Survival rate of honeybee (*Apis mellifera*) workers after exposure to sublethal concentrations of imidacloprid

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Imidacloprid is a commonly used systemic insecticide which can induce several sublethal effects. Previous research has not shown any increased mortality in bees that were fed with sublethal doses. However, there is very little research conducted with the focus on survival rate of honeybees in the field. The aim of this study is to assess the influence of imidacloprid on the survival rate of honeybees under field conditions. Honeybees from different colonies were administered a single dose of imidacloprid of 0, 0.07, 0.7, 7 or 70 ng per bee. From each concentration one group was kept in the laboratory to assess lethal effects and one group was returned to the hive to assess possible sublethal effects. The surviving bees were counted regularly during 4 weeks. Analysis has shown no difference in survival rate between treatments in the laboratory. There is a difference between the 70 ng treatment and the control group in the field ($P<0.0005$), but because this treatment was conducted on only two colonies, this result can not be generalized. Therefore, increased mortality due to sublethal effects of imidacloprid is not shown in this study.

Keywords: honeybee, *Apis mellifera*, sublethal effects, survival rate, imidacloprid, systemic insecticides

The past decades show an obvious decline in honeybees. From 1947 to 2008 the honeybee population in the United States decreased with 60% (vanEngelsdorp *et al.* 2008). Also in Europe there is an obvious decline (Biesmeijer *et al.* 2006). There does not seem to be a specific cause for the almost worldwide decline of honeybees, but there are probably multiple factors that contribute to the weakening and increased mortality of colonies. Possible causes are the introduction of exotic parasites, such as varroa, that undermine the vitality of the colonies and indirectly increase mortality (Martin 2001), decline of foraging resources (Biesmeijer *et al.* 2006) and the use of pesticides in agriculture (Oldroyd 2007, Bortolotti *et al.* 2003).

New pesticides are regularly developed to protect crops against parasites and diseases. Before the introduction of a new product on the market, the lethal effect on bees will be measured by determining the oral and contact LD_{50}-values in laboratory tests and during semi-field experiments (EPPO 1992). However, this LD_{50}-value only gives information about acute toxicity in comparison to other pesticides, but does not say anything about possible adverse long-term effects. It is known that low concentrations of pesticides do not induce acute mortality, but may result in sublethal effects.

Products from a relatively new and currently commonly used group of insecticides, neonicotinoids, are far more toxic than formerly used insecticides, so a much lower concentration is needed to induce the same effect. Because the used concentration is very low, these products are not instantaneously toxic to honeybees in the field. Low concentrations can, however, cause sublethal effects which are usually not seen in laboratory LD_{50}-tests. A well-known insecticide from the group of neonicotinoids is imidacloprid, commonly used as a seed dressing in agricultural field crops or as drench in greenhouses.

Imidacloprid is a systemic insecticide and is therefore, after being absorbed by the roots, translocated to all parts of the plant, including the nectar and pollen. Since the introduction in 1994 it has been used as seed treatment for several crops, like sunflower and maize. Several beekeepers in France noticed unusual honeybee mortality after foraging on sunflowers, and blamed the use of the new systemic pesticides. Because of this accusation, several studies focussed on the sublethal effects of imidacloprid on honeybees, such as impaired olfactory memory (Decourtye *et al.* 2004) and learning performance (Ramirez-Romero *et al.* 2005), and decreased mobility of bees (Medrzycki *et al.* 2003). Such effects are not likely to be detected in the current registration procedure.

Although research has not yet given a complete answer to the question wether imidacloprid is responsible for increased honeybee colony losses, most researchers agree that the registration procedure must be adapted to the new generation of pesticides. Because sublethal effects are not assessed in laboratory tests but are likely to lead to mortality under field conditions, it is important to develop a method to measure mortality due to sublethal effects and integrate such a method in the registration procedure for new insecticides. A possible method is to focus on the survival rate of honeybees in the field, after exposure to imidacloprid. The aim of this study is to assess the influence of imidacloprid on the survival rate of honeybees under field conditions.

