STATEMENT OF EFSA

Assessment of the scientific information from the Italian project "APENET" investigating effects on honeybees of coated maize seeds with some neonicotinoids and fipronil [1]

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ABSTRACT

The European Food Safety Authority was asked by the European Commission to assess the scientific information on some neonicotinoids (*i.e.* thiamethoxam, clothianidin and imidacloprid) and fipronil gathered by the Italian authorities with a funded project named "APENET" and to identify whether this new scientific information might require a change in the assessment of these substances as regards their effects on bees. APENET is a multidisciplinary monitoring and research project, mainly aimed at evaluating the bee health status, the dust dispersal during the sowing of maize coated seeds with thiamethoxam, clothianidin, imidacloprid and fipronil, the lethal effects on bees exposed to this dust, and homing behaviour and orientation effects. Potential synergism between clothianidin and bee pathology was also considered. EFSA evaluated in particular the scientific information as reported in the project report from 2011 (APENET, 2011), which was brought to the attention of the European Commission. Overall, due to some deficiencies in the study designs, weakness in the statistical analysis as documented and incompleteness in the reporting of results, it was not possible to draw a definitive conclusion on all the scientific information. However, within this project some potential concerns such as lethal effects on bees exposed to dust, sub-lethal effects and interactions between clothianidin and pathogens were identified suggesting that a change in the assessment of the substances thiamethoxam, clothianidin, imidacloprid and fipronil as regards their effects on bees might be required.

KEY WORDS

honeybees, neonicotinoids, fipronil, maize coated seeds, dust exposure, lethal and sub-lethal effects

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SUMMARY

Following a request from the European Commission, EFSA assessed the scientific information on some neonicotinoids (*i.e.* thiamethoxam, clothianidin and imidacloprid) and fipronil which the Italian authorities gathered with a project named "APENET". APENET is a multidisciplinary monitoring and research project, mainly aimed at evaluating the bee health status, the dust dispersal during the sowing of treated maize seeds and the lethal and sub-lethal effects on bees exposed to this dust; potential synergism between some neonicotinoids and bee pathology was also considered. This project was funded by the Italian authorities following the losses of honeybee colonies reported in Italy since spring 2008. The project was launched after the temporary suspension in Italy, as precautionary measure, of the placing on the market of maize seeds treated with plant protection products containing thiamethoxam, clothianidin, imidacloprid or fipronil.

The research undertaken within APENET was described and reported in 3 different reports: APENET 2009, 2010 and 2011. However, the most comprehensive report was the APENET 2011, which was brought to the attention of the European Commission. Therefore, as requested by the European Commission, EFSA performed an in-depth evaluation of the latter and considered the other reports as background documents.

To evaluate the bee health status, within APENET a monitoring network was set up. Hives situated in different geographic areas were monitored by means of periodic sampling and laboratory analysis of different matrices like dead bees, live bees, brood, honey, wax and pollen. Both pathogens and chemicals were analysed. In relation to the monitoring of pathogens EFSA identified gaps in terms of the data provided (e.g. the total number of stations, apiaries and hives). The reasons behind the sampling plan chosen and the conclusions drawn were presented without describing the levels of representativeness and uncertainty of the estimates obtained. Some important pathogens have not been included in the sampling plan. In relation to the chemicals analyses, the concentrations found are reported as range and not per active substance. An appropriate analysis of the results was also difficult due to the lack of environmental characteristics such as the agricultural landscape around the sampling points and the weather conditions.

Several trials were performed to measure the dust dispersal of thiamethoxam, clothianidin, imidacloprid and fipronil. These trials were conducted with a precision pneumatic seeder machine equipped with deflectors further modified (*i.e.* Prototype 1 and Prototype 2) to investigate the dust drift reduction. EFSA noted some deficiencies on the reporting of the results. EFSA concluded that a detailed analysis of these results could not be performed, but some general trends could be observed. In particular, the dust and therefore the deposition of residues in the off-crop area decreased with the distance; however, no decrease with the distance was apparent in the air concentration. This was attributed by the authors to the very fine fractions of the dust. The reduction in dust deposition at soil level for imidacloprid was 89% for Prototype 1 and 95.4% for Prototype 2. The overall reduction reached for the other active substances for the Prototype 2 deflector system was 74.4% for clothianidin, 88.6% for thiamethoxam and 94.8% for fipronil. The reductions in air concentration of imidacloprid were 53.1% and 72.4% for Prototype 1 and Prototype 2, respectively. The reductions in air concentration were 86% for clothianidin, 90% for thiamethoxam and 96% for fipronil with the Prototype 2.

Field tests to evaluate the effects on bees directly exposed to dust produced during the sowing of coated maize seed were performed. Different trials were carried out by using different protocols: 1) bees inside cages placed at different heights were exposed to dust produced during the maize clothianidin-coated seeds sowing with a "modified" machine (Prototype 2); 2) free flying bees were exposed to dust produced during the sowing of maize coated seeds with thiamethoxam, clothianidin, imidacloprid and fipronil; 3) bees inside mobile cages were exposed to dust during the sowing of maize coated seeds with thiamethoxam, clothianidin and imidacloprid. For trials under points 2) and 3) an unmodified seeder machine was used for sowing.

As regards the tests with bees inside cages and the Prototype 2, EFSA noted that in each trial only a small number of bees was used and the number of repetitions was very small, therefore the data available on mortality were considered insufficient for a robust statistical assessment, although some data analysis was performed by the authors. Furthermore, some mortality occurred also in the untreated control group, which may have added uncertainty to the data analysis. Due to such deficiencies, it was difficult to make any firm conclusion. Although the authors concluded that the mortality rates were higher in all treated groups than in the untreated control, they also argued that the results could not be generalised and represented worst case exposure conditions for flying bees with respect to sowing line and wind direction.

As regards the tests on free flying bees with unmodified machine, it was concluded that forager bees are at risk if they fly through dust clouds emitted by seeders sowing maize seeds coated with either fipronil, thiamethoxam, clothianidin or imidacloprid (in these trials no deflector system was used). The elevated air humidity increased the mortality rate of the bees that had been exposed to dust containing fipronil, thiamethoxam, clothianidin or imidacloprid. EFSA considered these conclusions supported by the data provided.

As regards the tests with bees inside mobile cages with unmodified machine it might be concluded that the level of dust emission is in the range where detrimental effects on bees cannot be excluded. However, it was noted that the exposure in these trials was unrealistically high.

Effects of contaminated dust were reported on the short and long-term learning and olfactory memory abilities of bees for all the four molecules tested at concentration levels found with both unmodified and modified machines located at 5 m from bees. With the use of modified machines emitting between 80 and 90% less dust than unmodified machines, it was unclear why the authors considered a worst-case exposure for clothianidin alone (*i.e.* 20% of contaminants in dust *versus* 10% of contaminants in dust with thiamethoxam, imidacloprid and fipronil) which might have under-estimated the effect of dust exposure with the three other tested molecules. However, the observed effects on bees could not be validated because, from the information provided, it was not possible to guarantee that the protocol was developed in fully controlled conditions and with appropriate statistical testing.

The sub-lethal effects observed and measured on bees in studies dealing with orientation and homing behaviour were regarded more as proposals for implementing the current protocols rather than definitive testing studies because of the small number of bees tested (e.g. study with the simple labyrinth), the exploratory nature of the study design (e.g. study with different exposure scenarios for testing homing and foraging in the field and study on orientation in a complex labyrinth) or simply because the authors mentioned that the study was still ongoing. The incompleteness of the description of these studies and their results did not allow a proper assessment of the methodology and data presented. Nonetheless, the proposed protocols were found innovative and interesting because they attempted to take into account the variability found in the environment of bees (e.g. different exposure scenarios, different field conditions). Such studies warrant further development and fine-tuning for the testing of the effects of pesticides on bee behaviour in field conditions.

