2.3 The effects of fenoxycarb in a chronic *Oomen* feeding test – results of a ring-test*

Johannes Lückmann[1], Stephan Schmitzer[2]

[1] RIFCON GmbH, Goldbeckstraße 13, 69493 Hirschberg a. B., Germany,
e-mail: johannes.lueckmann@rifcon.de
[2] IBACON GmbH, Arheilger Weg 17, 64380 Rossdorf, Germany, e-mail: stephan.schmitzer@ibacon.com
* on behalf of the Oomen-brood method ring test group of the AG Bienenschutz (Germany)

Abstract

Background - The Oomen bee brood feeding test (Oomen *et al.* 1992)[6] is recommended by the EFSA (2013)[2] as one method to investigate potential effects of plant protection products on honeybee brood (*Apis mellifera* L.), with the 'brood termination rate' as the key endpoint. In 2013 the test method of Oomen was adapted to a chronic feeding scenario including current methods (OECD GD 75, improvements by Pistorius *et al.* 2012[7]) and was subsequently ring-tested in 2013 and 2014.

Results - The results were compared to data of acute feeding studies. Overall the obtained results of the chronic Oomen feeding studies indicated that the design is a robust and reliable test method with low brood termination rates in the control and a sufficient exposure of the brood to the reference item.

Conclusion - Nevertheless, based on current experiences and recent publications adaptations are proposed concerning dosing of the test item, assessment intervals and methodology (digital brood assessments). Moreover the test method was compared to the bee brood test according to OECD GD 75 and several advantages were pointed out.

Key words: honeybees, chronic Oomen bee brood feeding test, ring-test, EFSA

1. Introduction

According to the 'Guidance Document on the risk assessment of plant protection products on bees' (EFSA 2013)[2] the Oomen bee brood feeding test (Oomen *et al.,* 1992)[6] is recommended, next to the OECD Guidance Document 75 (2007)[5] as one possibility to refine the risk assessment on honeybee brood if a potential concern is raised. In contrast to a single application foreseen in the Oomen test, EFSA proposes to extend the feeding period of a sucrose solution spiked with the test item over a period of 9 days. But EFSA (2013)[2] lacks to give detailed information, *e.g.* dosing and concentration of the test and reference item, was not up-dated (*e.g.* timing of Brood area Fixing Days, hereafter BFD) and included questionable endpoints, *e.g.* pupal weight and pupal deformations. Moreover the original method of Oomen gives only a rough description of the test, was not validated and is not in line with current methods on bee brood testing (*i.e.* number and intervals of the BFDs, using digital brood evaluation etc.). Therefore in 2012 a sub-group of the German AG Bienenschutz was founded with the aim to collect and evaluate historical data of 'Brood Termination Rates' (hereafter BTRs) of Oomen tests with a single feeding. These data will be called hereafter 'acute data' and were presented by Lückmann & Schmitzer (2013)[4]. In spring 2013 the group developed a ring-test protocol for a chronic feeding test under field conditions and in 2013 and 2014 a larger number of tests using the ring-test protocol were conducted.

The present paper shortly describes the method, analyses the data of the ring-test, and discusses them in the light of the acute feeding data, lines out the advantages and disadvantages of the chronic Oomen feeding test compared to the OECD GD 75. Finally, on the basis of our obtained results, we try to identify factors influencing BTRs and give some additional recommendations for further testing (based on the studies carried out in Germany and Switzerland between 2013 and 2014).

2. Experimental Methods

Testing was performed with free flying and similar sized honeybee colonies with 10,000 to 20,000 bees per colony, at least 4-6 brood combs containing eggs, larvae and capped cells and a sufficient supply with pollen. Excessively nectar/honey stores in the colonies and mass-flowering crops in the vicinity were avoided to limit a dilution of the test item in the hive and to ensure feeding solution was taken up in a timely manner. A quantity of 0.5 L freshly prepared sugar solution was placed in each hive once per day over 9 days of feeding. Food uptake was assessed daily. The control colonies were fed with untreated sugar solution and the insect growth regulator fenoxycarb was used as a reference item. Four colonies per treatment group (exception: 1 study with 3 replicates) were used. The daily concentration was 1/9 of the rate of 300 g a.s./ha in 400 L water which corresponds to 42 mg a.s./0.5L/colony/day. Adult and pupal mortality was daily assessed via dead bee traps for a period of 28 days. Shortly before the initial feeding 200 cells with eggs, young and old larvae were selected (BFD 0). The development of these cells was assessed 4 to 5, 10 (±1), 16 (±1), 22 (±1) and 28 (±1) days after BFD0 according to the method described by the OECD GD 75. The conditions of the honeybee colonies, *i.e.* colony strength, area with brood stages (*i.e.* eggs, larvae, pupae) and food was determined at BFD 0, 10 and 28. As main endpoints the BTR and the pupal mortality were evaluated. In total seven studies with a total of 27 replicates (colonies) were performed by four different German contract laboratories: BASF SE (1 study), BioChem agrar (1 study), Eurofins Agroscience Services (2 studies), IBACON GmbH (1 study) and RIFCON GmbH (2 studies).

