presentation highlights effects of neonicotinoids, pyrethroids and additional PPPs with emphasis on endocrine disruptive activities of these compounds. Together, our studies indicate that molecular effects are highly sensitive tools that can be incorporated in existing or new test guidelines.

1.5 Practical experiences with a syrup feeding study design based on a new MRL guideline SANTE11956/2016 rev.9 (2018)

Yotam Cohen, Christian Berg, Gundula Gonsior, Silvio Knäbe

Eurofins Agroscience Services Ecotox GmbH

Eutinger Strasse 24, 75223 Niefern-Öschelbronn

E-Mail: GundulaGonsior@eurofins.com

DOI 10.5073/jka.2020.465.005

Abstract

A new study design, according to the guideline SANTE11956/2016 rev:9 (2018), was established to determine the maximum residue level (MRL) of plant protection products in honey. The guideline describes a syrup bee feeding study designed as a worst-case scenario for transferring plant protection products into honey. Previously, field and semi-field studies designs were used. The objectives of this study were to validate the suitability of this feeding semi-field studies according to the new guideline.

Maximum Residue Levels, MRL, Honey, Honey Bees, Consumer Safety

Introduction

Feeding studies could be a cost-effective and standardized way to determine residue levels of plant protection products in honey. The basic idea of the feeding study described in the SANTE11956/2016 rev:9 (2018) guideline, is to feed a solution containing the highest amount of pesticide residue that has been found in “aerial parts of plants” that were applied/sprayed with a pesticide. Usually, the maximum residue that has been found in nectar samples is used. Since practical experiences with this study design are to a large extent missing, different materials and different methods concerning the creation of the artificial honeybee swarms were compared.

Materials and Methods

To examine different methods, four swarms (10,000 bees each) have been prepared with the artificial swarm technique (also known as "shook swarm method"). The colonies, two containing wax foundations and two containing drawn-out combs, were held in a dark cool (<15°C) in the basement for 48 hours and were fed with commercial sugar solution. The bees were then transferred into empty hive bodies in tunnels without any crop.

In addition to the four swarms kept in the dark and cool place, a colony containing brood and food storages was placed in a tunnel under field conditions. Once the swarms have been transferred into the hive bodies containing wax foundations or drawn-out combs in the tunnels, the fifth colony was also transferred into a hive body containing drawn-out combs.

After the set-up of the hives, the five colonies were fed with sugar solution containing a blue additive. During the first two feeding occasions, a 5 % dye sugar solution was provided. For the following two feedings, a 2.5 % dye sugar solution was provided. Subsequent feedings with uncolored sugar solution were done until honey stores (capped honey or honey containing less than 20 % water) were available.

Preparation of the Colonies

Four artificial swarms of honeybees (*Apris mellifera*) with at least 10,000 bees each, were prepared by using the "shook swarm” method (Waite *et al*. 2013). Before the start of the study, each colony was visually inspected for a healthy egg-laying queen, healthy brood nest and no visible symptoms of viruses or diseases. The swarms, along with their caged queen, were placed in a dark and cool

place for 48 hours and fed with commercial sugar solution. The food supply as well as the swarms' condition was checked twice during the 48 hours period.

Additionally, a "natural hive" containing more than 10,000 bees, all brood stages, food, and queen was installed in a tunnel tent at the field site and was later transferred directly to new material.

Feeding

In order to test different study setups, the hives were divided into three treatments:

2 tents with each a "shook swarm" hive containing ten wax foundations
2 tents with each with a "shook swarm" hive with five wax foundations and five drawn-out combs in the middle
One tent with one "natural hive" (without dark and cool place period) with drawn-out combs that was transferred directly to new material.

Each tent had a size of 60 m^2 and was placed on bare soil.

The experiment was conducted near Pforzheim in Germany in April 2019.

The bees were transferred into the magazines and set up in the tunnels directly before the start of the first feeding. The queen cages were opened to release the queen.

The hives were fed with commercial sugar solution colored with a blue additive as surrogate for a test item. Food consumption was monitored, and additional feeding was made once the feeders were empty from leftovers of the previous feeding. The first two feedings consisted of a full concentrate colored solution (5 % blue dye (w/v)), the following two feedings consisted of a half concentrate colored solution (2.5 % blue dye (w/v)). Thereafter, continuous feedings with uncolored sugar solution was done until enough honey stores were available.

