Bulletin of Insectology ***56*** *(1): 91-96, 2003*
ISSN 17218861

Honey bee brood ring-test in 2002: method for the assessment of side effects of plant protection products on the honey bee brood under semi-field conditions

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Abstract

Based on the presented test methods in the past, to evaluate possible side-effects of plant protection products on the honey bee brood and the recent OEPP/EPPO No.170 guideline, the "Arbeitsgemeinschaft Bienenschutz" developed a new test method in the semi-field. According to that method 5 semi-field trials were carried out at different locations in Germany during 2002.

Each trial consisted of 2 treatments; i.e. the reference substance Insegar 25 WG, which is known as an IGR and the water treated control treatment. Separate tunnel tents (at least 40 m^2 crop area per tunnel) with flowering *Phacelia* (*Phacelia tanacetifolia* Benth.) were used for the different treatments. The crop was sprayed in both treatments on the same day during bee-flight activity of the bees. The effect of the reference substance Insegar 25 WG was examined on small bee colonies ("Mini-Plus-Beuten") placed in the tents before the application. The bee colonies were exposed to the treated crop for 7 days.

Special attention was turned on the assessments of the condition of the colonies and the development of the bee brood during the trials. The assessments were carried out at least once before the application and four times after the application on fixed dates. The time schedule of the assessment dates was chosen in order to check the bee brood at different expected stages during the development. For the evaluation of the condition of the colonies the strength of the colony, presence of a healthy queen, areas with the pollen and nectar on combs and areas containing eggs, larvae and pupae on combs were assessed. The development of the bee brood was evaluated by using transparent acetate sheets to mark single cells on brood combs with its content on different assessment dates during each trial. Additionally the flight intensity in the tunnels and the mortality was checked before as well after the treatment.

In all trials of the reference treatment an increased number of dead pupae was noticed in the dead bee traps with malformations as typical for the active substance fenoxycarb (Insegar 25WG). The pupae mortality occurred approximately 14 days after application and was on a different level in the trials. For special interests on the honey bee brood development the detailed assessments with transparent acetate sheets were evaluated by calculation of brood termination-rates in % and brood indices. The termination rate of brood ranged from 94% to 100% in the reference treatment of the trials, which confirms the sensitivity of the test method. In the control treatment a wide range in the termination-rate was noticed in the trials (8% to 43%). The increased brood termination in the control treatment in single trials could be explained by weather conditions in Germany in 2002. It was rainier than in the previous years and therefore the food supply for the bee brood during the exposure in tents was reduced. It should be discussed, above which level of control mortality the test should be repeated.

Key words: *Apis mellifera*, brood, colony development, pupae mortality, tunnel test, Insegar 25 WG, fenoxycarb, Mini-Plus-Beuten.

Introduction

According to the decision-making scheme for the environmental risk assessment of plant protection products presented in the recent EPPO guideline (OEPP/EPPO, 2001) a brood test is required if it is presumed that a product effects the bee brood development.

The purpose of the ring-test was to create, evaluate and validate a test method under semi-field conditions for determination of adverse effects of products on the honey bee brood.

Several laboratory test methods were presented, e.g. by Rembold and Czoppelt (1982), Czoppelt (1993), Wittmann (1981 and 1982), and discussed so far. Based on the presentation of de Rujiter and van den Eijnde at the ICP-BR meeting in Braunschweig (1996) it was concluded, that these kinds of methods are not reproducible as standard laboratory tests under GLP.

The "In-hive field test" published by Oomen *et al.* (1992) is a qualitative test method with an extreme exposure of the plant protection product to the honey bee brood. An extrapolation of the produced data to the natural conditions is limited.

The aim of the members of the "Arbeitsgemeinschaft Bienenschutz" in Germany was to fulfil the lack in the sequential testing scheme with the development of a test method under semi-field conditions and to produce a quantitative data-set as basis for an evaluation. This method based on the publications of Oomen *et al.* (1992), Mühlen (1996), Tornier (1999) and the recent OEPP/EPPO No. 170 guideline.

Material and methods

Test design

In the ring-test 6 trials were carried out at different locations in Germany during 2002. Each of the trial consisted of 2 treatments: The water-treated control and the reference substance treatment. As reference substance Insegar 25 WG was chosen, because it's known as a product with insect growth regulating properties. The application rate was 0.6 kg product in 400 L water per ha.

For each treatment one tunnel tent was built up. The used crop was *Phacelia tanacetifolia* as recommended in the recent EPPO guideline for honey bee tests in the semi-field. One tent covered a crop area of at least 40 m^2.

