Toxicity of imidacloprid feedings on honeybee colonies

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In Europe, weaknesses or losses of honeybee hives have been noticed for many years. Many factors influence hive population: environmental conditions including weather and pollutants, pathogens and use of bee races more or less adapted to local conditions. In France, the systemic insecticide Gaucho^{NT} is used as seed coating since 1994. Its active ingredient – imidacloprid – and its metabolites have been accused to be responsible of bee mortality. In order to test this hypothesis, colonies have been fed with various concentrations of imidacloprid in syrup during a full year and their summer development and winter survival have been followed and compared. Groups of 8 hives each were fed with: syrup alone, syrup supplemented with imidacloprid at a concentration of 0.5 ppb, or syrup supplemented with imidacloprid at a concentration of 5 ppb. A fourth negative control group was not fed.

Parameters to assess hive activity were: number of returning bees at 2 p.m., presence or absence of pollen brought back by bees, bee mortality outside entrances, colony weight, honey production, capped brood area size, hive population evaluated with the number of inter-frame occupied by adult bees. The other factors were: brood quality, presence of eggs and queen cells, pathologies such as acarapisosis, nosemosis, varroosis, american foolbrood, european foolbrood, chalkbrood, chronic bee paralysis. Multi-residual analysis were conducted in order to assess chemicals in wax. Specific analysis was conducted in honey and syrup to quantify imidacloprid.

These parameters followed the same trend in all groups. At the beginning of experiment (July), activity at hive entrance was high (ranging from 10 to 44 entering bees per minute) and then decrease to 3 to 30 bees per minute in December. Colony population increased until early August ranging from 8 to 19 occupied inter-frames and then decrease to a range of 7 to 11 inter-frames in September. An overall decrease of capped brood area size was observed from 10.5 to 79.8 dm^2 at the beginning to 0.5 to 19.8 dm^2 in late October. The statistical analysis did not indicate any negative influence of syrup supplementation with imidacloprid despite the use of very high dose (5 ppb). Other parameters – mortality, colony weight, pathologies including varroosis number – did not exhibit any significant difference between treatments.

Imidacloprid determination using HPLC and GC/MS in several matrices

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Imidacloprid is a new insecticide-acting active principle with a wide range of action. In fact, it is used not only to kill sucking insects –such as whiteflies and aphids– and chewing insects, but also to control termites and insects infesting domestic animals. Honey bees are very sensitive to this substance, so, in order to verify its possible presence in cultivated lands, two techniques allowing to detect it in several matrices both of animal and vegetable origins have been developed. According to the first one, honey bees (dehydrated or in their natural state) are extracted with dichloromethane, by ultrasonic extraction; the extract is concentrated in a rotavapor concentrator and purified in a small column containing florisil®. Then, an elution is carried out with two solutions: ethyl acetate/n-hexane (80/20) and acetonitrile that is dried in a rotavapor concentrator and to which 2 ml of acetonitrile are added. The extract obtained is analyzed by HPLC with a UV detector. The quantification of imidacloprid is determined through the external standard method, with a detection limit of 50 ppb.

However, developing another technique has been necessary because, as widely reported in the literature, imidacloprid can be often found in the environment not as a simple molecule but as a group of degradation products, many of which are toxic. These observations have led to the application of a method able to quantify all these species as oxidation products. Thanks to the results achieved, it is now possible to determine the initial quantity of imidacloprid through a conversion factor. According to this method, imidacloprid and its degradation products are extracted from the matrix (filters made from paper, bees, grass, flowers, etc.) by using a water-methanol mixture in acid conditions by sulphuric acid. Then, if necessary, the extract is cleaned of the lipidic fraction by hexane extraction. The sample is further cleaned up in a column filled with XAD 4 resin. Then, it is oxidized at a high temperature by potassium permanganate in basic conditions by sodium hydroxide. In these conditions, both imidacloprid and its degradation products are completely oxidized to 6-chloronicotinic acid that is then extracted with methyl *tert* - butyl ether (MTBE), dried on a rotavapor and to which is added acetonitrile. With the gas chromatography-mass spectrometry detection it is necessary to derivatize the molecule with MSTFA (N-Methyl-N-(trimethylsilyl) trifluoroacetamide). The compound obtained 6-chloronicotinic acid trimethylsilylester can be

easily identified in the chromatogram due to the characteristic fragmentation.