The study of the interaction between DWV prevalence in bees and clothianidin exposure was in line with some recent findings showing the potential interaction and/or synergy between various factors involved in bee health (e.g. *Varroa* and DWV or *Nosema* and pesticides). The authors claimed that drs-GFP expression was significantly reduced in *Drosophila* larvae when exposed to clothianidin at LD_{50} concentrations. Nonetheless the insufficient quality of the reporting did not allow an appraisal of the methodology used and the conclusions drawn by the authors could not be supported. In addition the mechanisms investigated in *Drosophila* still need to be demonstrated in honeybees. The underlying mechanisms involved in the interaction between pesticides and infection level in bees would merit further investigation.

Overall, it was not possible to draw a firm conclusion on all the scientific information in the APENET report, due to some deficiencies in the study designs and weakness in the statistical analysis and

conclusions drawn as reported, or due to the incompleteness in the reporting of the results. However, within this project some potential concerns such as lethal effects on bees exposed to dust, sub-lethal effects and interactions between clothianidin and pathogens were identified suggesting that a change in the assessment of the substances thiamethoxam, clothianidin, imidacloprid and fipronil as regards their effects on bees might be required.

EFSA recently received a mandate from the European Commission for scientific and technical assistance and was requested to provide an EFSA conclusion with an updated risk assessment to bees for the neonicotinoids thiamethoxam, clothianidin, imidacloprid, acetamiprid and thiacloprid. The results for the neonicotinoids investigated in the APENET project might be re-considered, within this mandate, provided the identified deficiencies of the reports will be addressed. For this purpose the papers mentioned in the report and in the process of being published, might be useful. However, since fipronil does not belong to the neonicotinoids, it will not be considered in the new mandate.

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TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

On 23 March 2012 EFSA received a request from the European Commission to provide a statement assessing the scientific information submitted by the Italian authorities justifying their suspension of the use of maize seeds treated with plant protection products containing clothianidin, thiamethoxam, fipronil and imidacloprid and identifying whether this new scientific information might require a change in the assessment of the substances as regards their effects on bees.

The agreed deadline for providing the statement is 23 June 2012.

ASSESSMENT

1. Introduction

Following the losses of honeybee colonies reported in Italy since spring 2008, the Italian authorities have temporarily suspended, as precautionary measure, the placing on the market of maize seeds treated with plant protection products containing the neonicotinoid active substances clothianidin, thiamethoxam, imidacloprid or the active substance fipronil.

In order to further clarify the honeybee colony losses, the Italian authorities launched in December 2009 a specific national monitoring and research project named "APENET". This project was coordinated by the "Consiglio per la Ricerca e la Sperimentazione in Agricoltura" (CRA). The research and experiments were reported in 3 reports, *i.e.* APENET (2009, 2010, 2011). The report published in October 2011 was brought to the attention of the European Commission. In this report concerns on the use of seeds treated with thiamethoxam, clothianidin, imidacloprid or fipronil were raised.

The European Commission requested EFSA to assess the scientific information submitted by the Italian authorities (APENET, 2011) and to identify whether this new scientific information might require re-assessing the risk of these substances to honeybees.

2. Overview of the APENET project

The APENET is a 3 year monitoring and research project including several activities and experiments with the aim of evaluating the honeybees' health status, the effects of contaminated dust drift (which is generated during the sowing of coated seeds), and the potential synergism between some neonicotinoids and bee pathology. The investigations were conducted on maize coated seeds. Some experiments were carried out in field conditions, others in laboratory conditions.

Bee monitoring

A national monitoring network was established in order to gather information on the health status of the honeybee colonies. Hives situated in different geographic areas were monitored by means of periodic sampling and laboratory analysis on dead bees, live bees, brood, honey, wax and pollen. The monitoring network is described in the 2009 and 2010 reports, while the main results are reported in the 2011 report.

Bee exposure to dust and assessment of effects

Dust drift during the sowing of maize seed coated with clothianidin, thiamethoxam, imidacloprid or fipronil was investigated. The sowing was performed with modified or unmodified seeder machines. The active substance concentrations in soil and in air were measured at different distances from the sowing point. All the experiments, investigating dust drift, were carried out in 2009, 2010 and 2011 and reported in the related reports.

Effects on bees exposed to dust drift during sowing were also investigated.

Assessments of effects in laboratory were conducted. Tests were carried out to investigate lethal and sub-lethal effects on honeybees for clothianidin, thiamethoxam, imidacloprid and fipronil. Some trials were performed in 2009, but most in 2010 and 2011.

The synergistic interaction between the sub-lethal dose of clothianidin and other stress agents was investigated. This was only reported in APENET 2011.

Guttation

Measurement of the concentration of thiamethoxam, clothianidin, imidacloprid and fipronil in guttation droplets from maize were carried out. These experiments were performed in 2009 and 2010 but not in 2011 and therefore only briefly considered in this statement.

In the experiments from 2009, the presence of residues of the above active substances was measured in leaf guttation fluid and in droplets collected from the calyx of container-sown maize. Very high concentrations were detected for all the 3 neonicotinoids but not for fipronil as reported in Table 14 and 15 of the APENET 2009. The range was 16.22-345.76 mg/L for leaf guttation fluid and 2.93-134.66 mg/L for guttation droplets. Clothianidin residues in guttation droplets were also measured in field grown maize plants. Values were found to be appreciably lower than those detected in container-grown maize. The foraging activity on guttation was also investigating in the field. However, the number of bees observed was too low to draw any conclusion (3 bees observed, 1 on leaves not collecting guttation droplets).

In the experiments from 2010, neonicotinoids in guttation were measured in different situations: 1) on field grown maize, 2) on maize grown in tunnel and under different treatment regimes and 3) on maize grown in different soil types. The investigation of bee foraging activities on guttation was also repeated in 2010 and the authors concluded that bees foraging on maize guttation droplets in environmental conditions where the investigations were carried out, was nil or negligible.

Agronomic trials

In addition to the above mentioned research, some trials not directly related to the risk assessment for bees were performed. These trials were not considered in this statement, but they are mentioned for completeness. Some trials concern the assessment of the incidence of plant viruses in different susceptible maize varieties from coated seed and uncoated seed. Other trials regarded the maize production of coated seeds with clothianidin, thiamethoxam, imidacloprid and the fungicide "Celest" (containing the active substances fludioxonil and metalaxyl) *vs* coated seeds with the fungicide "Celest" alone (APENET 2009, 2010, 2011). It is noted that also the persistence of the active substances in plant tissues at different growth stages was detected. The results indicated that an appreciably reduction of the levels of thiamethoxam, clothianidin, imidacloprid and fipronil in leaves occurred from the 2nd-3rd leaf stage. It is also mentioned that in the same trials, residues in pollen were investigated and were not detected for any of the 4 active substances (<LOD 0.2µg/kg). However, no sufficient details are reported in the APENET 2011 to fully validate these findings.

3. The monitoring network

3.1. Description

The sampling plan for the national monitoring network developed within the project and described in the report was composed of 20 surveillance modules, every module consisted of 5 stations (94 apiaries in total), each of which is in turn made up of 10 hives (total of 940 hives). In the following paragraphs details are given concerning the material and methods, results and conclusions in relation to the winter mortalities recorded in 2010/2011; the identification of pathogenic agents focused on *Nosema apis*, *N. ceranae* and several other pathogens.

The aim of this monitoring network was to gather information on the health status of the bee colonies contained within the modules, by means of periodic surveys and subsequent laboratory analyses performed on the different matrices collected. In addition to routine analyses, the programme also specified that special surveys, sample collection and analyses were carried out when abnormal mortality was reported.

Chemical analyses were carried out on bee samples, wax and pollen to measure the residues of several active substances belonging to organophosphate, organochlorate, carbamate and neonicotinoid pesticides. Nectar was not investigated. The results are reported in the Tables 17, 18 and 19 of the APENET 2011.

The monitoring network is further supported by the reporting system, which makes it possible to notify the authorities of abnormal events occurring in hives even if the hives in question did not form part of the network. By means of the reporting system, bee-keepers send a notification of any abnormal mortality to the Veterinary Service of the Health District authorities. The Veterinary Service

is then responsible for conducting an on-site inspection, collecting samples, ensuring appropriate storage (-20°C) and shipping of the samples to the laboratory of the Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe), where analyses were performed in cooperation with the APENET network.

