

ORIGINAL CONTRIBUTION

Aerial powdering of bees inside mobile cages and the extent of neonicotinoid cloud surrounding corn drillersV. Girolami¹, M. Marzaro¹, L. Vivan¹, L. Mazzon¹, C. Giorio², D. Marton² & A. Tapparo²¹ Dipartimento di Agronomia Animali Alimenti Risorse Naturali e Ambiente - Entomologia, Università di Padova, Legnaro, Padova, Italy² Dipartimento di Scienze Chimiche, Università di Padova, Padova, Italy**Keywords***Apis mellifera* L., colony losses, drift of dust evaluation, driller modification, seed dressing**Correspondence**Vincenzo Girolami (corresponding author),
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Abstract

Sudden losses of bees have been observed in spring during maize sowing. The death of bees has been correlated with the use of neonicotinoid-coated seed and the toxic particulates emitted by pneumatic drilling machines. The contamination of foragers in flight over the ploughed fields has been hypothesized. The airborne contamination has been proven, both with bees inside fixed cages around the field and in free flight near the driller. A new trial involving mobile cages has been established and consists of making rapid passes with single bees inside cages fixed to an aluminium bar. The bar was moved by two operators at different distances from the working drilling machine. A single pass was shown as sufficient to kill all the bees exposed to exhaust air on the emission side of the drill, when bees were subsequently held in high relative humidity. The extent of toxic cloud around driller was evaluated at the height of 0.5, 1.8 and 3.5 m and proved to be about 20 m in diameter, with an ellipsoidal shape. The shape may be influenced by working speed of the drill and environmental parameters, and is easily shown by adding talc powder to the seed in the machine hopper. A new driller equipment was evaluated consisting of two tubes inclined towards the soil that direct the exhaust air towards the ground. The survival rate of the bees was not substantially increased using the modified drill and was lower than 50%. Chemical analyses show up to 4000 ng of insecticide in single bees with an average content around 300 ng. Similar quantities were observed at increased distances from the modified or unmodified drillers. This new evaluation of bee mortality in the field is an innovative biological test to verify the hypothetical efficiency (or not) of driller modifications.

Introduction

In recent decades, in Europe and North America, the colonies of honey bees (*Apis mellifera* L.) were subject to catastrophic losses (Le Conte et al. 2010; Neumann and Carreck 2010) characterized by a common set of specific symptoms such as the rapid loss of worker bees without a related quantity of dead worker bees being found, both within and

surrounding the hives, but with excess brood in relation to adult bee populations. This syndrome was called colony collapse disorder, or CCD (vanEngelsdorp et al. 2009), and it is linked to interactions between different causes such as parasites, in particular *Varroa destructor* Anderson et Trueman (that also induce viral infections), and environmental factors including agricultural insecticides (Maxim and van der Sluijs 2010).

The death of bees in the maize-growing area of Northern Italy has been linked in the first place to attacks by *Varroa destructor* particularly where the autumn and winter deaths were concerned. However, serious losses have been observed at the same time as the spring sowing of maize seed in a distinctly different time frame to those caused by varroa; losses that are not strictly attributable to CCD in as much as large accumulations of dead bees were often found in front of the hives.

The cause of the rapid death of thousands of bees during the sowing of the maize seed coated with neonicotinoids has been associated with foragers coming into contact with particles emitted from pneumatic drilling machines. The contamination was thought to have come from fragments falling on the vegetation at the edges of the fields (Greatti et al. 2003, 2006), but chemical analysis showed the presence of rather low (p.p.b.) concentrations of insecticides (Greatti et al. 2003). This hypothesis was formulated following the heightened deaths that were observed in the spring of 2000 in north-east Italy and, although contested as a possible cause of the deaths (Schnier et al. 2003), has been widely accepted up to now (Pistorius et al. 2009; Krupke et al. 2012).

