppb produced 40% fewer nest cells per day. Sex ratios of F1 generation were skewed toward male in the 50 ppb treatment group with 50% fewer females. The number of cells and pre-pupae per nest, as well as the weight of pre-pupal cocoons did not vary among exposure levels. There were no mortality effects at 20% soil moisture for any level of imidacloprid, but at 40%, mortality of females was >50% at all levels of imidacloprid. These results suggest that acute exposure to imidacloprid residue in soil can have negative impacts on soil-interacting bees, and the effects may be relative to the degree of soil moisture.

1. Introduction

Neonicotinoids are a class of pesticides which function as nicotinic acetylcholine receptor agonists, causing neuronal hyperexcitation and death in insects (Matsuda et al., 2001). Imidacloprid, one of the most commonly used neonicotinoid insecticides worldwide (Bass et al., 2015), is often applied as a soil drench in forest, nursery, and orchard systems to control bark and wood-boring beetles, as well as other pest insects (Felsot et al., 1998; Cowles et al., 2006; Francis et al., 2009). Mason bees in the genus *Osmia* are an economically important group of solitary bees that are often managed for tree pollination in various orchard systems e.g., almond, apple, blueberry, cherry, and pear (Bosch and Kemp 2000, 2002; Bosch et al. 2000, 2006; Monzón et al., 2004; Sampson et al., 2004). *Osmia* species utilize existing holes or cavities in deadwood for nesting, and collect mud to create partitions in their nests. Thus, adult female *Osmia* have frequent contact with soil (Phillips and Klostermeyer, 1978). Soil drench applications of imidacloprid often take place in spring to be taken up by the plants' vascular system (Oliver et al., 2010; Coots et al., 2013). *Osmia* are a spring nesting genus, and females actively seek soil for their nest partitions in early spring (Bosch and Kemp, 2000), leading to a potential

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for contact with recently drenched soils. Further, as the vast majority of wild bees in North America are soil-nesting species (Michener, 2007), mason bees may be surrogates for understanding effects of contaminated soil contact for other important groups.

The current pollinator risk assessment for imidacloprid by the Environmental Protection Agency (EPA) does not consider contact exposure for soil treatments, as honey bees do not generally have direct contact with soil (U.S. Environmental Protection Agency, 2016). Risk assessment profiles of solitary cavity-nesting bees indicate that pollen and nectar exposure studies alone are not adequate to understand the impacts exposure routes related to interaction with nest-building materials (Kopit and Pitts-Singer, 2018). Contact with nest substrates and nesting materials create a unique risk framework for solitary bees which cannot be adequately addressed by honeybee studies (Sgolastra et al., 2018b). A recent risk assessment found risk thresholds from soil-based exposure were exceeded for the ground nesting solitary bee *Peponapis pruinosa* for three different neonicotinoid pesticides (clothianidin, imidacloprid, and thiamethoxam) in cucurbit fields treated eight weeks prior (Willis Chan et al., 2019). However, no direct assay studies have been conducted to date to determine the effects of acute soil-based exposure to imidacloprid on adult nesting females exposed while collecting soil, nor have any studies examined residues based on soil drench treatments for adult nesting females.

Soil drench applications involve pouring an imidacloprid suspension directly on the soil around the base of a tree. This method is a common practice for pest management in forestry, ornamental nursery, and orchard settings (Blalock and Oliver, 2014; Addesso et al., 2016; McCarty and Addesso, 2019; Thompson et al., 2020). Imidacloprid is applied to soils in limited areas in forestry applications to control invasive forest pests such as the hemlock woolly adelgid (*Adelges tsugae*) and emerald ash borer (*Agrilus planipennis*) (Benton, 2016). Reapplication is needed annually for emerald ash borer (Herms et al., 2019) and every 5–7 years for hemlock woolly adelgid (Benton et al., 2015). In ornamental nursery and orchard systems, imidacloprid is often applied as soil drench after planting young trees and may be re-applied every three years to protect trees from boring insects (Blalock and Oliver, 2014). Imidacloprid can also be applied to soils through chemigation in field crop, orchard and nursery settings, though maximum acreage restrictions are the same for all application methods (Reding, 2008; Donnarumma et al., 2011; U.S. Environmental Protection Agency, 2014). Imidacloprid binds to organic material in soil (Liu et al., 2006a, 2006b), so while residues can migrate away from the application site in lower concentrations over time, higher concentrations of the material persist in the area immediately around where the suspension was poured (Knoepp et al., 2012). Chemigation applications residues are generally limited to 30 cm radial distance around the emitter (Felsot et al., 2002). Hence, risks of soil drench treatments to *Osmia* species and other soil interacting bees would be greatest in or near soil application sites.

