

Field Trial for Evaluating the Effects on Honeybees of Corn Sown Using Cruiser[®] and Celest xl[®] Treated Seeds

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Abstract A first field study was conducted to investigate the possible adverse effects that seeds dressed with neonicotinoid insecticides pose to honeybees during sowing. It was observed that in the exposure hives bee mortality increased on the day of sowing and that the number of foraging bees decreased the days after the sowing. The corn sowing posed a significant threat to honeybees, with thiamethoxam being the most probable toxic agent. A theoretical contact exposure was calculated for a bee when flying over the sown fields, revealing a dose of 9.2 ng bee⁻¹ close to the contact LD₅₀ of thiamethoxam.

Keywords Neonicotinoid insecticides · Honeybees' health · Pesticide exposure · Colony collapse disorder

Since the late 90s in the US and in Europe widespread events of a winter-spring honey bee disappearance were registered (vanEngelsdorp et al. 2007; Laszlo 2008), and attributed to a complex pathology of honeybee called Colony Collapse Disorder (CCD) (Hileman 2007). Many

causes were considered: pathogens (Ribiere et al. 2008; Higes et al. 2009), environmental stresses, including climate change, (Le Conte and Navajas 2008) and pesticides (Decourtye et al. 2004; Johnson et al. 2009).

Of the pesticides, great attention was placed on neonicotinoid insecticides, such as acetamiprid, imidacloprid, thiacloprid and thiamethoxam, as these all act on the insect nicotinic (acetylcholine) receptor (nAChR) (Buckingham et al. 1997). Iwasa et al. (2004) revealed that the nitro-substituted compounds were the most toxic (LD₅₀ values of 18 ng/bee for imidacloprid, 22 ng for clothianidin, 30 ng for thiamethoxam, 75 ng for dinotefuran and 138 ng for nitenpyram) in comparison to the cyano-substituted chemicals (LD₅₀ values of 7.1 and 14.6 µg/bee for acetamiprid and thiacloprid, respectively).

Neonicotinoid insecticides are used in agriculture for soil and foliar treatment and as a seed dressing in many crops, such as corn and sunflower. When used as a seed dressing, neonicotinoid insecticides can effect pollinating insects as powder dispersion during the sowing procedures (Greatti et al. 2003, 2006). These authors demonstrated that pesticide contaminated powders are being dispersed to air, during sowing operations when pesticide-dressed seed was used. The pesticide containing powders can subsequently deposit on local soil and vegetation, posing an exposure risk to foraging honey bees and other pollinating insects. As to date there have been very few field investigations conducted on field scale to establishing the science behind CCD (Iwasa et al. 2004).

In order to investigate this issue, this research focuses on the possible adverse effects on honeybees of a corn sown using commercial seeds dressed with Cruiser[®] and Celest xl[®] (two common commercial products containing the neonicotinoid insecticide thiametoxam and the fungicides fludioxonil and metalaxyl-M as active ingredients, respectively).

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Materials and Methods

The study was conducted in an agricultural farm situated in the agricultural plain south-east of Milan, Italy. In this farm a second sowing of corn was performed on a single area of 7 hectares at 11 am on 24th of June 2008. Corn seeds, dressed with Cruiser® and Celest xl®, were sown with a Kinze 3,000 seed drill (pneumatic type). Cruiser® contains the insecticide thiamethoxam and Celest xl® contains the fungicides fludioxonil and metalaxyl-M as active ingredients. Pesticide active ingredient concentrations in the two commercial products were 350 g L⁻¹ of thiamethoxam in Cruiser® and 25 and 10 g L⁻¹ of fludioxonil and metalaxyl-M, respectively in Celest xl® (www.syngenta.it). Corn was sown using approximately 70,000 seeds hectare⁻¹, corresponding to 21 kg of seeds (mean seed weight is 0.3 g). Seeds were dressed at the recommended dose of 100 mL of each product for every 100 kg of seeds (www.syngenta.it), equating to a total mass of active ingredient per hectare of 7.35, 0.525 and 0.21 g for thiamethoxam, fludioxonil and metalaxyl-M, respectively.

Control and exposure hives were deployed within the experimental area at two sites. The control hives were sited inside the farm garden approximately 200 m away from the treated fields, and the exposure hives located at the field hedge boundary at the test field. Two days prior to the planned sowing, experimental hives were selected ensuring the presence of a similar number of adult bees and a comparable colony consistency between hives, to ensure acquisition of representative data throughout the exposure period. Homogeneous colony consistency was assessed using the Liebefeld method. This resulted in the selection of six experimental hives comprising two exposure hives and four control hives.