In the spring of 2008, all 185 reports proved to have been concomitant with maize sowing, and of the 132 samples gathered and analysed, 57.5% tested positive for the neonicotinoids used in maize seed coating. In 2009, three reports were notified, two of which were official and submitted to the Veterinary Service during the maize sowing period, while the third was not submitted by the official route but reported directly to CRA-API. All three of these cases were found to be linked to non-authorized use of coated maize seeds. With regard to the spring of 2010, reports (Table 20 of APENET 2011) did not involve maize-growing areas. It should also be noted that in 16 out of the 21 cases reported, the Veterinary Services of the Local Health district (ASL) was involved for further investigation. Analogous to the previous year, in spring 2011, no report came from maize-growing areas, and 14 out of the 16 reports registered until the end of June 2011 were official. Between May and September 2011, further reports of bee die-offs were received by the three institutes involved in the APENET project (i.e. CRA-API, IZSVe and DiSTA-UNIBO). The details of each report are shown in Table 21 of APENET 2011.

The authors highlighted that the APENET project was officially terminated at the end of March 2011, together with the associated reporting system. The subsequent reports are thereby fruit of voluntary service.

3.2. Results and evaluation

Considering the data and information provided, uncertainties and data gaps were identified. It is considered important to describe better how the study design was prepared and based on which criteria. The presence of different pathogens, with different characteristics and prevalence has been assessed. It is relevant to describe the percentage of the population that was sampled, as well as the total number of modules, apiaries and hives. It should be considered that the actual prevalence of each pathogen searched may affect the significance of the results obtained. It is not explained how the geographic representativeness of the areas of each Region was defined for the hives. Also information related to the frequency of the periodic surveys and the number of times the special survey was conducted is missing. It could be useful for the future studies and samplings to describe data on the probability of isolating the different agents in the different matrices collected.

According to the report, winter mortalities in 2010/2011 were estimated to be 22.48% (78 dead colonies on 347). Winter colony losses estimated by means of the COLOSS European network questionnaire were 13.44% (1850 colonies on 13770).

Open questions are whether this difference (almost double) can be explained because of different sampling plans/designs and what is the uncertainty associated with both estimates. The number of colonies sampled is very different. COLOSS collected 40 times more colonies. In conclusion, the representativeness of the study conducted is unclear.

The analysis conducted to identify pathogenic agents, according to the APENET 2011, "concentrated on *Nosema apis*, *N. ceranae* and viruses. Results showed endemic spread of the fungus (Microsporidia) *N. ceranae* throughout all Italian regions. This fungus has almost completely replaced the species previously present (*N. apis*), with the exception of one apiary in the province of Bolzano, where both species were detected. Thus the investigation, which is still on-going, confirmed the first reports that date back to 2007 indicating the presence of *N. ceranae* in Italy as well. Findings obtained so far have allowed a clearer picture of the spread of this pest over the different areas of Italy. The samplings carried out in 2010 in the APENET network confirmed the presence of *N. ceranae* only: *N. apis* was not identified in any sample."

It would be interesting to have more information concerning the reasons for the differences found, which might be linked to different sampling plans. Also the levels of uncertainty associated with the estimates should be provided. Taking into consideration the recent situation, it is not clear why were other important pathogens like *Varroa* were not included in the analysis.

"Among viruses, in the 2009 sampling, the presence of the Deformed Wing Virus (DWV), the Black Queen Cell Virus (BQCV), the Sacbrood Virus (SBV), the Chronic Bee Paralysis Virus (CBPV) and the Acute Bee Paralysis Virus (ABPV), either individually or in varying combinations, was confirmed. In none of the hives on which the analyses were performed, the presence of the Apis Iridescent Virus (AIV), the Kashmir Bee Virus (KBV) or the Israeli Acute Paralysis Virus (IAPV) was detected. Our findings show that the main bee viruses are present in Italy, similarly to their presence throughout Europe, but the presence of DWV and BQCV is particularly marked in Italy. In the samples collected in 2010, the same viruses as the previous year were detected with the addition of KBV and IAPV, the latter found in 3 apiaries in Sardinia, Lazio and Tuscany. Of the 378 samples analysed in 2010, 12 resulted negative, while the prevalence of each virus in the remaining 366 samples was 96% for BQCV, 78% for DWV, 60% for SBV, 29% for ABPV. With the exception of AIV, which was never detected, the prevalence of the other analysed viruses was below 10%. It is important to note that this is the first nation-wide investigation based on biomolecular techniques undertaken in Italy to examine the presence of bee viruses. Previous studies, which date back to a considerable number of years ago, were not only limited to just a few regions, but were also based on electron microscope and serologic methods, which at that time were the only techniques available to test for the presence of these pathogens. The new knowledge acquired on bee virus distribution is of considerable interest and represents a valid starting point for further research."

From the available information it cannot be concluded that the absence of KBV and IAPV in 2009 is really an absence and not related to the sampling plan/design. It is an open question what is the certainty associated with the conclusion that these 2 viruses are absent (not detected) in 2009. Finally, it is unclear whether there was a sampling for evaluating the presence of the viruses in 2011 as this report concerns 2011 (at least until March, when the project finished).

As regards the chemical analysis of pesticides, it was noted that the method of analysis, the LOQ and LOD were not reported. The concentrations found are reported as range and not per active substance. It was noted that some concentrations were very high. Only thiamethoxam and imidacloprid were detected in some pollen samples. In the same samples other active substances were detected and the reported range was 16-1619 ng/g and 99-363 ng/g. An appropriate analysis of the results was also difficult due to the lack of environmental characteristics such as the agricultural landscape around the sampling points and the weather conditions.

4. Dust dispersal during coated maize seed sowing with modified seeders

4.1. Static trials

4.1.1. Description

Fixed point tests were conducted in order to control the efficiency of deflectors and to gain experience for the further developments of more efficient prototypes. In this context, deflectors mean any type of technical solutions, modifications of the drilling machine that are used to mitigate the emitted dust.

A large, covered experimental site was used for these trials, where a controlled, constant artificial wind was generated. Maize sowing was simulated in a small area by a precision pneumatic seeder. To capture the dust drift during these operations, Petri dishes were placed on the ground and air samplers at 2 m height downwind at various distances from the seeder. To control the reduction of the emitted dust, the simulated sowing trials with treated seeds for all the four active substances with or without employing deflector systems were completed. Two prototypes of deflector systems were compared with a conventional seeding technique:

- Prototype 1: The air exhaust is recycled into the airtight hoppers using plastic pipes. The excess air can only exit via a filter (activated carbon anti-pollen filter) fixed on the lid of the hopper. This prototype was tested only for imidacloprid.

- Prototype 2: The air exhaust is channelled first into a collecting tube and from there channelled into the hoppers. Again, the excess air can only exit via a filter, but in this case the activated carbon anti-pollen filter is equipped at the lower side of the collecting tube, close to the soil level.

The efficiency of the filter and the efficiency of the whole device of Prototype 2 was controlled with some measurements during the simulated sowing trials for imidacloprid. These evaluations were carried out by sampling air exiting from the seeder pneumatic system being with or without modifications.

All Petri dishes and air samplers were analysed for residues.

4.1.2. Results and evaluation

The results of efficiency measurements revealed that a high filtering effect can be achieved by the tested devices. The efficiency of the activated carbon filter was found to be 95.20% and the efficiency of the whole system of Prototype 2 was 97.57% when compared with unmodified machinery. These figures refer to the reduction of imidacloprid residues in sampled air. Since the air exited was sampled directly at the outlet (in a closed pipe) in static conditions, it has to be noted that these results do not reflect the whole emission of the machinery or the whole potential emission of the drilling process. The number of repetitions of these measurements or the duration of an air sampling was not reported. Additional preliminary results suggested that with decreasing size of dust particles the ratio of non-captured dust increases.