Additionally in summer 2014 a call for acute Oomen feeding studies not yet considered by Lückmann & Schmitzer (2013)[4] was made to broaden the data base for evaluation. Control and reference item data of further four studies were provided and thus a total of 21 studies with up to 65 replicates per developmental stage were available for data analysis (Table 1). Studies were performed in Germany or Switzerland and derived from BASF SE, Bayer CropScience, BioChem agrar, E. I. DuPont de Nemours and Company, Eurofins Agroscience, IES, IBACON GmbH and RIFCON GmbH.

Table 1 Number of Oomen bee brood feeding studies and replicates

Test group	Number of replicates [n] in Acute feeding (21 studies)			Chronic feeding (7 studies)		
	Eggs	Young larvae	Old larvae	Eggs	Young larvae	Old larvae
Control	65	62	62	27	27	27
Reference	63	60	60	27	27	27

Calculation of descriptive statistics was performed for both kinds of feeding studies. Whereas overall medians and means were determined based on the mean BTRs of each study, standard deviations, minima, maxima were determined from all replicates (colonies). For statistical analysis of BTRs between the respective brood stages in the reference item group, *i.e.* eggs, young and old larvae U-test (chronic feeding) and Fisher-test (acute feeding) were performed ($\alpha = 0.05$). Chi^2-tests were performed to analyse dependence of BTR and colony strength ($\alpha = 0.05$).

3. Results

Data of the ring-test (chronic Oomen feeding studies) were compared to updated data of acute Oomen feeding studies to see differences between the methods.

3.1 Chronic vs. acute feeding

For the chronic feeding, the results indicate that the test method worked, as mean BTRs in the control were quite low, whereas those in the reference item were distinctly increased (Figure 1). For the different brood stages in the control mean BTRs for eggs, young and old larvae were

determined to be 14.7%, 12.6% and 7.6% compared to 71.5%, 35.3% and 30.2% for the respective stage in the reference item group. The decreasing BTRs with increasing age of the brood indicated a decreasing sensitivity. This is underlined by the statistical analysis in the reference item group which showed that BTRs of young and old larvae were significantly lower compared to the eggs (U-test, $p<0.001$).

A comparison of the chronic feeding versus the acute feeding shows that both feeding approaches resulted in comparable results.

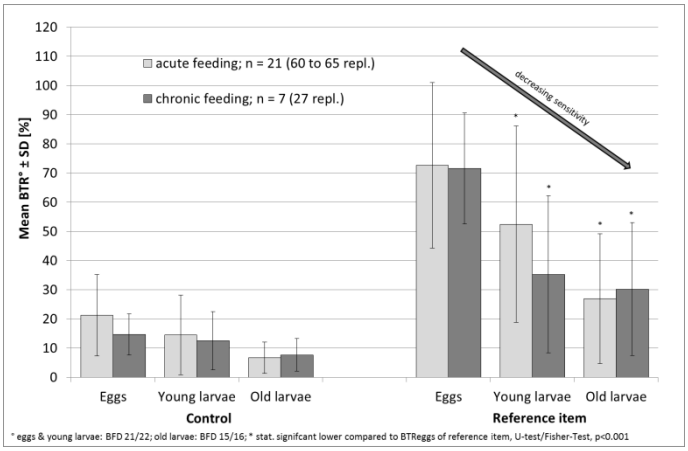

Figure 1 Mean BTRs of chronic and acute Oomen bee brood feeding studies

A summary of the descriptive statistics of the BTRs of the chronic and acute Oomen feeding studies is given in Table 2.