The exposure period in the tents lasted for approx. 3 days before the treatment and for further 7 days after application. After the exposure in tents the colonies were placed in areas where no flowering main crops were available to insure that the contaminated food in the test colonies will not thin down.

Test colonies

As test colonies the "Mini-Plus-Beuten" were used. This special bee hive is smaller than a commercial hive and is made of polystyrene. All nuclei were produced at the same time with sister queens to guarantee uniform bee hives in each trial. The colonies were prepared with at least 2 bodies including more than 3 brood combs, 1 food comb and approximately 6000 worker bees at start of the test. A good food supply of the colonies during the time before start of the test should be guaranteed. The hives were introduced into the tents approximately 4 days before the planned application to enable the bees getting familiar with the environment and to lower mortality, which usually is increased at start of the exposure in tents after the transport.

Application

The application was carried out with a portable boom sprayer that simulates a commercial application in the tents. The treatment was done during full flowering and a bee flight activity higher than 10 bees per m^2 *Phacelia* to insure the exposure of the bees and their brood to the treated nectar and pollen from the crop.

Mode of assessment

Mortality in front of the hive

Dead bee traps with gauze on bottom and on top were attached to the entrance of the hives in order to register those dead bees, which were carried out of the hives. Furthermore, the mortality was recorded in an area of 2 m^2 directly in front of the entrance of the hives. Therefore the *Phacelia* was removed prior to the set-up of the hives and waterpermeable linen sheets were spread out on the soil. The time schedule of the assessments is given in table 1.

After exposure period in the tents, the mortality in the dead bee trap was recorded for further 2 weeks outside of the tents. The numbers of dead bees recorded during assessments were separated in number of dead adult worker bees, larvae, pupae and males per assessment.

The assessments were done earlier in the morning to avoid the loss of dead adults and pupae due to e.g. the undertaking behaviour of worker bees and predators (wasps, birds).

Table 1. Evaluation of mortality

Time of the test	Evaluations of mortality*
Over at least three days before the application	Once a day at the same time of day in the morning
On the day of application	• Shortly before application • 2 h after application • In the evening after daily flight activity of the bees
During exposure period in tents	Once a day at the same time of day in the morning
Up to day +22 after BFD (out of the tents; only in bee traps)	Once a day at the same time of day in the morning

* Remark: At each evaluation date the dead bees were counted and removed.
BFD = Brood Area Fixing Day

Table 2. Evaluation of flight intensity (number of bees/m² flowering *Phacelia*).

Time of the test	Evaluations of flight intensity
Over at least three days before the application	Once a day during flight activity of the bees
On the day of application	• Shortly before application • At least 4 times in the first hour after application • 2 h after application • 4 h after application • 6 h after application
On the following day after application	Three times during flight activity of the bees (morning, midday, evening)
During exposure period in tents	Once a day during flight activity of the bees

Flight intensity

At each assessment time the number of bees that were both, foraging on flowering *Phacelia* and actually flying over the crop were counted on 3 different places (each of 1 m²) per tent.

The observations of the flight intensity were carried out according to the scheme given in table 2.

Bee brood assessments

Condition of the Colonies:

The condition of the colonies was checked once before the application and five times after application (see table 3).

In order to record effects of the reference substance, the following parameters were assessed:

- Strength of the colony (number of combs covered with bees)
- Presence of a healthy queen
- Areas with pollen and nectar
- Areas containing eggs, larvae and capped cells

Table 3. Assessment of the condition of the colonies

Assessment dates
BFD
Application on +2 days (± 1 day) after BFD
+5 days (± 1 day) after BFD
+10 days (± 1 day) after BFD
+16 days (± 1 day) after BFD
+22 days (± 1 day) after BFD
+28 days (± 1 day) after BFD

BFD = Brood Area Fixing Day

The estimation of the areas containing brood and food was done according to appropriate methods (e. g. Imdorf *et al*., 1987). This was done for all combs (both sides) per hive. So that areas covered with brood (pupae, larvae and eggs) and food (nectar and pollen) could be determined from the total area, which was available for the bees in the colonies.