In the context of the general uncertainty of the effect of neonicotinoids on bees, even today (Cresswell 2011), we were unconvinced of the contamination caused by falling fragment, and a new hypothesis was formulated that unknown sources of lethal poisoning could be connected with the sowing of the maize. One of the first theories formulated was that toxic guttation drops produced by the seedlings of maize could be responsible (Girolami et al. 2009); however, the infrequent visits of foragers to such exudations did not lead us to consider guttation as the cause of such frequent and extensive deaths. It was thus thought that bees could come into contact with the particulates, not after they had fallen on the vegetation, but directly, in flight. The experimental method by which this powdering came to light was initially to expose bees contained in fixed, single and small cages connected to poles to the dust emitted by the maize seed drill (Marzaro et al. 2011). Subsequently, bees were conditioned to fly over fields destined for maize by using a dispenser of sugary solution to attract them (Girolami et al. 2012).

Both of these methods had their limitations, inasmuch as the first obliged us to keep the bees exposed to the drill emissions for long periods. The second quite faithfully reproduced field conditions in

which foraging bees made repeated flights over fields, where maize was being sown to visit spring flowering (dandelion, rape and orchards); nevertheless, it did not furnish answers to the questions as the distance from the drill sufficient to kill bees, or how many flights were necessary before death ensued.

Thus, the theory that bees could come into contact with the powder emitted from the drill when contained in cages was tested. Finally, a new method was applied to test the hypothetical usefulness of modifications to the drilling machine. Currently, the efficiency of the modifications to this machine, whereby the dust emitted is reduced and directed to the ground, have been evaluated (Nikolakis et al. 2009; Pistorius et al. 2009; Biocca et al. 2011; Donnarumma et al. 2011) even though the results seem difficult to compare (Forster 2009). What is missing, however, is experimental verification of bee mortality in relation to the modified emission of dust. In the present study, we aim to establish experimentally whether modifications to drilling equipment reduce mortality in honey bees flying in proximity to the drilling process.

Material and Methods

Experimental sites

Field trials took place at the experimental farm of the Agricultural Faculty (University of Padova) located in Legnaro. The plot was 50 m wide by 70 m long (coordinates: 45°20'41. 19"N-11°57'16.22"E). The meteorological data reported were collected and processed by ARPAV (Regional Agency for Environmental Protection of Veneto). The data come from the meteorological station located in Legnaro and placed at about 200 m from the plot. The wind speed measurement reported was recorded at a height of 10 m.

Insect origin and holding

The Padova Beekeeping Association (A.P.A. Pad) supplied 12 hives. For the trials (with caged bees), the insects were caught with a net in front of a single colony. The bees were kept in tulle mesh cages 20 × 20 × 20 cm, fed at honey drops on the top of cage and, where possible, freed in the evening and replaced each day. Later, at the time of the tests, caged bees were captured (from the 20-cm cage) in a test tube and placed in smaller cages with a cubic steel skeleton of 5 cm and all the six sides entirely in

tulle (with mesh of 1.1 mm) and again fed with drops of honey placed on the top, as reported in the study by Marzaro et al. (2011).

Seed employed

Two batches of seed were used for the trials: one produced in 2009 and the second in 2010, called '2009 or 2010 coating', respectively. The coatings (hybrid employed in 2009 was X1180D 964890, and PR32G44 in 2010; both from Pioneer Hi-bred Italy) were: Celest XL[®], containing only fungicides (Fludioxonil 2.4% and Metalaxyl-M 0.93%; Syngenta, Basel, Switzerland), Poncho[®] (clothianidin 1.25 mg/seed; Bayer Cropscience AG., Leverkusen, Germany) (Altmann 2003; Andersch and Schwarz 2003), Gaucho 350FS[®] (imidacloprid, 0.5 mg/seed; Bayer Cropscience AG.) (Elbert et al. 1990) and Cruiser[®] 350FS (thiamethoxam 1 mg/seed; Syngenta International AG) (Maienfisch et al. 2001; Robinson 2001). The seed was supplied by A.I.S. (Italian Seed Association, Bologna, Italy) courtesy of MiPAAF (Ministry of Agriculture, Food and Forestry), a departure from the suspension of the use of neonicotinoids for maize seed coating in Italy for the research project Apenet. The 2009 and 2010 seed batches have a quantity of dust abrasion under the limit of 3 g per 100 kg seeds. The quantity was tested with the Heubach test, considered the method that best allows standardization of dust abrasion measurements within the seed industry (Apenet, 2009, 2010, 2011; Nikolakis et al. 2009).