Imidacloprid residues in soil vary widely based on soil conditions, as clay content and organic material can affect the movement and concentration of the chemical within the soil matrix (Cox et al., 1998; Felsot et al., 1998; Liu et al., 2006a, 2006b), which creates a high variability in concentrations even within an immediate treatment area. Average concentrations have been found as high as 1200 ppb six months after soil injection treatment, with concentrations as high as 4000 ppb in the immediate treatment area within 1 m of the tree (Knoepp et al., 2012). Soil drench applications in citrus orchards have detected median concentrations ranging from 290 to 1280 ng cm^3 at the soil surface 18 days after application (Fletcher et al., 2018). *Osmia* adults frequently interact with wet soils, as they require moist clay soils for their nest partitions. Imidacloprid readily partitions in water, and is stable to hydrolysis under acidic and neutral conditions (Zheng, 1999). Hence, soil moisture levels may also need to be considered when assessing the risk of exposure to soil residues for this group of bees.

Our research objectives were to determine how acute contact with imidacloprid residues with variable soil moisture levels impacts: 1) female nesting activity; 2) nest cell production rate; 3) reproductive output; and 4) mortality of O. *lignaria*, which is a widely available commercial species. We conducted two experiments using laboratory-reared *O. lignaria* females exposed to three different concentrations of imidacloprid in soil in a Petri dish for 20 min (concentrations were 0, 50, 390 and 780 ppb, referred to as control, low, mid, and high concentration, respectively). The goal was to emulate conditions in the soil adjacent to soil drench applications. The effects of imidacloprid exposure on nesting activity, nest cell production rate, and reproductive output were assessed in the field in Experiment One. Mortality was assessed in Experiment Two in a laboratory, where adult females were released in test cages after exposure to imidacloprid treated soil at the same concentration levels at both 20% and 40% moisture levels.

2. Materials and methods

2.1. Soil imidacloprid treatments

For the soil medium, Madison sandy clay loam soil (A-layer) was collected from cleared forest stands in the Whitehall Forest, University of Georgia in southwestern parts of Clarke County, Georgia (33°53′05.0″N 83°21′27.0″W). Soil was homogenized, air dried, and filtered using USA Standard testing sieve (No. 10, 2 mm) to remove debris and larger sand particles. Sixty four standard petri dishes (100 × 15 mm) were each filled with 50 g of dried filtered soil. The 64 petri dishes were divided into four imidacloprid treatment groups (16 replicates per treatment). PESTANAL® analytical grade imidacloprid was obtained from Sigma Aldrich International GmbH. A 100 ppm stock solution of imidacloprid (90 mg imidacloprid/900 mL DI H_2O) was mixed for 12 h in the dark using a magnetic stirrer. Serial dilutions were then created by spiking 0, 0.25, 2.5 and 5 mL of the 100 ppm stock solution into vials containing 200 mL of deionized H_20. After the serial dilutions were mixed, 20 mL of serial dilution was added to each the 50 mg soil petri dishes in replicates of 16 at each level, and mixed well to create a slurry. The end concentrations of imidacloprid expected from these mixtures was 0, 35, 350 and 700 ppb in slurry. These concentrations were selected to reflect concentrations which have been detected in soil at or near the application site in the first six months after a soil drench or chemigation application (Felsot et al., 1998, 2002; Knoepp et al., 2012).

Eight petri dishes from each imidacloprid concentration treatment were randomly selected for imidacloprid residue analysis to verify imidacloprid concentrations. Insecticide residue verification by liquid chromatography tandem mass spectrometry (Villanova University, Villanova, Pennsylvania). Final concentrations in soil averaged (±SE) as: 1) 0 ± 0 ppb for the control group; 2) 53 ± 8.6 ppb for the low treatment group; 3) 386 ± 33.2 ppb for the mid treatment group; and 4) 784 ± 47 ppb for the high treatment group.

For Experiment One, a soil moisture level of 30% was used consistently in all petri dishes. For Experiment Two, 20% and 40% soil moisture levels were chosen for comparison. The beginning soil moisture after adding the 20 mL solution was 40% [50 g soil and 20 g (mL) solution]. Twelve of the soil petri dishes (three from each level) were placed in a freezer at 0 °C immediately to maintain the 40% moisture level. Another random set of 12 petri dishes were

allowed to dry in the dark and weighed every hour until weight of soil reached 60 g, indicating that 10 mL of water had evaporated, and soil moisture had reached 20%, after which they were placed in the freezer to maintain that level. The remaining 24 soil petri dishes for use in Experiment One were allowed to dry to 65 g, indicating that soil had reached ~30% moisture, and placed in the freezer and maintained at -1° Celsius. The consistency of clay soil at 20% moisture is tacky/sticky, while 40% is very muddy but will still adhere to form a ball. Although there are not adequate studies on what moisture level is preferred by *Osmia* spp., soil must have sufficient surface tension to adhere and create a ball. This range of moisture levels from 20 to 40% was selected as a representation of what *Osmia* bees would likely interact with in the field.

2.2. Experiment One: nesting assessment

2.2.1. Osmia lignaria emergence and treatment

For Experiment One, O. lignaria adult cocoons were obtained from a commercial source (Mason Bee Company, Deweyville, Utah) in February 2019 and were kept in cold storage at 4 °C until ready for emergence. Four hundred cocoons were emerged in the laboratory at ambient temperature (25 °C) between 11–14 March 2019. Emerged bees were returned to cold storage until ready for use. Once 120 females and 180 males had emerged successfully, the females were randomly distributed into four treatment groups of 30 females per group and individually marked in the cold room at 4 °C. Each female was marked on the thorax with two dots of enamel paint, one to indicate the treatment group and the second to identify the individual female.