Development of the Bee Brood:

The assessment of the development of the bee brood in individual marked brood cells was carried out by using acetate sheets. At the assessment before the application (Brood Area Fixing Day = BFD) a brood comb was taken out of each colony to mark areas with at least 100 cells containing eggs. The exact situation of each cell and its content was marked in the acetate sheet. The sheet was fixed with needles on the wooden frame and the position on the frame was marked. This allowed placing the sheet exactly in the same position on each of the following observation dates. Therefore the development of each individually marked cell throughout the duration of the study could be determined (pre-imaginal development period of worker honey bees typically averages 21 days).

The time schedule of the brood assessment dates was chosen in order to check the bee brood at different expected stages during the development (see table 4). The application in tents was performed 2 days (± 1 day) after BFD.

Evaluation of the test results

For the validation of the test method, the evaluation was done by comparing the results in the reference substance treatment to the water treated control and furthermore by comparing the pre- and post-application data:

- Mortality in the dead bee trap and on the linen in front of the hives (number of dead adult bees and pupae)
- Condition of the colonies (average brood areas per hive, strength of the colonies)
- Brood development (brood-index, brood termination-rate in %)

Brood development

Brood-index:

The assessed contents in single cells on brood combs were transformed in categories for further calculations:

Category 0: Termination of the development
Category 1: Egg stage
Category 2: Young larvae (L1 – L2)
Category 3: Old larvae (L3 – L5)
Category 4: Pupae stage (capped cell)
Category 5: Empty after the hatch

Starting with eggs marked on BFD (category 1) an increase of the category during the following assessments can be observed, if a normal development of the brood is presumed. If the brood was terminated before a successful development the cell was titled with 0. Afterwards the mean values were calculated for each observation date and colony. At the last assessment date (BFD +22) the cells should be empty after hatch (category 5) or again filled with eggs (category 1) or small larvae (category 2) when the first marked eggs followed a successful development. Cells filled with nectar and pollen were titled as "N" and "P" and the respective cells were not included in further evaluations (except if the brood was terminated).

The values of all cells in each treatment, assessed at the same date, were summed up and divided by the number of observed cells in order to obtain the brood-index.

Brood termination-rate:

For the calculation of the brood termination-rate in % the observed cells were divided in 2 categories:

- Bee brood in the cell reached the expected brood stage at the different assessment dates and was empty or egg containing after hatch of the adult bee on BFD +22 was evaluated as a successful development
- At one of the assessment dates the expected brood stage was not reached or food was stored in the cell during BFD +5 to +16 was evaluated as termination of the bee brood development

Afterwards one mean value was calculated per colony and treatment.

Table 4. Assessment of the development of the bee brood.

Assessment date	Determined brood stage in marked cells
BFD	Egg
Assessment date	Expected brood stage in marked cells
+5 days (± 1 day) after BFD	Young to old larvae
+10 days (± 1 day) after BFD	Capped cells (pupae)
+16 days (± 1 day) after BFD	Capped cells shortly before hatch
+22 days (± 1 day) after BFD	Empty cells or egg/young larvae containing cells

BFD = Brood Area Fixing Day

Results and discussion

Mortality

The total number of dead adult bees and dead pupae before and after the treatment in the trials are summarized in table 5. In all trials of the reference treatment an increased number of dead pupae was noticed in the dead bee traps with malformations as typical for the active substance fenoxycarb (Insegar 25WG). This pupae mortality occurred in the trials around 2 weeks after the treatment and lasted for a few days (for detailed results trial P4 see figure 1). The pupae mortality in the reference treatment was on a different level in the trials (table 5).

Bee Brood

Condition of the colonies

At the start of the trials the colonies in the treatments were in good condition (all brood stages available, queen-right colonies) with a comparable strength between the colonies of one trial at start of the test.

In figure 2 the results of the estimation of the areas covered with brood and food of one trial are presented.

The covering rate with the brood stages and food is stated in % from the total area per hive and assessment date in the treatments. At start of the test (BFD) the colonies showed an equal distribution of the different brood stages and the total covered area was also on a similar level in the two colonies. On BFD +6 a lack in the larval stage was noticed in the reference treatment by the termination of the development of the eggs noticed on BFD. This caused at a later assessment date (BFD +16) a decreased area covered with capped cells (pupae) compared to the control treatment.

Brood development

Brood-index:

In the reference treatments of the trials a strong effect with a decreased brood-index was noticed during the entire test periods (see table 6). The colonies were not able to recover during the assessment dates after the treatments. The control treatments of the trials showed increasing brood indices from BFD to BFD +10, but in several trials the expected brood-index on the assessment dates after the treatment was not reached. Especially at a later stage of the trials the index values of the control treatments were on a lower level. This was caused by the termination of the brood in marked cells because of the bad weather conditions during summer 2002 in Germany. It was rainier than in the previous years and therefore the food supply for the bee brood during the exposure in tents was reduced.