Drilling machines and sowing

A Monosem NG Plus (Monosem, Largeasse-France) drilling machine was used for all the sowing operations. Normally 73 000–74 000 seeds per hectare were sown (75 cm between rows, 18 cm between seeds in the row). The drill moves at 4–6 km/h with a seeding width of 3 m and requires 30 min to sow 1 ha. The air exhaust pipe is situated on the right hand side of the machine and expels air (and dust) at approximately 65 l/s (under real sowing conditions), at a height of 1.8 m and an angle of 45° upwards.

A modified vacuum pneumatic drilling machine was also used where the airstream, generated by the fan (as above described) to maintain the suction pressure, which in the unmodified driller was ejected from one single outlet, was divided into two tubes (dual pipe) of 10 cm diameter and the air released close to the surface of the ground (about 20 cm). The modification adopted is similar to that

reported for a Monosem drilling machine in a Syngenta document (Syngenta International AG 2010).

Sowing was carried out in two modes: mobile or static. The mobile mode is the standard field method, while the static mode envisages the use of two tractors. The first is usually used to raise the drill above the ground and provides the power to move the air fan; the second tractor moves the drilling machine at the required speed, which in turn distributes the seed; in this mode, the drilling machine, while still static, functions in a similar way to the usual methods, and emerging seeds are collected in four bowls under the machine.

Direct dusting in mobile cages and influence of relative humidity

The influence of a brief dusting to simulate that of bees flying near a drilling machine in action was evaluated by means of an aluminium bar 4 m long, to which cages, each containing a single bee, were attached at every 0.4 m (10 in total). The cages were numbered taking account of the progressive distances from the drill. The bar was supported at each end by a vertical pole of 2.5 m. The bar was passed by two people at a fast walking pace (6–8 km/h) by the side of the drilling machine.

Once the bees had been exposed to the insecticide dust in the field, they were transferred (inside the same cage) to a room at a controlled temperature ($22 \pm 1.5^\circ\text{C}$).

In trial 1 (table 1), with the drill in static mode, passes were made on the right side where the dust was expelled, with the proximal side of the bar at a minimum distance of 2, 4, 6 and at a 1.8 m height from the ground. The bar was held perpendicular to the longitudinal axis of the tractor. Two passes were made with a total of 20 bees for each of the three distances. To evaluate the influence of relative humidity in this trial, half of the cages were kept at the relative humidity of the laboratory lower than 70% (with the use of dehumidifier if needed). The other half of the cages were kept at a relative humidity close to saturation (>95%), hereafter designated as high humidity. To obtain conditions of high humidity, caged bees were held in plastic boxes with plexiglass on the top and a moistened paper on the bottom (according to Marzaro et al. 2011). All the bees were fed with drops of honey, periodically renewed, on the top of the cage. The even-numbered cages were placed in conditions of high humidity, and the odd-numbered cages in laboratory humidity.

Table 1 Details of field trials carried out to evaluate the toxicity on caged bees

Number of trial – date	Starting time – sowing method ¹ - driller equipment ²		Active ingredient ³ and coating year ⁴		Meteorological conditions				No. bees tested	Humidity conditions ⁵ after exposure
					t (°C)	RH (%)	Wind			
							Direction	Speed (m/s)		
1 – 16/7/09	11.00-s ¹	Un ²	C ³	09 ⁴	27	75	ESE	3.4	60	L-H ⁵
2 – 5/8/09	15.00-s	Un	C	09	30	63	SSE	2.8	160	H
3 – 24/8/09	15.00-s	Un	C	09	28	65	SSE	2.3	160	H
4 – 26/8/09	15.00-s	Un	C	09	27	72	SSE	2.2	190	H
5 – 03/5/11	10.00-m	Un-M	F + M	10	20	57	ENE	1.1	80	H
6 – 04/5/11	10.00-m	Un-M	I	10	17	51	ENE	5.9	80	H
7 – 11/5/11	10.00-m	Un-M	I	10	24	38	ONO	2.3	80	–
8 – 20/5/11	10.00-m	Un-M	C	10	24	49	S	1.4	80	H
9 – 19/6/11	10.30-m	M	I	10	21	68	ENE	4.9	40	H
10 – 29/6/11	10.00-m	Un-M	T	10	28	47	S	2.8	80	H

¹s = static mode; m = mobile mode.