Regarding the fixed point tests (simulated sowing trials) the analysis of the results in the APENET 2011 report was only partial and for clothianidin, thiamethoxam and fipronil data from a few repetitions only were available (the total number of repetitions was not clearly reported). The only reported results were the reduction of dust drift expressed in percentage (%) based on the overall mean values calculated from regression curves of each active substance. The regression curves (deposition of residues in dust or the measured air concentrations *vs* distance from the seeder) were graphically illustrated with low resolution. In case of imidacloprid, the plotted values were corrected by the results of a set of repeated measurements that was conducted due to a bias with the first measurements. No details were reported regarding how the corrections of these data were made.

Due to these deficiencies, a detailed analysis of these results could not be performed, but some general trends could be observed:

- The dust, therefore the deposition of residues in the off-crop area, decreases with the distance from the drilling machine.

- Regarding the air concentration, no decrease with the distance was apparent. This was attributed by the authors to the very fine fractions of the dust that were not captured by the filter. This fraction might be persistent in the air.

- Reduction in dust deposition at soil level for imidacloprid was 89% for Prototype 1 and 95.4% for Prototype 2 compared to the conventional seeder. The overall reduction reached for the other active substances for the Prototype 2 deflector system was 74.4% for clothianidin, 88.6% for thiamethoxam and 94.8% for fipronil.

- Reductions in air concentration of imidacloprid were 53.1% and 72.4% for Prototype 1 and Prototype 2, respectively. Reductions in air concentrations were 86% for clothianidin, 90% for thiamethoxam and 96% for fipronil with the Prototype 2.

- Clothianidin air concentration, as well as the clothianidin deposition was markedly higher than for the other active substances.

4.2. Field sowing trial

4.2.1. Description

The aim of the field sowing trials was to get experience on field performance of the deflector system Prototype 2 and to assess whether the emitted dust is lethal for bees flying over the seeding area.

The trials were performed at an approximately square-shaped field measuring 4 hectares. Maize seeds coated with clothianidin were sown by a precision pneumatic seeder equipped with Prototype 2 deflector system (see paragraph 4.1.1). The hoppers were loaded on the edge of the field, with 12 sacks of coated seeds. To monitor the distribution of dust in the area adjacent to the sowed field, 2 series of 9 Petri dishes with adsorbents were placed at 1 and 5 m from the first seeding line downwind. Four air samplers placed at 5 m distance downwind from the edge of field and 2 m height were also used. Wind speed and wind direction were continuously monitored during the operations.

4.2.2. Results and evaluation

The wind direction was not found to be constant during the trials and the wind speed was fairly low (total average wind speed was reported to be 0.63 m/s), suggesting that dust dispersal far from the sampling area was limited. The results of the chemical analyses of the Petri dishes and the air samplers did not allow concluding any trend for dispersion and deposition of the dust in the off-crop area. The location of the positive samples (residues > LOQ) appeared to be concentrated in a single isolated plot within the sampling area.

The authors concluded that this spot contamination was not connected with the sowing, but rather with the seed loading and other preliminary seeding operations.

5. Field assessment of effects on bees exposed to dust during the sowing of maize coated seeds

Field tests to evaluate the effects of bee directly exposed to dust produced during the sowing of coated maize seed were investigated. Different trials were carried out by using different protocols: 1) bees inside cages placed at different heights were exposed to dust during the sowing with a "modified" machine (Prototype 2) of maize coated seeds with clothianidin; 2) free flying bees were exposed to dust during the sowing with an unmodified machine of maize coated seeds with thiamethoxam, clothianidin, imidacloprid or fipronil; 3) bees inside mobile cages were exposed to dust during the sowing with an unmodified machine of maize coated seeds with thiamethoxam, clothianidin or imidacloprid.

5.1. Effect assessment during field sowing trial with "modified" seeders

5.1.1. Description

During the trials described in paragraph 4.2, forager bees of same age and size were collected and placed in small cages provided with feeders. For each trial, 10 cages with a single bee were hung to a horizontal bar and the bar with the cages was kept in the sowed field. The protocol used in each trial was quite similar with some slight variations:

- Cages were placed directly behind the seeder at 2.5 m height and followed the machine during the sowing operation

- Cages followed the seeder at 4 m behind the tractor and at 0.5 m height to intercept the dust close to ground level

- Cages followed the seeder at 4 m behind the tractor at 1.8 m height, and placed laterally, downwind to the tractor, so as to intercept better the drifting dust cloud

- Cages followed the seeder, but the seeder was not equipped with Prototype 2 deflector system. It is noted however that the air exhaust was channelled down to soil level behind the coulters.

- Cages placed at 1.8 m height followed the seeder being without seeds at 4 m behind the tractor. This trial served as untreated control.

For some trials two repetitions were completed, while for some others there was only one run. In all trials the bees in the cages followed the drilling machine for 200 m length resulting in an exposure time to the dust cloud of 270 s on average.

After the sowing trials, the cages with the bees were placed into laboratory conditions for 24 hours and the mortality was checked. At the end of the monitoring period, dead bees were collected and sent for chemical analysis.

5.1.2. Results and evaluation

It has to be noted that the scenario modelled in these trials (270 seconds in the dust cloud) is unlikely to occur in realistic field conditions. However, bees can fly through dust clouds several times a day in situations where the sowing field is between the hive and the foraging area. Also, dust clouds can drift into the foraging area from the neighbouring fields.

In each trial only a small number of bees were used (10 or 20), therefore the data available on mortality are not considered enough for a robust statistical assessment, although some data analysis was done by the authors. Relatively high mortality (7 out of the 10 bees) occurred in the first repetition of the first trial (cages placed directly behind the seeder at 2.5 m height), but the mortality was only 3 out of the 10 bees when the trial was repeated 10 minutes later. The authors argued that some mortality in the first repetition may be attributed to the loading of the seed hoppers and to other preliminary seeding operations. On this basis, they considered this trial repetition as an outlier. However, as mentioned above, the number of bees and the number of repetitions was very small, therefore it is not justified to underpin the exclusion of this trial. Furthermore, some mortality occurred also in the untreated control group; 3 out of the 20 bees (15%), which makes the data analysis difficult.

Due to these deficiencies, it is difficult to make any firm conclusion, but some general trends could be observed:

- The mortality rate was higher in all treated groups than in the untreated control groups.

- The mortality rate was lower in all treated groups compared to the groups from the trial where the Prototype 2 deflector system was not applied

From these results, it might be concluded that the Prototype 2 deflector system is efficient, but the level of dust emission is still in the range where detrimental effects on bees cannot be excluded if they are under considerable exposure to dust clouds.

Clothianidin residues were detected in some dead bees from the trials where Prototype 2 deflector was used. However, from the trials where air exhaust was only channelled down to soil level, but the Prototype 2 deflector system was not used, relatively high (22-291.5 ng/bee) clothianidin residues were detected in all dead bees. Moreover, clothianidin residue (18 ng/bee) was found in one of the dead bees from the control trial.

5.2. Effect assessment during field sowing trial with "unmodified" seeder

5.2.1. Free flying bees, description

Forager bees of four hives were trained to visit an artificial feeder that was placed about 100 meters from the hives. A small field was located between the hives and the feeder. Trials for fipronil, thiamethoxam, clothianidin or imidacloprid coated maize seeds were sown in the same field but in different periods during 2009 and 2010. A small, but widely used seeder machine without deflector system was used for these trials. The air exhaust (150 L/s) discharged at a height of 1.8 meter. The sowing operations lasted about 45 minutes and were performed in intensive foraging periods that were checked visually. At the beginning of the sowing and subsequently at 15 minute intervals up to an hour (up to only 30 minutes for thiamethoxam), bees were captured near the feeder. At each occasion, 24 bees were captured and placed in small cages supplied with a feeder. All together, 120 bees were assayed for each trial except for thiamethoxam where this number was 72. The captured bees were transported to the laboratory and monitored for mortality for 24 hours. During this period, half of each bee groups were kept in normal laboratory humidity conditions, while the other half was kept under humidity conditions approaching saturation (> 95%).