2.2.2. Nesting assessment

Nesting activity was measured by observing pollen and mud provisioning activity of exposed female bees after release in flight cages enclosing rabbiteye blueberry shrubs. On 21 March 2019, treated soils in petri dishes were removed from the freezer and allowed to thaw to room temperature in the dark for about an hour. Marked females were removed from the cold room and treated with contact exposure to the soil by placing five females in each Petri dish with one of the four concentration treatments (control, low, mid, and high imidacloprid) for 20 min. Female interaction with soil in petri dishes involved walking or resting on the soil, no digging or mandibular manipulation was observed. There were six petri dishes for each concentration treatment. Six completely enclosed flight cages made of polyethylene mesh (natural amber color) measuring 6 m × 6 m x 2 m were set up at Durham Horticulture Research Farm, University of Georgia in Watkinsville, Georgia, each enclosing 5–6 flowering Rabbiteye blueberry shrubs. Five females from each of the four imidacloprid treatment levels (total = 20 females) were released simultaneously with 30 untreated males on 21 March 2019 in each large tent. Each tent contained a Styrofoam nest block with 36 nesting holes filled with 8 mm diameter paper tubes. To facilitate tracking individual female nests, nest holes were arranged in a 6 × 6 matrix and uniquely identified with columns marked 1–6, and rows marked A-F. A 15 cm deep by 15 cm wide untreated mud pit was created in each tent and watered daily.

Cell production rate was measured by tracking dates of female nest initiation to completion. Nests were monitored daily to look for signs of females initiating nesting activity, including entering nests with pollen in the scopa and/or bringing mud to nest holes. Formal nesting activity observations began on 27 March 2019, and continued daily except for days when weather conditions precluded active foraging (days <12 °C and/or raining) through 10 April 2019. Nest observation consisted of 20 min of observing a nest block and logging all active observations of females bringing pollen or mud to a nest hole (i.e., "nesting activity"). When nesting activity was observed, the insecticide treatment (left dot) and individual (right dot) were noted along with the alpha numeric nest hole in which the female was active (A1 to F6). Observations included documenting the first date of a female initiating a nest and the date of sealing the end of the nest. A total of 20 h of formal observation was conducted over 14 days during the peak of blueberry bloom with each tent receiving 3 h and 20 min of observation in 11 separate 20 min observation periods.

Nesting activity waned significantly after 7 April 2019, and nest usurpation behavior indicated that resources within the tents were likely diminishing. Nest usurpation in solitary bees occurs when a female will attempt to take over a nest started by another female - competitive behaviors ensue (i.e., fighting between females) and multiple cells are often destroyed within the nest as a result of the competitive process (Tepedino and Torchio, 1994). Hence, on 10 April 2019, nesting tubes were removed for examination. Reproductive output was measured by total number of cells produced per nest, numbers of pre-pupal cocoons per nest, weight of pre-pupal cocoons, and gender ratio of F1 generation adults. An otoscope was used to determine number of cells in each nest tube. Nest tubes which had clearly been usurped as indicated by observation or destroyed cells (a total of eight nest tubes) were discarded from analyses due to inability to assign all offspring to a single individual. Number of cells in combination with the amount of time from nest initiation to nest completion was used to assess nest cell production rate.

During 2–5 June 2019, pre-pupal cocoons were removed from the nest tubes, number of pre-pupal cocoons per nest were counted, and cocoons were individually weighed to the nearest 0.0001 gm with a microbalance scale, placed in clear 1.5 mL microcentrifuge tubes for storage, labeled, and incubated at 27 °C in the lab for the remainder of the summer. Temperature was reduced to 22 °C in early September. On 1 November 2019, F1 generation cocoons were placed in cold storage at 4 °C. F1 generation adults did not successfully emerge when removed from cold storage in late March 2020. Thus, F1 generation adults were cut from their cocoons in mid-April 2020 and sexed.

2.3. Experiment Two: adult mortality

Mortality was not directly considered in the flight cage assay, due to the inability to find dead bees within the large tents, and thus a second experiment was designed for assessing mortality in a laboratory setting. A group of 168 adult female *O. lignaria* were emerged, using the same methods described in Experiment One, and divided into groups of seven females. The experiment had eight different treatments: the same four imidacloprid concentrations each at two different moisture levels, each treatment had three replicate petri dishes. Each group of seven adult females was exposed to soil in petri dishes for 20 min and released into rectangular insect test cages measuring 60 cm × 30 cm x 30 cm. Twelve rectangular insect dorms were utilized for the experiment where tests for 20% moisture were conducted during 22–25 April 2019, followed by tests for 40% moisture during 29 April-2 May 2019.