²Un, unmodified drilling machine; M, modified drilling machine.

³C, clothianidin; I, imidacloprid; T, thiamethoxam; F + M, fludioxonil + metalaxyl-M.

⁴09 = 2009 seed batch; 10 = 2010 seed batch.

⁵L = laboratory humidity condition; H = high humidity condition.

The mortality was noted every 3 h, and the data reported refer to a 24-h period. A bee was considered dead if both the arching of the abdomen and wing block were present (Girolami et al. 2009).

Extent of the toxic cloud

To make the cloud emitted by the drilling machine visible, 200 g of talc powder was added to one of the seed-containing hoppers during the sowing. The extent of toxic cloud, containing solid fragments of the seed shell surrounding the drilling machine, while in a static mode and set in a south–north direction, was evaluated using the movable bar from trials 2, 3 and 4 (table 1). The moveable bar was passed perpendicular to the longitudinal axis of the tractor, on the left and right side of the machine, and parallel to the same axis at the front and back of the machine (all four sides of the drill). These passes were made at 4-m intervals up to 16 m (four passes each with 10 cages, each with a single bee) at three different heights: 1.8 m in trial no. 2, 0.5 m in trial 3 and 3.5 m in trial 4. At each height, four samples of 40 bees were tested, behind, in front of, and at both sides of the tractor, giving a total of 480 bees for all the three trials. A further 10 bees were exposed to the emissions over the tractor. After the trial, the caged bees were taken to the laboratory, and all placed in high humidity. To evaluate the duration of the toxic cloud (in trial. 4), 4 and 8 min

after the drill had been turned off, the bar, with 10 cages attached, was moved at 1.8 m high, along the right hand side of the drill at a distance of between 2 and 6 m.

Driller modifications and bee poisoning

In trials 5, 6, 8, 9 and 10, with the drill in mobile mode, the bees were exposed (for about 30 s) to the emission of the driller (unmodified or modified with dual-pipe deflector) with the aluminium bar perpendicular to the longitudinal axis of the tractor. The people with the bar followed and passed the tractor on the right hand side (in the first 30 m of the plot). The tractor then reduced speed and waited while the people with the bar made a U-turn and again passed the machine, once more at working speed, on the left hand side. In this way, the bees were twice exposed to the cloud in a similar way to foragers in free flight making a round trip over sowing area. A first pass was made between 1 and 5 m from the side of the tractor, and a second pass, with another 10 bees, was made between 5 and 9 m from the tractor. The cages were numbered taking account of the progressive distances from the drill. After the exposure, all the bees were fed with drops of honey on the tops of the cages, which was periodically renewed, and all were placed in conditions of high humidity in the laboratory. Three neonicotinoids and a single fungicide used to coat maize seed were tested.

Content of insecticide in bees

For chemical analysis (in trial 7), after exposure, the caged bees were immediately placed in a refrigerator at 2–4°C for 15 min until complete immobility ensued. Later, they were placed in a vial in a freezer at –80°C. To evaluate separately the powder intake on the left and right hand side of the unmodified and modified drill, the bees were exposed in a similar way to the trials above described, but after the U-turn, passes were made either on the right hand side or on the left hand side. The bees collected in trial no.7 (table 1) were individually analysed to determine the content of neonicotinoids. The analytical procedure, based on lyophilisation, solvent extraction and instrumental analysis by ultra high performance liquid chromatography (UHPLC-DAD) is described in Tapparo et al. 2012.

Of the 20 bees analysed, the distances from the drilling machine and the side of the exposure have been taken into account.

Statistical analysis

To compare the mortality in different samples of bees, we tested the null hypothesis that the frequency of mortality occurred independently of considered parameters using a chi-squared goodness-of-fit test.