Mortality in front of the hives was also monitored for 24 hours. Some dead bees collected in front of the hives or around the feeder and bees died in the laboratory were sent for chemical analysis.

A similar sowing trial was also conducted using only fungicide seed treatments.

5.2.2. Free flying bees, results and evaluation

All the bees captured at the beginning of the sowing (just before they could be exposed to dust) survived the 24-hour period in the laboratory without showing effects of intoxication. Practically no mortality (either 0 or 1 died out of the 12 bees in each group) was observed when only fungicides were tested. Regarding the trials with insecticides, all the bees captured after the end of the sowing operations (30 minutes after the beginning of the sowing for thiamethoxam) and kept under humid conditions died. The mortality rate was variable in the other groups, but it was always higher (in one case equal) in the group maintained in the humid conditions. In one trial with clothianidin, all the bees kept in normal laboratory humid conditions survived, while all the bees kept in elevated humid conditions died.

Detailed information was only reported for the mortality observed in front of the hives or for the residue analysis of dead bees. In general, several hundred bees were counted in front of the hives after the sowing operations. Relatively high residue levels (not rarely >> 100 ng/bee) were found in the dead bees for which these data were available.

From these showing trials the following can be concluded:

- Forager bees are at risk if they fly through the dust clouds emitted by seeders sowing maize seeds coated with either fipronil, thiamethoxam, clothianidin or imidacloprid (in these trials no deflector system was used)

- The elevated air humidity increases the mortality rate of the bees that had been exposed to dust containing fipronil, thiamethoxam, clothianidin or imidacloprid

5.2.3. Bees in mobile cages, description

Bees were confined in small cages and the cages were hung to a horizontal bar at 40 cm intervals. For each trial, 10 cages with a single bee were used. The exposure of the bees to the dust cloud was ensured by the two operators who hold the bar and followed the machine during the sowing operation parallel with the direction of the tractor in such a way as to intercept the dust cloud. Two sets of trials were conducted and for both two repetitions were conducted. In the first set up the front-part of the bar was located in line with the tractor, so the cages with the bees were located from 0 to 4 meters behind

the tractor. In the second set up, the front-part of the bar was located 4 meters behind the tractor, so the cages with the bees were located from 4 to 8 meters behind the tractor. The distance or the time during which the cages followed the machine was not reported, but it was indicated that the exposure lasted for a forward run of the tractor and one return way. After this simulated exposure, the bees were transported to the laboratory and monitored for mortality for 24 hours at high humidity conditions. These trials were conducted with seed dressing products of thiamethoxam, clothianidin and imidacloprid (Cruiser® 350 FS, Poncho® and Gaucho®). Moreover, trials treated with fungicide only were also conducted. Details on the doses, on the seeder or whether a deflector was used, were not reported.

In separate trials with imidacloprid, bees were exposed in a similar way as described above at different distances from the seeder, either on the right side or on the left side of the seeder. Some bee samples from these trials were subject to chemical analysis.

5.2.4. Bees in mobile cages, results and evaluation

Some important methodological details (e.g. collection of bees, machinery, duration of exposure) were not reported, what makes the evaluation of these trials difficult. Since these trials are reported under the same section of the APENET 2011 as the previous trials (free flying bees), it can be assumed that these trials were conducted in the same small field with the same machine without deflector as described in paragraph 5.2.1. It is likely that duration of the exposure of the bees that was modelled in these trials, was too long compared with realistic field conditions. However, bees can fly through dust clouds several times a day. Also, dust clouds can drift onto the foraging area from the neighbouring fields.

Only one out of the 40 bees died from the trials where only fungicides were used, while considerable mortality (11 to 20 dead bees out of the 20 exposed) was observed in the trials with the insecticides. In the trials with clothianidin and imidacloprid, more bees died from the group exposed at 0-4 meters behind the seeder than in the groups exposed at 4-8 meters behind the seeder. In the case of thiamethoxam, the mortality rate in these set-ups was the same.

The chemical analysis of the imidacloprid exposed bees revealed higher residues in bees exposed on the right side of the seeder compared with the residues in bees from the left side. The residue data measured in bees showed a decreasing trend with the distance from the seeder. It is noted that the air exhaust of the seeder used in the trials described in paragraph 5.2.1 was placed on the right-hand side of the machine.

From these results, it might be concluded that the level of dust emission is in the range where detrimental effects on bees cannot be excluded if they are under considerable exposure to dust clouds.

6. Sub-lethal effects of neonicotinoids and fipronil

The effects of sub-lethal doses of neonicotinoids such as clothianidin, imidacloprid and thiamethoxam as well as the effect of fipronil on honeybees were investigated on different behavioural endpoints and with several tests: (i) learning and olfactory memory with the proboscis extension reflex (PER) test, (ii) orientation in a simple labyrinth, (iii) homing and foraging in the field and (iv) orientation in a complex labyrinth.

The PER tests were conducted on all four molecules, the orientation ability was tested with thiamethoxam in the simple labyrinth and with clothianidin in the complex labyrinth, and the homing and foraging ability were assessed with fipronil and clothianidin.

Several of the protocols described in this section were initiated in previous years and more data were collected and described in the previous reports (APENET 2009, 2010). For example for the PER test, data on contaminated dust were collected as early as 2009. In addition, the analysis of some of the data

presented in this report (2011) is not yet finalised. For example, the analyses of the data from the test on the homing behaviour and on in-hive bees are still in progress.

6.1. Learning and olfactory memory assessed with the proboscis extension reflex (PER) test

6.1.1. Description

In the 2011 APENET report, two PER studies were described to report the effect of clothianidin, imidacloprid, thiamethoxam and fipronil administered as contaminated abrasion-dust on honeybees. The first study (p25-30, section 3) was conducted with an unmodified sowing machine and from estimates taken from the APENET 2009 trials whereas the second study (p70-76, section 6.2) was conducted with a modified machine (i.e. equipped with a deflector to reduce dust emission) and from estimates taken from 2010 and 2011 trials.

The protocol used in each study was quite similar with some slight variations:

- In each study, about 10 foraging bees coming from a single hive were introduced in cages of a volume of 56.7 cm². In the first study, the number of bees tested (9 to 11) and the number of repetitions (3 to 4) varied among treatments. In the second study, a total of 210 bees were tested (160 treated bees in 4 lots of 10 bees for each of the 4 molecules tested and 50 control bees in 5 lots of 10 bees each).

- Contaminated dust to be used for the experiment was extracted from a Heubach cylinder. In the second study (with modified machines), the amount of dust emitted was found to be 80-90% less than in the first study (with unmodified machines). Therefore, in the second study the quantity of a.s. dispersed by dust was estimated to be 10-20% of the quantity of a.s. found in the first study.

- The concentrations found in dust per surface area at 5 m from the sowing machine were used as reference values for the four a.s. tested. Based on these estimates and knowing the volume of the cage where bees were maintained, the amount of a.s. to be used per cage (in µg/m² or ng/cm²) was calculated. Finally, for the second study, knowing the amount of final dust emitted by modified machines, the total amount of a.s. to be introduced in the cage could be calculated. Both studies used 0.01 g of contaminated talc to be spread at the bottom of the cages (*i.e.* in a Petri dish).

- The bees were maintained for 3 h in the cage, in the darkness, in the presence of the contaminant at 26°C and had access to sugar syrup for the first 2 h of captivity. Then, bees were tested for the PER with the same odours (citronellol associated with the reward, *i.e.* a sucrose solution; peppermint associated with the punishment, *i.e.* a saline solution) at 60 min, 180 min and 24 h. Preliminary odour recognition was conducted with an alternate presentation of the reward- and punishment-associated odours for the first study and with a semi-random sequence presentation of the reward- and punishment-associated odours for the second study. In the first and second study, 10 and 6 presentations of each odour were used, respectively.

- Finally, after the PER test, observations were made on bees locomotion in both studies.

- The results were analysed with a one-way ANOVA.