Table 2 Descriptive statistics of BTRs of acute and chronic Oomen bee brood feeding studies

Parameter	Acute feeding (n=21)			Chronic feeding (n=7)		
	Eggs	Young larvae	Old larvae	Eggs	Young larvae	Old larvae
Control						
Median	16.7	9.6	5.9	15.0	13.0	5.5
Mean	21.3	14.5	6.7	14.7	12.6	7.6
SD°	17.7	20.9	8.4	13.4	16.2	9.1
Min.°	2.5	0.0	0.0	2.0	0.0	0.0
Max.°	92.6	93.3	48.0	48.0	61.8	39.8
Reference item						
Median	85.6		16.8	62.9	31.5	29.3
Mean	72.7	52.4*	26.9*	71.5	35.3*	30.2*
SD°	31.9	36.4	25.9	25.0	30.7	25.4
Min.°	1.0	2.0	0.0	29.2	0.3	0.3
Max.°	100	100	98.4	100	98.5	77.5

n=number of studies, ° calculated from all replicates (colonies), * statistically significant lower compared to BTR_{eggs} in reference item (Fisher-test for acute feeding, U-test for chronic feeding, $p<0.001$)

3.2 Reliability of the test method: control

To evaluate the reliability of the test system in the control, the proportion of replicates below/equal a BTR-threshold of 30% was analysed (Table 3). The results show for the chronic feeding that a very high proportion of replicates was below or equal to a BTR of 30% indicating a high reliability and a low variability of the test method. Moreover this proportion increased with the age of the brood.

These findings also count for the acute feeding with the exception that the proportion for the eggs was slightly lower.

Table 3 Reliability of the test method (control)

Proportion of replicates with BTRs ≤30% in					
Acute feeding			Chronic feeding		
Eggs (n=65)	**Young larvae (n=62)**	**Old larvae (n=62)**	**Eggs (n=27)**	**Young larvae (n=27)**	**Old larvae (n=27)**
75.4%	87.1%	98.4%	85.2%	85.2%	92.6%

n=number of replicates (colonies)

3.3 Reliability of the test method: reference item

To evaluate the reliability of the test system in the reference item, the proportion of replicates equal/above a BTR-threshold of 70% was analysed (Table 4). The results show for the chronic feeding that approximately 50% of the replicates with marked eggs displayed BTRs ≥70%. Those replicates with a BTR_{eggs} <70% showed a daily pupal mortality being >6 dead pupae/day which is more than 168 dead pupae during the entire post application period. This proved first that the double field rate of the reference item is a suitable concentration, whereas the single does not cause reproducible dose-related effects (Hecht-Rost et al. 2014)[3]. Secondly the increased BTR_{eggs} and/or the increased pupal mortality verify a sufficient exposure of the brood indicating a high reliability of the test method.

The proportion of replicates with BTRs ≥70% for a respective brood stage decreased distinctly with the age of the brood indicating a decreased sensitivity of young and old larvae compared to the eggs.

These findings also count for the acute feeding (Table 4).

Table 4 Reliability of the test method (reference item)

Proportion of replicates with BTRs ≥70% in					
Acute feeding			Chronic feeding		
Eggs (n=63)	**Young larvae (n=60)**	**Old larvae (n=60)**	**Eggs (n=27)**	**Young larvae (n=27)**	**Old larvae (n=27)**
68%	40%	8%	52%	15%	4%

n=number of replicates (colonies)

3.4 Analysis of potential BTR driving factors in the control

An analysis of potentially BTR driving factors in the control shows that neither for the chronic nor for the acute feeding a correlation between the time in the year when a study is started and the BTR_{eggs} was found (Figure 2). Thus the performance of the Oomen feeding is study is possible during the entire bee season.

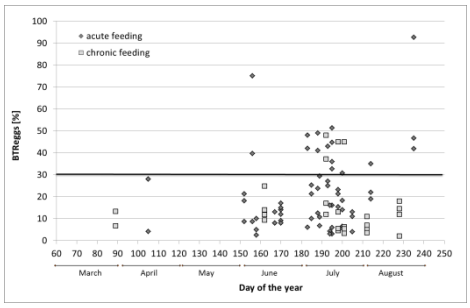

Figure 2 Correlation between day of the year (BFD 0) and $BTR_{eggs\ in}$ chronic and acute Oomen bee brood feeding studies

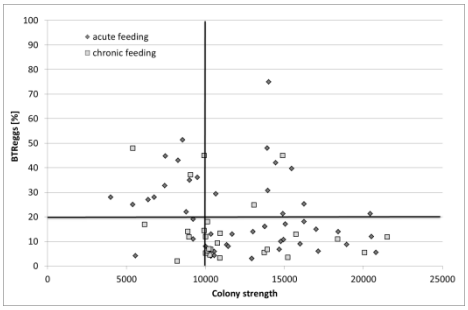

Figure 3 Correlation between colony strength at BFD 0 and $BTR_{eggs\ in}$ chronic and acute Oomen bee brood feeding studies

For the colony strength, no statistically significant correlation was found for both feeding regimes, if a trigger of 30% was assumed. But if a trigger of BTR_{eggs} 20% was assumed, it was found for the acute feeding that colonies with ≥10 000 bees displayed statistically significant more frequent BTR_{eggs} ≤20% than smaller colonies (Chi^2-test, p=0.002). For the chronic feeding this analysis was not possible as the number of replicates was too low, but it can be assumed that this finding is also applicable. Thus, the proposed colony strength given in the ring-test protocol is confirmed.