Once the honey was available for sampling (13 to 20 days after start of feeding), hives were relocated outside the tunnels and colony assessment was performed to estimate the colonies' strength (estimation adapted from Imdorf & Gerig, 1999, and Imdorf et al., 1987).

Calibration for the analysis of blue dye concentration in the sampled honey

To be able to analyze the content of blue dye in the honey samples, a calibration was done. The absorption of known concentrations of blue dye in the feeding solution was measured at a wavelength of 620 nm by using a spectrophotometer (Unicam UV 500). The absorption values of five different blue dye concentrations from 5% down to 0.31% were tested and a linear calibration curve was calculated (see Figure 1).

Sample preparation and absortion measurement

The samples taken from the combs in the field were brought to laboratory. The wax that was present in the samples was removed by centrifugation of the samples. The samples were diluted with deionized water and the absorption of the samples was measured at a wavelength of 620 nm by using a spectrophotometer (Unicam UV 500).

The content of the blue colour in the samples was calculated using the following formula:

$$x\ [\%] = \frac{(\mathrm{A}-\mathrm{b})}{\mathrm{s}}\,\mathrm{d}$$

x = blue colour content [%]
A = Absorption
b = axis intercept
s = slope
d = Dilution factor

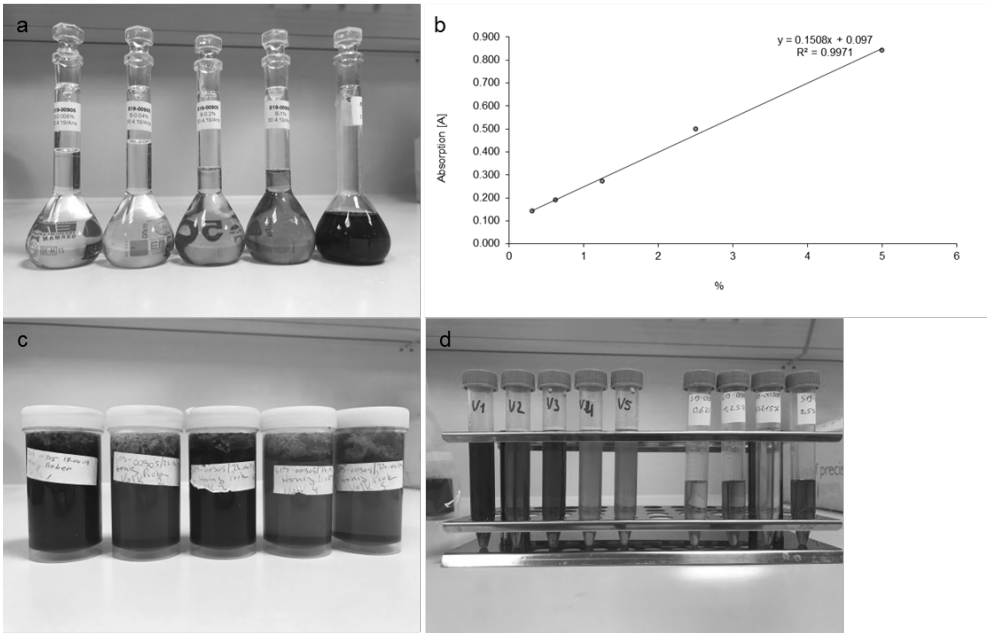

Figure 1: Calibration and sample preparation for the analysis of blue dye content in artificial honey. **a**: Solutions used for calibration, containing 0.31 % to 5 % of blue colour. **b**: Linear calibration curve obtained by measuring the absorption of solutions shown in a. **c**: Artificial honey samples taken from the test colonies before preparation and removal of wax. **d**: Prepared artificial honey samples after removal of wax and dilution by a factor of 1.6.

The analysis of the absorption was done for all honey samples taken from each hive. The measurement of the absorption allows a direct comparison of the blue dye found in the samples compared to the blue dye that was mixed into the original feeding solution.