In this process, the quantification is carried out by the external standard method, as well. The chromatographic analysis could detect quantities of 10 ppb derivatized 6-chloro nicotinic acid. The linearity was excellent (R=0,999) over a wide concentration range (10 ppm ÷ 10 ppb).

The data obtained from the analyses of several matrices (filters made of paper applied to the sowing machine, grass, flowers, bees, etc.) have allowed us to determine imidacloprid dispersion in the environment during the period of the sowing of imidacloprid-treated corn.

A method to feed bees known amounts of pesticides

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To study the effect of pesticides on bees in the laboratory, bees must be individually fed measured amounts of test solutions. We devised a method to feed bees individually, and tested it against two other available methods on three bee species, *Apis mellifera* L., *Osmia lignaria* Say and *Megachile rotundata* (Fabricius).

Method 1. Film canister - bees were individually transferred to black film canisters with a small hole drilled on the side of the canister near the base. The test solution was pipetted onto a microscope slide next to the hole.

Method 2. Glass vial - bees were individually transferred to 12-ml glass vials with plastic snap caps. The test solution was injected into a segment of plastic tubing, fitted snugly into the plastic snap caps.

Method 3. Flower - a tiny ampoule, made of polyethylene tubing was inserted into the calyx of a flower, whose reproductive column had been previously removed with forceps. The test solution was pipetted into the ampoule. Flowers and bees were individually housed in ice cream cups covered with a lid. To facilitate flower manipulation, we used large flowers with open corollas (*Prunus avium*, *Vinca minor* and *Convolvulus arvensis*).

In all three methods, a 10 µL-drop sucrose test solution (25% vol.) was offered to the bee for one hour. We tested each method under four different light conditions: 1. Natural light – outdoors; 2. Artificial light – 15W Cool White Sylvania® fluorescent tube; 3. Light stimulating plant growth – 20W Gro-Lux/Aquarium Standard Sylvania® fluorescent tube; 4. Complete darkness.

A. mellifera workers were captured in the morning at the hive entrance and brought to the laboratory, where they were chilled for a maximum of half an hour at 4°C prior being assigned to the different feeding methods. *O. lignaria* and *M. rotundata* female cocoons were incubated until emergence. Upon emergence, females were starved overnight and then assigned (no chilling was necessary) to the different feeding methods. For each bee species, 20 individuals were tested with each feeding method (3) and light regime (4).

For all bee species, Method 3, the flower, was the most effective: 90-95% of the bees fed under natural light, 80-95% under artificial light, 75-100% under light stimulating plant growth, and 45-70% in darkness. Percent success was 0-60% with the glass vial method, and 0-50% with the film canister method.

In conclusion, Method 3, the flower with the polyethylene tubing insert, is a simple, yet superior, feeding device to very successfully feed bees known amounts of test solutions. Because a trained worker can prepare 100 test flowers in 40 minutes, and, because of its success rate, the method actually saves time and bees. This method could help standardize oral tests on the effect of pesticides on bees in the laboratory.

A tentative method to evaluate behavioural effects of pesticides on honey bees: the proboscis extension response

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Risk assessment of chemical pesticides on honey bees is based on a sequential scheme including laboratory, semi-field and field evaluation. These methods mainly take into account the survival of bees exposed to pesticides. Besides mortality, various aspects of the honey bees behaviour may be affected by sublethal doses of pesticides. Among the bees of a colony, foragers are the most likely to be exposed to chemicals. The foraging behaviour is known to rely on a conditioning process, floral cues being associated to the food, memorized and used for flower recognition during the following trips. The conditioning process occurring on the flower can be reproduced under laboratory conditions, using the olfactory conditioning of the proboscis extension response (PER), on restrained individuals. The classical odour conditioning of the PER is based on the temporal paired association of a conditioned stimulus (odour) and an unconditioned stimulus (sucrose solution). Bees can exhibit the

PER as a conditioned response to the odour alone after even a single odour-sucrose solution presentation. This procedure allows standardised recordings of behavioural responses and provides information on learning and memory processes.

