Title: Honey bees and neonicotinoid-treated corn seed: contamination, exposure, and effects

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Abstract (250 words max)
Most corn (*Zea mays*) seeds planted in the US in recent years are coated with a seed treatment containing one of two neonicotinoid insecticides: clothianidin and thiamethoxam. Abrasion of the seed coating generates insecticide-laden dust that disperses through the landscape during corn planting and has resulted in a number of 'bee-kill' incidents in North America and Europe. For three years we documented the presence of corn seed treatment insecticides in bee-collected pollen and elevated honey bee mortality during corn planting in central Ohio, a landscape dominated by corn agriculture. In 2015 honey bee colonies were tracked at ten sites representing a gradient in corn agricultural intensity and it was found that total pollen contamination with seed treatment neonicotinoids was positively correlated with corn area. Additionally we conducted a semi-field experiment small colonies experienced a significant increase in worker mortality when fed contaminated pollen collected during corn planting. Despite the clear acute effects on honey bee worker survival during corn planting we did not observe significant effects of seed treatment neonicotinoid exposure on brood production, food storage, or winter survival of the colonies in the subsequent year. The lack of long-term colony-level effect may be explained by the low persistence of insecticide residues in stored pollen and the potential confounding effect of abundant late-season forage in Ohio's agricultural landscape. Exposure to seed treatment neonicotinoids during corn planting has clear short-term detrimental effects on honey bee colonies being kept in corn growing areas and has the potential to seriously affect the economic viability of beekeeping operations that are dependent on maximizing honey bee populations in the springtime.

Keywords: clothianidin, thiamethoxam, *Apis mellifera*, *Zea mays*, dust, pollinator, seed treatment, planting, talc

Introduction
It is estimated that 90% of corn (*Zea mays*) in the United States is grown from seed treated with neonicotinoid insecticides. The predominant neonicotinoids used in corn seed

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treatments are clothianidin (Poncho®) and thiamethoxam (Cruiser®) at rates between 0.25 and 1.25 mg per seed (Douglas and Tooker 2015). Assuming a seeding rate of 54,~~340~~ 000 – ~~81~~82,~~510~~ 000 seeds per hectare (Thomison 2015), up to 100 g/hectare of insecticide active ingredients are applied to sown fields each year. These broad spectrum insecticides are highly toxic to many insects, including honey bees (*Apis mellifera* L.), to which they are lethal in nanogram quantities (as low as 0.003 µg/bee for oral LD_{50} and 0.02 µg/bee for contact LD_{50} over 48 hr) (Decourtye and Devillers 2010, Laurino et al. 2013).

A link between observations of honey bee mortality and the planting of neonicotinoid-treated corn was suspected as early as the late 1990s, when researchers in Italy noted a rise in colony damage reports coinciding with spring corn planting (Bortolotti et al. 2009). In subsequent years, similar patterns of honey bee mortality were observed in Italy (Schnier et al. 2003, Greatti et al. 2006, Bortolotti et al. 2009), France (Giffard and Dupont 2009), and Slovenia (Alix et al. 2009, van der Geest 2012, Žabar et al. 2012). In 2008, a large-scale bee kill in Germany and neighboring parts of France was attributed to the planting of neonicotinoid-treated corn after an extensive investigation found neonicotinoid residues in dead bees, bee bread, and plant samples collected from the affected area (Forster 2009, Nikolakis et al. 2009, Pistorius et al. 2009, Chauzat et al. 2010). Since then, additional incidents of honey bee mortality during corn planting have been reported in Slovenia and neighboring Hungary (van der Geest 2012), the United States (Krupke et al. 2012; L. Keller, personal communication, 2016) and Canada (Health Canada 2013).

While these reports clearly establish a link between honey bee mortality and the planting of corn with a neonicotinoid seed treatment, the route through which bees are exposed to lethal levels of seed treatment insecticide is difficult to ascertain. During the planting process, seed treatment material sloughs off the seed surface in small particles that are available to disperse in the environment (**Fig. 1**). Bees may encounter these particles as dust deposited on flowers (Krupke et al. 2012), nectar and pollen contamination via uptake from the soil (Long and Krupke 2016), contamination of surface water (Samson-Robert et al. 2014; Schaafsma et al. 2015), contamination of guttation fluids (Tapparo et al. 2011), and in-flight contact with aerial dust (Girolami et al. 2012). Soil containing insecticides residues left over from previous years of planting may also become airborne during planting and contribute to bee exposure during this period (Forero et al. 2017). While each of these routes may contribute to honey bee exposure, identifying the most significant route or routes is crucial for designing mitigation strategies.

To better understand the association between corn planting, neonicotinoid residues in honey bee-collected pollen, and honey bee mortality, we conducted a three-year field study (2013 - 2015) in Ohio, one of largest corn-growing states in the United States. Each year, we measured worker mortality and pesticide contamination of bee-collected pollen prior to, during, and after corn planting.

In 2015, we analyzed neonicotinoid residues in nectar and pollen at 10 apiary locations across a gradient of corn-planting intensity. Colony development was monitored throughout the summer and after the following winter. Additionally, a semi-field experiment was conducted in which small colonies were provisioned with insecticide-laden pollen collected by honey bees during corn planting to determine the effect of pollen consumption on bee mortality.

Results

Worker bee mortality

Increased numbers of dead bees at hive entrances were consistently observed around the time

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when corn was being planted. The daily worker mortality index, calculated from dead bees counted at the hive entrance and standardized by month-average mortality of the colony (see **Eq. 1** in Methods), was significantly and consistently higher during corn planting than the non-planting periods for the same colonies for all years (2-tailed paired t-test comparing mortality of the same colony averaged during planting vs. non-planting periods; 2013: $t = 2.79$, $df = 11$, $P = 0.0175$; 2014: $t = 3.02$, $df = 23$, $P = 0.0061$; 2015: $t = 10.25$, $df = 37$, $P < 0.0001$) (**Fig. 2a**). Additionally, mortality during corn planting was consistently greater than zero (the expected value if the variation in standardized mortality index is random) for all years (**Fig. 2a**), indicating the association between the elevated mortality observed and corn-planting.