6.1.2. Results and evaluation

The exposure of bees in the first study to concentrations 10, 100 and 1000 times higher than the concentration found at 5 m from the machine corresponded to particular conditions (e.g. when bees fly through a dust cloud containing high amounts of dusts as described in paragraph 5.2). In the same study, the exposure of bees to the amount found at 5 m from the machine corresponds to field conditions with unmodified machines. The reduction of dust with modified machines is in the range of

80 to 90%. While the choice of a quantity of a.s. corresponding to 20% of the quantity found with unmodified machines corresponds to a worst case scenario, only clothianidin was tested at this level (the three other molecules were tested at 10%). Therefore, it is expected that the effects observed on bees for the three remaining molecules may have been under-estimated.

Results on locomotion were only described for the first study. These data, as described by the authors, showed that the PER test could not have been influenced by impairment of locomotion, since no such effects were observed at the end of the experiment.

Although the amount of contaminated talc was lower in the second experiment (corresponding to the uses of modified machines) than in the first study (corresponding to the use of unmodified machines), the authors claim that significant effects were still observed on the learning and olfactory memory of bees. Such effects were reported as early as 60 min after exposure and still visible on the longer term (at 180 min and 24 h) for imidacloprid, thiamethoxam and fipronil. The same trends were obtained for clothianidin.

The two studies share two major methodological flaws: the lack of a fully controlled setting (e.g. no indication on the randomization of the bees assignment to the treatments, no measurement of the bees learning/olfactory memory just prior to the start of the test, no description of the way the performance of bees was scored), and the difficulty to appraise the appropriateness of the statistical methodology because of the poor reporting. Therefore, no conclusions could be drawn out of them.

6.2. Orientation in a simple labyrinth

6.2.1. Description

Due to time constraint, the study was only conducted on 4 bees, although it was initially intended to be conducted on 3 lots of 10 bees. The bees were issued from the same hive, from one frame with one queen and brood linked to a free flight chamber and a Y-shaped labyrinth. A sucrose solution reward was placed in the labyrinth, opposite to the hive.

The training experiment was conducted into two steps. The first step aimed at marking the bees which succeeded to go to the reward located in a colourless chamber of the labyrinth. The second step aimed at training the marked bees to associate colours with odours corresponding to either the reward (sugar solution and blue colour) or the punishment (saline solution and red colour), each device located in one of the two arms of the T-labyrinth. Six randomised trials were conducted for this training phase. For each bee, the colour choice and time were recorded.

After the training, bees were contaminated with dust containing thiamethoxam following the procedure and experimental conditions described for the PER test. Then, the bees were released in the free flight chamber and if they did not manage to get to the labyrinth within 5 minutes, they were put in the common chamber of the labyrinth and allowed 5 minutes to make a choice. This operation was repeated twice per bee. Bees were then fed and maintained in the dark for 24 h at 26°C and afterwards were represented to the labyrinth for a second test repeated as above.

6.2.2. Results and evaluation

There is no indication on the level of contamination to which bees were exposed. It is assumed that the contamination level is the same as the one described for the PER test. However, different concentrations were used in the PER test and therefore it is not possible to know what was the contamination level used in this study.

The results of this study are based on a small number of bees (n=4). In addition, no control group is used and there is no mention of any statistical analysis performed, given the small sampling size.

Given the above weaknesses and incomplete dataset (the authors mentioned that the study was still ongoing), it is not possible to support the conclusions drawn from the authors as reported at page 77 "however, the data indicate that bees contaminated with thiamethoxam dust experienced considerable difficulty in recovering the correct memory of the colour associated with reward… individuals treated with thiamethoxam recover memory of the wrong colour at the moment of making their choice".

6.3. Orientation in a complex labyrinth

6.3.1. Description

The study was conducted from June to August 2011. Bees were tested for effects on memory after oral treatment to clothianidin. For this experiment, bees were tested in a labyrinth with a 50% sucrose solution reward associated with a colour recognition (the distance between the hive and labyrinth was 50 m, in outdoor conditions).

Five trials were conducted on bees in three phases (*i.e.* a training phase with a sucrose-reward solution, a treatment phase where marked bees were administered 10 µL in trial 1 and 40 µL in trials 2-5 at 10 µg/L of clothianidin in trials 1-4 and at 20 µg/L in trial 5, corresponding to doses of 0.1 ng a.s./bee for trial 1, 0.4 ng a.s./bee in trials 2-4 and 0.8 ng a.s./bee for trial 5). The five trials were assayed to determine the best protocol for this test.

- **Trial 1**: bees which managed to get to the dispenser were marked and transferred in the lab where they were stunned with CO_2. Four groups were formed (2 treatment and 2 control groups). These bees were then placed in cages (15-20 bees/cage) and provided sucrose solution for 3 h. Then two groups of 125 bees each (control and treated) were released to detect any immediate effects and two other groups of 20 bees each (control and treated) were released 24 h later.

- **Trial 2**: No CO_2 was used to reduce stress in bees and the training was conducted as for trial 1. Therefore, the first 14 bees arriving at the dispenser (containing pure sucrose solution) to feed were marked as control bees and the 17 next feeding bees arriving at the dispenser (containing contaminated sucrose solution at 10 µg/L) were marked as treated bees.

- **Trial 3**: the training was conducted as in trial 1 and treatment as in trial 2. Bees which had fed at the dispensers (17 control bees and 19 treated bees) were collected, brought back to the lab and stunned with CO_2 for 30 minutes and marked. Bees were released in the labyrinth after 90 minutes. The total trial duration from capture to release was 120 minutes.

- **Trial 4**: the training was conducted as above and contamination as in trials 2 and 3. Bees which had fed at the dispensers (19 control bees and 20 treated bees) for 30 minutes, were collected, brought back to the lab and stunned with CO_2 for only 10 minutes and marked. Bees were released in the labyrinth after 30 minutes. The total trial duration from capture to release was 75 minutes.

- **Trial 5**: the training and contamination procedures were conducted as in trial 4. Bees which had fed at the dispenser (33 control bees and 34 treated bees) were collected, brought back to the lab and stunned with CO_2 for only 10 minutes. Bees were released in the labyrinth after 30 minutes. The total trial duration from capture to release was 75 minutes.

The time required to reach the labyrinth and the time to reach the dispenser in the labyrinth were recorded just after release and for a period of 3 h as well as after 24 h of release. A X^2 test was used to test the number of bees of the treatment and control groups which were lost, disoriented or which arrived at the dispenser. Differences among treatment groups were analysed by means of the t-test (or Mann-Whitney test).

6.3.2. Results and evaluation

This protocol is an adaptation of the protocol used and developed by Decourtye *et al.* (2009) in a view to improve it (*i.e.* to reduce time effect between treatment and control groups; to increase its suitability to warmer conditions such as those found in Italy, e.g. the setting needed to be moved outside the tunnel). This new protocol and trials allowed determining more optimal conditions for this test, *i.e.* to use a higher number of individuals of bees (30-70) and to reduce the duration of stress induced by CO_2 treatment and by confinement.

The doses provided to bees (in one single time) are comparable to the a.s. concentration found in drops of either dew or nectar (i.e. 15 µg/L). However the bees were fed in group and not individually. Although the concentration of the ingredient in the sucrose solution was fixed, it is not possible to state which dose was taken up by each bee also considering the variable size of the groups (15-20).

In trial 1, the marking of bees (control and treated and those released after 3 h and 24 h) was not distinct. Therefore, bees after 24 h were stunned again to be re-marked which may have induced a bias (additional stress) in the outcome of the results. A large number of trained bees, after contamination, failed in returning to the labyrinth after their release (60-80%) and therefore could not be included in the observations made in the labyrinth. For the remaining bees, no significant differences were found between control and treatment groups at the two times of observation (just after release and 24 h after) as well as no significant differences were reported in frequencies of bees returning to the labyrinth and time taken to return.