The PER bioassay has been adapted to the screening of the effects of a range of chemicals at sublethal concentrations. It was applied to 15-day-old bees surviving a diet contaminated with pesticide over 11 days. This is an attempt to simulate what young hive bees could experiment when feeding on contaminated stored food, before becoming foragers. The PER procedure allowed to establish threshold concentrations above which a significant decrease in olfactory learning abilities is observed. The PER assay also enables comparative studies of responses to different chemical treatments (organochlorine, organophosphorus, pyrethroid, imidazole, phenylpyrazole, carbamylpyrazole).

The laboratory conditions can be considered as worst case conditions, which do not reflect the natural conditions. Therefore, we were concerned about testing the PER after more realistic exposure conditions in a standard crop protection agronomic system. Bees foraging on treated and control crops have shown differences in a PER assay under laboratory conditions. These preliminary results indicate the possibility to subject the bees to the PER assay after an exposure to chemical pesticides under agronomic conditions.

Acute contact toxicity on honey bees – two answers for two questions

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To assess the acute contact toxicity of pesticides to Honey Bees, OECD Guideline 213 or EPPO 170 Guideline for the evaluating of side effects of plant protection products are taken into account. The recommendation of the guideline is to apply test items to the dorsal side of the thorax of the bees at a volume of 1 µL.

One of the aims of our experiments was to investigate, whether there is a difference in sensitivity of dorsal as compared to ventral application to the honey bees. The other experiments should give us an answer to the question whether there are differences in the impact of chemicals and the applied droplet size.

Two independent sets of experiments were conducted in summer 2001. One set of experiments with a dorsal and ventral application of 4 doses of Dimethoate in parallel. The second set of experiments with applications of 3 different droplet sizes (1, 2 and 5 µL). Each experiment was conducted twice. Perfekthion EC (400 g/L Dimethoate) was used as the reference substance (containing 1 % wetting agent) at 4 dose rates (0.30, 0.20, 0.15 and 0.10 µg a.i. per bee). During application the bees were anaesthetised with CO_2. Control bees were anaesthetised and treated with water/wetting agent only. During the test the bees were exposed in incubators at 25 °C and 48 – 60 % rel humidity. Mortality was assessed after 2 hrs, 24 and 48 hrs following the applications.

According to the present results, applying a droplet to the ventral side of the bee thorax seems to have a stronger effect to the bees than to the dorsal side. On the other hand the droplet size does not influence the extent of the effect of chemicals.

The effects of Insect Growth Regulators on honeybee populations

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The effects of IGRs on colonies is usually assessed over a relatively short period and investigates the toxicity to honey bee larvae or pupae. The aim this project was to assess the longer term effects of fenoxycarb, a juvenile hormone agonist, and diflubenzuron, a chitin synthesis inhibitor, fed to honey bee colonies on the fate of the treated colonies over the following year. Assessments were also made on the effects on the viability of immature queens, i.e. egg production.

IGRs were diluted with sucrose to a rate equivalent to their maximum application rate on flowering crops. The effects of exposure to the treatments was assessed in brood up to 8 weeks after treatment. In addition all colonies were generally assessed prior to the day of test item application and 2 weekly after the application until November and then monthly until a year after treatment. The mean replacement/removal for the eggs marked over the 5 week period was 19% in the controls, 47% (max 60%) in the fenoxycarb treated and 44% (max 95%) in the diflubenzuron treated. Significantly greater numbers of sealed pupae did not emerge in the fenoxycarb treated colonies and in many cases these cells were present for several weeks. Colonies treated with fenoxycarb declined during the season earlier than the control and started the season slower; one fenoxycarb treated colony failing to survive over the winter. The fenoxycarb treatment resulted in significantly lower numbers of bees and brood in the month after treatment and affected the development of the colonies in the following spring. Colonies treated with diflubenzuron resulted in a short term reduction in the numbers of adult bees and brood after treatment when compared with controls. There was no significant effect on development of brood the following spring but there did appear to be a slower expansion when compared with

controls.