MATERIAL AND METHODS

Apiary

For this study four honeybee (*Apis mellifera mellifera*) colonies were used (A, B, C and D). The colonies, held in 10-frame hives, were installed at the research site

of Wageningen University and Research centre with 10 m distance between the hives. Four days before the start of the experiments, each hive was provided with a Muenstertrap (Illies *et al*. 2002), to collect dead bees that were removed from the colony by undertaker bees. The colonies had not been treated against parasites or diseases for at least 6 weeks before the start of the study, nor during the study. The colonies were fed with extra sugar dye ad libitum.

Animals

From each colony four groups of 100 bees were collected. From two colonies each 100 extra bees were collected (colonies C and D). The bees were held in a flight cage for at least 1 h, then they were put in small metal cages in groups of 10 bees per cage. Each group was fed with a certain concentration of imidacloprid. After feeding, the bees were sedated with carbon dioxide to ease handling. From each group 50 bees were transferred to small cardboard cages (10 bees per cage) in a climate room (25 ± 2 °C and 80% relative humidity) and 50 bees were marked with acryl paint on their thoraxes and returned to the colony. The experiment started with one colony every week, for 4 weeks, on: 28 April, and 5 , 11 and 20 May 2009.

Experimental feeding

Admire® (70% imidacloprid) was administered to four replicate groups of 100 bees by ingestion at four concentrations: 0.07, 0.7, 7 and 70 ng imidacloprid per bee per group. Each concentration was administerd as a single dose (10 µl per bee). The concentration of 70 ng per bee was administered to bees of only two colonies. The feeding solution for control groups was 50% sucrose, the same solution was used for dissolving Admire® to various concentrations for the experimental groups. The bees were provided clean sucrose solution ad libitum after at least 30 min after they consumed the entire experimental solution.

Observations

The remaining marked bees in the colonies were visually counted at several times after the treatment for 4 weeks. Dead bees in the Muenstertrap and in the cages in the climate room were counted as well. Dead bees in the cages in the climate room were removed after each observation. The bees were counted three times a week for the first 2 weeks after starting the treatments, and two times a week for the last 2 weeks.

Statistical analysis

The Cox proportional hazard model was used to compare data (*i.e.*, hazard rate) from treated groups with control groups. Dead bees that were found in the Muenstertrap were recorded as censored, whereas bees that were found alive in the Muenstertrap were recorded as failures. Bees that died during handling or that escaped from the cages in the climate room, were recorded as censored. Data for laboratory and field observations were analysed separately, treatment and colony were entered as covariates.

RESULTS

After feeding the experimental solution, sublethal effects such as decreased mobility, trembling and black-out were observed in the 7 and 70 ng treatments. The survival probability for bees in the laboratory is shown in Fig. 1A. Colony A was not analysed for the laboratory observations. Due to a problem with leaking feeding tubes in the cardboard cages, too many bees had to be entered as censored events. The results for the remainder colonies showed no significant differences between treatment and control groups.

Significant differences between the control group and treatments were found in the colonies in the field. The P-values and hazard ratio's are shown in Table 1, Fig. 1B shows the survival probability for the bees inside the colonies in the field. Over all colonies, a significant difference was only found between the 70 ng treatment and the control group (P<0.0005) with a hazard ratio of 3.069. Marked bees from all treatments except 70 ng were seen carrying pollen loads.

Table 1. P-values and hazard ratio's for significant differences between treatments.

Colony	Treatment	P	HR
B	7 ng / control	0.012	0.532
C	70 ng / control	0.006	1.945
D	7 ng / control	0.039	2.033
D	70 ng / control	<0.0005	6.191
ABCD	70 ng / control	<0.0005	3.069