In trial 2, significant differences were found between control and treatment groups for the number of bees returning to the labyrinth (higher number in controls). However, the marking of the bees during the feeding phase at the dispenser may have been disturbing for the bees because the authors hypothesised that the marking may have started before the bees had finished filling up their honey sac. This bias may have resulted in an underestimation of the differences between control and treated bees at the dispenser.

In trial 3, many bees failed in returning to the labyrinth as in trial 1 and the authors explain this result by the use of CO_2 which disturbed the bees. However, effects seemed reversible within 6 days (bees from control and treatment groups returned to the dispenser after that period of time). No analysis was conducted on time taken to return (sample size was too small, n = 1).

In trial 4, a greater number of bees returned to the labyrinth (62%). The authors explained this result by the shorter duration to CO_2 treatment and experimental confinement. However, no significant differences were found between control and treatment groups in frequency and time to return to the dispenser.

In trial 5, a greater number of bees returned to the labyrinth (52%). No significant differences were found between control and treatment groups in frequency and time to return to the dispenser.

This study should be rather seen as a proposal for implementation rather than a definite testing protocol. Several factors may have caused bias in the obtained results (e.g. stress caused by CO_2 treatment) and there were restrictions linked to the protocol (e.g. due to inappropriate timing of the marking). Further investigation and refinement of the trial settings would be needed to make firm conclusions on the effects of the tested pesticides on bee orientation.

6.4. Homing and foraging in the field

6.4.1. Description

This study is the continuation of a previous study conducted in 2010 (*i.e.* two groups of bees exposed to 0.7 and 0.47 ng/bee of clothianidin) where effects were observed on foraging ability (time to return to the hive and visit frequency).

In this study, bees coming from one colony (6 frames of which 2 brood frames) were trained to search for a 40% sucrose solution placed in an artificial dispenser and bees were marked. Then, the dispenser was gradually moved to 150 m away from the hive and for 40 minutes, the number of visits per bee to the dispenser was recorded and 5 lots of 10 bees *i.e.* assiduous visitors feeding at the dispenser were captured for the testing.

Four protocols were run mimicking different scenarios of field exposures (from extremely low to high exposure):

- **Low exposure.** In the first protocol, each bee was administered 0.092 ng of contaminants in 5 µL of 40% sucrose solution and was allowed to fill in the rest of the honey sac with uncontaminated sugar solution. In this protocol, bees were immediately released and recaptured upon their return to be re-administered the same amount of contaminants as long as the bee managed to return to the dispenser.

- **Medium exposure.** In the second protocol, each bee was administered 0.47 ng of contaminants in 5 µL of 40% sucrose solution all at once, but the bees were not allowed to fill in the rest of the honey sac with uncontaminated sugar solution.

- **High exposure.** In the third protocol, each bee was administered 0.47 ng of contaminants in 5 µL of 40% sucrose solution in three times, but the bees were not allowed to fill in the rest of the honey sac with uncontaminated sugar solution.

- **Extremely low exposure.** In the fourth protocol, each bee was administered 0.552 ng of contaminants in 30 µL of 40% sucrose solution. In this protocol, bees could empty their honey sac after they had return to the hive.

In the first protocol, only frequencies of visits and returns were recorded. In the second and third protocol, the same parameters as in the first one were observed and in addition, flight duration and behaviours at the nest and at the dispenser were recorded. The observations made in the fourth protocol were not described. Time intervals at which observations were made after treatment were only described for protocol 3 (60 min, 180 min and 24 h after treatment). Video recordings were made on behaviour in protocol 3. Controls were fed with uncontaminated sucrose solution.

6.4.2. Results and evaluation

The design of this study is interesting and innovative. The environment in which bees forage is highly variable and trying to incorporate this variability to estimate variable exposure levels is certainly valuable. The remark in the conclusion on the inference of the effects observed at the individual levels to the whole colony, especially when it is massively involved in foraging activities, requires further evaluation. However, the conclusions made on the observed effects are not supported by statistics (no test and small sampling size).

Due to time constraint, only data for protocols 1 and 2 were presented and these data are incomplete (observations on behaviour in protocol 2 are still under analysis). No statistical procedure is described to analyse these data. However, on p 82, the homing behaviour recorded with a video camera is described and analysed with a Mann-Whitney U test that most probably, although not fully clearly, refers to protocol 2 since in Table 34 no controls are available for protocol 3.

Table 34 presents the number of bees (control *versus* treated with clothianidin) for the four protocols. These numbers do not correspond to the numbers presented in the methods (10 treated bees and 10 control bees/protocol). For protocols 3 and 4, these numbers are lower probably due to time constraints as stipulated by the authors. However, for protocol 1, these numbers are higher without explanation provided.

Authors reported that no abnormal foraging behaviours were noticed in controls. However, no quantitative data were shown.

Tables with raw data on frequencies of visits from protocols 1 and 2 are shown. However, protocol 1 does not present controlled conditions in terms of the treatment dose given to the bees and specification of the endpoints. Furthermore no statistical tests were performed on these data so it is not possible to conclude on the results obtained although sharp differences on total number of frequencies are reported between controls and treated groups (6.3 visits for treated groups versus 20 for controls and decrease of visits in time from 86.7% to 16.7% after 1 h).

Three graphs were made to show the frequencies of visits after 60 min, 180 min and 24 h for protocol 2. They present the median of the forage frequency for control and treated groups at the various timelines. The authors showed that foraging bees treated with clothianidin did not perform any foraging flight at 1 h after the treatment. A Mann-Whitney U test was mentioned as the statistical method used to analyse the data but no numerical results were reported (although reported for protocol 3, it is very likely that it refers to protocol 2). Authors claim that significant differences were found between treated groups and controls at 60 min and 180 min, while no significant differences were shown after 24 h, probably because of recovery. However, the sample size (9 treated/10 controls) is likely to be insufficient to allow the detection of any difference between control and treated groups. In addition, because of the poor reporting of the data collection and results of the tests, it is not possible to make any firm conclusions on these results.

The designs of the various protocols, as they stand, do not present controlled conditions. They seem to be still in an exploratory phase for the implementation of methods on the testing of effect of sub-lethal dose of pesticides on bee homing and foraging behaviour.

7. Synergistic interactions between stress agents and bee colony collapse

Observations made both in the field (bee mortality) and laboratory (amounts of genomic copies of DWV, Deformed Wing Virus) are summarised to highlight the possible synergism between bee mortality and pathogens such as DWV through disturbance of the bee immune defence under the pressure of nutritional deficiencies and sub-lethal doses of pesticides. However, data are shown on the effect of clothianidin on honeybees and *Drosophila* in laboratory conditions only.

The objective of this study is to determine the impact of clothianidin on honeybees' infection over time and to explore the underlying mechanisms on *D. melanogaster* (*i.e.* the immune response).

7.1. Study on honeybees

7.1.1. Description

The study on honeybees investigated the effect of topical exposure of clothianidin on bee mortality and DWV prevalence at 12, 24 and 48 h after treatment. In total, 8 groups of 30 bees were used (6 treated groups: 1 µl of acetone containing 3, 10, 20, 30, 40 and 50 ng of clothianidin/bee and 2 control groups: 1 µl of water and 1 µl of acetone/bee). All bees originated from a healthy colony and were taken at emergence from a brood frame placed in an incubator (34°C, 80% RH). Bees were provided with food (protein-containing sugar syrup provided *ad libitum*). DWV levels were quantified by RT-PCR on 5 live bees taken from each group at 12, 24 and 48 h after treatment and expressed as the mean of viral copies in each bee. In addition, mortality was recorded at 12, 24 and 48 h after treatment. The overall study was repeated twice. The experimental data were analysed with the Kruskal-Wallis and Dunn's Multiple Comparison Test.

7.1.2. Results and evaluation

Results on the interaction between clothianidin and the number of copies of DWV at the three different time periods highlighted a significant increase in DWV with increasing time at 10 and 20 ng/bee. Although the results were significant, the statistical test used to compare treated and control

groups was inappropriate (Kruskall-Wallis) since it is meant for comparing 3 or more groups (while only 2 are available here). Furthermore, it was not possible to assess how these conclusions were reached since the description of the analysis and testing was only provided in graphics and not in tables and numbers.