3.5 Recommendations for future chronic feeding studies

The results of the ring test showed that the test designs works. Only minor adaptations are regarded necessary:

1. The assessment of young and old larvae is not necessary at BFD 0; it is sufficient to assess eggs and follow their development up to hatching, as results showed that eggs are the most sensitive brood stage and the chronic feeding covers also older brood stages.
2. Detailed brood assessment should be carried out at BFD 3-4, BFD 10 (after feeding), BFD 16, BFD 22. There is no need for assessments on BFD 28 as this is already part of the 2nd brood cycle.
3. The number of colony assessments should be reduced to avoid disturbance of colonies which may result in an adverse impact on the brood development. The assessments should be carried out on BFD 0, 10 (after feeding) and 22 (end of study/1st brood cycle). Again there is no need for assessments on BFD 28 as this is already part of the 2nd brood cycle.
4. The selection of 200 cells with eggs is sufficient.

3.6 The chronic Oomen feeding test compared to bee brood test according to OECD GD 75

In Table 5 a comparison of chronic Oomen feeding test compared to the brood test according to OECD GD75 and the evaluations of Becker et al. (2014)[1] is given.

In the chronic Oomen feeding test, bees are exposed artificially in a worst case scenario for a period of at least nine days to a defined concentration of a chemical in sugar solution. In OECD GD 75 bee brood tunnel studies, the exposure scenario is much more realistic with an exposure to contaminated pollen and nectar. Here, exposure period depends on the flowering length of the treated flowers, the degree of storage of contaminated food in the hive and the food consumption and overall with decreasing residue levels over the time.

The advantage of the chronic feeding test compared to OECD GD 75 is that a defined concentration of a chemical can be adjusted to current needs, *i.e.* the application rate or residue level. Although the feeding test should not be carried out during mass flowerings in the vicinity of the colonies to limit a dilution of the test item in the hives, bees forage on non-target plants or

crops and thus dilute the chemical concentration, this also happens in OECD GD 75 if bees forage on newly blossomed and therefore untreated flowers. Additionally, with the Oomen test method, herbicides which are taken up by the leaves and lead to rapid fading of the crop can be tested. Moreover there is no 'caging effect' which leads to a higher variability of BTRs and the performance of the test is less dependent on climatic, seasonal and crop conditions, *i.e.* flowering stage of the crop (BBCH). The last two points. *i.e.* absence of a 'caging effect' and less dependence on climatic seasonal and crop conditions may be important reasons why mean $BTRs_{eggs}$ in the control are distinctly lower and reliability of the test system is distinctly higher compared to current OECD GD 75 bee brood studies (Becker *et al.* 2014)[1].

The advantage of studies according to OECD GD 75 is that bees are exposed via oral and contact route to realistic and declining concentrations of a chemical both in pollen and nectar.

Table 2: Comparison of chronic Oomen feeding and OECD GD 75

Topic	Chronic Oomen feeding	OECD GD 75
Exposure scenario	Artificial, worst case, oral	Realistic worst case, oral and contact
Chronic exposure	Exposure to constant residue level for at least 9 days; longer duration depends on storage and consumption rate	Duration depends on flowering period of treated flowers, storage of contaminated food in the hive and food consumption; decreasing residue level over the time
Exposure of bees to a defined concentration in nectar	+	Application rate can be adjusted to a certain degree to obtain a defined residue level, but residue level declines over the time
Exposure of bees to a realistic concentration in pollen	-	+
Exposure of bees to a realistic concentration in nectar	Can be adjusted based on residue data	+
Foraging on non-target plants/crop	+ (but study should not be carried out during mass flowerings)	After exposure phase
Testing of herbicides intended for dicotyledonous plants	+	herbicide mode of action may led to methodological problems in feasibility (rapid fading of crop possible)
'Caging effect'	-	+
Dependency on climatic, seasonal and crop conditions	-	+
Mean $BTRs_{eggs}$ in the control	14.6%	32.9%
Reliability of test system (control BTR_{eggs}; replicates ≤30%)	85.2%	55.6%

4 Conclusions

In 2013 the test method of Oomen *et al.* (1992)[6] was adapted to a chronic feeding scenario including current methods and was subsequently ring-tested. The obtained results indicated that the chronic feeding design is a robust and reliable test method with low BTRs in the control and a sufficient exposure of the brood to the reference item. Nevertheless adaptations are necessary concerning assessment intervals, selection of appropriate brood stages, number of selected cells etc.

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