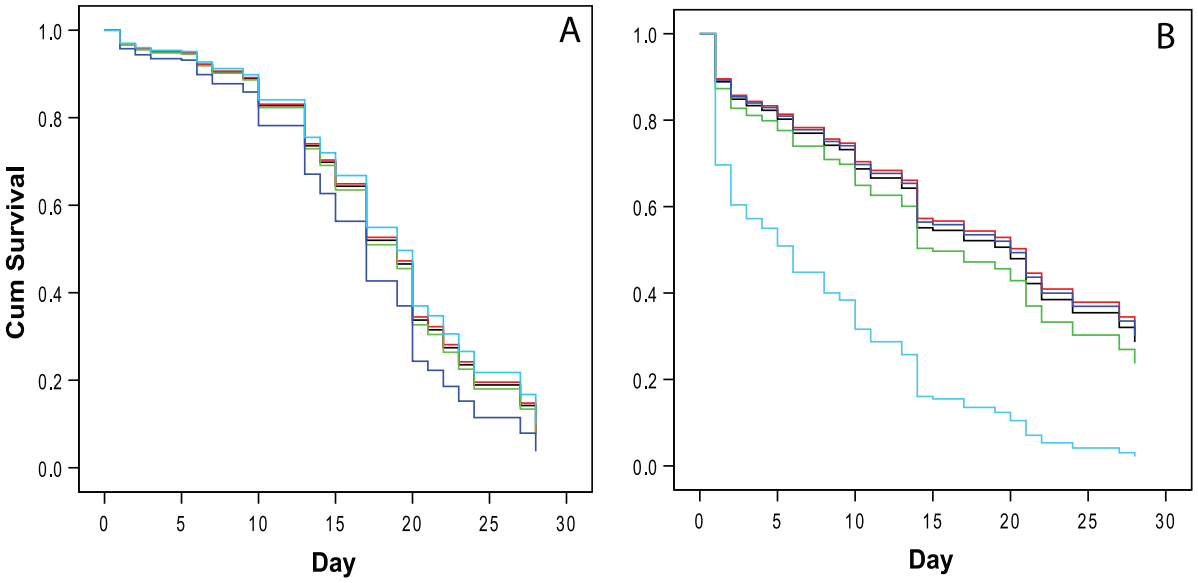

Figure 1. Survival rate for bees in (A) the laboratory, and (B) the field. Black, control; red, 0.07 ng; green, 0.7 ng; dark blue, 7 ng; light blue, 70 ng.

DISCUSSION

The LD_{50} value for imidacloprid can vary between 3.7 and >81 ng per bee (Schmuck *et al*. 2001, Nauen *et al*. 2001). Most researchers, however, assume 4-40 ng per bee to be general. In this experiment bees in the laboratory did not die with 70 ng. To be sure that these results were not imputed to the used product, an LD_{50} test following EPPO guideline no. 170 was conducted with the same concentrations and the used product and a product from a newer batch. In this test no difference in death rate was observed between the two used products. The oral 48-h LD_{50} in this study was estimated to be >70 ng per bee.

A significantly lower survival rate was found in the treatment of 70 ng per bee. This mortality was not observed in the laboratory and is therefore ascribed to sublethal effects. However, this treatment is conducted on only two colonies and the result can therefore not be generalized. Although research has shown that leaf guttation drops from corn plants germinated from imidacloprid-coated seeds can contain such concentrations or even more, bees did not seem to be attracted to drink from field-collected guttation drops (Girolami *et al.*, 2009).

All colonies were infested with varroa. In colony A many bees with deformed wing virus have been observed. If the colonies were weakened by parasites and other pathogens, they might show a deviant interaction with imidacloprid.

Imidacloprid was administered only once and the bees were returned to the hives at the end of the day. If bees collect contaminated nectar and pollen, they would be chronically exposed to imidacloprid. According to Suchail *et al*. (2004) the half-life of imidacloprid in the honeybee ranges between 4.5-5 h, which indicates that most of the effects could be expected during the first few hours after consumption. These effects cannot be seen in this study, because most bees were in the hive at least till the next morning. If bees consume contaminated nectar or pollen in the field, they possibly suffer directly from sublethal effects (Bortolotti *et al*. 2003).

From this study it cannot be concluded that a single dose contamination of food induces a higher bee mortality in the field. Even though bee mortality due to sublethal effects cannot be demonstrated with this study concept, it is nevertheless very important to develop a practical method to assess both lethal and sublethal effects of pesticides, and integrate this method in the registration procedure for pesticides.

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