Queens reared from grafted larvae were allowed to pupate in the reared colonies and each pupa was then transferred to an Apidea supplied with fondant containing the IGR at a rate equivalent to the application rate. Two to three weeks after emergence the number of mated queens was determined by recording egg production. The number of queens which successfully mated and laid eggs was 6/11 in the control, 9/12 in the diflubenzuron treated and 0/11 in the fenoxycarb treated. In the fenoxycarb treated 9/11 queens were present but showed virgin queen characteristics, e.g. small abdomen, suggesting they had not been mated.

The results so far show effects of fenoxycarb on the colony both in the short term and the following spring. Fenoxycarb also appears to have severe effects on queen viability. Diflubenzuron has effects on colonies in the short term but effects the following spring are less severe than after fenoxycarb exposure and no effects were observed on queen viability. The next stage is to assess the impacts of the ecdysteroid agonist tebufenozide and the ecdysteroid antagonist azadirachtin. This will ensure comparable data on effects are collated for the major types of IGR pesticides which are or may be used in the UK.

Sub-lethal effects in honeybees: their significance and use in pesticide risk assessment

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There is increasing concern, particularly amongst beekeepers, that sub-lethal effects may have significant impacts on honeybee colonies. Current assessment of risk to honeybees is based on mortality in laboratory studies and, for more toxic compounds, mortality in semi-field or field studies. The design of studies is the subject of both OECD and EPPO guidelines with emphasis on the mortality of adult bees and confirmation of bees foraging in the crop. There is no guidance on what sub-lethal effects should be recorded and what significance they might have in risk assessment.

A wide variety of sub-lethal effects have been reported in bees following exposure to pesticides, many occurring at doses well below estimated exposure levels following field application at recommended rates. Such effects include developmental and morphogenic effects in larvae and adults, reduced egg laying, failure to re-queen, reduced longevity, reduced foraging, changes in communication of food sources and homing behaviour, alterations in nest-mate recognition resulting in exclusion of returned foragers from the hive and repellency. The effects are dependent on the dose and route of exposure.

To be able to take sub-lethal effects into account in risk assessment the correlation between laboratory observations and effects in semi-field and field studies and longer term consequences of these changes e.g. effects of reduced lifespan on over-wintering survival need to be more fully understood.

Greater attention should be paid to sublethal effects in the laboratory. This is particularly important for compounds which may not otherwise undergo higher tier testing, due to their low acute toxicity or low application rates, but may result in effects at the colony level. Semi-field and field studies should routinely include observations of behaviour and activity levels at the hive entrance, full colony assessments both at the termination of the trial and delayed effects as well as the behaviour of foraging bees as all of these impact on colony development and survival. The longer term consequences of sub-lethal changes in colonies e.g. over-wintering survival, should also be assessed.

Microencapsulated fenitrothion and chlorpyrifos ethyl on *Apis mellifera* L.; a synopsis of research carried out on apples