Insect test cages included feeders made from 10.6 cm × 3.8 cm diameter 'desert foam' dry foam discs into which four Fisherbrand™ 1.5 mL conical microcentrifuge tubes were inserted. Each tube was then filled with 2 mm diameter plastic straws and surrounded by silk flowers of various colors which were pinned into the Styrofoam. The microcentrifuge tubes with straws were then filled with 1 mL sugar/water/pollen mixture. The mixture consisted of 250 mL sugar, 250 mL water, and 2 g pollen, and the solution was mixed for 1 h. Four tubes were included in each Styrofoam disk, for a total of 4 mL of food for each group of bees. Bee mortality was

assessed daily at 4 p.m. for three days.

3. Statistical analyses

For all statistical treatments, α at 0.05 was used to determine statistical significance. All analyses were conducted using R statistical software (RStudio RStudio Team, 2019).

3.1. Nesting activity

All bees (n = 30, each group) were scored as active or inactive daily. Observations of nesting activity were analyzed utilizing logistic regression, with imidacloprid concentration exposure as a fixed effect and block as a random effect. The model assumes that the number of days active for the kth bee in the jth treatment in the ith tent is binomial with sample size of 14 (days) and probability p_{ijk}.

3.2. Nest cell production rate

Nest cell production rate considers the number of cells produced per day per female. A total of 48 filled nest tubes with single parentage were used for the production rate analysis (n = 16 control, n = 19 low, n = 8 mid, n = 5 high). Nest cell production rate (number of cells produced per day per female) was analyzed using a generalized linear model (GLM) with negative binomial distributions, using number of cells produced per nest as the response variable, offset by the number of days until completion of the nest. Negative binomial models were run utilizing the MASS package in R statistical software (Venables and Ripley, 2002).

3.3. Reproductive output

The difference in average cells per female, and number of pre-pupal cocoons per female among imidacloprid treatments were analyzed using generalized linear models with negative binomial distributions, utilizing the MASS package in R statistical software (Venables and Ripley, 2002). For both pre-pupal weight and gender ratio, all 183 offspring produced by each treatment group were considered (n = 55 control, n = 80 low, n = 34 mid, n = 14 high). Owing to the fact that multiple nests only contained males, per nest estimates of pre-pupal weight by gender and gender ratio were not possible for all nests. Offspring pre-pupal weight was compared between treatments utilizing Analysis of Variance tests, considering males and females separately. Levene Test was used to verify assumptions of homogeneity of variance, and normality was tested by visual inspection of q-q plots. Gender ratio of the F1 generation was examined using Chi-square analysis, considering observed frequencies of females and males in each treatment group, compared to expected frequencies of male and female for each group. Expected frequencies were calculated based on Fisher's (1930) Sex Ratio and Investment Theory and formulas utilized in Torchio and Tepedino (1980), where the expected sex ratio (ESR) is based on the ratio of mean female weight (MFW) and mean male weight (MMW) for each treatment group, whereby ESR = MFW/MMW. Then we can predict the expected percentage males (EPM) = N(ESR/ESR+1), and the expected percentage female (EPF) = 1-EPM.

3.4. Adult mortality

Survival data were analyzed using Cox proportional hazards regression, using the 'Survival' package in R (Bosch and Kemp, 2003; Therneau, 2020). Deaths per day, or incident rate, was the response variable and concentration of imidacloprid, soil moisture, and the interaction between concentration of imidacloprid and soil moisture were the fixed effects. Hazard ratio is computed in this model as exp(β) for each treatment and interaction compared to the reference (control with low moisture). The assumption of proportional hazards was verified by plotting Schoenfeld residuals. To compare the effects of moisture at each imidacloprid concentration separately, Kaplan-Meier estimation was used to generate survival curves for each imidacloprid concentration at high versus low soil moisture, utilizing the "Survival" package in R (Therneau, 2020). Log rank tests were used to determine significant differences between low and high soil moisture survival curves for each level of imidacloprid treatment.

4. Results

4.1. Experiment One results: nesting

4.1.1. Nesting activity

A total of 138 nesting activity events (female entering nest with pollen on scopa or mud in mandibles) were observed over the course of the two-week observation period. As compared to the controls with no imidacloprid treatment, female bees were 42%, and 66% less active in mid ($z = -2.144$, $P = 0.032$) and high ($z = -3.696$, $P < 0.001$) imidacloprid treatment groups, respectively [Fig. 1A]. The low imidacloprid treatment group exhibited a slight increase in activity as compared to the control, but it was not significant ($z = 1.601$, $P = 0.11$). For both the mid and high groups, only six individual bees were observed nesting, out of 30 for each group. For control and low, 15 and 20 individuals were observed out of 30, respectively.

4.1.2. Nest cell production rate

Nest cell production rate (the number of cells per female per day) for the high imidacloprid treatment group was 40% lower than control, low, or mid groups ($z = -2.095$, $p = 0.04$). The high group had an average nest cell production rate of 0.6 cells per day, while control, low, and mid groups were all at or slightly above one cell per day [Fig. 1B].