Brood termination-rate in %:

In all trials nearly all eggs marked on BFD of the reference substance treatment did not reach the expected stages up to BFD +22. The termination rate ranged from 94% to 100% in the reference treatment of the trials, which confirms the sensitivity of the test method (see table 7). In the control treatment a wide range in the termination-rate was noticed in the trials (8% to 43%). The high control mortality in trial C1, C2 and H3 could be explained by weather conditions in Germany in 2002. It should be discussed, above which level of control mortality the test should be repeated.

Table 5. Mortality during the experimental phase of the trials in the treatments

Trial number	Treatment	Σ dead bees during the observation period before the application		Σ dead bees during the observation period after the application	
		Adult bee	Pupae	Adult bee	Pupae
C1	Control	74	0	85	3
	Reference substance	110	0	193	114
C2	Control	30	0	173	1
	Reference substance	70	1	353	36
H3	Control	3	0	46	0
	Reference substance	4	0	113	3
P4	Control	8	0	41	3
	Reference substance	16	3	109	47
M5	Control	16	0	52	2
	Reference substance	26	0	74	118

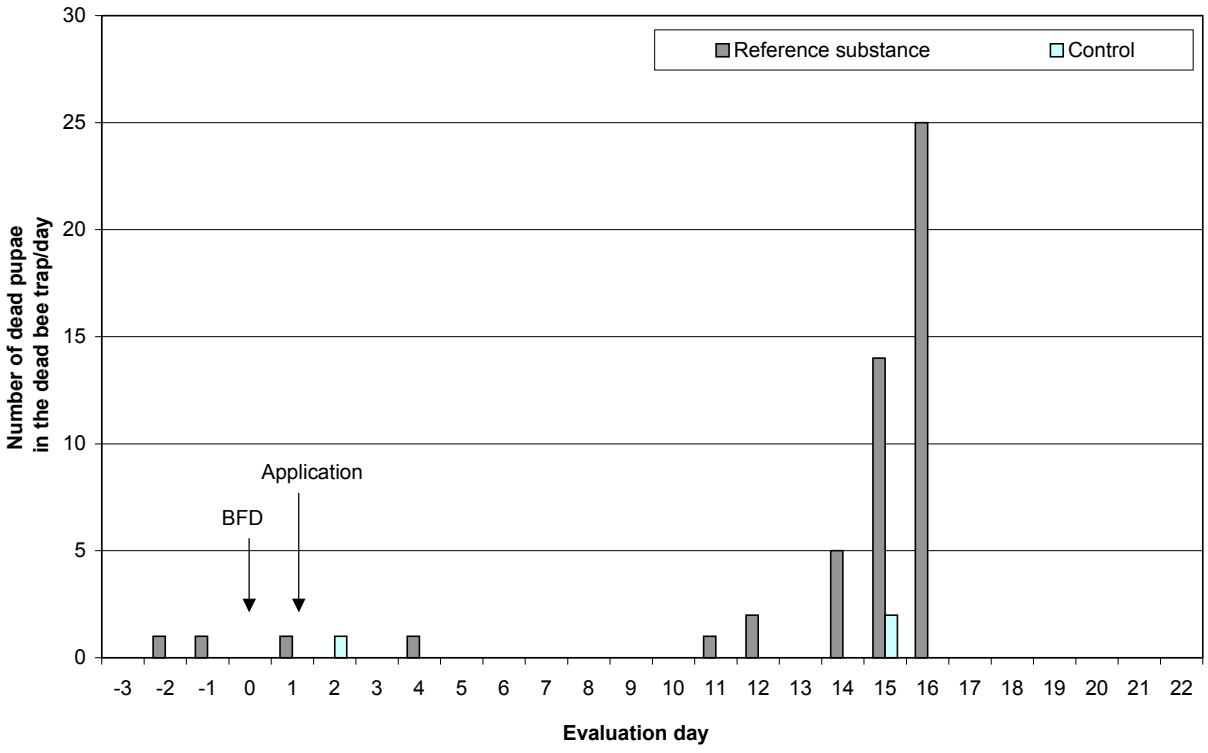

Figure 1. Number of dead pupae counted in the dead bee traps of the treatments in trial P4 during the observation period.