Direct mortality in the hive area and foraging activity intensity were chosen as the key health parameters for assessing the possible effects of the corn sowing with pesticide dressed seeds on honeybees. These two simple bee health parameters (the number of dead bees within the hive and the foraging activity) can be considered as an integrated diagnostic tool of the bee health, because the first one depends mainly to the direct lethal effects of pesticides inside the hive or in front of it, while the second one will serve as an indicator of sub-lethal effects far from the hive too, such as the failure of the foraging activity or loss of orientation.

Direct mortality in the hive area was monitored using of traps called ‘underbaskets’, which were located on the ground outside the entrance to each hive in order to collect dead bees that were eliminated from the inside or dead just in front of the hive. Each underbasket was 50 × 100 × 10 cm in dimension and comprised of a wooded tray sealed

at the base by an iron net able to retain falling dead bees and on the top by a second net of a mesh size that allowed dead bees to pass through, but prevented removal of bees via predation. Bee mortality measurements were conducted over a 6 day period and commenced 2 days prior to seed sowing (22nd of June), finishing on 28th June. Here the number of dead bees in each underbasket was counted and removed at 4:00 p.m. each day in order to give a measure of the number of bees that had died in the hive or in front of it on the previous day.

The foraging activity of honeybees was monitored each day at 11:00 a.m. inline with the bee mortality study period, however, for this investigation foraging was assessed on an additional 2 days on the 3rd and 9th of July. Only foraging bees entering the hive with pollen were counted during the 1 min sampling period. For each hive, this process was repeated three times, enabling a mean daily foraging value to be calculated. Three minutes for each hive is a very short period, but our measurements and previous evidence found this time efficient for having sufficiently stable and comparable data. Before the corn sowing, statistical analyses do not find significant differences among hives. The duration of the monitoring of the two bee health parameter was limited by the whether conditions. In fact, both parameters can be affected by them and can be considered comparable only under stable and comparable whether conditions. The measurements began when the whether stabilise and were conducted for a period in which it was stable, sunny and without evident wind interference. The statistical analyses do not find significant differences of both parameters among hives and between days before the corn sowing.

Statistical analyses comprised: Kolmogorov–Smirnov non-parametric test used in order to verify the normal distribution of the data, Student *t*-test used for single comparisons, ANOVA used for one-factor-multi-comparisons, and General Linear Model unvaried analysis (GLM) with post-hoc Bonferroni and Duncan tests used to assess the interaction between time with respect to the sowing and the hive distance from the treated fields. Statistical analyses were performed using SPSS ver. 17.0 software.

Results and Discussion

Figure 1a presents the number of dead bees and the foraging activity, during the corn sowing period, for both the exposure and control hives. Whilst it is likely that both sets of hives were potentially subject to pesticide exposure, the exposure hives located alongside the treated fields are more likely to have been directly impacted by the sowing activity than the control hives located away from the treated area. It can be observed that the two hive groups