Pollen contamination

Analysis of pollen samples showed that clothianidin and thiamethoxam, the insecticides present in corn seed treatments, were consistently the most abundant neonicotinoid insecticides detected in bee-collected pollen in all years of our study and the detection of these compounds occurred more frequently (Fisher's Exact Test, $P < 0.0001$) and at higher concentrations during corn planting periods (**Table 1, Fig. 2b**). Neonicotinoid insecticides that are not used for corn seed treatments, but applied to other crops in Ohio (imidacloprid, nitenpyram, dinotefuran, and thiacloprid) were detected in pollen samples, but the timing of detection for other neonicotinoids was not related to corn-planting (**Supplemental Material S2**).

The relationship between neonicotinoid concentration in pollen and the area of corn grown within the 2km foraging range was evaluated for 10 apiary sites studied in 2015. During corn planting, pollen collected from sites with more surrounding cornfields contained higher concentrations of clothianidin (Pearson's $r = 0.65$, $P = 0.040$) and thiamethoxam ($r = 0.62$, $P = 0.056$). The sum concentration of clothianidin and thiamethoxam together was significantly correlated with cornfield area ($r = 0.68$, $P = 0.030$). No correlation between cornfield area and clothianidin or thiamethoxam concentrations were detected outside the planting period ($P > 0.4$).

Relationship between pollen neonicotinoid concentration and worker mortality

Worker mortality was consistently higher on dates when clothianidin and thiamethoxam were present in pollen samples (Fig. 2c) although the difference was only statistically significant in 2014 and 2015 when more colonies were monitored (2-tailed paired t-test; 2013: $t = 2.13$, $df = 11$, $P = 0.0565$; 2014: $t = 3.82$, $df = 23$, $P = 0.0009$; 2015: $t = 8.13$, $df = 33$, $P < 0.0001$). To determine if there was any interactive effect between insecticide exposure and cornfield area in the landscape, we further tested for correlations between the number of dead bees and clothianidin and thiamethoxam concentrations in pollen on the same sampling dates for each apiary along a gradient of corn intensity in 2015. Positive correlations between the insecticide concentrations and mortality were detected at sites with more corn fields (> 30% within 2-km radius from the apiary) in the surrounding landscape (**Table 2**).

Insecticide residues in in-hive samples

In 2015, we analyze stored pollen and nectar for corn seed treatment insecticides taken from inside honey bee colonies. Honey and pollen were sampled from combs in two colonies at seven apiaries (DS, SC, IB, HR, TV, BG, MM) at four separate time points (before planting, during planting, post-planting, and two weeks post-planting). The total clothianidin and thiamethoxam concentrations in bee bread were generally low before planting (< 13.5 ng/g, mean 4.0 ng/g;

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Supplemental Material S2). The levels increased in samples collected during corn-planting (4.8 - 42.3 ng/g, mean 21.6 ng/g) and immediately post-planting (4.5 - 60.8 ng/g, mean 23.6 ng/g). Total clothianidin and thiamethoxam concentrations in stored pollen collected two weeks after planting returned to a lower level (< 11.6 ng/g) except for a sample collected at TV (35.76 ng/g), averaging at 11.6 ng/g for this sampling time. Mean concentrations differed significantly among samping times (one-way ANOVA $F_{(3, 24)} = 3.23$, $P = 0.04$) with the lowest concentration level in bee bread sampled before planting.

Neonicotinoid concentrations for in-hive stored pollen were were compared with concentrations detected in corbicular pollen trapped at the entrance of the same hive for the four sampling periods. A significant correlation between the two was detected immediately post-planting ($r = 0.87$, $P = 0.0098$) but not in other sampling periods (Fig. 3).

Concentrations of clothianidin and thiamethoxam in honey were low (< 0.76 ng/g total neonicotinoid), though there were more positive detections for honey sampled during and after corn-planting, the mean values did significantly differ across sampling time.

Closed nuc experiments
To test for a direct association between clothianidin and thiamethoxam contamination of pollen and worker mortality, we conducted a semi-field experiment where small honey bee colonies, enclosed in nucleus hive equipment and denied the ability to freely forage, were fed with corbicular pollen collected from our study apiaries in 2015 around corn planting and contaminated with known concentrations of clothianidin and thiamethoxam. An increase in worker mortality correlated with the total concentration of clothianidin and thiamethoxam in pollen was observed over four days (Pearson's $r = 0.81$, $P < 0.0001$; Fig. 4).

Post-planting colony development.
To address the question of whether seed treatment neonicotinoid exposure in May had long term consequences for colony growth, we tracked six hive health metrics (adult bees, pollen stores, nectar stores, open brood, and capped brood, measured by frame area) at four time points (April, May, June, and August) in 2015. Neonicotinoid exposure in May was correlated with a reduction in the relative population growth of the hive (as measured by area of bees and seams of bees) over the earliest time interval (late April to late May; area of bees: $t = -3.61$, $P = 0.01$; seams of bees: $t = -2.50$, $P = 0.04$). However, there may have been a recovery in the second time interval, from late May to late June, as hives exposed to greater neonicotinoid levels in May had a larger increase in adult bee population during this time (for seams of bees only; $t = 2.47$, $P = 0.04$). Finally, in the third time interval, from late June to early August, hives located in areas where neonicotinoid exposure during planting was greatest had more stored pollen ($t = 3.35$, $P = 0.01$). Area of cornfield surrounding apiaries was also positively correlated with increases in stored pollen (Pearson's $r = 0.79$, $P = 0.007$) and honey ($r = 0.68$, $P = 0.03$) during the summer period.