Results

Direct dusting in mobile cages and influence of high humidity

The bees exposed in single cages with rapid passes near the drilling machines, using the new exposure method, were lethally poisoned (by clothianidin) if they were subsequently held in the laboratory in high humidity (table 2).

Some mortality was also observed, in bees exposed to the most intense dusting at 2 m from the drilling machine, even though kept in laboratory humidity (table 2). Lethal effects were observed both in round-trip test (with two passes) and in a single pass in the trial carried out to evaluate the extent of the toxic cloud (reported in fig. 1).

Extent of the toxic cloud

A rapid visualization of the cloud emitted by the driller was obtained putting some talc into the seed hoppers during sowing process (reported in fig. 2).

Trials 2, 3 and 4 attempted to quantify the extent of the dust cloud of particulates, emitted by the drilling machine (in static mode), with concentrations sufficient to kill bees in a single rapid pass in a mobile cage and afterwards held in high humidity conditions. Fig. 1 shows results that are obviously relative to the model of the drilling machine used, in which the air is expelled on the right side at a height of 1.8 m. The bees that passed on the right side of the machine up to a distance of 6 m all died, and a very high mortality was reported up to 12 m, at all the heights tested. Mortality was encountered on the left hand side up to 8 m distance, and mostly up to a height of 2 m (fig. 1 top). Including the deaths of those bees flying above the machine, the toxic cloud extends up to 20, 10 m on either side of the drilling machine. In the direction of travel (fig. 1 below), at a height of 1.8 m, the (total) lethal zone extended beyond 12 m. The toxic cloud, surrounding the drilling machine, showed a flattened, ellipsoidal body of some 2–3 m high and 20 m wide. The cloud is slightly shifted to the right hand side where the air is released and to the rear of the tractor where the drill is placed. The predominating wind was blowing in a SSE direction with a wind speed averaging <10 km/h (table 1).

At the end of trial 4, the dust cloud remained for almost 4 min after the machine was switched off as

Table 2 Numbers of dead and surviving bees (in groups of 10), exposed individually in mobile cages to the emissions of the drilling machine, moved with rapid passes at the right side of the drill, at progressive distances, taken to the laboratory and placed in varying conditions of RH

No. – date of trial	Insecticide	Exposure distance from the drill	Mortality at different distances from drilling machine				Probability*
			Lab humidity		High humidity		
			Dead	Survived	Dead	Survived	
1 – 16/7/09	Clothianidin	2 m	3	7	10	0	0.001
		4 m	0	10	10	0	0.0001
		6 m	0	10	9	1	0.0003

(*) The probability is referred to statistical differences at χ^2 test within the same row (**P ≤ 0.01).

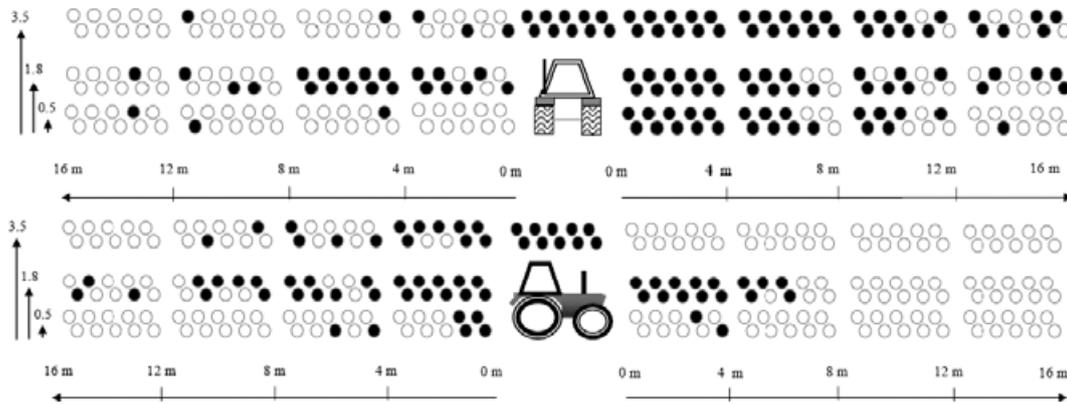


Fig. 1 The black circles represent dead bees within 24 h after a single rapid pass in mobile cages, at varying heights and distances from the drilling machine. The sowing was carried out with clothianidin-coated maize seed. The top diagram shows the dead bees at the side of the machine, and the bottom shows those in the direction of travel.