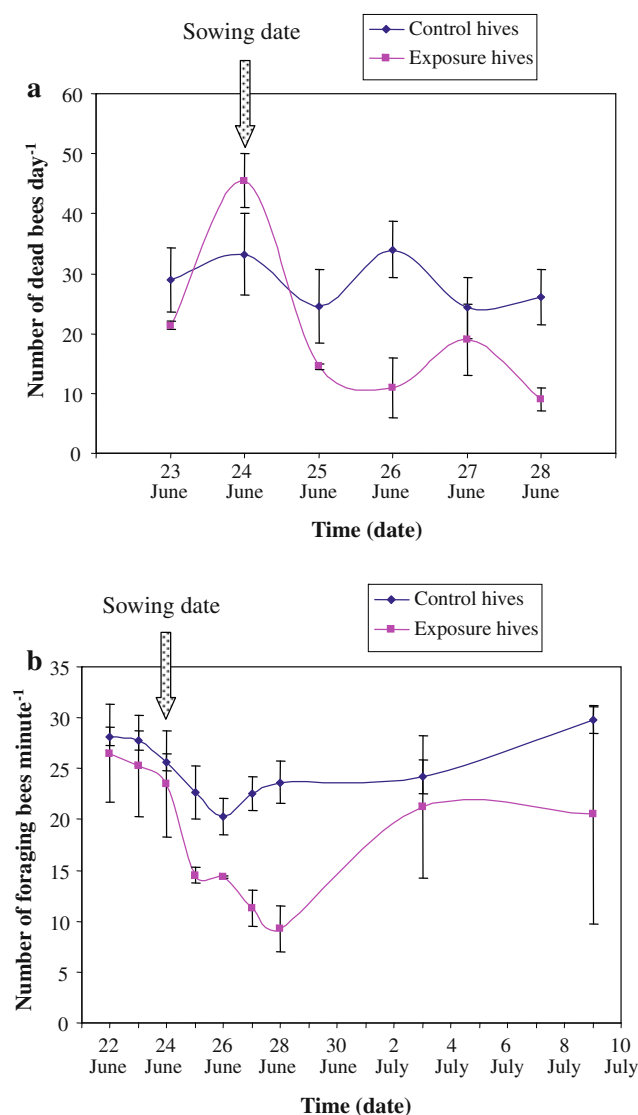


Fig. 1 Mean number of dead bees *per day* during the sowing period in the exposure and control hives (a), and mean number of foraging bees currying pollen and entering in the hive in 1 min during the sowing period in the exposure and control hives (b). Bars refer to the interval of the mean $\pm$ standard deviation

(Fig. 1a) had a similar number of dead bees before the sowing ($t = 0.951$; $d.f. = 4$; $p = 0.395$). However, on the day of sowing, bee mortality in the control hive did not differ significantly to that of pre-treatments levels, whilst in the exposure hives bee mortality increased from 20 before pre-treatment to >40 on the day of sowing. Interestingly shortly after the sowing period, bee mortality in the exposure hives decreased to values of approximately 10. Mortality data for both sets of hives, both pre and post pesticide exposure were evaluated by GLM once normal distribution was confirmed (Z di Kolmogorov–Smirnov = 0.737; $n = 36$; $p = 0.649$). Both time and hive group were found to be statistically significant in

determining the number of dead bees (p values of 0.020 and 0.024, respectively). This datum indicates that the number of dead bees is largely dependent on the location of the hive (i.e. distance from fields sown with treated seed) and the time period since sowing. In exposure hives, the average number of dead bees was 45.5 on the 24th of June (the day of the sowing) compared to that of 21.3 and 14.5 for the day before and after, respectively. The number of dead bees observed on 24th at 4:00 p.m. relate to those which died in the previous 24 h, coinciding with the period of sowing with treated corn seed. (sowing activity started on 24th June at ~ 11 a.m. for a duration of 3 h).

The relationship between ‘time’ and the number of the dead bees was only found to be significant in the exposure hives (ANOVA: $F_{5,6} = 12.4$; $p = 0.004$). Bee mortality in the underbasket measures the number of dead bees within the hive and in front of it. In the case of toxic exposure, bee mortality in the underbaskets will either increase or decrease dependent on whether the exposure episode acts. If a toxic substance directly enters in contact to the hive, it is probable that the number of dead bees recorded in the underbasket will increase. Whereas if a toxic substance is acting far from the hive, the number of dead bees in the underbasket is likely to decrease as the number of foraging bees returning to the hive is reduced and therefore the number of bees that died in the hive at the end of their life cycle. In fact, the foraging activity is performed by the last age class of honeybees which is constituted by the oldest bees; after a certain time of this activity bees naturally die. Foraging activity is defined as the harvesting of pollen, nectar and water from an average radius of 5 km from the nest (Colin et al. 2004). Moreover, the foraging activity is a complex phenomenon comprising a sequence of coordinated actions such as moving, sense perception, orientation, information acquisition and memory, social regulation such as communication dances and food exchange. It is believed that all these activities could be affected by exposure to sub-lethal doses of pesticides, in addition to acute toxicity effects (Colin et al. 2004).

The data presented in Fig. 1a, suggests that in the exposure hives a significant toxic event occurred during the day of sowing inside the hive or in front of it, and additional toxic events occurred far from the hive the days after sowing. In fact, an increase in the number of dead bees in the underbasket was registered the day of sowing, and a decrease in the number of dead bees was registered after the sowing.