Results on mortality data and survivorship showed no survival after 48 h at concentrations of 30 ng/bee and above.

Results on mortality data and the deduced contact LD_{50} of clothianidin on honeybees showed a value of 18.89 ng/bee (*vs* 44.26 ng/bee as described in European Commission, 2005).

As reported by the authors in the introduction, *Varroa* influences the prevalence of the DWV, from innocuous latent infections to viral explosion. Its role in colony collapse needs to be elucidated as recently highlighted by Martin *et al*. (2012). The interaction between *Varroa*, DWV and pesticides such as the one studied here (i.e. clothianidin) merit further investigation.

7.2. Study on *Drosophila* transgenic lines

7.2.1. Description

The study on *Drosophila* investigated the Toll/IMD pathway (which mediates the immune system) by quantifying the fluorescence emitted by GFP expressed in transgenic lines of *D. melanogaster* treated topically with clothianidin. The GFP proteins are under the control of promoters of different genes coding for antimicrobial peptides (AMP). In the *Drosophila* transgenic line the GFP protein is fused to the AMP drosomycin which is under the control of the drosomycin gene (drs-GFP). An absence of reduction of the fluorescence produced by GFP is an indicator of an inhibition of the Toll pathway. For this experiment, third-instar larvae taken from a wild population reared at 25°C on an artificial diet were treated individually with 1 µl of acetone containing various concentrations of clothianidin (1-100 ng/larva) to define the LD_{50} (i.e. dead individuals counted after 24 h). Then, following the same protocol, new third-instar larvae were treated with clothianidin at a concentration = pre-defined LD_{50}, infected by mould with a tungsten needle, placed at 21°C for 4 h and finally observed by epifluorescence microscopy. Differences in GFP expression (expressed as percentage of larvae with intense fluorescence) were analysed with a Chi-square statistical test.

7.2.2. Results and evaluation

The sampling size (i.e. the number of larvae tested in total and by treatment group) was not described.

Results on mortality and the deduced contact LD_{50} of clothianidin on *Drosophila* showed a value of 42.53 ng/larva.

Results on the drs-GFP expression show that only one clothianidin concentration (i.e. 40 ng/larva = LD_{50}) tested, showed a significant difference with controls. However as in the study on honeybees, few details were reported on the experimental settings, the statistical analysis and the numerical results making difficult the appraisal of the internal validity of the study.

This study concluded in favour of a clear effect of clothianidin at 40 ng/larva on the immune response of *D. melanogaster*.

Although the mechanisms reported in this model are very interesting, it was unclear how they could apply to honeybees.

Finally, an interesting point raised by the authors was the variability of responses obtained by honeybees treated by pesticides which may be explained by different levels of bee infection before tests are conducted.

CONCLUSIONS

Following a request from the European Commission, EFSA evaluated the scientific information carried out within the Italian funded project named APENET. APENET is a multidisciplinary project, including monitoring activities and several scientific researches with the main objective to investigate the dust emission generated during the sowing of maize coated seeds with thiamethoxam, clothianidin, imidacloprid or fipronil, the bee exposure to this dust and to evaluate the effects following this exposure.

As regards the monitoring network for the bees health, EFSA noted several gaps in the data provided (*i.e.* the total numbers of stations, apiaries, hives were not presented). Moreover, the reasons behind the sampling plan chosen and the conclusions taken without presenting the levels of representativeness and uncertainty of the estimates obtained was not reported. Some important pathogens have not been included in the sampling plan. The chemical analysis of pesticides in different matrices was not reported per each active substance. An appropriate analysis of the results was also difficult due to the lack of environmental characteristics such as the agricultural landscape around the sampling points or the weather conditions.

As regards the dust dispersal during coated maize seed sowing, several deficiencies were observed in the data as provided in the APENET 2011, and it was not possible to analyse in detail the results and to conclude on their robustness. Nevertheless, it was noted that the dust, and as a consequence the soil deposition of thiamethoxam, clothianidin, imidacloprid and fipronil residue in the off-crop area, decreases with the distance. No decrease with the distance was apparent in air concentrations. When seeder machine were modified to reduce the dust release, the reduction of the dust deposition onto soil was in the range of 74.4-95.5%, while the reduction of air concentration was in the range of 53.1-96%.

When honeybees were exposed in field to dust from a conventional seeder, lethal effects were observed. Due to deficiencies in study design with the modified seeder (*i.e.* prototype 2), it was not possible to make any firm conclusion as regards the effects on honeybees exposed to dust from this machine.

When learning and memory abilities were tested on bees exposed to contaminated dust emitted from unmodified and modified machines, significant effects were observed, but data gaps identified in the study and statistical designs did not allow to validate these conclusions. Finally, regarding studies assessing orientation and homing behaviour in the field or in labyrinths, they were seen still as in an exploratory phase from which no firm conclusion could be drawn. However, it was recognised that the proposed protocols were innovative and deserved further development.

The interaction between DWV and clothianidin highlighted the need for further investigation, in particular on the underlying mechanisms. However, based on the data provided, the observed significance of the trends obtained could not be confirmed.

Overall, it was not possible to draw a firm conclusion on all the scientific information in the APENET report, due to some deficiencies in the study designs and weakness in the statistical analysis and conclusions drawn as reported, or incompleteness in the reporting of results. However, within this project some potential concerns such as lethal effects on bees exposed to dust, sub-lethal effects and interactions between clothianidin and pathogens were identified suggesting that a change in the assessment of the substances thiamethoxam, clothianidin, imidacloprid and fipronil as regards their effects on bees might be required.

EFSA recently received a mandate from the European Commission for scientific and technical assistance and was requested to provide an EFSA conclusion with an updated risk assessment to bees for the neonicotinoids thiamethoxam, clothianidin, imidacloprid, acetamiprid and thiacloprid. The results for the neonicotinoids investigated in the APENET project might be re-considered, within this mandate, provided the identified deficiencies of the reports will be addressed. For this purpose the

papers mentioned in the report and in the process of being published, might be useful. However, since fipronil does not belong to the neonicotinoids, it will not be considered in the new mandate.

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ABBREVIATIONS

µg	microgram
µL	microlitre
a.s.	active substance
ABPV	Acute Bee Paralysis Virus
AIV	Apis Iridescent Virus
AMP	antimicrobial peptides
ANOVA	ANalysis Of VAriance
ASL	Azienda Sanitaria Locale
BQCV	Black Queen Cell Virus
CBPV	Chronic Bee Paralysis Virus
COLOSS	Prevention of Colony LOSSes network
CRA-API	Consiglio per la Ricerca e la Sperimentazione in Agricoltura – Unita di Ricerca di Apicoltura e Bachicoltura
CRA-ING	Consiglio per la Ricerca e la Sperimentazione in Agricoltura – Unita di Ricerca per l'Ingegneria Agraria
d	day
DAR	Draft Assessment Report
DiSTA-UNIBO	Dipartimento di Scienze e Tecnologie AgroAmbientali – Universita di Bologna
drs	drosomycin
DWV	Deformed Wing Virus
EU	European Union
FS	flowable concentrate for seed treatment
g	gram
GAP	good agricultural practice
GFP	green fluorescent protein
h	hour(s)
ha	hectare
IAPV	Israeli Acute Paralysis Virus
IMD	immune deficiency
IZSVe	Istituto Zooprofilattico Sperimentale delle Venezie
KBV	Kashmir Bee Virus
kg	kilogram
L	litre
LD_{50}	lethal dose, median; dosis letalis media
LOD	limit of detection
LOQ	limit of quantification
m	meter
mg	milligram
min	minute
mL	millilitre
MS	Member State
ng	nanogram
NOEL	no observed effect level
PER	proboscis extension reflex
ppb	parts per billion
RH	relative humidity
RT-PCR	reverse transcription polymerase chain reaction
s	second
SBV	Sacbrood Virus
wk	week
yr	year