Of the 38 colonies monitored, one colony died in late summer and three were relocated to another location and were excluded from monitoring over winter. A total of 34 colonies were prepared for overwintering at the end of September 2015 and 31 of those colonies (91%) were still alive at the end of March, 2016, although one of the surviving colonies was queenless. No significant correlation was observed between survival over winter and the level of corn seed treatment neonicotinoids in pollen or percent corn area in the surrounding landscape across the

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10 apiaries (Spearman's rank correlation tests, $P > 0.36$ for all tests).

Discussion

Mortality, exposure, and corn planting

For three years, we consistently observed elevated mortality in adult honey bee workers during corn planting. This pattern of mortality coincided with our finding that clothianidin and thiamethoxam, the insecticidal component in corn seed treatment, were detected more frequently and at higher concentrations in pollen collected by honey bees during corn planting. Additionally, our study showed that seed treatment insecticides can consistently be detected in bee-collected pollen during planting, indicating that the release of seed treatment particles during corn planting is ubiquitous and that released particles are subject to aerial transport, in agreement with previous studies (Krupke et al. 2012, Schaafsma et al. 2015).

Together, these lines of evidence strongly indicate a causal connection between elevated honey bee mortality and seed treatment insecticides emitted during planting. This conclusion is further corroborated by recent work in Italy, where reports of honey bee mortality during corn planting have decreased significantly since the suspension of use of neonicotinoid seed treatments in corn (Sgolastra et al. 2017). Nevertheless, the level of mortality observed in our study was not sufficient to cause detectable long-term impacts on colony health. To the contrary, corn area was associated with more pollen and honey accumulation by colonies later in the summer. This observation may be associated with food sources such as clover and other summer wildflowers that thrive in roadsides and field margins, and with the flowering of soybean, which is often planted in rotation with corn, and which may serve as a nectar source for honey bees (Vanderlinden 1981, Sponsler and Johnson 2015, Sponsler et al. 2017).

One inconsistency must be considered, though. The concentrations of neonicotinoid insecticides detected in pollen samples were below those that would be expected to cause acute mortality, yet adult mortality was observed in free-flying colonies observed during corn planting and in confined colonies fed pollen collected during corn planting. Based on a range of acute oral LD50s for adult workers of 1.11 - 6.76 ng/bee (Laurino et al. 2013) and predicted pollen consumption of nurse bees 6.5 mg/bee/day (Rortais et al. 2005), substantial mortality would only be expected at concentrations greater than 171 µg/kg in pollen. However, neonicotinoid residues detected in bulk pollen samples may not meaningfully reflect doses received by individual bees (Sponsler and Johnson 2017). For example, a bulk pollen concentration of 20 µg/kg clothianidin could reflect a uniform distribution of insecticide or it could reflect a skewed distribution in which one or a few pollen pellets carry very high concentrations while the rest of the pollen is relatively uncontaminated. These two distributions would have the same mean concentration, but would result in different effects on the colony. Given the observed pattern of mortality we can infer that neonicotinoid contamination is not evenly distributed throughout all pollen since an increase in bee mortality is reliably observed.

Post-planting colony development

Although we found strong evidence associating the surge in honey bee worker mortality with the exposure to corn seed treatment insecticides emitted as planter dust during planting, we did not detect negative long-term impacts on colony growth or survival over winter. The observed lack

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of long-term effects at the colony level may be explained by the dynamics of clothianidin and thiamethoxam once these compounds had entered the hives. While high levels were detected in bee bread and honey sampled immediately after corn planting, levels in bee bread and honey declined to the pre-planting level the following week for most sites. Contaminated food stored inside the hives were likely consumed or diluted to lower concentration as uncontaminated pollen and nectar were brought in after planting.

The sub-lethal effects of the exposure remain unclear and require further investigation. The sudden loss of adult bees occurred at a critical time when colony populations were building up for early-summer honey production and smaller colonies may not be able to recover from the loss (Khoury et al. 2013). Beekeepers that depend on early spring buildup to make splits and sell nucleus colonies could be especially hard hit by a loss of bees during corn planting. Exposure to neonicotinoids during corn planting could affect queen quality as the planting season coincides with queens rearing for the swarm season (Tsvetkov et al. 2017). Clothianidin and thiamethoxam can also affect honey bee's immunity against viral diseases (Di Prisco et al. 2013) or reduce survival when colonies are under nutritional stress (Tosi et al. 2017).

Conclusion

Our study confirms that seed treatment insecticides are released during corn planting and that these insecticides contaminate pollen collected by bees. Honey bee colonies experience elevated adult mortality due to seed treatment insecticide exposure. The level of mortality we observed, however, was not sufficient to cause detectable long-term effects on colony health, though long-term health effects were likely confounded by the positive effects of the agricultural landscapes on colonies over the summer.

Methods

Study sites

A total of 13 apiaries located throughout the corn-growing region of central Ohio were monitored prior to, during, and after corn-sowing from late-April to end of May in 2013 (3 apiaries), 2014 (6 apiaries) and 2015 (10 apiaries). Four apiaries were studied in multiple years (Supplemental Material S1). Apiaries were located at least 4 km from each other and were selected to represent a range of agricultural intensity, including one suburban apiary in 2015, with minimal corn agriculture within foraging range. Apiaries consisted of between 4 - 20 colonies. Two to four healthy, actively foraging colonies, varying in sizes and queen ages, were monitored for worker mortality (see Supplemental Material S2 for colony information). All colonies were housed in eight- or ten-framed Langstroth hives.