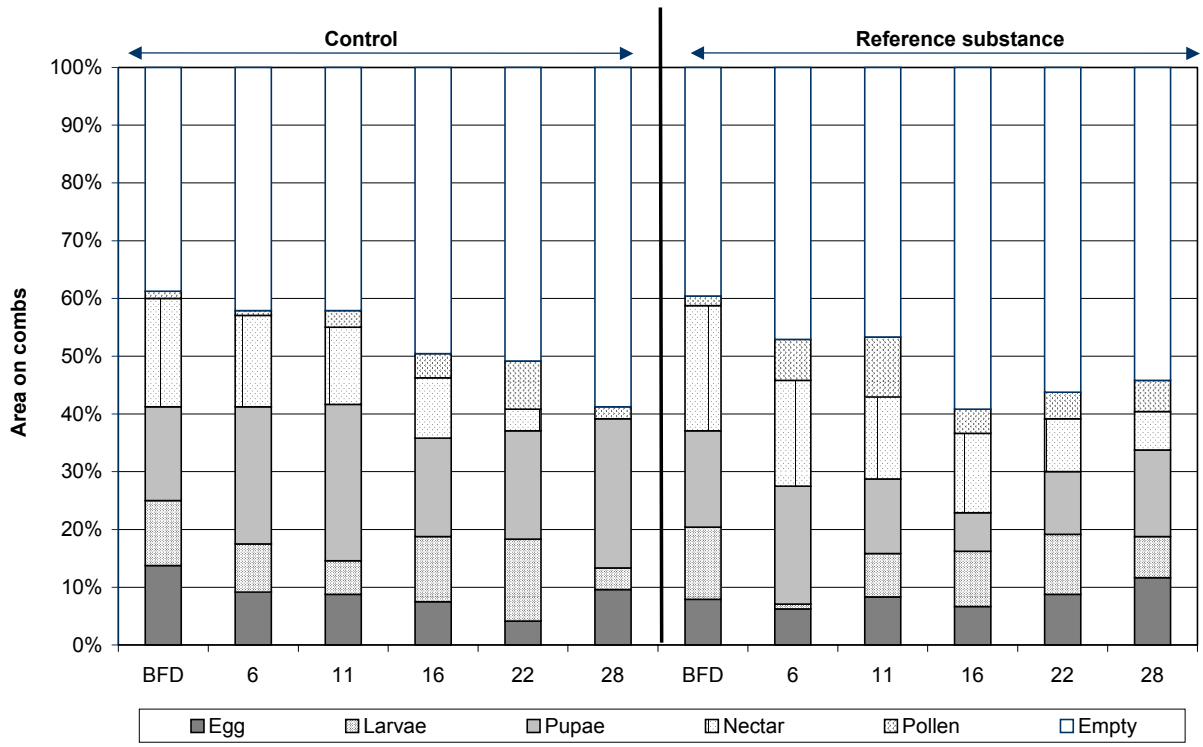

Figure 2. Average areas on combs covered with the different brood stages, nectar and pollen of both treatments on the assessment dates during the experimental phase in trial P4.

Table 6. Brood indices in the colonies of the treatments.

Trial number	Treatment	Brood indices during the observation period				
		BFD	BFD +5 (±1 day)	BFD +10 (±1 day)	BFD +16 (±1 day)	BFD +22 (±1 day)
--	Expected indices	1	2 – 3	4	4	5 or 1 – 2
C1	Control	1.00	2.22	2.33	2.78	2.42
	Reference substance	1.00	0.14	0.00	0.00	1.60
C2	Control	1.00	1.95	2.97	3.37	2.31
	Reference substance	1.00	0.00	0.00	0.00	0.00
H3	Control	1.00	1.59	3.14	3.57	3.35
	Reference substance	1.00	0.01	0.00	1.28	2.54
P4	Control	1.00	3.25	3.74	3.68	1.52
	Reference substance	1.00	0.75	0.85	1.11	2.15
M5	Control	1.00	3.21	3.91	3.06	3.18
	Reference substance	1.00	2.06	1.98	1.28	1.56

Table 7. Brood termination rate of the marked cells of the colonies from BFD to BFD +22

Trial	C1		C2		H3		P4		M5	
Treatment	C	R	C	R	C	R	C	R	C	R
Brood termination rate in %	43	100	39	100	32	100	18	100	8	94

Conclusion

The test method with the described parameters gives us the possibility to evaluate a plant protection product based on a quantitative data set. A decision could be made whether a product may cause effects on the bee colonies and their brood under "natural" conditions or not.

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