Fig. 2 Visual image, obtained by introducing talc into the hopper, of the cloud emitted by an unmodified drill (left) and a drill with dual-pipe equipment (right). The modification pushes the cloud closer to the ground but is still consistent.

all 10 bees died. No further toxicity was reported after 8 min.

Driller modifications and bee poisoning

Using the mobile cage in trials no. 5–10, it was possible to evaluate the influence of the modifications made to the machine in the poisoning of bees. When seed coated solely with fungicide was employed, no significant acute poisoning occurred when the bees were exposed to the emissions from the drills, for all distances and machines tested, even when they were subsequently held in high humidity conditions (trial 5, table 3). In all the remaining experiments (trials 6, 8, 9 and 10; table 3), a relatively elevated level of mortality occurred, above 50%, in bees passing both modified and unmodified drills at various distances. No significant differences in mortality resulted whether or not the drills were modified, with the exception of trial no. 10 where, at a distance of 5–9 m, employing a drill with a dual-pipe modification, a higher survival rate was observed. Amongst the different neonicotinoids tested, clothianidin appeared to be the most toxic,

inasmuch as it caused total mortality in the range of 1–5 m (trial 8) and at least 80% in the other trials employed. These trials were not carried out at the same time, and this requires that further experiments are carried out under identical environmental conditions. Using the same method, the extent of the toxic cloud was evaluated.

Insecticide content in bees

In the analysed bees (trial 7 – table 1), when the distances from the drill were taken into account, very large quantities of insecticide were found. For instance, considering the bees exposed to the emission of unmodified driller, the sample that was powdered on the left hand side at a distance of 1 m showed a content of 4786 ng of clothianidin per bee (table 4). The quantity of insecticide generally diminished in relation to the distance of exposure from the drill, not, however, in a linear manner. The minimum for the right side was 142 ng/bee at 4.5 m. As expected, the quantity of insecticide was less on the left hand side of the drill, but was still elevated and superior to the DL_{50} –18 ng of clothianidin (Iwasa

Table 3 Surviving and dead bees (groups of 20) exposed in single pass to the emission of unmodified or modified drilling machine with different insecticides at different distances

No. – date of trial	Insecticide	Exposure distance from the drill	Drilling machine equipment				Probability
			Unmodified (standard)		Modified (dual pipe)		
			Dead	Survived	Dead	Survived	
5 – 3/5/11	Fludioxonil + Metalaxyl-M	1–5 m	1	19	1	19	–
		5–9 m	2	18	0	20	–
6 – 4/5/11	Imidacloprid	1–5 m	16	4	13	7	0.298 ns
		5–9 m	11	9	16	4	0.095 ns
8 – 20/5/11	Clothianidin	1–5 m	20	0	17	3	0.071 ns
		5–9 m	17	3	16	4	0.688 ns
9 – 19/6/11	Imidacloprid	1–5 m	–	–	14	6	–
		5–9 m	–	–	8	12	–
10 – 29/6/11	Thiamethoxam	1–5 m	13	7	8	12	0.118 ns
		5–9 m	13	7	4	16	0.004**

(*) The asterisks indicate statistical differences at χ^2 test between modified and unmodified driller in the same row (**P < 0.01).

Table 4 Insecticide content in bees (nanograms/bee) powdered employing unmodified and modified drilling machine

Driller equipment	Insecticide	Sampling side	Insecticide content (ng/bee) at different distances from drilling machine					Mean
			1 m	2.25 m	4.5 m	6.75 m	9 m	
Unmodified	Imidacloprid	Right side	4786	457	142	523	199	1221
		Left side	<LOD*	410	110	98	33	162
Modified (dual pipe deflectors)	Imidacloprid	Right side	2372	424	134	1778	500	1042
		Left side	<LOD*	<LOD*	<LOD*	25	<LOD*	25

*LOD: lower than the limit of detection (10 ng/ape – Tapparo et al. 2012).

et al. 2004). The exception was the distance of 1 m, which showed no insecticide, probably because the flow of air was hampered by the cab of the tractor.