4.1.3. Reproductive output

Reproductive output was assessed by total numbers of cells per nest, total pre-pupae per nest, weight of pre-pupal cocoons, and gender ratio of F1 generation. A total of 219 cells were produced by all nesting bees, 183 of which yielded pre-pupal cocoons. No differences were found between each treatment group and the control for total numbers of cells per female (control vs treatments: $P \geq 0.31$) [Table 1]. Similarly, there were no differences in F1 pre-pupal cocoons per nest between the control versus all treated groups ($P \geq 0.4$). There were no differences in F1 pre-pupal weight between treatments for male ($P = 0.3$) or female ($P = 0.7$) offspring. Gender ratio differences were significant at the low imidacloprid treatment level ($X^2 = 12.14$, $P < 0.001$), with ~53% of the nests containing only F1 males [Table 2].

4.2. Experiment Two results: mortality

Bees were exposed at the same four imidacloprid concentrations as Experiment one, but at two different soil moisture levels (20% and 40% moisture) to assess mortality for three days in insect test cages. Mortality at day three was <30% for all groups at 20% (low) soil moisture. At 40% (high) soil moisture, mortality at day three remained the same for the control group, but increased to 62% in the high imidacloprid treatment, and 81% for low and mid imidacloprid treatments. Results of multivariate Cox regression analysis revealed high hazard ratios (>9) for the interacting effects

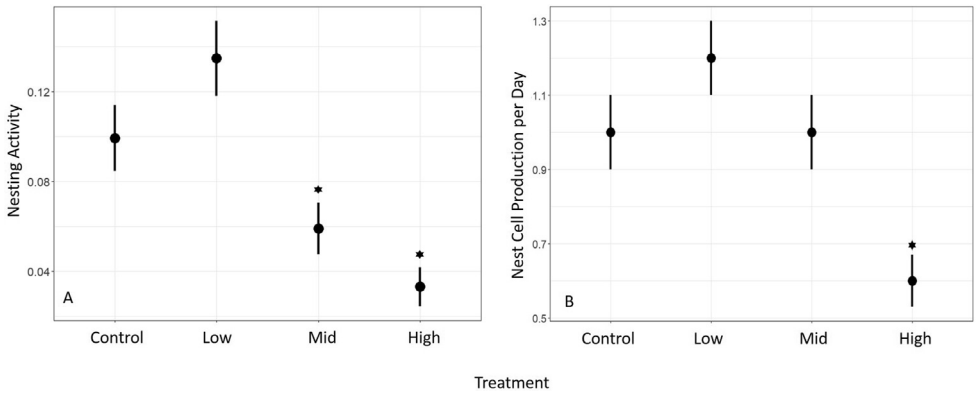

Fig. 1. Nesting activity logistic regression (A) and nest cell production rate negative binomial regression (B) effects plots. Star represents significant difference compared to control and bars indicate 95% confidence limits. Y axis for (A) is probability of individual bee exhibiting nesting activity at the indicated treatment level in a given tent during a given observation period (N = 30 individuals, each level). Y axis for (B) is, the mean number of nest cells produced by active females per day [N = 16 (control), 19 (low), 8 (mid), 5 (high)). Concentrations in soil: control = 0 ppb, low = 50 ppb, mid = 390 ppb, high = 780 ppb.

Table 1
Mean measurements ± standard error for each reproductive output measure for each treatment group. Concentrations in soil: control = 0 ppb, low = 50 ppb, mid = 390 ppb, high = 780 ppb.

Treatment	Total offspring	Cells per female	Pre-pupae per female	Mean male weight (MMW) (g)	Mean female weight (MFW) (g)
Control	56	7.7 ± 1.6	6.2 ± 1.5	0.108 ± 0.004	0.171 ± 0.008
Low	80	4.8 ± 0.5	5.3 ± 1.0	0.108 ± 0.003	0.169 ± 0.01
Mid	34	4.4 ± 0.9	6.8 ± 1.7	0.098 ± 0.004	0.179 ± 0.009
High	14	3.2 ± 0.7	2.8 ± 2.2	0.11 ± 0.01	0.183 ± 0.016

Table 2
Chi square test results of observed vs. expected counts of male and female offspring for each treatment group. Expected counts based on expected sex ratio (ESR) = mean female weight (MFW)/mean male weight (MMW), whereby expected percentage males (EPM) = N(ESR/ESR+1), and the expected percentage female (EPF) = 1-EPM. Star represents significant difference in observed vs. expected values. Concentrations in soil: control = 0 ppb, low = 50 ppb, mid = 390 ppb, high = 780 ppb.

Treatment	Observed Female	Observed Male	Expected Female	Expected Male	X^2	P
Control	15	40	21	34	3.179	0.08
Low	16	64	31	49	12.14	0.0005*
Mid	11	23	12	22	0.105	0.72
High	5	9	5	9	0.031	0.89