The timing of corn sowing activity was identified through direct observation of planting activity near apiary sites and communication with farmers, and were in line with state-wide agricultural statistics for each year ("USDA - National Agricultural Statistics Service" n.d.). The bulk of sowing activing in this region occurred between May 5 – 16 in 2013, May 5 – 10 in 2014, and May 2 – 8 in 2015. Less intensive corn planting continued beyond this period in all years, but was particularly drawn out in 2014 when high rainfall resulted in planting and re-planting through the end of May.

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Landscape characterization

The landscape surrounding each apiary was characterized within a 2-km radius around the apiary. Visual ground-truthing supplemented by satellite imagery (Google OpenLayers), was used to classify landscapes into crop field, forest, treeline, herbaceous strips in field margins and roadsides, and residential lots. Crop type was determined by a second visual inspection in early summer and the USDA crop data layer (USDA-NASS-RDD n.d.). All landscape data were analyzed and visualized using QGIS software (QGIS Development Team 2015). Immediately prior to corn planting, each agricultural field was visually assessed to determine the abundance of in-field flowering weeds and scored as "abundant", "sparse", or "absent".

Apiaries in 2013 and 2014 were surrounded by a high proportion of corn fields within 2 km of the apiaries, ranging from 31 – 45% corn in 2013 and 21 – 51 % in 2014. In 2015, a wider gradient of corn area was in the foraging range, 0 - 49% corn.

Worker bee mortality

Under-basket style dead bee traps (102x51x15 cm; (Human et al. 2013) were placed in front of each colonies being monitored. Dead bees in traps were counted and removed every 2 – 4 days, starting in late April each year, approximately one week before corn planting, until 1 - 2 weeks after planting activities had ceased. Averaged number of dead bees per day was calculated for each sampling interval as the number of dead bees in the trap divided by number of days elapsed since the last sampling. Because large colonies eject more dead bees than small colonies, we converted the daily dead bees count for each hive to a mortality index, denoted by M_i, using the following formula:

$$M_i = (N_i - N_a)/N_a \qquad \textbf{Eq. 1}$$

where

N_i is the number of dead bees per day on a given date i

N_a the average number of bees per day for a colony collected over the entire sampling period

The standardized mortality, M_i is the difference between daily mortality and the overall mortality for a given colony over the sampling period. If mortality is consistent throughout the sampling period, then M_i is expected to be zero. To compare bee mortality between planting and non-planting periods, we took the *means* of M_i values for each hive during planting and non-planting periods respectively. Each hive has two values representing mortality during the planting and non-planting periods respectively. Paired-sample t-tests were performed to compare mean mortality of the same hives during planting vs. non-planting periods. A separate analysis was performed for each year.

Commented [LEH1]: This mortality index is equal to the number of means away from the mean and is equal to -1 if Ni=0. It is better to have a mortality index that is equal to 0 if Ni=0 and is equal to 1 if Ni=max(Ni). That is, the mortality index ranges from 0 to 1 for all site-years. I propose the mortality index, M2(i) = N(i)/max(Ni). If mortality is constant, then M2(i)=1 versus M(i)=0. If mortality N(i) is equal to X for period t and 0 for all other periods, then M2(i) = 1 for period t and 0 for all other periods. Given p days for period t and T total days, M(t) = (X/p-X/T)*T/X= T/p-1 which can vary by site and year. For example, let T=100 days, p=5 then M(t)=19. Suppose for a second site, let T=100 and p=4, then M(t)=24. The test statistic based on M(i) is highly sensitive to the peak values which are not standardized.

Commented [LEH2]: How are the planting and non-planting periods defined in the t-test? Does the planting period correspond to May 5 – 16 in 2013, May 5 – 10 in 2014, and May 2 – 8 in 2015?

Commented [LEH3]: Before performing a separate analysis by year, one should combine the data across sites and years and test for an interaction with site and year. the statistical analysis should also consider the proportion of corn fields within 2 km and pollen collected from returning bees as covariates in the statistical model.

Sampling and pesticide screening

Incoming corbicular pollen was collected for pesticide screening from bees returning to two strong colonies in each apiary using bottom-mounted pollen traps (Sundance I, Ross Rounds, Inc.). Pollen was collected every 2-4 days, pooled by site and date and stored at -20 °C until further analyses. In 2015 additional samples were collected for pesticide screening, including: dead bees from dead bee traps and in-hive samples of bee bread, honey, larvae, and live nurse bees. In-hive samples were collected from two queen-right, overwintered colonies at seven

apiaries (DS, SC, BG, HR, TV, MM, Supplemental Materials S2). The in-hive samples were collected during four sampling periods: before planting (April 27 - 30), during planting (May 5 - 7), immediately after planting (May 12 - 13), and two weeks after planting (May 20 - 22). Honey and bee bread were collected from uncapped cells peripheral to the brood area where bees were actively depositing food. Nurse bees on the brood area and 25 - 30 late-stage larvae in open cells were collected from each hive. In-hive samples were pooled by apiaries and stored at -20 °C until further analyses.

Five grams of pollen from each site and sampling date were extracted using a modified QuEChERS protocol for 2013 - 2014 samples (Camino-Sanchez et al. 2010). Samples from 2015 were extracted following a method by Yáñez et al. (2014) except ethyl acetate was used instead of dichloromethane. In all years extracts were analyzed for neonicotinoid insecticides (clothianidin, thiamethoxam and imidacloprid) using liquid chromatography tandem mass spectrometry (LC-MS-MS) methods. Analysis was performed by the USDA-AMS lab in Gastonia, North Carolina (2013 - 2014 samples) and EPA National Exposure Research Laboratory in Athens, Georgia (2015 samples). All residues were reported as mass-mass concentration (µg/kg).