The effect eventually occurred far from the hive were better recorded by the second analysed parameter: the mean number of foraging bees entering the hives in the time of 1 min (Fig. 1b). This number was not found to be significantly different ($t = 0.704$; $d.f. = 3.28$; $p = 0.528$) between the two hive groups prior to sowing with treated

corn seed (relief of 22nd and 23rd of June). Also on the day of sowing this number was similar and in line with numbers observed in the previous 2 days. It must be remembered this parameter was measured every day at 11:00 a.m., which coincided with the commencement of sowing. Thus it is expected that on the day of sowing, bee foraging data would not be affected by pesticide exposure, with the full effect of being observed on the 25th. On the 25th of June the mean number of foraging bees decreased in both sets of hives, compared to that of previous days (Fig. 1b). The observed decrease was more evident in the exposure hives, where the mean number of foraging bees decreased from a mean value of 25 to just 9.3 the day after sowing. In the control hives, the mean number of foraging bees decreased from a mean value of 28 to 23 in the same days. Measurement of foraging activity 15 days after sowing revealed that in control hives values had recovered to levels similar to that of before sowing. On the contrary in exposure hives an opposite result was observed: one hive had recovered to 'normal' levels, while the other still presents altered conditions. These contradictory results gave the high variability ranges of the last two data in exposure hives (Fig. 1b). Foraging data for the exposure and control hives over the whole study period was tested by GLM after the check of the normal distribution of the data (Z di Kolmogorov–Smirnov = 0.847; $n = 54$; $p = 0.471$). The GLM test revealed that the two considered independent variables, time and hive group, were both statistically highly significant ($p < 0.001$) in determining the number of foraging bees entering the hives in 1 min. Control hives too were subject to adverse effects far from the hives (the number of foraging bees entering in the control hives significantly changed over time; ANOVA: $F_{8;27} = 3.6$; $p = 0.006$).

Results of this study clearly show that sowing operations with Cruiser®- and Celest xl®-dressed seeds affected exposure hives, while control hives located 200 m away from the test site and protected by a vegetation barrier were much less affected by the pesticide toxicity. After the sowing period in the exposure hives, an increase in the number of dead bees was registered. It is likely that this group was probably affected by the direct dispersion of fungicide/insecticide containing powders within the hive site. Therefore, for an acute toxic effect to be observed, the insecticide has to be in contact with the bees at a concentration close to or exceeding their LD_{50} value. In contrast foraging bees can be affected also far from the hives by either lethal or sub-lethal effects.

In order to assess the relative contribution of the different active ingredients, their toxicities toward honeybees were considered. The insecticide thiamethoxam has a contact LD_{50} of tens of $ng\ bee^{-1}$, in comparison to that of the two fungicides that exhibit LD_{50} values greater than

hundreds of $\mu g\ bee^{-1}$ (EU 2002, 2007). Contact LD_{50} values of 30 and of 24 $ng\ bee^{-1}$ were reported for thiamethoxam by Iwasa et al. (2004) and Senn et al. (1998), respectively. In addition the mass of insecticide used in the sowing process is 10 times higher than that of the two fungicides applied (total mass of chemical applied to sown area was 51.45, 3.675 and 1.47 g for thiamethoxam, fludioxonil and metalaxyl-M, respectively). Assuming similar dispersion of the different products to the air during sowing, the atmospheric concentration of the insecticide would be much greater than that of the two fungicides. In addition to being present at a higher atmospheric concentration, the insecticide thiamethoxam exhibits a greater toxicity to bees than the two fungicides, therefore its toxic potential during the sowing can be considered much greater than that of the two fungicides. The two fungicides when in a mixture with a neonicotinoid insecticide might give synergistic effects as suggested by Iwasa et al. (2004). Synergistic effects between neonicotinoid insecticides and fungicides were observed with P450 inhibitor fungicides, particularly with the cyano-substituted neonicotinoids (acetamiprid and thiacloprid) as these compounds are degraded by the P450 metabolic system resulting in no active metabolites (Iwasa et al. 2004). The synergic effect of several fungicides was demonstrated to increase the toxic effects of cyano-substituted neonicotinoids from levels of tens of $\mu g\ bee^{-1}$ to that of tens of $ng\ bee^{-1}$, resulting in effects typical of nitro-substituted neonicotinoids. On the other hand, the nitro-substituted neonicotinoids, such as imidacloprid and thiamethoxam, showed a minimal synergistic effect of P450 inhibitors (Iwasa et al. 2004). Therefore, the possibility of synergistic effects of fungicides on thiamethoxam toxicity can be considered improbable on the up to now knowledge. Given the above considerations, it is highly probable that the observed adverse effect on the bees of the exposure hives is likely to be attributed to the dispersion of the active ingredient thiamethoxam alone.