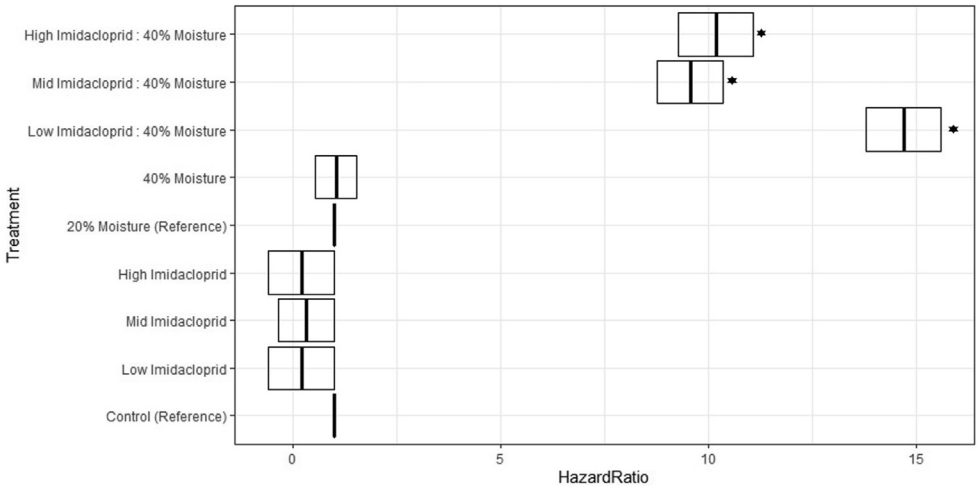

Fig. 2. Results of multivariate Cox regression, showing hazard ratios [(expβ), represented by solid vertical bars) ± SE (boxes) for hazard associated with imidacloprid exposure concentration + moisture + interacting effects of imidacloprid concentration and moisture. Reference levels for hazard ratios are control (no imidacloprid) and 20% moisture. Stars represent significance as compared to control at 20% moisture. Concentrations in soil: control = 0 ppb, low = 50 ppb, mid = 390 ppb, high = 780 ppb.

of high soil moisture (40%) and imidacloprid treatments at low, mid and high levels [Fig. 2]. Comparisons of the survival curves between high and low moisture at each imidacloprid treatment level reveals that increase in mortality at 40% moisture compared to 20% moisture were significant for all treatment groups (low: $X^2 = 21.5$, $P < 0.001$; mid: $X^2 = 18.8$, $P < 0.001$; high: $X^2 = 12.9$, $P < 0.001$),