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Laboratory and field trials have been carried out to evaluate the side effects of the microencapsulated insecticides Fenitrocap, IPM-400 (a.i. fenitrothion) and Pyrinex (a.i. chlorpyrifos ethyl) on *Apis mellifera* L.. Preliminary analysis of the characteristics of microencapsulated insecticides show the following aspects: the average size of capsules diameter was 12.9 micron for IPM400/Fenitrocap and 9.8 for Pyrinex; with regard to <10 micron class, the highest number of capsules is obtainable. The drift of capsules (IPM400) in the air was observed up to 15 m distance from the treated orchards; furthermore the spore-traps indicated that the capsules remained in the air for about 7 hours after the treatment. On microscope slide the persistence of IPM400 and Pyrinex microcapsules was detected within 18 days after treatment whereas on treated vegetation it was observed up to 12 days after treatment. When there was no blooming in the field, the treated microcapsules were not attractive towards foraging honeybees. The pollen collected by foraging honeybees became contaminated by microcapsules only when the bees foraged on flowers previously sprayed with the microencapsulated formulations. When the bees foraged on treated flowers, capsules could

be detected in their digestive apparatus up to 14 days after the treatment.

In laboratory foraging honeybees have been exposed to Fenitrocap and Pyrinex by ingestion and topical treatments, and regression line and LD50 have been accordingly determined. Field investigations have been conducted applying the microencapsulated insecticides on a *Phacelia tanacetifolia* crop before and during blooming, and on an apple orchard with and without mowing before treatment and with and without a repellent.

In laboratory exposure to the encapsulated formulations Fenitrocap and Pyrinex has resulted to be less toxic both topically and by ingestion compared to the exposure to an emulsifiable fenitrothion formulation. Open field applications of insecticide on *P. tanacetifolia* crop have shown that adult honeybees mortality rate was lower before blooming whereas during blooming it was higher than usual only within 48 h after treatments. Field investigations on apple orchards treated with IPM400 and Pyrinex have shown a remarkable adult mortality and the reduction of some productive parameters only when lawn was blooming. Further field investigations on apple orchards treated with microencapsulated and a repellent during blooming are discussed.

Poisoning incidents involving honeybees in Germany (1999-2002) and new problems for beekeeping

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The number of poisoning incidents involving honeybees which have been reported in Germany within the last 10 years was on average 82 per year. This is only 25 % of the yearly reported poisoning incidents 25 years ago. About 25 % of the damages are caused by deliberate poisoning and for another 25 % there is given no information about the cause of the incident. The share of these groups of the total number of incidents remains constantly. Therefor it is supposed, that the number of yearly happening incidents in Germany cannot be lowered importantly.

The incidents, which are caused by the use of plant protection products are distributed among different cultures. But only in rape and fruit cultures there are happening yearly important poisoning incidents, while the involvement of other cultures (vine, potatoes, broad beans, peas, cereals) changes from year to year. While incidents in potatoes are increasing those in vine are decreasing. Depending on changes in the registration of plant protection products in Germany the involvement of active substances in the origin of the incidents is also changing.

Although the number of poisoning incidents in Germany has remained on a low level in the last years, there is arising a new problem for beekeepers: residues of plant protection products in honey. Most of the beekeepers are keeping their honeybee populations in order to yield honey. Honey is a foodstuff of a special reputation and any residues are unwelcome. In 2001 there were detected residues of plant protection products in honey samples originating from fruit and rape growing areas. In Germany honey is belonging to animal foodstuff and not to vegetable foodstuff. Therefor for most of the active substances of plant protection products are existing no maximum residue limits regarding honey. Because of that the regulations of the decree for maximum residue limits are not valid for honey, as well honey is not considered in the different EEC-guidelines which are concerned with residues of plant protection products in vegetable foodstuff. According to the regulations of the German law for foodstuffs and household essentials honey has to be prepared that way that consumers are not put at risk. But in the mostly bad informed sensational press every residue in honey is put on a level with poisoning, but in fact the level of the detected residues is without any importance for human health. As consequence beekeepers have problems to sell their honey and they are asking for investigation of residues in honey when they are indicating poisoning incidents.

Although there had been no risk for consumers up to now by residues of plant protection products in honey BBA has started 2001 a testing programme in rape and fruit which shall lead to fixed maximum residue limits in honey.