Statistical analyses

Mortality and pollen contamination associated with planting
At the colony level, paired t-tests were performed to compare mortality with in averaged during planting vs. non-planting periods within the same colonies. The same tests were also performed to compare mortality of the same colonies recorded on dates with vs. without detectable clothianidin and thiamethoxam residues in pollen.

Non-parametric Fisher's Exact tests were performed to compare the frequency of positive detection of clothianidin and thiamethoxam residues in pollen collected during planting vs. non-planting periods. T-test assuming unequal variance was performed to compare clothianidin and thiamethoxam concentrations in pollen collected at each apiary during planting vs. non-planting periods.

Association between mortality and pollen contamination
Non-parametric Fisher's Exact Tests were performed to test the hypothesis that mortality was higher on dates when detectable levels of clothianidin and thiamethoxam were present in pollen. Pairwise correlation between mortality and clothianidin and thiamethoxam concentration in pollen collected on the same sampling dates was evaluated for each site.

In-hive samples
The averaged concentration of clothianidin and thiamethoxam in corbicular pollen trapped during the week prior to each in-hive sample collection were calculated. Pairwise correlations between the averaged concentrations in corbicular pollen and bee bread were analyzed separately for each sampling date.

Closed colony experiments
To test the link between insecticide contaminants in pollen and worker mortality, we conducted

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Commented [LEH5]: These tests to compare the frequency of positives or insecticide concentrations between planting and non-planting periods do not honor the experimental design. It is incorrect to assume that the measurements taken at multiple times at the same site are independent. For simplicity, suppose that there are 12 sites and at each site, one measurement is taken during non-planting period and one measurement is taken during planting period. Then one should use a paired t-test to compare the mean difference between the two periods. When there are multiple measurements taken during each period at the same site, the measurements do not represent a random sample. The two-independent sample t-test is not valid because the data violate the assumption of independence. Same for fisher's exact test .

Commented [LEH6]: See above comment. the data do not represent a random sample and violate the assumption of independence which is required for Fisher's exact test and the test for correlation. The statistical analysis does not fully utilize the temporal structure of the data to examine a lagged response to insecticide exposure and the seasonal pattern of mortality.

an experiment where enclosed honey bee colonies were fed with corbicular pollen collected from the study apiaries during corn planting. The colonies were confined in Bee Brief Nuc boxes (NOD Apiary Products, Frankford, Ontario, Canada), each containing a pollen feeder frame and two frames with drawn wax combs, one of which had approximately 100 cm^2 of capped brood for stabilizing the colony, one frame of nurse bees, and a laying queen that was 3 - 5 weeks post-mating. The equipment was weighed prior to adding bees and again after bees were shaken into the box to obtain the net weight bees per colony. Two hundred grams of corbicular pollen were pushed into two 96-well cell culture plates (Biotix #AP-0350-9CU, San Diego, California) affixed to the pollen feeder frame. Treatments were selected from the different apiaries to include a wide range of concentration of naturally contaminated clothianidin and thiamethoxam. Fresh sugar syrup (50% w/w) was provided liberally. A total of four trials with 4 - 7 colonies were performed (20 colonies total). Each trial also contained a "positive control" treatment of which a relatively clean pollen sample (< 10 ppb) was spiked with of clothianidin. The colonies were enclosed and kept in a dark room with average daily high temperature of 24.4 °C (daytime) and low temperature of 19.5 °C (nighttime) for 96 hours. Then, dead bees in the box were counted and the plates were weighed to estimate how much pollen was consumed. Five grams of pollen were sampled for pesticide analysis. Pearson's correlations were performed to evaluate the association between the number of dead bees and neonicotinoid concentrations. Two of the colonies contained less than 100 g of bees and were excluded from the analysis.

Post-planting colony growth

To address the question of whether exposure to corn seed treatment insecticides in May is linked to long-term consequences in colony growth, we tracked the colonies from April 2015 through February 2016. Four detailed colony inspections were performed using a modified Liebfelder method (Delaplane et al. 2013) on April 28 – 30 (before planting), May 20 – 22 (after planting), June 19 - 24, and August 14 - 19. During the inspection each frame was removed from the monitored colonies to record the area of coverage with the following components: adult bees, brood (open and capped), pollen, and honey. Additionally, the total adult bee population was estimated by looking up and down frame spaces to estimate "seams" of bees. All colonies were managed using standard beekeeping practices. *Varroa* mites were controlled by applying formic acid (Mite Away Quick Strip, NOD Apiary Products) in June and vaporized oxalic acid in November. Plain baker's fondant (Dawn Food Products, Inc., Jackson, MI, USA) and Dadant AP23 winter patties (Dandant & Sons Inc., Hamilton, IL, USA) were fed to the colonies, as needed, through the winter.

We examined whether the relative change in each colony variable through time was associated with neonic concentrations measured in pollen in May. Relative change for each variable was calculated as:

$$Relative\ change\ (\%) = \frac{final - initial}{initial} * 100$$

We considered each interval between inspection dates, as well as the interval between the first and last inspections. To determine whether neonic loads in pollen were significantly associated with colony growth through time, we constructed linear regression models with relative change as the response and mean clothianidin and thiamethoxam concentrations in pollen in May as the predictor. We also included the relative change in colony pollen coverage over the same time

interval as a covariate, to account for the potential that the negative effects of neonic exposure could be partially offset by the positive effects increased food supply. If the pollen change covariate was not a significant predictor, we dropped the term and refit the model.

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Table 1. Clothianidin & thiamethoxam in pollen

Frequency of positive detections (Det./n, n = total number of samples) of clothianidin and thiamethoxam residues, and range of concentration when detected, in bee-collected pollen. T-tests assuming unequal variance were performed to compare the means of planting vs. non-planting periods.