Considering the bees exposed to the emission of modified machine on the right hand side, the quantities of active ingredient were still high, with values of half those of the unmodified drill at 1 m, similar values for samples at intermediate distances and decidedly higher approaching 9 m. On the left hand side of the drill, only those bees exposed at 6 m showed the presence of insecticide. During this trial, a wind speed of 4 m/s (table 1) was blowing WSW carrying powder from the opposite side towards the tractor.

Discussion

Direct dusting in mobile cages

The test in which fixed cages were exposed to the dusting on the margin of the sowing area (Marzaro et al. 2011) could have been influenced by the movement of air caused by the sowing machine, or

by wind, more than and not by the flight of the bees. The adopted test method using mobile cages allowed an exposure to the dust emitted by the drill and simulated more realistically the conditions of a bee encountering a drill in flight. Another advantage is that the exposure of the bees can be evaluated with more precision in relation to free flight, given that both the flight path and length of exposure can be controlled. The mobile cage method also assists in the evaluation of successive influences of powdering in flight in the laboratory, given that the bees are already contained in cages.

The influence of high humidity in increased mortality of exposed bees has also been further confirmed with this new system of exposure and showed no substantial differences when compared with the results obtained with the bees exposed in fixed cages (Marzaro et al. 2011), or in free flight (Girolami et al. 2012).

Riley and Osborne (2001) reported: ‘...that in calm conditions, ...bees typically flew with a ground speed of circa 7 m/s and we visually estimated their height of flight to be about 2 m’. Our findings agree

with this reported data: the flight of bees over the ploughed area varied from 0.5 m to 4 m, but was most regularly at around 2 m (Girolami V, unpublished data). Thus, during the sowing, bees flew over the ploughed field at a height that corresponds to the toxic cloud which extends around the tractor. The exposure of the bees in mobile cage can, with reason, correspond to the exposure of a single forager in free flight when encountering a maize drill.

The speed of a bee in free flight (approximately 7 m/s, equal to about 25 km/h) is about twice that of the operators who exposed the bees in mobile cages. However, this longer exposure time seems to not affect in significant manner the extent of the powdering, given that bees in free flight died in similar numbers (Girolami et al. 2012). In brief, the new method adopted has allowed us to suggest that a single return flight in the vicinity of a sowing machine seems sufficient to kill a foraging bee and, on the basis of the experimentation on the extent of the toxic cloud, even a single trip. Nevertheless, the difference in the speed of the exposure, the influence of the mesh of the cages and, indeed, the stress induced in the tested bees could result in some modification of behaviour and survival. Such aspects need further study to evaluate mortality. The final aim of this work should be to determine the minimum contamination that bees can tolerate without compromising their survival.

Extent of the toxic cloud

The cloud rendered visible by the emission of talc easily documented with a camera (fig. 2) may be considered a good indication of the cloud of air that contains, in suspension, the fragments of seed shell that caused the death of bees as reported in fig. 1. Taking into account that talc, a silicate with a specific gravity of 2.7, is heavier than the organic material that constitutes the shell fragments (Tapparo et al. 2012), consequently, the cloud containing the fragments of shell could be somewhat larger and last longer than that of talc. However, no substantial differences seem to exist in relation to the toxic cloud evaluated, with the mobile cage method, which clearly showed how a large lethal cloud in the order of 20 m in diameter can form around a drill in action (fig. 2), passing through which, a bee could be potentially poisoned with a fatal dose. The evaluation of the extent of the cloud was carried out with a static drill to establish the size of the cloud without the complication of the effect that forward movement would have on the emissions shape. The data

obtained can form the experimental basis for further trials that take account of the speed of the drill as well as other variables such as wind speed and thermal inversion, which in our observations seems to influence the thickening and the duration of the cloud at lower atmospheric levels.