Studies to improve the performance of dead honey bees collection traps for monitoring bee mortality

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The number of dead bees found in collection traps is an important criterion for estimating the hazards of pesticides to bees. In spite of this fact, the dead bee count through the use of this traps is underestimated because the number of dead bees lost in the field and during the return flight to the hive is not known. The solution would consist in having a continuous mode of monitoring by electronic devices, but the high cost of using these devices limits their application.

The dead bee collection traps are usually used in bio-

surveys of pesticides pollution, where the mortality rate is controlled on a weekly basis. Our research group, with the aim of avoiding the dead bees disappearing from these traps due to predators, has been studying for some time, a different type of trap that may solve this problem. Such trap (named "barrier trap") applied directly to the hive's entrance is divided into two parts. The upper one, a V shaped structure directly attached to the hive's entrance, impedes the export of dead bees by the undertaking bees, forcing them to pass through 6.5 mm diameter holes 5 cm from the floorboard. This way the undertaking bees are obliged to carry the dead bees into the underneath section of the devise, designed to collect dead bees and allow the alive ones to exit, through the 6 mm holes. Twelve hives were used in the experiment. Six were placed in a rich spontaneous botanical species area, a suitable refuge for a numerous small animals (complex environment), while the other six hives were placed in a cultivated area (simplified environment). Three hives from each area were fitted with the experimental traps "barrier traps" and other three with the "underbasket" cages, consisting of a wooden frame (50 x 100 x 10 cm) covered above and below with wire mesh. The top meshes had a hexagonal configuration with a side length of 2 cm and the bottom ones had rectangular with a 3 mm side length. The trials were carried out over two weeks in the Spring and Summer periods. The next trials will take place in Autumn. For each day of the trials, 20 dead bees marked with a different colour were introduced to each hive. The weekly count of dead bees per trap was made on the eighth day.

The average efficiency of the two traps, "barrier trap" *vs.* "underbasket", in the two environments, was respectively, for the Spring period, of 84.4% *vs.* 53.8% in the complex environment and 77.0% *vs.* 56.8% in the simplified environment. For the Summer period, the results were respectively: 88.1% and 29.5% in the complex environment and 77.4% and 4.8% in the simplified one.

This preliminary data allows us to forecast an efficient performance of the "barrier traps" in comparison to the "underbasket". Subsequent trials are being made to increase the devices' application and efficiency.

Method to evaluate the interference of pesticides on pollination activity of *Bombus terrestris* in protected cultures

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Present work is referring to a new method, used by the authors, to verify the effect of pesticides on pollination activity of *Bombus terrestris*, in protected cultures.

Results obtained from some experimental trials devoted to evaluate limits and capabilities of use of the following insecticides imidacloprid, indoxacarb and spinosad, in presence of the above mentioned pollinator, are reported.

Tomato is the species that, for several aspect, demonstrates to be the most suitable for such trials.

Some fundamental aspects on which is based the proposed methodology as, minimum area requested to the trials, choice of cultivar, synchronized opening of all hives used during experiments, continuous monitoring of flight activity, are discussed.

The effect of antagonistic micro-organisms on the brood of honeybees and bumblebees

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Several plant pathogenic fungi enter the plant trough open flowers. Spores of antagonistic micro-organisms present on the flowers can successfully compete with the possible pathogens. Honeybees and bumblebees can be used for transporting these antagonistic micro-organisms from the hive into flowers in order to prevent infections of the flowers. It is unavoidable that this antagonistic material also enters the brood nest of the bees. Since a healthy brood nest is an essential stimulant for the foraging activities of the bees and knowing that fungi and yeast may be able to cause infections of the brood, the effect of two antagonistic micro-organisms is tested on the honey bee and bumble bee brood.

Eggs and larvae were contaminated and during the larval and pupal phase, samples were taken and checked for the presence these antagonistic micro-organisms.

The antagonistic micro-organisms were not found in the samples and no significant changes in the broodnest were observed. These results show that antagonistic micro-organisms that were used do not develop in the brood nest of honeybees and bumblebees and that these antagonistic micro-organisms can safely be spread by honey bees and bumble bees to flowers to prevent infections.