		Det./n, range (µg/kg)		Mean±se (µg/kg)		
Year	Residues	Planting	Non-planting	Planting	Non-planting	t-test
2013	Clothianidin	12/12 (100%) 4.8 – 35.5	2/18 (11%) 3.9 – 6.9	16.63 ± 2.96	0.57 ± 0.40	t = 5.38, df = 11 **P = 0.0002**
	Thiamethoxam	8/12 (67%) 1.6 – 9.1	1/18 (6%) 2.2	3.67 ± 1.01	0.12 ± 0.12	t = 3.50, df =11 **P = 0.0048**
	Total	12/12 (100%) 4.8 – 44.6	3/18 (17%) 2.2 – 6.3	20.3 ± 3.75	0.69 ± 0.41	t = 5.19, df = 11 **P = 0.0003**
2014	Clothianidin	5/18 (28%) 12.0 – 18.4	0/55 (0%) N/A	4.15 ± 1.64	0	t = 2.53, df = 17 **P = 0.0219**
	Thiamethoxam	5/18 (28%) 5.6 – 9.3	0/55 (0%) N/A	1.94 ± 0.78	0	t = 2.49, df = 17 **P = 0.0233**
	Total	8/18 (44%) 5.6 – 21.1	0/55 (0%) N/A	6.09 ± 1.89	0	t = 3.24, df = 17 **P = 0.0048**
2015	Clothianidin	40/40 (100%) 2.2 – 91.9	23/50 (46%) 1.2 – 8.0	15.15 ± 2.67	1.65 ± 0.33	t = 5.02 , df = 40 **P < 0.0001**
	Thiamethoxam	36/40 (90%) 1.4 – 46.5	24/50 (48%) 1.1 – 14.4	6.07 ± 1.39	1.73 ± 0.42	t = 3.41 , df = 46 **P = 0.0013**
	Total	40/40 (100%) 3.6 – 138.4	31/50 (62%) 1.2 – 17.4	21.86± 3.78	3.39 ± 0.60	t = 4.82, df = 41 **P < 0.0001**

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Table 2. Interactive effect of site & pollen insecticide concentrations on mortality
Summary of Pearson's correlation test between same-day data of worker mortality and neonicotinoid (clothianidin and thiamethoxam) concentrations in bee-collected pollen for the apiaries monitored in 2015. Sites are presented in the order of corn area (in %) within a 2 km radius centering the apiary.

Site	% corn	r	P	Time series intervention model
DS	1	0.5358	0.1371	Mortality = 0.05+0.1396* thiamethoxam_lag1, R2=0.78
SD	8	0.4702	0.2015	Mortality = 0.27+0.0322* thiamethoxam+ (0.0147-0.0147*ISC3-0.0118*ISC4-0.0122*ISC17-0.0133*ISC48)*clothianidin+ 0.36*I128, R2=0.52
MB	19	0.435	0.2419	Mortality = 0.03+0.0388*clothianidin+ 0.0259* clothianidin_lag1+0.25*I131, R2=0.60
BR	22	0.313	0.4121	Mortality = 0.34+0.0287*clothianidin*IBG59+ 0.58*I122-0.23i>145, R2=0.67
IB	22	0.4883	0.1823	Mortality = 0.03+0.0632*clothianidin+ 0.29*I>142, R2=0.48
WB	30	0.3764	0.318	Mortality = 0.22+0.0225*clothianidin_lag3+ 0.0131* thiamethoxam+0.23*IWB13-0.26*I120, R2=0.42
HR	30	0.937	0.0002	Mortality = 0.24+0.0526*thiamethoxam+ 0.19*IHR5-0.16*IHR6-0.14*HR20, p=0.21,R2=0.85
TV	31	0.8698	0.0023	Mortality = 0.05+0.0038*clothianidin+ 0.0089*clothianidin_lag1+ 0.0350*thiamethoxam+0.16*ITV62, R2=0.96
MO	39	0.9197	0.0004	Mortality = 0.30+(0.0254+0.0122*IMM39)* clothianidin+0.0123*clothianidin_lag1+ 0.16*I131-0.17*IMM16-0.35*IMM39, R2=0.76
FSR	49	0.8179	0.0071	Mortality = 0.03+0.0076*clothianidin+ 0.0114* thiamethoxam_lag1+0.28*I143, R2=0.87

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Commented [LEH10]: Report r to 2 decimal places, p-value to 3 decimal places. Best correlation with nitenpyram and mortality lag 1 (0.67), thiamethoxam and mortality lag 1 (0.59).

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Commented [LEH12]: Best correlation with thiamethoxam and mortality (0.41), clothianidin and mortality lag 2 (0.32).

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Commented [LEH13]: Best correlation with clothianidin and mortality lag 1 (0.51).

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Commented [LEH15]: Best correlation with thiacloprid and mortality lag 3 (0.42), nitenpyram and mortality lag 3 (0.44), clothianidin and mortality (0.34).

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Figure 1. Corn seed & planter dust images