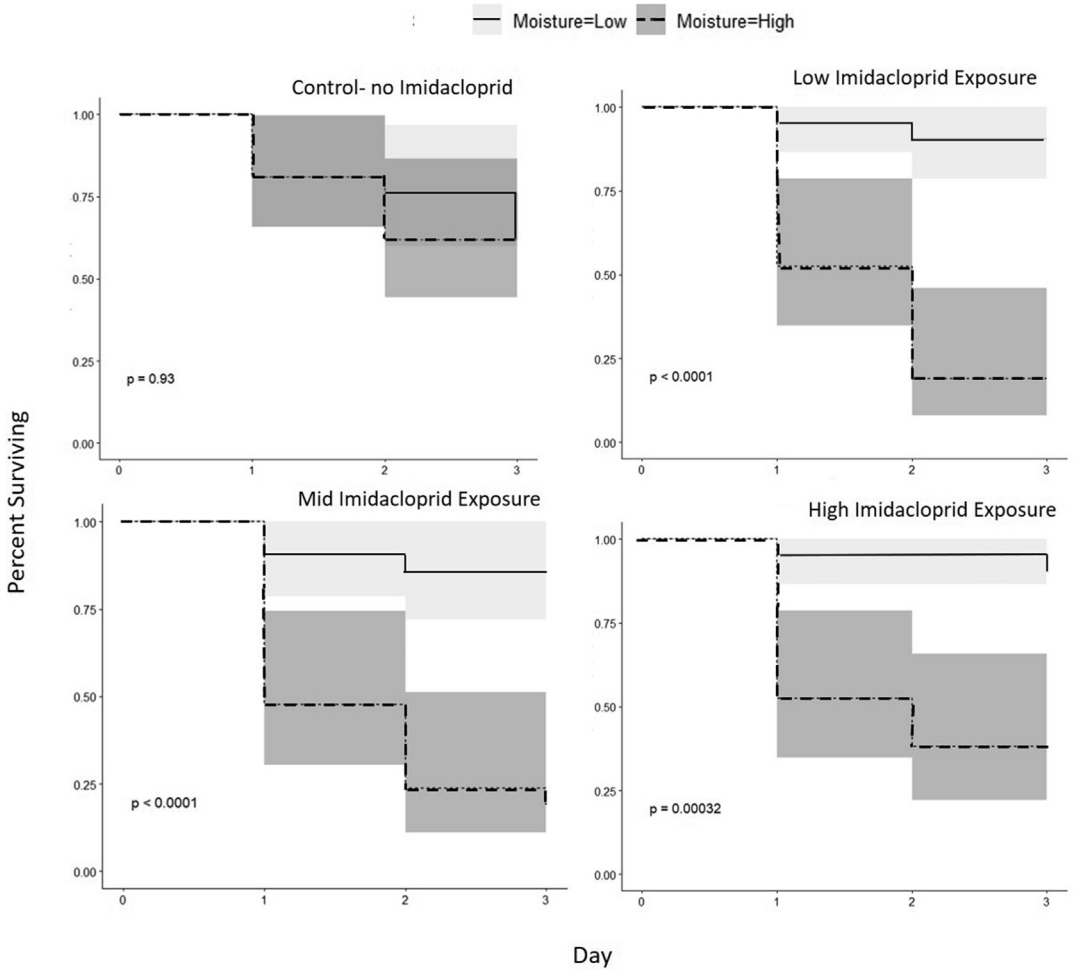

Fig. 3. Kaplan-Meier survival probability curves for comparison of each imidacloprid treatment group at two moisture levels: low moisture = 20% (light grey) and high moisture = 40% (dark grey). Curves being compared in each figure are percent survival over a three-day time period of each treatment moisture level, with shaded areas showing 95% confidence limits. Concentrations in soil: control = 0 ppb, low = 50 ppb, mid = 390 ppb, high = 780 ppb.

while no differences were found for the control group ($p = 0.9$) [Fig. 3].

5. Discussion

Imidacloprid binds to nicotinic acetycholine receptors (nAChRs) on the post-synaptic cleft causing excitation of the neuron. The nAChRs are a major component of the mushroom body in the insect brain; the center for memory, learning and sensory integration (Zars, 2000). Sub-lethal effects of imidacloprid and other neonicotinoids therefore often manifest in impairment of sensory processing, learning and memory functions (Palmer et al., 2013; Peng and Yang, 2016). These impairments of central nervous system functions can result in a myriad of observable behavioral effects in bees and other insects, such as impaired orientation and navigation (Fischer et al., 2014), mating (Tappert et al., 2017), nesting behavior (Crall et al., 2018) and foraging activity (Gill and Raine, 2014; Tan et al., 2014).

Our study documents reduced nesting activity of *O. lignaria* after acute exposure to imidacloprid in soil. Delayed nest initiation and reduced nesting activity have been documented with chronic oral exposure to imidacloprid in bumble bees (Wu-Smart and Spivak, 2017; Crall et al., 2018; Leza et al., 2018). However clear effects on nesting activity in *Osmia* have minimal documentation, studies on low level (<5 ppb) chronic dietary exposure did not show effects on nesting activity in *Osmia bicornis* (Dietzsch et al., 2019). In our study only six bees were observed nesting in both the mid and high treatment groups, suggesting the remaining bees either experienced mortality or failed to initiate nesting. Reduced pollen foraging efficiency in bumble bee workers (Feltham et al., 2014), and impaired foraging behavior for both bumble bees (Gill and Raine, 2014) and honeybees (Tan et al., 2014) have been documented. Impaired foraging could lead to a slower nest cell production rate and could also partly explain lower activity levels for bees exposed to the higher concentrations in soil.

Other studies have found reduced fecundity resulting from imidacloprid dietary exposure in bumble bees (Laycock et al., 2012) and honeybees (Wu-Smart and Spivak, 2016). Clothianidin, another neonicotinoid pesticide, slows ovary maturation in *Osmia bicornis* (Sgolastra et al., 2018a). While lower nesting activity and nest cell production rates were detected in the current study, an effect of reduced fecundity due to treatment was not observed, as our metrics of reproductive output including total number of cells per nest, number of pre-pupae per nest, and average pre-pupal weight did not show differences between imidacloprid treatments and control. Due the fact that our experiment had to be stopped after two weeks of active nesting, we were therefore unable to determine any longer-term fecundity effects which might have emerged toward the end of the nesting cycle. Averages for the number of cells and pre-pupae per nest were numerically lowest in the high

treatment group (Table 1), but were not statistically different from control. Pupal weights of offspring were numerically highest at the high imidacloprid treatment level, although this difference was not significant. it is possible that lower production rates at this level were a function of females spending more time provisioning each offspring, as provision sizes are directly correlated to offspring size in *Osmia* (Bosch and Vicens, 2002, 2006). This observation may merit more research.

Male bias in offspring as a result of trace level (<3 ppb) dietary exposure in *Osmia bicornis* has been documented though not replicated (Sandrock et al., 2014), and extreme offspring male bias in *Osmia cornuta* as a result of dietary exposure has also been documented (Strobl et al., 2019). In our study, this effect was only found at the low treatment level (50 ppb). Non-linear responses to imidacloprid exposure have been documented in studies on bumble bees, honeybees, and *Osmia* (Tosi et al., 2016; Potts et al., 2018; Anderson and Harmon-Threatt, 2019), though typically these effects are hormetic i.e., at low levels there is a fitness benefit, while at higher levels fitness decreases. The low treatment group produced more offspring overall, had higher nesting activity measures and production rates. This could be suggestive of a hormetic effect, however these measures were not different from the control group. Nonetheless, it is possible that the increased production rates and higher numbers of offspring in the low treatment group might be accounted for by an increased investment in males. Male offspring are a lower investment, as female *O. lignaria* average 31 and 35 provisioning trips for male and female offspring, respectively (Phillips and Klostermeyer, 1978). Investing more in males therefore would allow the female to produce more offspring faster, but at the expense of a balanced sex ratio. Low levels of imidacloprid exposure have been found to effect mating behavior in other insects, often improving reproductive output (Yu et al., 2010; Ayyanath et al., 2013; Rix et al., 2016) but also including reduced mating duration of treated females (Haddi et al., 2016). Unsuccessful mating could explain why many females in the low treatment group produced only males and no female offspring, as almost half of the nests in this group contained only males. As a haplo-diploid species, males can be produced by females without successful mating.