Timing	Activity
2DBF	Building and storing of four artificial swarms in cold and dark room. Setup of fifth hive, contains brood and food stores, in a tunnel tent.
0DBF	Transfer of the swarms placed in cold and dark room into empty magazines to the field site, either with wax foundation (hives 4 and 5) or drawn-out combs (hives 1 and 2). Direct transfer of bees and queen (without cold and dark period) into a magazine with drawn-out combs (Hive 3). Feeding of all colonies with 2 L colored sugar solution (5 % w/v).
1DAF until honey is available	Feeding of: 2 L colored feeding solution (5 % w/v). 2 L colored feeding solution (2.5 % w/v). 2 L colored feeding solution (2.5 % w/v). 2 L feeding solution (uncolored). (Feeding was only made once the feeders were empty from leftovers of the previous feeding.)
Every 3rd day	Beekeeper check to record development of the colonies.
When honey available	Sampling (capped honey or honey containing less than 20 % water)
Up to 7 days after sampling	Colony assessment.

Figure 2: Activities during the course of the study and the corresponding timing

Honey Sampling

Samples of honey were taken from all hives during the study. The first sample was taken 13 days after start of the initial feeding whereas the last sample was taken 20 days after start of the initial feeding.

Results

Results are presented in Table 1.

The highest concentration of blue day was found in Hiv1, in which the sample of the honey was taken 13 days after the start of the feeding, 6 to 7 days before the samples have been taken in the other hives. The content was 7.67 % of blue dye, which corresponds to 153.4 % of the blue dye content of the original fully concentrated blue diet.

The 2nd highest concentration was found in the hive containing drawn-out combs (Hiv3), containing 4.34 % of blue dye. Here, the bees were transferred directly from another hive body into an empty one in the tunnel. The queen was apparently lost during the transfer process, so there was no egg-laying queen present when the feeding started.

Hiv2 showed the 3rd highest concentration with 2.95 % of blue dye, which had also drawn-out combs at the start of the feeding.

Hiv4 and Hiv5 had almost the same concentration of blue dye, 1.73 % and 1.78%. These two hives were equipped only with wax foundations at the start of the feeding.

Hive ID	Combs	Colony type	Day of honey sampling (DAF)	Dilution factor of analysed honey	Absorpion [nm]	Content of blue color in sample [%] (v/v)	Precent of feeding solution
Hiv1	Drawn-out	Artificial swarm	13	1.6	0.820	7.67	153.4
Hiv2	Drawn-out	Artificial swarm	20	1.6	0.375	2.95	59.0
Hiv3	Drawn-out	Direct transfer	19	1.6	0.506	4.34	86.8
Hiv4	Wax foundations	Artificial swarm	19	1.6	0.260	1.73	34.6
Hiv5	Wax foundations	Artificial swarm	19	1.6	0.265	1.78	35.6

Table 1: Absorbtion and content calculation of the blue color results of the honey samples collected from the hives enclosed in the tents
Note: DAF = Days after start of feeding

Discussion

Honey samples could be collected from all hives in this study. This indicates that the study design is suitable for the study purpose to collect artificial honey samples after providing a sugar feeding solution to the bee colonies transferred to new comb/hive material in tunnels with bare soil.

The timing of the sampling seems to influence the concentration of the blue dye. For example, Hiv1 sampled on 13DAF contained 7.67 %, whereas Hiv2, which was sampled 7 days later, had only 2.95 %. Since these two replicates had about the same number of bees and the same type of bee and comb material, the large difference indicates that the use of multiple replicates is recommended (the guideline suggests the use of four replicates.

A difference between the use of wax foundations and drawn-out combs was noticed. Indeed, samples from colonies equipped with wax foundations contained a lower concentration of blue dye. The building of combs seems to reduce the amount of blue dye in the artificial honey. The bees seem to metabolize more of the feeding solution and therefore more of the blue dye. This could be a result of the need of increased wax secretion and/or the additional work on the combs. The large difference between wax foundations and drawn-out combs, suggests that a standard method regarding the combs should be used for this kind of studies in order to minimize variability originating from different hive set-up material used.

Hiv3 (direct transfer, drawn-out combs, loss of queen during transfer) had a relatively high concentration of blue dye in the honey samples, probably also because drawn-out combs have been used. The queen loss might have affected the onset of brood rearing and therefore the concentration of blue dye, but more replicates would be needed to test this. How the study design relates to more realistic scenarios like semi-field or field studies where bees collect directly from plants, needs to be investigated in future. It might be too worst-case and conservative to feed four liters of feeding solution containing the highest concentration that was found in aerial parts of the plants followed by another four liters containing half of the initial concentration.