Regarding the persistence of the toxic cloud around the driller, the simple experimentation reported allows us to consider that the cloud has relatively short duration on the air, in the order of few minutes. This aspect agrees with the toxic cloud dimension, which is a consequence of a dynamic process with continuous production and falling of particulates.

The ellipsoidal dimension and compactness of the cloud assessed with the drill stationary in calm wind conditions and a hot sunny day do not necessarily correspond to the shape the cloud would take during normal sowing. Nevertheless, the 20 m diameter of the cloud may be considered a realistic approximation, in that, were the drill in motion, the cloud would have a narrower and more elongated shape and given a wind would be further lengthened and irregular, not centred on the drill, but logically lengthened in one direction. As a consequence, the probability of a bee encountering the cloud would increase in relation to the situation shown with a static drill.

Driller modifications and content of insecticide in bees

The evaluation of the extent of the toxic cloud reported in fig. 1 was obtained by exposing caged bees to the cloud at various distances and heights. To make the contact with particles more realistic, the trials were planned simulating a return flight of foraging bees.

The results obtained show that the method can also be employed to verify the effectiveness of various modifications made to drilling machines. It became evident that the advantages, by simply directing the ventilated air towards the ground, universally accepted as useful in the survival of bees (Pistorius et al. 2009), did not contribute in any meaningful manner to reducing deaths connected with the use of drilling machines employing coated seed. The hypothetical benefits brought about by the use of a deflector clearly contrast with the results of all the trials using a modified machine, given the mortality rate still above 50% (table 3). The modifications seem, however, to bring about a small increase in survival when compared with the

unmodified machine although of little relevance to the aim of defeating bee mortality. The validity of the test adopted to assess the influence of the modifications to the machine was confirmed in the chemical analysis of the caged bees passed at varying distances from the machine that were dusted with very high doses of insecticide (table 4). Clear differences of contamination with neonicotinoids arose amongst the various distances and directions in relation to the drill. The chemical contamination is not conflicting with the survival results of the bees. For example, on the right hand side of the drill, doses higher than 142 ng of imidacloprid could induce the death of all the bees in conditions of high humidity. This was seen in trial 1 (table 2) for bees exposed (on the right side) and then held in high humidity with total mortality. The passage of a single bee at a distance of 1 m accounted for a quantity of 4786 ng, sufficient to kill hundreds of bees, given that DL_{50} of contact with imidacloprid is 18 ng/bee (Iwasa et al. 2004), which is 200 times less than the quantity encountered.

The modified machine has not substantially changed the values of dusting in relation to the unmodified machine. Although the quantities recovered from a single sample at 1 m were halved, the values for other distances were generally higher for bees exposed to the modified machine. In drill equipped with a 'dual pipe' (and also in the other models tested – unpublished data), the exhaust air, directed towards the ground, seems to displace but not reduce the toxic cloud (fig. 2). The mobile cage test adopted refers to a single return flight, which simulates an actual foraging flight of a bee in the vicinity of a functioning drill. It allows greater possibilities for improvement, simply, for example, exposing bees for longer and in down-wind conditions to drill emissions. Moreover, the mobile cage test is a simplification in relation to the free flight test (Girolami et al. 2012) while still maintaining all of its validity.

At all events, the evaluation reported is a biological test based on the mortality of bees in the field and is therefore an innovation in relation to the simple hypothetical expedient of off-crop ground deposition (Nikolakis et al. 2009) or of the powdering attributes of various batches of seeds using the Heubach test.

All the work reported is a further proof to explain that bees become lethally contaminated in flight. It is not necessary to take under consideration particles falling on the soil with consequent contamination of vegetation. This suggestion is also confirmed in a recent innovative paper (Schneider et al. 2012),

reporting that 'At field-relevant doses for nectar and pollen no adverse effects were observed for either substance.' (imidacloprid and clothianidin) on foraging behaviour bees.

Lastly, if contaminated dust persists in the body hair of foragers, they can contaminate other bees and the hive as a whole. Additionally, the pollen may be directly tainted by the brushing activity, which produces the pollen masses. This aspect makes the relationship between the presence of neonicotinoids in pollen and the visits of foragers to contaminated vegetation on the margin of the cornfields questionable.

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