Results from Experiment Two indicated that bee mortality can occur with an acute exposure to contaminated soil even at low levels, but the mortality was a function of soil moisture. At the 40% moisture level, we saw >50% bee mortality at all imidacloprid treatment levels but minimal mortality at 20% moisture, and hence, exposure to dry soil may be less of a concern than mud. Imidacloprid is stable to hydrolysis, and the effects of photolysis are slowed in wet soil (Graebing and Chib, 2004). Further, water content naturally increases surface tension in soils and thus adhesion of soil particles to the insect body, possibly increasing bioavailability of the chemical to the insect. All of these factors in combination may contribute to wet soils posing a higher danger of exposure to soil-interacting bees than dry soils. It is notable that bees exposed at the low imidacloprid level (50 ppb) in Experiment One, where soil moisture was 30%, were equally if not slightly more productive than control bees, albeit with heavier investment in males, while in Experiment Two bees exposed at the same concentration experienced >75% mortality when soil moisture was 40%. It seems that exposure to these lower levels of soil residue may have highly variable effects depending on soil moisture. Since *Osmia* spp. generally interact with wet soils, this may be a concern for this group, and highlights the need to better understand various ways in which wild bees may be exposed to contaminated water or moisture in their environment and during their life cycles.

Further work is needed to fully understand the risks to *Osmia* and other bees in field realistic scenarios, as risk must take into account the likelihood of exposure (U.S. Environmental Protection Agency, 1992; Artz and Pitts-Singer, 2015). In the current study we forced females into a 20 min exposure, however the amount of time a female would spend in contact with contaminated materials in field scenarios, and if females may avoid contaminated materials is currently unknown but is being investigated. It is also notable that the levels we have tested would be found within the immediate treatment area, not broadly dispersed within the landscape. Hence, the total area treated relative to the foraging area of bees in the landscape should be considered when assessing risk. Other variables to consider for risk would include the degree of soil moisture, whether the timing and interval of the treatment is concurrent with nesting bee activity, whether soil interacting bees are present within the immediate area, and whether soils are exposed, mulched, or covered with leaf litter after treatment, as mulch and leaf litter would limit contact by this group of bees.

While field-level risk is yet to be determined, this study provides the first assay-based evidence that exposure to contaminated soil, in particular contaminated wet soil of 30% moisture or greater, may be considered a pathway of concern which merits more attention in research and inclusion in risk assessments. Our findings justify the need to include the soil contact pathway in risk assessments for imidacloprid, and potentially for other pesticides which persist or accumulate in soils. Considering that the vast majority of wild bee species make contact with soil in some manner, gaining a complete understanding of this risk pathway, the actual level of risk in a field setting, and potential risks to other soil-interacting bees is imperative. Future research might consider assessment of other species of soil-interacting bees, including ground nesting solitary and social groups, for a more complete understanding of this risk pathway to other wild bee species.

Author contributions

C.C.F., E.M., and K.J.K.G. designed research; C.C.F. performed research; C.C.F analyzed data; and C.C.F., E.M., and K.J.K.G. wrote the paper.

Declaration of competing interest

The authors declare that they have no competing financial interests that could have appeared to influence the work reported in this paper.

Appendix 1

Overall design for Experiments 1 and 2. Nesting parameters for experiment 1 included: nesting activity, nest production rate, total reproductive output measured by number of cells per female and number of pre-pupae per female, and gender ratio of F1 generation.

	Methods schematic						
	Soil Concentration	Soil moisture	Individuals (female)	Cages	Individuals per cage	Perameters measured	Length of experiment
Experiment 1	Control	30%	30	6 (blocks)	5	Nesting	21 days
	Low (50 ppb)	30%	30	6 (blocks)	5	Nesting	21 days

(*continued on next page*)

Appendix 1 (*continued*)

	Methods schematic						
	Soil Concentration	Soil moisture	Individuals (female)	Cages	Individuals per cage	Perameters measured	Length of experiment
	Mid (390 ppb)	30%	30	6 (blocks)	5	Nesting	21 days
	High (780 ppb)	30%	30	6 (blocks)	5	Nesting	21 days
Experiment 2	Control	20%	21	3 (replicates)	7	Mortality	3 days
	Low (50 ppb)	20%	21	3 (replicates)	7	Mortality	3 days
	Mid (390 ppb)	20%	21	3 (replicates)	7	Mortality	3 days
	High (780 ppb)	20%	21	3 (replicates)	7	Mortality	3 days
	Control	40%	21	3 (replicates)	7	Mortality	3 days
	Low (50 ppb)	40%	21	3 (replicates)	7	Mortality	3 days
	Mid (390 ppb)	40%	21	3 (replicates)	7	Mortality	3 days
	High (780 ppb)	40%	21	3 (replicates)	7	Mortality	3 days

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