Apart from the material used for the hives, the protein supply could influence brood rearing and therefore, metabolism and food logistics, resulting in higher or lower residue levels. There might be differnces if milled pollen, pollen patties or frames containing bee bread are being used as protein supply.

References

SANTE11956/2016 rev.9 (2018) Technical guidelines for determining the magnitude of pesticide residues in honey and setting Maximum Residue Levels in honey

WAITE, R.J., M.A. BROWN, H.M.THOMPSON, M.H. BEW, 2003. Controlling European foulbrood with the shook swarm method and oxytetracycline in the UK. Apidologie **34**, 569–575.

IMDORF, A., G BUEHLMANN, L. GERIG, V. KILCHMANN AND H. WILLE, 1987: Überprüfung der Schätzmethode zur Ermittlung der Brutfläche und der Anzahl Arbeiterinnen in freifliegenden Bienenvölkern, Apidologie 18 (2), 137 – 146. [A Test of the Method of Estimation of Brood Areas and Number of Worker Bees in Free-Flying Colonies].

IMDORF, A. AND L. GERIG 1999: Lehrgang zur Erfassung der Volksstärke, Schweizerisches Zentrum für Bienenforschung. [Course in Determination of Colony Strength].

1.6 Impact of an Oomen feeding with a neonicotinoid on daily activity and colony development of honeybees assessed with an AI based monitoring device

Gundula Gonsior[1], Frederic Tausch[2], Katharina Schmidt[2], Silvio Knäbe[1]

[1]Eurofins Agroscience Services Ecotox GmbH, Eutinger Str. 24, 75223 Niefern-Öschelbronn, Germany

[2]apic.ai GmbH, Rintheimerstraße 31-33, 76131 Karlsruhe, Germany

E-Mail: GundulaGonsior@eurofins.com

DOI 10.5073/jka.2020.465.006

Abstract

Feeding experiments are standard tools in the pollinator risk assessment. The design (Oomen *et al*. 1992) was developed to test insect growth regulators and herbicides. In recent years there was an update (Lückmann & Schmitzer 2015) on the outline in order to also focus on the advantage of different rates making a dose response design possible where exposure levels are known. Additionally, this design gives the possibility to test different rates for honey bee colonies foraging in the same landscape.

The main objective of the experiment presented here was to determine the natural variability of foragers losses of hives fed with a sub-lethal neonicotinoid concentration compared to an untreated control. Other objectives were to see if the neurotoxic exposure results in any observable sub-lethal effects and to find out if losses can be correlated to hive development. This was assessed with traditional methods and a novel, visual monitoring device.

Keywords: Artificial Intelligence, traditional methods, Oomen feeding, colony development, novel method, hive monitoring, bee counter

Introduction

In order to prove that a substance used in agriculture will bring no harm to pollinators, extensive testing must be performed on the active ingredients of plant protection products. There are several different testing protocols available. However, since there is a wide range of possible outside influences, tests run with free flying bees are always subject to uncertainty. One of the methods currently applied to compare bee mortality between different treatments is the use of dead bee traps. Regarding this method, potential uncertainties are known, *e.g*. correlation of the total number of dead bees and the number of dead bees in the dead bee trap and the limited number of data sets which can be collected during testing. Furthermore, as the bees are foraging freely, it is very difficult to determine their level of exposure. Therefore, a realistic dose response design is not possible with spray application. The only test design, which gives the possibility to test different rates in the same environmental conditions, is the Oomen test design. The design presented was extended to include a digital hive monitoring device using computer vision and deep learning beside traditional mortality assessments. The device recorded all bees entering and leaving their hives with a camera, thus enabling the constant near-time observation of hive development and bee activity throughout the year. Deep learning analysis of the footage recorded made it possible to count the number of bees entering and leaving throughout the day and to calculate the losses of foragers over selected periods of time.

Materials and Methods

To test the applicability of the approach, the study compared the hive development and losses of foragers from hives exposed to a neonicotinoid with a control group. Eight hives were monitored during the study. The colonies contained all brood stages (eggs, larvae and capped cells). Four