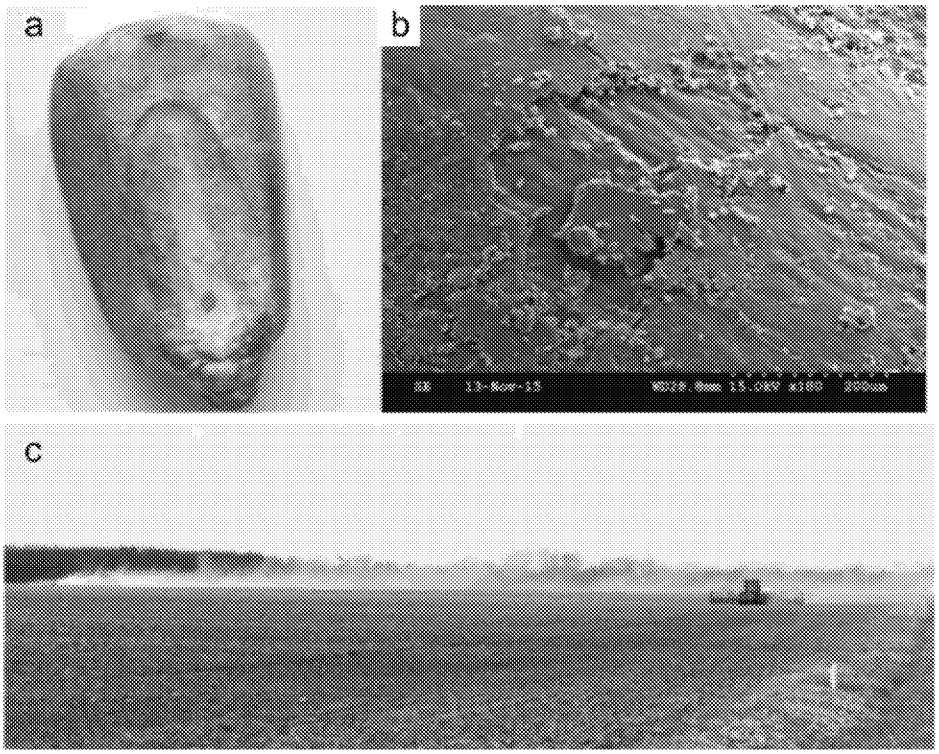
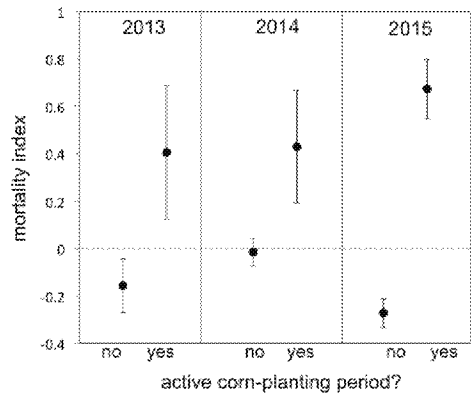


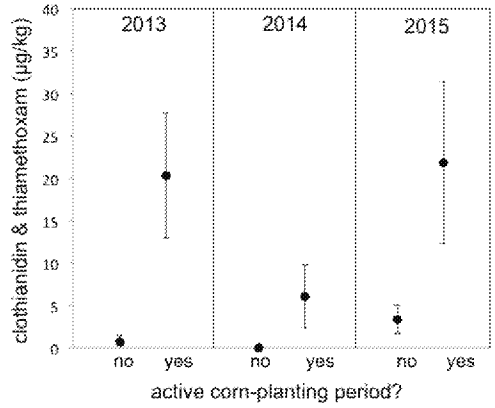
Figure 1. Seed treatments are applied to seeds as flowable solids that dry to form a coating. In corn, this coating results in visibly patchy coverage of the seed (a). The seed treatment forms particles of varying size on the surface of the seed as captured using scanning electron microscopy (SEM) (b). The striated surface visible in the center of the micrograph is the seed surface. Particles of the seed treatment coating are then emitted as planter dust during the sowing process (c). Macrophotography was performed by M. Spring, and SEM preparation by K. Kaszas.

Figure 2. Associations between honey bee worker mortality, clothianidin and thiamethoxam residues in bee-collected pollen, and corn-planting. (a) Honey bee worker mortality (per hive) during planting and non-planting periods. Dashed line represents the hypothetical value as if mortality is consistent across sampling periods. (b) Concentrations of clothianidin and thiamethoxam detected in pollen samples (per site) collected during planting and non-planting periods. (c) Honey bee mortality (site average)

(a)



(b)



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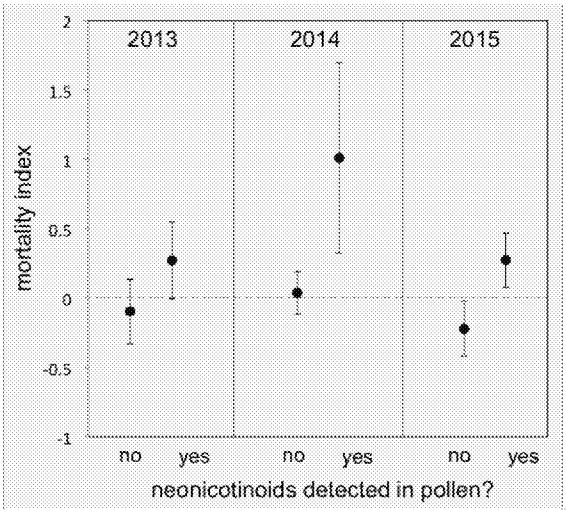

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Figure 3. Summed clothianidin and thiamethoxam concentrations in bee bread and trapped corbicular pollen before, during, and after corn planting.