In order to assess the possibility that thiamethoxam resulted in a toxic action during the corn sowing, it is essential to evaluate the dispersed mass of chemical and calculate a theoretical dose to honey bees. Due to absence of field data relating to thiamethoxam dispersion during corn sowing, data from a study by Greatti et al. (2003, 2006) on imidacloprid was selected as a surrogate. In this study the dispersion of Gaucho®, a commercial seed dressing containing the active ingredient imidacloprid, was investigated. Chemical residues were measured by putting filter papers 2 cm in front of the fan hole of the pneumatic seed drill, revealing imidacloprid concentrations of between 70 and 120 μg per minute depending on the dressing products. Furthermore the concentration of the active ingredient in the vegetation in field borders, adjacent to the test site, were found to range from 20 to 120 $ng\ g^{-1}$.

Even if it is difficult to derive the percentage of insecticide dispersed during the sowing process from the data set of Greatti et al. (2006), it can be hypothesised that a low percentage of the total mass of chemical present in the dressed seeds was dispersed to the environment.

In our case, considering by hypothesis 1% of thiamethoxam dispersion, this would equate to a 514.5 mg of the active ingredient dispersing into air. Generally, ‘drift’ phenomena during agricultural treatment determines the deposition of pesticides within a small distance from the field edge (Ganzelmeier et al. 1995). Given that atmospheric conditions at the time of the study were stable, it can be inferred that pesticide residues dispersing to air, from the seed drill, would mix up to a height of 5 m with the air package residing nearby the sown fields. However, it is likely that concentrations of insecticide in the air will be greater the closer the proximity to the seed drill (visible cloud of dust) and much lower further away from the point source due to both chemical dispersion and deposition processes. It can be hypothesised that the volume of air for consideration should correspond to the surface of the sowing area and a uniform height of 5 m, corresponding to 350,000 m³ of air. Given uniform dispersion of the studied insecticide in this air package, a concentration of 1.47 µg m⁻³ is obtained. Using this theoretical air concentration an insecticide exposure level for bees flying to and from their foraging areas can be calculated. If a bee crossed the test site during sowing operations, an exposure can be calculated by using the estimated contact air volume and the concentration of the insecticide in the air. The volume of air that a bee would come into contact with during foraging activity can be calculated by multiplying the flight distance over the treated fields (even 500 m of trip) by the front surface area of the bee (12.5 mm² derived from the approximation of the bee body to a cylinder with the dimensions of 12–13 mm of length and 4 mm of height). This equates to an air exposure volume of 0.00625 m³. Considering that a bee’s anatomy is characterised by thick hairs on the body, that will serve to efficiently trap airborne particulates, it is not unrealistic to calculate chemical exposure to the bee by multiplying the air exposure volume by the insecticide air concentration, resulting in a dose of 9.2 ng bee⁻¹.

This theoretical exposure model serves to indicate that the calculated exposure is lower than the lethal dose (contact LD₅₀ of 24–30 ng bee⁻¹), but close enough to it to induce sub-lethal effects. In a study by Aliouane et al. (2009), behavioural effects of honeybees were observed after exposure to thiamethoxam for 11 days, equating to a dose rate of 1 ng day⁻¹ bee⁻¹. The investigation was able to establish an oral dose of 0.1 ng day⁻¹ bee⁻¹ as no-observed-effect-concentration (NOEC). However, in a study by el Hassani et al. (2008) it was reported that a

single sub-lethal dose of 1 ng bee⁻¹ had no effect on gustatory, motor and mnemonic functions of honey bees. These conflicting data sets therefore confirm the necessity for novel research into the sub-lethal effects of neonicotinoid insecticides toward bees.

The data set obtained in this study can also be used to estimate pesticide deposition to both ground and vegetation. Assuming that all the dispersed thiamethoxam mass (514.5 mg) is deposited on the sowing area itself, a mean insecticide deposition of 7.35 µg m⁻² (0.73 ng cm⁻²) is obtained. If we consider that the pesticide may deposit on the wood hedge in front of the hive opening (average dimension of 50 × 7 cm), this would equate to a deposition mass of 257 ng of chemical, consistent with a lethal dose by contact for a certain number of bees. During the investigation direct mortality within the hive area was observed for the exposure hives only.

The observed effects of this study were too much limited to be conclusive for supporting the pesticide cause of CCD events, but they can be considered as a first field proof of the possible toxic effects that seeds dressed with neonicotinoid pose to bees during sowing. Furthermore, as this field trial was performed during June when corn sowing in this area was limited, honeybees are therefore likely to come into contact with higher concentrations of this pesticide during spring sowing. Therefore, the effects of neonicotinoid insecticides on honeybees and other general pollinator insects must be considered carefully in future agricultural policy (sowing modalities and dressing doses) in order to avoid more severe effects.

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