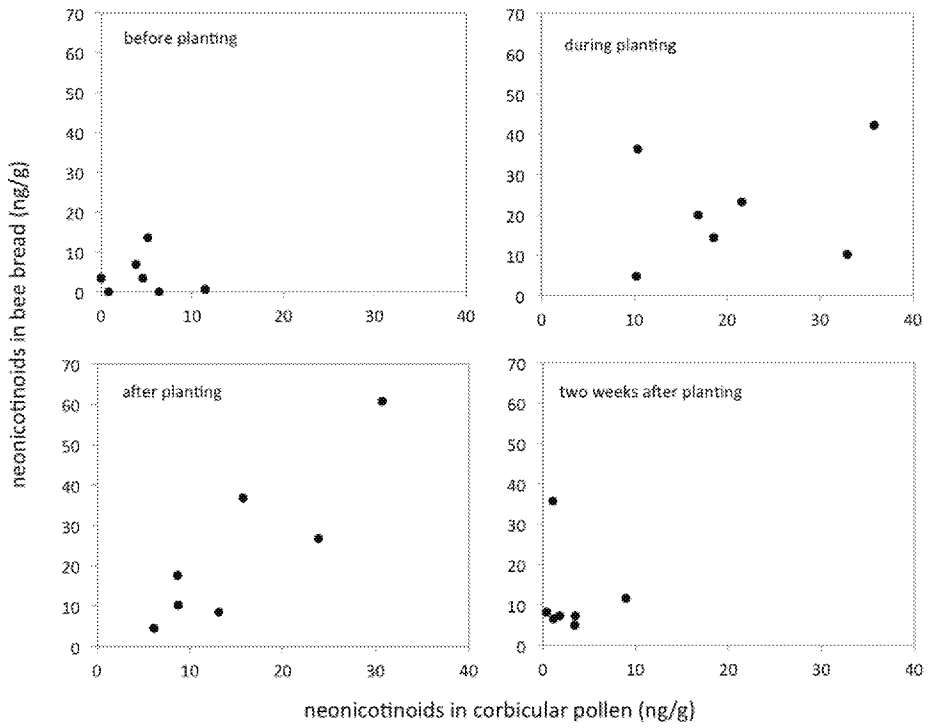


Figure 4. Number of deadworkers and clothianidin and thiamethoxam concentration (ng/g) in enclosed colonies.

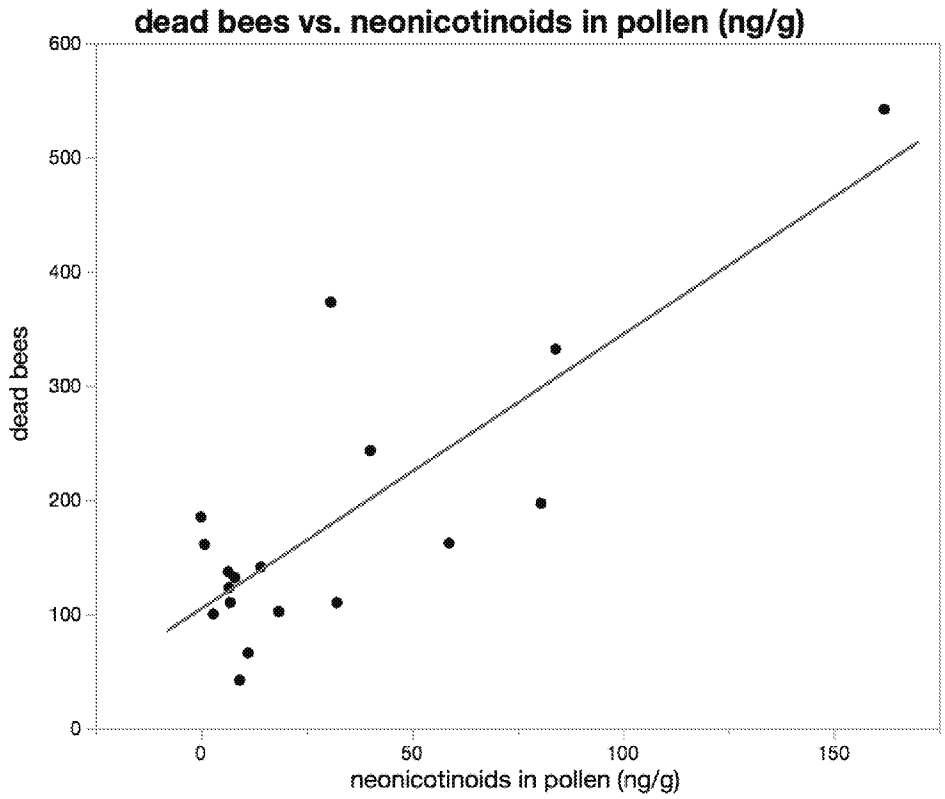
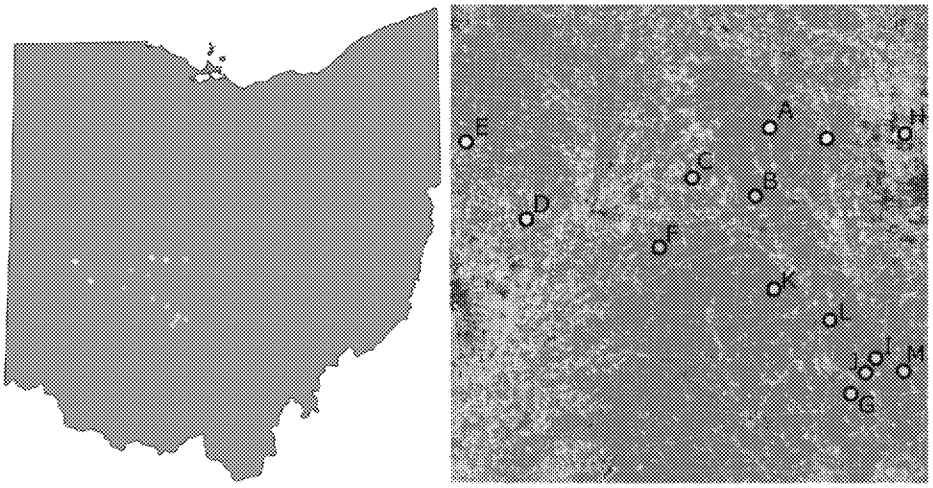


Figure 5: Study apiaries distributed throughout central Ohio. Map of apiaries used in 2014 (yellow), 2015 (blue) and both years (green). Central Ohio landscapes are characterized by field crops (brown) with urban areas (pink and red), forest (green) and pastures (yellow) in the National Landcover Database map (Homer et al. 2015).

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